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Transcriptional and chemical-genetic profiling of yeast response to chloroquine

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This work is dedicated to my parents,

John K and Frances Newman

and to the memory of my indomitable grandmother,

OM (Fimrite) Stickney (1914-2003)

and my angelic and enchanting great-aunt

Mary Faulkner Stickney (1908-2006)

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Transcriptional and chemical-genetic profiling of yeast response to chloroquine

Victoria L Newman

Abstract

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Among the five species infecting humans, *P. falciparum* is most lethal, though its range is restricted to the tropics. *P. vivax*, responsible for up to 40% of disease episodes, can be found in temperate zones as well and is a serious cause of morbidity and reduced economic well-being. Cumulatively, parasite species precipitate in excess of half a billion malaria episodes each year, a million fatal.

The lifecycle of *Plasmodium* parasites is complex, encompassing multiple sexual and asexual stages specialized to inhabit a preferred tissue-type in host or vector. Disease symptoms and pathology, however, stem from the asexual intraerythrocytic cycle, during which parasites repeatedly invade and proliferate within red blood cells; these blood-stage parasites are the target of antimalarial chemotherapy, and the development during the 20th century of novel antimalarial drugs reduced the proportion of the world's population at risk of malaria from over 70% in 1900 to 40% today. However, acquisition of resistance to control measures has led to a recent increase in disease incidence and nearly complete loss of efficacy of the former frontline antimalarial chloroquine.

While pathogenic resistance to chemotherapeutics is often conferred by the mutation of their target proteins, chloroquine's toxic effect to *Plasmodium* parasites stems from its impairment of an essential but immutable process, that of the crystallization of host-derived heme liberated from hemoglobin during amino acid

scavenging. In *P. falciparum*, resistance to the drug arises from selection of mutations in the *P. falciparum* chloroquine resistance transporter (*PFCRT*), but other *Plasmodium* parasites exhibit independent modes of chloroquine resistance acquisition. *Plasmodium*'s genetic intractability precludes comprehensive *in vivo* examination of the mechanics of chloroquine resistance. We have expressed *PFCRT* in *S. cerevisiae* for chemical-genetic profiling in the presence and absence of chloroquine and PfCRT; we have also investigated yeast's short-term transcriptional response to chloroquine treatment. We find that chloroquine impairs yeast vacuolar homeostasis, protein trafficking, and ability to sense nutritional repletion, and that expression of chloroquine-resistant *PFCRT* can partially suppress these defects.

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Chapter 1: Introduction

Plasmodium *identification and characterization*

Malaria's geographic extent and cause. Malaria is a parasitic vector-borne disease caused by species in the genus *Plasmodium* and disseminated by female *Anopheles* mosquitoes. Five *Plasmodium* species infect humans [1, 2]; *P. falciparum* is most virulent, and causes death predominantly among Sub-Saharan African children under 5 years of age. *P. vivax* is second in its impact and, while infrequently deadly, inflicts significant morbidity and economic burden in areas of endemicity [3-5].Worldwide, 2.6 billion people inhabit areas of endemic or seasonal malaria transmission; more than 600 million incidents of falciparum malaria occur each year, a million fatal, while vivax malaria accounts for nearly 400 million additional disease episodes [6].

Although human malaria today impacts tropical and subtropical regions, historically its reach extended far into the temperate zones. In 1900, 90% of malaria mortality occurred external to Sub-Saharan Africa; indeed, much of the initial characterization of (endemic) parasites was performed in the Mediterranean and Canada [2, 7]. *P. falciparum*'s range is constrained by its inability to complete sexual reproduction, which occurs in the arthropod vector, at temperatures below 18 degrees Celsius [7-9]. *P. vivax*, in contrast, has been occasionally observed as far north as the Baltic and the St Lawrence River Valley. Successful eradication of disease in temperate regions combined antimalarial prophylaxis and DDT-based vector control strategies [3, 10].

Despite reports of increasing malaria incidence and fear that climate change will drastically enhance the parasite's impact, newer analysis suggests that urbanization or containment in areas of seasonal transmission will stem the tide of disease [5, 11, 12].

However, in the tropics, transmission rates (primarily for falciparum malaria) of 700 to 1500 infectious bites per year [3, 10] have forestalled parasite elimination, and prophylaxis and mass drug-administration campaigns succeeded in selecting for parasite antimalarial-drug resistance rather than reducing disease [13]. Antimalarial treatment failure and infectious recrudescence are common, confounded by prevalence of mixed-strain or mixed-species infections and the possibility of zoonoses from wild parasite reservoirs [14-16]. Additional complexity is introduced by consideration of vector behavior and insecticide-resistance, environmental factors contributing to parasite transmission intensity, and the human immune system [16]. The disease remains devastating in regions of endemicity and its vagaries little understood, despite a century of effort expended on research as well as control measures.

Plasmodium *identification*. *Plasmodium* parasites were first noted in the blood of infected individuals, posited to cause malaria, and shown to be curable by quinine, in 1880. Within two decades sexual stages had been discovered and anopheline mosquitoes confirmed as vectors by work on three continents; during this time inroads were also made in classification of three separate etiological agents of human malaria (*P. vivax*, *P. falciparum*, and *P. malariae*) by definition of their distinct pathologies [2, 17]. The parasites are now known to belong to the phylum *Apicomplexa*, comprising both extracellular and obligate intracellular species, and so-called from their actin-based motility system, which depends on the “apical complex” of organelles. In addition to primates and rodents, *Plasmodium* can infect avians, amphibians, and lizards [1-3, 18]. Human malarias are thought to have originated as zoonoses from primates [3, 15].

Plasmodium *comparative phylogeny. Plasmodium* species probably first emerged about 18 million years ago (Mya), with mammalian-specific Plasmodia diverging from progenitors by 13 Mya. Although speciation of *P. falciparum* and the closely related chimpanzee species *P. reichenowi* was thought to have occurred contemporaneous with speciation of their respective hosts' most recent common ancestor [19], it now appears likely that *falciparum*-like parasites represent a recent zoonosis from West African gorillas based on sampling of both nuclear and mitochondrial DNA from fecal samples of numerous primate species [15]. Parasite time of departure from Africa has also been contentious, with some groups favoring the idea that *P. falciparum* populations were small until the inception of modern agricultural societies [20, 21] and others postulating that parasites accompanied humans from Africa more than once before undergoing a rapid expansion close to the end of the last ice age [22]. A great-ape reservoir of *falciparum*-like strains entering human populations at different times could account for apparent historical bottlenecks; human *P. falciparum* parasites appear of lower genetic diversity than related gorilla parasites, supporting limited cross-species transfer [15]. The potential for human-primate parasite transfer could have important implications both for dissemination of antimalarial resistance and for disease control and/ or elimination or eradication efforts [5].

Human malaria parasites. *P. falciparum* causes the deadliest form of human malaria and has been extensively studied in both laboratory and field. Severe disease pathology results from parasites' ability to sequester in the microvasculature during mature asexual blood stages; parasite aggregates cloistering themselves in cerebral capillary beds to evade immune surveillance can lead to coma, while placental seclusion may cause

abortion or impaired fetal growth [3, 23, 24]. Hemolytic anemia is a frequent hallmark of disease and results from repeated cycles of erythrocyte invasion and lytic egress during an infection [24]. In Sub-Saharan Africa hyperendemicity stems from a long season, coupled with astronomical rates, of transmission. Multiply infected individuals are the norm, and repeated parasite exposure leads to a form of immunity (premunity) that modulates the severity and duration of, rather than preventing, illness [3, 16, 25]. Significant disease transmission occurs from asymptomatic, premune, individuals; production of gametocytes refractory to many antimalarial drugs late in infection may facilitate the propagation of drug-resistance [13]. Although falciparum malaria may suppress parasitemia due to other *Plasmodium* parasites during mixed-species infections, it is also proposed that chronic *P. malariae* infection could reduce the severity of *P. falciparum* disease in endemic regions [14].

P. vivax is responsible for as many as 40% of all malaria infections and is the predominant malaria parasite outside Africa [5, 26]. *Vivax* parasites invade reticulocytes rather than mature erythrocytes [18, 26]. Although these non-sequestering parasites are less lethal than *P. falciparum*, *P. vivax* engenders significant morbidity, not least due to production of dormant liver stages, or hypnozoites, that can reactivate to initiate or reinitiate infection months to years after the transmission event [27, 28]. Gametocytogenesis occurs early in an infection, reducing the likelihood of transmitting drug-resistance, but hypnozoite-derived parasites in relapsing individuals may be selected by subtherapeutic levels of antimalarials remaining in circulation from treatment of the initial malaria episode [26]. *Vivax* parasitemia is suppressed by co-infection with *falciparum*, but clearance of *falciparum* parasites often causes hypnozoite reactivation

[6]; PCR-based detection methods suggest that *vivax* and *falciparum* co-infection may occur in as many as 30% of malaria cases [4]. Differences in *Anopheles* vectors permit *vivax* propagation in areas not congenial to *P. falciparum*, and seasonal transmission and low rates of *vivax* incidence tend to enhance the severity of each disease episode. *P. vivax* parasites cannot be maintained in laboratory culture [5].

P. knowlesi is genetically similar to *P. vivax*, though it invades mature erythrocytes and lacks a hypnozoite stage [29]; its asexual cycle is 24 hours in duration [3]. Although causing severe disease in humans, primarily in SE Asia, *knowlesi* parasites' usual host is a macaque; however, they can infect numerous Old and New World primate species [30]. In the macaque disease tends to be chronic and low-level, apparently through immune-suppressive molecular mimicry [29, 30]. A rhesus macaque model is available for laboratory study and parasites can also be cultured *in vitro*; transfection methodology likewise exists and *knowlesi* parasites are more amenable to genetic manipulation than *falciparum*. *P. knowlesi* has been proposed as a possible model for *P. vivax* malaria [30].

Plasmodiums ovale and *malariae* cause less-severe and more infrequent disease than other *Plasmodium* parasites, but can form part of mixed-species infections with each other and with *P. falciparum* or *P. vivax* in malaria-endemic regions [14]; both species undergo gametocytogenesis early in infection [13]. *P. malariae* attains 15-30% prevalence in children in portions of Sub-Saharan African and Oceania and is seen at low levels in other malaria-endemic regions, including in S America, where humans may have introduced it into primate populations; it is responsible for "quartan" fever and has a 72-hour asexual replicative cycle [17]. *P. ovale* is noted more rarely, but also

predominantly infects children [14]; like *P. vivax*, it produces hypnozoites [13]. Recent work implies that *P. ovale* may actually comprise two distinct species of differing pathogenicity [31]. Both parasites remain sensitive to most antimalarial drugs; although of lesser clinical impact than other species, they may modulate host immune response to infection with other *Plasmodium* species, and *P. malariae* symptoms may be suppressed by co-infection with either *P. falciparum* or *P. vivax* [14]. As of this writing, neither parasite has been adapted to *in vitro* culture [17, 31].

Parasite genomics. Plasmodium species colonize a wide repertory of tissue types in both host and vector. The complete parasite lifecycle encompasses both haploid stages and a (brief) diploid phase, and likewise both free-living and intracellular forms, though parasite genomes are relatively small (23 Mb), comprising roughly 5500 ORFs over 14 chromosomes in addition to a mitochondrion and a relict plastid [23, 26, 29, 32].

Completion of the *P. falciparum* genome sequence [32-35], comprehensive proteomic analysis of life-cycle stages [36, 37], and characterization of the parasite's transcriptional profile during the intraerythrocytic developmental cycle (IDC) [38, 39] provided an important tool to pathogen study now supplemented by the publication of sequenced genomes to two other human malaria parasite species, *Plasmodiums vivax* and *knowlesi* [26, 29] and several rodent malarias used to model human disease [40, 41]. *P. falciparum* departs strikingly from most Plasmodia in the nucleotide composition of its nuclear genome (80% AT: 20% GC), but approximately 80% of ORFs are conserved across species; most interspecies differences result from host selective pressure and impact genes involved in cell invasion or fielding the immune system [42].

Each parasite encodes one or more gene families involved in antigenic variation; these genes, expressed during asexual stages to evade host immune surveillance, are encoded both subtelomerically and internal to the chromosomes in *falciparum* and *knowlesi* parasites, but confined to subtelomeric regions in *vivax* [26, 29, 32]. *Var*, *kir*, and *vir* genes (from *falciparum*, *knowlesi*, and *vivax*, respectively) comprise related families subject to regulation by interaction between promoter and intronic sequences conserved across parasite species. *P. knowlesi* encodes an additional means of subverting its native host's immune system, expressing peptides that mimic regulators of T cell proliferation and permit sustained low-level infection of macaques [26].

P. falciparum and *vivax* asexual-stage transcriptomes are induced in a continuous phasic cascade through the lifecycle [38, 43] and appear refractory to perturbation [44]. Genes nonsyntenic between species are transcribed at the initiation and conclusion of the *vivax* IDC, including (but not limited to) those encoding factors involved in erythrocyte ingress and immune evasion. In contrast to *falciparum* parasites [38], *P. vivax* expresses several antigenic variation genes concurrently at the beginning of its IDC, and during the schizont stages another, nonoverlapping, group is induced, perhaps due to an increased need for variety of surface antigens in a constantly circulating parasite [43]. Genes encoding components of metabolic pathways are, for the most part, similarly expressed in each parasite; however, hydrolases involved in hemoglobin degradation and the *P. vivax* v-ATPase are transcribed much later than their *P. falciparum* counterparts [43].

Transcriptional control in *Plasmodium* species is not well characterized. While the proteome of sexual and asexual stages differs more than that of asexual stages sampled at different times within the lifecycle [36, 37], the mechanics of stage-specific

expression are not fully elucidated. Regulatory motifs have been identified in some *falciparum* promoters [45], and conserved elements involved in sporozoite- and invasion-specific gene transcription are identified in *Plasmodiums falciparum*, *vivax*, *knowlesi*, and *yoelii* [29]. However, motif detection in *falciparum* is stymied by the extreme AT-richness (90%) of noncoding regions. Recently a family of transcription factors was identified in Apicomplexans that impart control over timing and stage-specificity of gene expression [45]. In addition, genome and proteome analyses identified some functional gene clusters, including one encoding hydrolases expressed during the asexual stages and a second comprising sexual-stage genes, possibly presenting an additional means for transcriptional regulation [37]. Translation in each lifecycle stage (asexual, sexual, and mosquito) is mediated by specific rDNA [32], although the functional consequences of the separate rDNA modules have not been described.

Genomic differences in P. falciparum *strains*. A more comprehensive picture of *P. falciparum* genome structure has emerged recently with the completion of several genome-wide association studies. First, among lab cultivars originally isolated in different geographical regions, smaller chromosomes are more polymorphic than larger, probably because highly recombinogenic antigenic variation gene clusters occupy proportionally more sequence; additionally, greater sequence diversity is found in the vicinity of known antigens, likely from host selection pressure [46]. Secondly, comparison of the 3D7 lab cultivar to a clinical isolate found increased genome size in the cultured strain. Additional comparison of these two *P. falciparum* strains to the genome of *P. reichenowi*, a chimpanzee parasite with marked similarity to *P. falciparum*, determined that genes under strong selection are expressed at low levels and tend not to

be constitutively induced during asexual stages; indeed, genes involved in merozoite invasion, localized to the apicoplast, or predicted to be exported were most mutable [47]. Finally, parasite genomes bear strong imprints of divergent drug selection schemata; comparison of twelve lab cultivars of worldwide origin found evidence of selective sweeps correlating with chloroquine resistance on chromosomes 5, 7, and 11, while selection by S/P reduced genetic diversity on regions of chromosomes 13 and 14, but not, as expected, on chromosomes 4 and 8 (which contain coding sequence for *DHFR* and *DHPS*, respectively). Linkage disequilibrium in African parasite strains extended over shorter distances than in Asian, likely due to the frequency of mixed infections and consquent high recombination rates [48]. Large-scale comparative genomics studies such as these can enrich our perspective on the disparate selection pressures operating on parasites from host, vector, and exterior environment, in addition to elucidating differences between field and laboratory adapted strains.

The Plasmodium *lifecycle*

Pre-erythrocytic stages. Clinical manifestations of malaria result from the intraerythrocytic developmental cycle (IDC), characterized by repeated cycles of host red blood cell invasion by haploid asexual parasites. Infection commences with subcutaneal injection of the motile sporozoite form of the parasite from the mosquito's salivary glands as the mosquito probes host dermis preliminary to blood meal ingestion [2, 3]. Sporozoites pass through or between epithelial cells by gliding motility, propelling themselves through host tissue to the endothelial wall using a non-canonical actin-myosin motor system [5, 18]. Upon entering the circulation, they quickly navigate to the liver to colonize hepatocytes, converting to obligate intracellular forms and massively

proliferating before synchronized rupture of host cells and release of invasive merozoites into the circulation—a total process of seven to ten days [17, 18]. Merozoite invasion of erythrocytes begins the IDC, which is characterized by cyclical fevers resulting from inflammatory responses to debris left behind upon the synchronized release (egress) of merozoites from a parasitized erythrocyte at the completion of the replicative cycle [2, 3].

The Intraerythrocytic Developmental Cycle. Initiation of the IDC by merozoite ingress into erythrocytes is necessarily an active process, as erythrocytes lack the capacity for endocytosis. After primary contact between a merozoite's coat and RBC surface proteins, the parasite reorients, apposing its apical organelles toward the RBC surface to form the "tight junction" [18]. The apical organelles, comprising rhoptries, micronemes, and dense granules, then initiate secretion of membrane-associated ligands that can interact with RBC surface adhesins (often transmembrane proteins connected to the RBC cytoskeleton through their cytoplasmic domains), as well as proteases and factors involved in the creation of the parasitophorous vacuole when invasion is complete. Secretory organelle contents are extruded onto the merozoite surface in a basally moving wave sequentially and in coordinated fashion. The cytoplasmic domains of membrane-integral ligands are associated with a noncanonical actin-myosin machinery, while extracellularly they bind receptors on the erythrocyte surface [18, 49]. The concomitant release and basal movement of merozoite adhesins attached both to propulsive machinery and to the erythrocyte surface results in forced engulfment of the parasite by RBC membrane. As the erythrocyte membrane closes around the parasite, "shaving" of the merozoite surface by proteolytic intra- or juxtamembrane trimming of adhesins forms a parasitophorous vacuole. The parasite now converts to an obligate intracellular form, activating

translation of certain stored mRNAs as well as the more or less invariant stage-specific transcriptional program that determines its development and proliferation [2, 17].

The IDC is characterized by extensive genome replication. Although other Apicomplexan parasites undergo endodyogeny or synchrous mitoses, in Plasmodia there is a wide distribution in the number of progeny produced (estimates range from 5-32 for *P. falciparum*). Mitosis is followed by genome partitioning into new merozoites, and the IDC is completed by parasite egress, the sequential, Ca^{2+}-dependent, proteolytic breakdown of the parasitophorous vacuole and RBC plasma membranes, accompanied by the explosive release of merozoites into the circulation [18, 50, 51].

The Sexual/ Arthropod Stages. Although the vast majority of progeny are competent to continue the IDC, in a process that is not well understood, but dependent on such environmental factors as the density of an infection, availability of nutrients, and antimalarial drug exposure, occasional parasite offspring exit the asexual platform to differentiate into sexual stages, or gametocytes [2, 13]. Both macro- (female) and micro- (male) gametocytes are produced; in a single infection, macrogametocytes prevail, but nearly equivalent numbers of macro- and microgametocytes are made when multiple genotypes are present to reduce the probability of selfing [52]. Gametocyte maturity is attained within approximately two weeks, after which they circulate, cell-cycle arrested, until their ingestion by a mosquito in a blood meal. In *P. falciparum* gametocytes are impervious to numerous antimalarials (though not primaquine or artemisinin) [13, 37].

The mosquito stage commences with the arrival of a blood meal in the midgut. A signaling pathway is initiated by the joint stimuli of temperature drop and exposure to the small molecule xanthurenic acid, resulting in three rounds of mitosis in the

microgametocyte. Macro- and microgametocytes then fuse, forming a zygote that rapidly undergoes meiosis and conversion to the ookinete form [53]. The ookinete, a motile form comparable to the sporozoite, moves from the mosquito midgut via gliding motility, passing through the midgut epithelium into the basal lamina. Here conversion to the oocyst is undergone and in this spore-like form the haploid genomes resulting from meiosis proliferate and convert into sporozoites that are released into the mosquito hemocoel and transported by the circulation to the salivary glands to recommence infection as the mosquito vector seeks a blood meal [2, 17].

Parasite metabolism. As newly invaded merozoites convert to obligate intracellular forms competent for replication and propagation of infection, they extensively modify the erythrocyte, including its plasma membrane. *Plasmodium*'s obligate intracellular lifestyle, bounded by three membranes, demands a complex mechanism for nutrient acquisition, and indeed, bioinformatics analyses predict export of 5-10% of the proteome to the parasitophorous vacuole, host cell cytoplasm, or plasma membrane [54, 55] to facilitate solute uptake from the host cell or the bloodstream [56]. *Plasmodium* parasites lack machinery for purine and amino acid biosynthesis [32], and during the asexual stages also depend on the host for glucose and lipids; to this end they traffic numerous channels and transporters to the RBC surface [32, 56]. The conductivity of parasitized RBCs alters within six hours of invasion with the incorporation of "new permeability pathways" into the RBC membrane [57, 58], and the plasma membrane is also deformed by the creation of knoblike protrusions to display antigens involved in immune evasion [37, 54, 59]. Modifications are effected via an elaborate exocytic system. Signal sequences specify import into the endoplasmic reticulum; the default trafficking pathway

appears to send proteins either to the parasite plasma membrane or into the parasitophorous vacuole (secondary signals define localization to internal compartments such as the apicoplast or DV) [60]. A further motif, the PEXEL sequence, demarcates proteins bound for the erythrocyte interior or plasma membrane, and is proteolytically processed by plasmepsin V and N-acetylated in the endoplasmic reticulum, presumably to facilitate interaction with transport machinery. Secreted proteins then transit through the exocytic pathway; upon release into the parasitophorous vacuole they are threaded through a translocon into the erythrocyte and enter a parasite-derived secretory pathway to reach their targets [54, 55, 59, 61-63].

Antimalarial Chemotherapeutics

Malaria eradication was first proposed in the 1950s, when chloroquine, an economical and highly efficacious antimalarial, became widely available; strategies of vector elimination by mass DDT application in conjunction with widespread drug administration were utilized and effected substantial reductions in transmission [3, 10, 13, 18, 64]. However, resistance arose to control measures both in the mosquito vector and in malaria parasites, leading to the abandonment of the goal by the 1960s [16, 65].

In 1998 the Roll Back Malaria initiative was launched by the World Health Organization (WHO), World Bank, and United Nations (UNDP and UNICEF) as a platform intended to reduce malaria deaths to a quarter of their then level by 2015 [66]; however, by 2004, malaria incidence had actually increased, seemingly due to donor noncompliance and poor enforcement of reduction strategies [66, 67]. A call for malaria eradication (i.e., total abolition from the field) [7, 65] was made again in 2007 at the joint behest of the WHO and Bill and Melinda Gates Foundation, although elimination

(discontinued local transmission) [7, 65] is considered more feasible in the absence of either transmission-blocking vaccines or drugs effective against gametocytes [68, 69]. Underfunding and fund misallocation are also hampering disease control [70], but, while reporting on malaria incidence remains problematic in some areas of Central Africa through lack of infrastructure and/ or population displacement due to conflict, in other areas of the continent falciparum malaria is in decline [64, 71, 72]. Current efforts in malaria reduction focus on insecticide-treated bednets; indoor, limited-scope DDT spraying; and chemotherapy, including intermittent preventive treatment of infants and pregnant women [3, 4, 10, 13]; it should, however, be noted that outside Africa vector feeding preference may reduce the efficacy of indoor residual spraying (IRS) [7, 73]. Complete malaria eradication will further require the development of vaccines, an expanded drug arsenal, and a repertory of insecticides utilizable to minimize infectious bites—and, not inconsequentially, infrastructure to target them adequately [7, 74].

Although antimalarial-resistance in *P. vivax* is not explicitly discussed here, it is worth noting that, though vivax malaria is commonly treated with chloroquine, *P. vivax* misdiagnosis, increased resistance to chloroquine, production of dormant stages, and unculturability all may hinder the efficacy of this course of action [10, 27, 75]; artemisinin combination therapy is currently being explored for this parasite species [6, 10]. Mechanisms for *P. vivax* chloroquine-resistance acquisition are unknown; resistance is not contingent on mutations in *PVCRT*-o, and sequence polymorphisms in *PVMDR1* are not always predictive of high-level resistance [27, 75], and identification of antimalarial-resistance determinants in this pathogen is unfortunately impeded by the difficulty of its culture. Moreover, some measures undertaken toward antimalarial vector

control, such as IRS, may be ineffective in reducing *P. vivax* transmission due to differences in vector behavior (exophily or exophagy) [6, 7, 13, 73], and only one antimalarial chemotherapeutic (primaquine; see below), beleaguered by compliance issues and side-effects, is licensed for hypnozoite clearance [76].

Falciparum malaria is treated with a number of chemotherapeutics that target varying aspects of parasite biology, including folate biosynthesis, mitochondrial respiration, heme anabolism in the digestive vacuole, and apicoplast inheritance; the WHO's recommended frontline treatment is currently artemisinin derivatives in combination with other antimalarials. Despite reports of delayed parasite clearance, artemisinin efficacy remains high, but counterfeiting is a major problem in some regions [4, 10, 73].

While resistance has arisen to most, if not all, antimalarial drugs currently in use, many processes impacted by these compounds are the focus of continued research [5, 77]. Components of the parasite's secretory pathway or proteolysis-based egress machinery have recently been proposed as viable blood-stage targets [5].

The mechanisms for resistance acquisition in *P. falciparum* have been well established for the antimalarials sulfadoxine/ pyrimethamine, atovaquone, and chloroquine, but remain lacking for quinine and artemisinin. Study of drug action and parasites' evolution of resistance is imperative due to reported tolerance in the field of all antimalarial chemotherapeutics approved for use. The following section discusses, where applicable, modes of drug action, as well as unifactorial and combinatorial resistance methods adopted by *P. falciparum* parasites.

Artemisinin. Artemisinin (ART), a sesquiterpene lactone endoperoxide, is isolated from the sweet wormwood *Artemisia annua* and has been used as an antimalarial in traditional Chinese medicine for thousands of years. ART derivatives are currently the WHO-recommended frontline treatment for antimalarial chemotherapy in combination with partner drugs whose use varies by country and known resistance phenotypes of parasites (ACT; partner drugs include lumefantrine, mefloquine, amodiaquine, and sulfadoxine/ pyrimethamine) [4, 10]; parasite clearance by ART derivatives occurs more quickly than that mediated by chloroquine or S/P [4]. Resistance to the ART metabolite dihydroartemisinin has been detected in a laboratory study in both SE Asian and African *falciparum* strains [78], and delayed parasite clearance is observed at certain Cambodian sites [73].

Artemisinin contains an unstable endoperoxide bridge predicted to be highly reactive toward Fe-S cluster-containing proteins and/ or ferrous iron associated with heme in the DV [5, 10, 79, 80]. Activated ART is thought to alkylate its targets; heme alkylation and DV impairment have been demonstrated in culture [10], as has ART covalent association with proteins in *P. falciparum* extracts [80]. Debate over the likelihood of activation by heme arises from the observation that CO treatment, which should inhibit the capacity of ferrous heme iron to interact with ART, does not reduce ART efficacy [79]. In support of a role for Fe-S cluster proteins in catalyzing reactivity, the compound is toxic against both late-stage parasites and earlier stages not catabolizing hemoglobin [10]. ART is also active against gametocyte stages [6, 13], although in combination with piperaquine may be less competent to reduce gametocyte burden [10].

Artemisinin resistance. Several studies with antimalarial-resistant field isolates and lab cultivars have shown low-level cross-resistance between ART and various compounds such as quinine, mefloquine, and halofantrine, probably mediated by amplification of or mutations in *PFMDR1* [81-84]; sequence changes in *PFMDR1* predicted to reduce gene product transport capacity appear to augment ART-sensitivity [85, 86] and fluorescent ART labels cytoplasmic structures, as well as the parasite-tubulovesicular network, suggesting that sequestration in the DV by PfMDR1 could be a means of detoxification [80]. A stepwise selection for ART-resistance in culture led to amplification of *PFMDR1* in some, but not all, genetic backgrounds [87]; cessation of pressure led to some *PFMDR1* deamplification in conjunction with a partial reduction in ART-resistance, suggesting the collaboration of multiple factors toward the trait [88].

Research has also focused on *PFATP6*, the *falciparum* homologue of the Ca^{2+}-dependent ATPase SERCA: when the protein was expressed in *X. laevis* oocytes, treatment with ART but not with quinoline antimalarials could inhibit its function comparably to known mammalian SERCA inhibitors such as thapsigargin; ART inhibition was specific to PfATP6, and did not affect either mammalian SERCA or the related, non-SERCA type Ca^{2+}-dependent ATPase PfATP4 [80]. Associations between SNPs and ART-resistance were also observed in field isolates, but further investigation suggested a high level of polymorphism in the coding sequence [10]. More recent work involving allelic exchange of the *P. falciparum* SERCA sequence with a modified mammalian allele (S263E) that does not interact with drug led to only subtle, non-significant alterations in parasite drug-resistance, perhaps suggesting a multigenic mode of parasite tolerance or resistance [79].

Sulfadoxine/Pyrimethamine. Sulfadoxine (SDX), a sulfonamide, inhibits dihydropteroate synthase (DHPS), and pyrimethamine (PYR), a diaminopyrimidine, dihydrofolate reductase (DHFR), enzymes in the folate biosynthesis pathway, to prevent DNA replication [89]; together the drugs show synergy [5]. Despite widespread resistance to S/P, folate synthesis remains druggable and is also targeted by cycloguanil, which is effective against DHFR, including some PYR-resistant forms (see below) [90]. Unfortunately S/P usage can stimulate gametocytogenesis, facilitating spread of resistant genotypes [13].

S/P resistance. PYR was initially used as monotherapy, and resistance arose almost immediately after its usage in a Kenyan mass drug administration campaign [89, 91]. Low-level resistance is conferred by DHFR mutation of serine to asparagine at position 108, and increases stepwise with subsequent alterations in up to four of five possible residues. SDX likewise selects mutations in as many as five residues of DHPS, with an initial change from alanine to glycine at position 437. Drug inhibition of each enzyme is competitive [89, 90]. In field isolates with lessened sensitivity to S/P, mutations in *PFDHFR* are frequently noted before those in *PFDHPS*, possibly because the presence of PYR prevents folate scavenging from the host, hence favoring mutation in *PFDHFR* [90].

A triple-mutant variant of *PFDHFR* has spread from Asia to Africa, while both double and triple mutants have been independently selected on each continent. Several other triple-mutant *PFDHFR* alleles have been detected within Asia and a quadruple-mutant hyperresistant allele is also seen in Asian field isolates [89, 91]. In a recent study

mutant *PFDHPS* alleles were found to have been selected *in situ* on different continents, presumably on the *PFDHFR* mutant background [92].

Atovaquone. Atovaquone, a naphthoquinone, is an inhibitor of respiration that targets the parasite mitochondrion by impeding electron transport at cytochrome *b*c(1); mutations in the *CYTB* gene can confer resistance [26]. As Malarone, atovaquone is co-prescribed with proguanil for antimalarial prophylaxis [4]; Malarone's prohibitive cost has generally restricted its use to tourists [73].

Proguanil/ Cycloguanil. Proguanil is a diguanide antimalarial metabolized in the liver to cycloguanil. While cycloguanil is a specific inhibitor of parasite *DHFR*, proguanil is believed to act against an independent target. Proguanil and atovaquone taken together are synergistic against falciparum malaria [4, 93].

*Mefloquine***.** Mefloquine (MFQ) is a 4-methanolquinoline. The mode of its action is unknown; although it has been postulated to prevent heme detoxification, resistance to the compound is frequently observed concomitant with increased expression of *PFMDR1* [10, 84, 94, 95], suggesting a cytoplasmic target. Cross-resistance is observed between MFQ and HF and may also correlate with *PFMDR1* sequence polymorphisms [81, 83, 94, 96]. Neuropsychiatric side-effects are commonly reported after treatment with the drug [97], and psychosis can occur in as many as 0.5% of patients [98]; however, the drug is supplied as a racemic mixture and removal of the (-) stereoisomer is proposed to circumvent these issues without loss of efficacy [99].

*Lumefantrine***.** Lumefantrine is an arylamino alcohol structurally related to MFQ, quinine, and halofantrine, and is currently in common usage in Africa with artemether as part of ACT. Mutations in or amplification of *PFMDR1* may contribute to establishment of

resistance; cross-resistance can be observed with mefloquine and halofantrine. Lumefantrine is highly active against chloroquine-resistant parasites [10, 84, 95], but its bioavailability is lessened if not taken with fatty food [98].

Primaquine. Primaquine (PMQ) is an 8-aminoquinoline used to clear liver-stage parasite infections, including *P. vivax* and *ovale* hypnozoites, or sexual-stage *P. falciparum* parasites. It has significant activity against *P. vivax*, but not *P. falciparum*, asexual stages [13], but sporadic resistance has been reported [26]. PMQ metabolites strongly engender oxidative stress, with attendant hemolysis, and thus the drug cannot be prescribed for pregnant women [26] or patients with glucose-6-phosphate dehydrogenase deficiency [4, 13], though side-chain modification appears to alleviate this effect somewhat in mouse models [76]. However, the mechanism of action remains unknown and is not clearly separable from redox effects, and concomitant with lack of a hypnozoite model, has hampered the design of replacement drugs [13, 76].

Halofantrine. Halofantrine (HF) is an arylamino alcohol similar to mefloquine and lumefantrine, with which it exhibits cross-resistance. In an *in vitro* study, HF-resistance was selected by mutation of *PFCRT* and corresponded to a loss of PfCRT-dependent chloroquine resistance (and augmented QN- and MFQ-resistance) [100, 101]. Mutations in or amplification of *PFMDR1* may also alter response to the drug [84, 96]. Although postulated to interfere with heme polymerization, HF's target is likely cytoplasmic based on determinants of resistance [101]. HF treatment can promote fatal cardiac arrhythmia in patients, usually as a result of underlying cardiac disease, and high parasite recrudescence may transpire after usage [101-103].

Amodiaquine. Amodiaquine (AQ) is a 4-aminoquinoline structurally related to chloroquine and thought to function analogously in impeding hemozoin crystallization [10]. It has been used in numerous regions, including S America, S Asia, and Melanesia, as monotherapy [104], and is currently in use in parts of Africa with artesunate as ACT [10]. Mutation in *PFCRT* or co-selection of mutations in *PFCRT* and *PFMDR1* contributes to AQ-resistance [104]; incomplete cross-resistance is seen with chloroquine [10].

Chloroquine. Chloroquine (CQ) is a 4-aminoquinoline and was the WHO's recommended frontline antimalarial for many years due to its extraordinary efficacy (parasite clearance within one IDC), low cost, and low human toxicity [4, 10]. CQ's site of action is the digestive vacuole, the parasite's hydrolytic compartment, in which it impedes sequestration of a toxic byproduct of hemoglobin digestion, resulting in parasite death by oxidative stress.

Over the course of the IDC, a parasite ingests and catabolizes as much as 60% of its host erythrocyte's hemoglobin [105]. The cytostome is an endocytic compartment in which erythrocyte cytoplasm is incorporated into transport vehicles and trafficked through the parasite tubulo-vesicular network to the DV; uptake is thought to maintain osmotic homeostasis and create *lebensraum* for parasite growth and division [106]. Within the DV, several hydrolases, most notably plasmepsins and falcipains, digest globin into amino acids or small peptides exported to the cytoplasm for parasite recycling [107]. However, liberated heme is extremely reactive, and is conjugated into hemozoin, a crystalline form whose unit cell is a β-hematin dimer comprising head-to-tail ferriprotoporphyrin IX moieties, to prevent redox stress [108].

While the forming crystal is autocatalytic for its own growth, the process of nucleation was not well understood for a number of years. Although enzymatic involvement in crystal construction was ruled out by evidence that the process was refractory to proteolysis and boiling, the rate of hemozoin formation in *in vitro* assays was much slower than that seen in the parasite, suggesting a parasite-derived catalyst. Two models were proposed: PfHRPII (histidine-rich protein, whose function is unknown) or peptides derived from it might act as a scaffold for crystallization, or crystallization could rather be lipid-catalyzed and protein-independent. Eventual localization of PfHRPII outside the DV, coupled with the observation that its deletion led to no loss of viability and did not prevent parasites from producing hemoglobin, ruled out a role for this protein in hemozoin formation [105, 107].

The current model for crystallization evolved from more exact recapitulation of intra-DV conditions *in vitro.* The process is now known to proceed inside lipid nanospheres, micelle-like structures within the DV possibly arising from recycling of the inner leaflet of trafficking vesicles after their docking at the DV membrane. Crystal nucleation occurs at the interface between the lipid-rich subcompartment and the aqueous DV lumen, and is pH-regulated due to the pH-dependent interconversion of β-hematin, the hemozoin building block, with the more frequently seen μ-oxo heme dimer, which lacks competence for crystal formation. Scanning electron microscopy detects the apparent initiation of crystallization at the edge of a micelle, as well as more mature hemozoin particles filling much of the interior. The role of proteins has not been defined: lipid nanospheres do not stain with Coomassie Blue, but proteins might participate at low stoichiometry. The means by which heme is transported into lipid nanospheres for

hemozoin formation and the means by which nanospheres themselves arise remain enigmatic [105, 107].

The CQ-hematin interaction has been extensively characterized and is known to be inbibited by plasmepsin inhibitors (ie, when heme release is prevented, parasite accumulation of CQ is reduced). CQ and other quinoline antimalarials display complex and specific interactions with heme; CQ binds dimeric heme, interacting (noncovalently) with the μ-oxo dimer as well as covalently with β-hematin, the hemozoin crystal's unit cell. QN interacts with monomeric heme; these interactions are pH-dependent and modulated by DV lipid composition [108].

Chloroquine resistance. Chloroquine, discovered in 1937, came into common usage after the Second World War and was used extensively for prophylaxis as well as chemotherapy. Regional or country-wide malaria prevention campaigns supplying CQ-spiked table-salt, and the ensuing subtherapeutic exposure, impelled the roughly simultaneous emergence of resistance at four to six independent foci in SE Asia, Melanesia, the Philippines, and S America. By the late 1970s resistance was pervasive in Africa and CQ-treatment failure is now common in most malarious regions [25].

Chloroquine is a lipophilic weak base, freely membrane-permeable when uncharged, but impermeant when its sidechain becomes mono- or diprotonated. In *in vitro* assays with CQ-sensitive parasites, the drug accumulates in the acidic DV to several thousandfold the concentration in the medium [109]. Conversely, resistant parasites accumulate less CQ, and likely possess a means for its exclusion or removal from the cell [25]. Similar affinities for CQ/ β-hematin binding among resistant and sensitive parasites suggest the latter [108].

Initial models for the mechanism of CQ-resistance acquisition suggested that drug trapping in the CQ-resistant parasite's DV was disfavored via altered DV homeostasis or a gain-of-function mutation allowing CQ export. Since drug-resistance might be effected by gain-of-function permitting proton leak from the compartment with coincident pH increase and reduced CQ trapping, these models are not mutually exclusive. However, evidence suggested direct export of CQ from the CQ-resistant DV. First, CQ-resistant parasites were chemosensitized to drug by verapamil; verapamil's function as a cation-channel blocker suggested that it might occlude a binding site for charged CQ present in resistant but not sensitive parasites. Secondly, selection studies with MFQ [94] and HF [100] demonstrated loss of CQ-resistance with increased tolerance of those compounds, while cross-resistance to QN was also found in some CQ-resistant strains [110]. Additionally, in *in vitro* assays, CQ analogs containing modified sidechains could bypass resistance [111-113].

The search for *PFCRT*. In the late 1980s a genetic cross was undertaken to identify the factor(s) responsible for CQ resistance. Gametocytes were raised from a CQ-resistant SE Asian strain (Dd2) and a CQ-sensitive Honduran strain (HB3), and fed to mosquitoes; after sufficient time for production of sporozoites, the mosquitoes transmitted parasites to a chimpanzee and infection was successfully established. Blood stages were isolated, cloned, and their resistance phenotypes characterized.

It was immediately apparent that, rather than exhibiting the spectrum of EC_{50}s predicted for a multigenic trait, parasites could be placed into discrete nonoverlapping subsets: resistant parasites' EC_{50} values were approximately eightfold those of sensitive parasites [114]. Microsatellite mapping observed segregation of a single region on

chromosome 7 with resistance. Initially this region was mapped to 400 kb [115], but establishment of finer-resolution genetic markers and extensive characterization of the locus among more than a thousand clonal progeny of the cross narrowed the resistance-determining region to 36 kb [116]. While PfMDR1, the orthologue of the mammalian multidrug-resistance transporter (Pgh-1), had been assumed to confer resistance to CQ based on the mammalian orthologue's interaction with the CQ chemositizer verapamil, it is encoded on chromosome 5. Indeed, characterization of recombinant progeny demonstrated random segregation of Dd2 and HB3 alleles of *PFMDR1* and no clear association between *PFMDR1* sequence and CQ resistance [114].

Scrutiny of the 36-kb region identified 8 genes predicted to encode proteins of at least 100 amino acids, seven of these transcribed during the IDC (to similar levels in CQ-sensitive and CQ-resistant progeny). Neighboring candidate genes (*CG*s) 1 and 2 possessed polymorphisms, though other ORFs did not. *CG1* sequence, unlike *CG2*, was not extensively dissimilar between the HB3 and Dd2 parents or between Dd2 and the S-American type CQ-resistant parasite 7G8. *CG2* was therefore hypothesized to be under CQ selection, with mutations in the ORF conferring CQ resistance [116].

Full characterization of these ORFs and examination of the association between *CG2* and CQ resistance necessitated substantial technological development, including that of transfection methodology; and true identification of the CQ-resistance determinant was not possible until allelic exchange could be undertaken. In the meantime, correlation between *CG2* and CQ resistance was probed both in lab cultivars and field isolates. *CG1* and *CG2* exhibited the same polymorphisms in all but one of a set of CQ-resistant parasites of Asian and African origin (differences between Asian and S American

parasites were regarded as evidence of their independent development of the trait). CQ-sensitive parasites had greater sequence diversity in the two ORFs, with the 106/1 Sudanese strain entirely identical to Dd2 for as much as 20 kb of the resistance locus, including the region encompassing both *CG1* and *CG2*. This incongruity was interpreted to imply additional determinants of CQ-resistance or the necessity of a "permissive genotype" for selection of resistance in at least this isolate; *CG2*'s role in CQ-resistance acquisition was assumed in the absence of more evidence to the contrary. However, models for *CG2*'s function in resistance remained incomplete, as immunofluorescence localized the protein to the plasma membrane or to vesicular structures external to the DV, not commensurate with its direct export of CQ from the DV [116]. The participation of *CG1* and *CG2* in CQ-resistance acquisition was eventually tested in 2000 by the replacement with HB3-type of Dd2-type polymorphisms in four CQ-resistant strains. Manipulation of either gene altered neither CQ resistance nor parasite chemosensitization by verapamil in the recombinant parasites [117].

Implicit in the tight linkage of *CG1* and *CG2* to CQ resistance in Dd2 x HB3 recombinants and field isolates was their close proximity to resistance determinants. Therefore, the Wellems group once again mined the 36-kb CQ-resistance locus, this time for ORFs encoding gene products smaller than their previous cutoff of 100 amino acids. This approach noted only a highly interrupted 3.1 kb ORF comprising 13 exons between *CG2* and *CG3*. The gene was transcribed during the IDC and translated to a protein of 424 amino acids. Bioinformatics predicted 10 transmembrane domains, and sequence comparisons among CQ-sensitive and CQ-resistant isolates found eight nonsynonymous polymorphisms within or proximal to the predicted transmembrane domains, evidence

that selection with CQ might confer altered transport properties on the gene product. S American CQ-resistant isolates varied slightly from Dd2 in their polymorphisms [118].

To determine whether the CQ-resistant allele of this new candidate could confer a dominant gain-of-function on CQ-sensitive parasites, 106/1 parasites were transfected with either the HB3-type CQ-sensitive or Dd2-type CQ-resistant allele and selected with CQ for the presence of episomes containing the gene. Only parasites transfected with the Dd2 allele were viable, with CQ application lethal both to HB3-allele and mock-transfected parasites. The episomally encoded Dd2-type allele therefore appeared to engender CQ resistance, a result confirmed by parasite transfection with CQ-sensitive, CQ-resistant, CQ-resistant revertant (with compensatory mutations, but T76K), and frame-shifted CQ-resistant versions of the gene selected with an unrelated drug; parasites were obtained from each transfection but only the CQ-resistant candidate episome augmented the strains' CQ EC_{50}. One selected strain was able to grow under continuous CQ pressure after losing its episome, having acquired a K76I mutation in the endogenous allele of the candidate gene. This mutation had occurred on the background of the numerous SNPs observed in the 106/1 CQ-sensitive allele, implying that the sequence of this allele is competent for single-step resistance acquisition. When introduced episomally into other strains the 76I mutation also conferred resistance. Charge-loss, rather than a specific mutation, at position 76 was therefore sufficient for the CQ-resistance phenotype. Antibodies to the gene product and construction of a GFP-tagged variant localized it to the DV membrane, in support of the models that changes in DV homeostasis or a direct means of CQ export bestowed CQ resistance, and the CQ-

resistance candidate gene product was christened the *P. falciparum* Chloroquine Resistance Transporter (PfCRT) [118].

Characterization of *PFCRT*. PfCRT's identification was the culmination of a brutal 13-year slog, in spite of which our understanding of CQ-resistance acquisition remains incomplete. However, it is now known that *PFCRT* encodes an essential gene placed by bioinformatics analysis within the Drug-Metabolite Transporter (DMT) Superfamily, a novel family of membrane-integral proteins comprising both channel and carrier entities. CRT orthologues, found in *Plasmodium* species and also in such disparate organisms as *Dictyostelium* and *Arabidopsis*, form a separate group within this family, but are most closely related to the Drug-Metabolite Effluxer branch (DME) [119], which constitutes mainly uncharacterized proteins from a number of plant and bacterial species [120]. A dearth of information on the DMT Superfamily and absence of a crystal structure for PfCRT constrain our understanding of its natural function and role in drug resistance acquisition. However, the DV's integral part in the process of hemoglobin digestion has led to the suggestion that PfCRT exports short peptides generated by globin catabolism from this compartment. Some experimental evidence exists to support this model [121]. Additionally, DME proteins can transport amino acids and their metabolites as well as organic cations and weak bases [119, 120]. The *Arabidopsis* orthologues of *PFCRT* have been proposed to function as a glutathione exporter from the plastid that maintains redox homeostasis [122], a model that has also been posited for PfCRT [123].

A hydropathy plot of PfCRT's 424 amino acids indicates that the protein assembles into ten transmembrane domains with N- and C-termini in the cytosol [119]; the topology has recently been experimentally confirmed [60]. PfCRT does not appear to

possess ATP-binding motifs, in contrast to PfMDR1's two, implying that it utilizes the electrochemical potential of the DV to transport substrate down its concentration gradient. This model is supported by experiments in *P. falciparum* investigating PfCRT's transport of CQ [124-126].

Post-translational modifications on *Plasmodium* proteins have not been extensively studied, although export to the erythrocyte requires N-terminal acetylation [59]. A recent study examining trafficking of PfCRT in asexual-stage parasites determined that its localization to the DV membrane is phosphorylation-dependent. Transport was brefeldin-A sensitive, suggesting entrance into the secretory pathway, but a putative lysosomal targeting signal could be deleted without affecting localization. Instead, phosphorylation of T416 in the protein's C-terminal cytoplasmic domain was necessary (but not sufficient) for correct trafficking. Mutation of the residue to alanine sent PfCRT to the plasma membrane, and partial rescue of the phenotype was seen with mutation to serine or the phosphomimetic aspartate. Mass spectrometry detected phosphorylation at T411 as well as T416, although mutation to alanine at 411 had no effect on trafficking [60]. Consistent with modification of PfCRT, a polyclonal antibody raised against the protein recognizes a doublet [83, 118]. However, the identity of the kinase and the contribution of other signals to PfCRT localization remain unknown.

There is significant homology between the first and second five TM domains of PfCRT, implying an internal duplication. Some extramembrane loops also preserve strong sequence similarity to each other: loops 3 and 8, and 4 and 9, are not divergent in sequence, and protein function and localization in DME family members is impaired by epitope-tag insertion in these regions [119], which according to the prevailing model are

cytosol-exposed [60]. In contrast, extramembrane loops 2 and 7, in the DV lumen, are dissimilar in sequence. Loop 7 is much longer than the others, and among PfCRT orthologues is well conserved. In other DMT Superfamily members, alterations in the length of this loop reduced substrate transport [119].

TM domains 5 and 10 mediate homodimerization in other DMT family members, although this process has not been studied in PfCRT. Within these domains, motifs comprising two glycines flanking a stretch of 6 hydrophobic residues may promote protein stability and appropriate assembly within the membrane. Domains 3, 4, 8, and 9 (principally 4 and 9) are important for substrate transit in other family members, and substitutions in these regions can reduce or abrogate transport; conserved prolines in TM domains 4 and 9 may facilitate interaction with substrate by distorting the transmembrane helix such that bonds are made with substrate molecules rather than between intrahelical sidechains [119]. In selection experiments with *P. falciparum* lab cultivars, acquisition of resistance to QN, HF, and AM was conferred by PfCRT mutations within TM domains 1, 4, and 9 [101, 127], and a labeled CQ-derived probe bound the intravacuolar loop between TM domains 9 and 10 [128], suggesting that these regions impart substrate specificity; a requirement for charge-loss at position 76 for CQ resistance [118, 129] and the observation that mutations within TM domain 1 modulate the degree of CQ resistance and verapamil reversibility provide further proof of this segment's role in substrate recognition [130]. The appearance of a mutation in TM domain 6 (A220S) in most CQ-resistant strains may also reflect cooperation of this region in substrate recognition or transport [119].

Additional contributors to chloroquine resistance. Although characterization of Dd2 x HB3 progeny observed a strict bimodal response to CQ congruent with a single gene driving drug response, it appears increasingly likely that multiple genes modulate the phenotype. Polymorphisms seemingly associated with resistance to both CQ and QN are found in numerous transporters [110], and PfMDR1 augments CQ resistance in lab cultivars from certain geographic regions—probably reflecting distinct drug selection histories on the different continents [104, 131]. Sequence polymorphisms and altered gene expression both contribute to drug resistance [48, 81, 132]. *P. vivax* parasites develop CQ resistance independent of mutations in their *PFCRT* orthologue [27], and multiple pathways to the trait are also consistent with PfCRT's being an escape route from CQ pressure rather than CQ's direct target (heme anabolism).

Transcriptional changes in chloroquine resistance. Short-term CQ perturbation moderately alters parasites' gene expression [133]. A recent study investigated transcriptional changes among the Dd2 x HB3 progeny and between their parents in order to identify heritable variations in gene expression impacting CQ resistance [132]. RNA from 33 Dd2-HB3 recombinants and the Dd2 CQ-resistant parent was compared by microarray to control nucleic acid from the HB3 parent and gene expression profiles mapped to expression level quantitative trait loci (eQTL) segregating independently among the strains. eQTLs involved in local and long-range transcriptional regulation were present on every chromosome and contributed singly or in combination to gene expression changes generally between two- and fourfold. The preponderance of transcriptional change was due to inheritance from the Dd2 parent of two to three copies of a chromosome 5 14-gene amplicon flanking *PFMDR1*: this region formed a regulatory

hotspot governing expression of 269 genes. Several candidate proteins with putative DNA-binding motifs are encoded in the expanded locus, which appeared to effect both gene induction and repression [132]. The size of this amplicon is known to differ extensively among CQ-resistant isolates, which may influence the degree of transcriptional influence exerted by the locus [134].

PfMDR1 modulation of chloroquine resistance. Preliminary attempts to associate PfMDR1, the homologue of the mammalian multidrug-resistance protein Pgh-1, with CQ resistance were of mixed success [135-137]; however, the protein appears to modulate PfCRT-mediated CQ resistance context-dependently [104, 131]. While *PFMDR1* did not segregate with resistance in the original Dd2 x HB3 cross, certain SNPs in the gene, notably N86Y, in a region between TM domains 1 and 2 and exposed to the DV lumen, associate with the trait (Table 2) [82, 136]. Additionally, the gene amplifies in some CQ-resistant strains (including Dd2); nevertheless, in an examination of uncloned progeny of the Dd2 x HB3 cross subjected to CQ pressure, CQ selection led to recovery of strains possessing the Dd2 allele of *PFCRT* and a sole copy of *PFMDR1* [114], and in an allelic exchange study *PFMDR1* knockdown had no effect on CQ EC_{50} [84].

The protein, localized to the DV membrane, includes 12 transmembrane segments created by internal gene duplication [138]; the cytosolic orientation of PfMDR1's ATP-binding domains and characterization of its activity suggest a role in substrate sequestration within the DV [85, 86]. MDR proteins lack stringent specifity, instead transporting compounds with a range of structures and sizes. Little is known about PfMDR1's function *in vivo* and its role in the parasite has been inspected only as regards its function in drug sequestration.

Two recent studies of recombinant strains have informed *PFMDR1*'s involvement in CQ-resistance acquisition. While the genetic cross between Dd2 and HB3 was intended to elucidate the determinant(s) of resistance and therefore involved a CQ-resistant and a CQ-sensitive parent, a second cross, between the S American 7G8 and Ghanaian GB4 strains, sought to parse variations in the phenotype and was therefore conducted with two CQ-resistant strains of differing provenance and *PFCRT* allele. S-American and SE-Asian type CQ resistance (which has swept through Africa and is maintained in GB4) are demonstrably dissimilar: SE-Asian type mutant PfCRT appears to impose a high fitness cost and *falciparum* parasites in Malawi, Kenya, and China have lost the allele in the absence of CQ pressure [3, 139, 140], whereas S-American type resistance is extant 50 years after cessation of CQ usage and even increasing in prevalence [141, 142], possibly from AQ administered as ACT [3, 10]. This additional cross was therefore intended to explicate the rationale for enhanced fitness with inheritance of S-American type CQ resistance [104].

Although the first analysis of cloned Dd2 x HB3 progeny demonstrated narrow bimodal distribution of CQ EC_{50} values suggestive of unifactorial resistance, this likely stemmed from the low-resolution approach taken in EC_{50} determination (microscopic smear counting) [114]. More quantitative reanalysis of these strains detected a range of EC_{50}s defining distinct CQ-resistant and CQ-sensitive populations: although drug resistance or sensitivity was governed by *PFCRT*, the most CQ-resistant strains consistently exhibited HB3-type *PFMDR1*, which departs from Dd2 sequence at three positions (Table 2). CQ-resistant strains with the Dd2 allele of *PFCRT* and either two or three copies of Dd2-type *PFMDR1* (single-copy Dd2 *PFMDR1* was not seen among the

progeny) were less CQ-resistant than parasites inheriting HB3 *PFMDR1*, though copy number variations in Dd2 *PFMDR1* did not contribute significantly to resistance. The Dd2-PfCRT/HB3-PfMDR1 combination was approximately 1.3-fold more CQ-resistant than the Dd2-Dd2, and 1.5-fold more AQ-resistant; these levels were statistically significant. *PFMDR1* sequence therefore appears to modulate the level of CQ-resistance [104, 131].

PFMDR1 influences CQ resistance in 7G8 x GB4 recombinants as well (Table 2). While strains with 7G8 PfCRT were only moderately CQ-resistant compared to those with the GB4 allele, inheritance of GB4 PFMDR1 reduced their resistance by half; inheritance of 7G8 *PFMDR1* with GB4 *PFCRT* increased by 1.5-fold the level of CQ-resistance. Each parental strain possessed one copy of *PFMDR1*, confirming that specific polymorphisms in, rather than expression level of, PfMDR1 can enhance CQ resistance though mutations in PfCRT determine phenotype [104]. Sequence changes in *PFMDR1* may reduce accumulation of CQ in the DV [85, 86] and/ or augment fitness in parasites with mutant *PFCRT* [143]. Co-selection of 7G8-type *PFMDR1* and *PFCRT* alleles is supported by additional evidence from an allelic exchange study introducing SNPs into *PFMDR1*; replacement of 7G8 *PFMDR1* with D10 sequence halved 7G8's CQ resistance, which was preserved when 7G8 sequence was replaced with cognate *PFMDR1* [81].

A requirement for additional factors? While interplay between PfCRT and PfMDR1 influences resistance level in multiple strain backgrounds, evidence is mounting for the imperative of a permissive backgound for introduction of CQ-resistant *PFCRT*. In a recent study several attempts to transfect CQ-sensitive and Dd2 or 7G8 CQ-resistant

PFCRT alleles into five CQ-sensitive strains originating on different continents recovered no Dd2-positive parasites, nor could 7G8 *PFCRT* be integrated into all strains. Moreover, introduction of 7G8 *PFCRT* into three different CQ-sensitive strains (3D7, GC03, and D10) resulted in significant variability in CQ resistance and verapamil-reversibility; interestingly, although CQ EC_{50} was only mildly altered with 7G8's introduction into the D10 CQ-sensitive background, resistance to the CQ catabolite mdCQ was considerably enhanced and EC_{90}s to both CQ and mdCQ augmented in all strain backgrounds expressing the 7G8 allele. Expression of 7G8 *PFCRT* also permitted sustained growth during (GC03) or eventual recrudescence (3D7, D10) after 6 days of growth in CQ at a concentration lethal to the CQ-sensitive parent strains [79]. GC03 is a recombinant strain derived from crossing Dd2 x HB3 and possesses the HB3 allele of *PFMDR1* (Table 2) [114, 115]; D10 and 3D7 encode wildtype *PFMDR1* [79]. These results, borne out by field evidence of CQ-sensitive PfCRT reintroduction [139, 140], strongly support the exaction of a fitness cost by CQ-resistant PfCRT and the necessity of co-selection at additional loci to augment parasite resistance or fitness. Higher glutathione levels and export of GSSG have been measured in the Dd2 CQ-resistant strain than in the 3D7 CQ-sensitive strain and may constitute an additional contribution to multidrug-resistance adaptation [123].

*Quinine***.** Quinine (QN), an alkaloid (structurally a 4-methanolquinoline) produced by the Cinchona tree native to the Amazon, was used as an antimalarial by the Inca. It was "discovered" by conquistadors and missionaries to the area—for a time it was known as Jesuit bark, reflecting this provenance—and exported to Europe to treat "Roman fever" from 1631 [144]. Its complete synthesis is extremely complex due to its 4 chiral centers

(and hence, 16 isomers) and was only solved in 2001 [145, 146]; the combined difficulties of its partial synthesis from intermediates and reduced access to Cinchona plantations in the Pacific during the Second World War were prime motivation in the testing of appropriate antimalarial drugs for campaigns throughout the Pacific and Asia that led to the adoption and widespread use of CQ. Prolonged prophylactic usage of quinine can result in neurotoxic effects, possibly through the drug's capacity for inhibition of tryptophan uptake [147].

Although it has been used for antimalarial prophylaxis and treatment for centuries in numerous geographical regions, only low-level resistance is reported and QN and its diastereomer quinidine remain in use to clear acute infections (usually in conjunction with another drug) [106]. QN resistance was reported only after commencement of prophylactic CQ use, and may have been facilitated by CQ-selected mutations in *PFCRT* [110]. CQ and QN have been reported antagonistic when co-administered; QN is thought to inhibit heme detoxification by binding monomeric heme (in contrast to CQ's binding β-hematin dimers) [108]. Although *P. falciparum* strains may exhibit resistance to either compound alone, CQ-sensitive strains often display low-level or no QN resistance, consistent with CQ selection contributing to QN resistance [110].

Quinine resistance. Potential contributions to QN resistance from multiple genomic loci have been investigated in the progeny of the Dd2 x HB3 genetic cross [143]. Although these strains were initially used to characterize CQ-resistance acquisition [114, 115], Dd2, with a QN EC_{50} approximately threefold greater than HB3's, possesses QN resistance comparable to many field isolates'. Principal QN-resistance determinants were inheritance from the Dd2 parent of the chromosome 7 locus surrounding *PFCRT* and a

380-kb region on chromosome 13. The chromosome 5 region flanking *PFMDR1* also enhanced QN resistance, but surprisingly only when contributed by HB3 [143]. Polymorphisms in the HB3 allele of *PFMDR1* may diminish or abrogate substrate transport [85, 86], perhaps thereby reducing QN accumulation in the DV to facilitate parasite hemozoin production.

Among the 100 genes in the chromosome 13 fragment associating with QN resistance were a subunit of the DV H^+-ATPase (involved in acidification of the organelle) and a Na^+/H^+ exchanger (*PFNHE1*). Both gene products might alter DV electrochemical potential and consequently drug partitioning in this compartment. No SNPs identified in either gene segregated with resistance, but asparagine- and aspartate-rich repeat regions within PfNHE1 correlated with enhanced resistance in Dd2 and field isolates from around the world. Co-inheritance of Dd2-type loci on chromosomes 9 and 13 and 6 and 7 increased the QN resistance of the offspring; strains with the HB3-type QN-sensitive locus on chromosome 6 were slow-growing, suggesting that co-selection of a region of chromosome 6 and *PFCRT* in the Dd2 backgound is advantageous to fitness [143]. A subsequent study on the same parasite strains associated higher cytosolic pH concomitant with PfNHE1 activity, or enhanced pH gradient between DV and cytosol, with increased QN resistance. High-level resistance was predominantly found in strains possessing Dd2-type polymorphisms on chromosomes 9 and 13; contributions from chromosome 6 and 7 regions or from *PFMDR1* appeared to operate independently [148]. *PFNHE1* knockdown also reduces QN resistance [144].

PfCRT's contribution to QN resistance, noted above, has been confirmed in several additional studies, but is not dependent on simple charge-loss at position 76 [106,

118, 129]; in a strain selected for CQ-resistance, mutation to 76I conferred QN hypersensitivity reversible by charge restoration in TM domain 1 or 9 [127]. While SE-Asian type PfCRT clearly drives CQ resistance in Dd2 x HB3 recombinants, these same strains exhibit graded response to QN [114, 115, 143]; inheritance of Dd2-type PfCRT defines the degree of verapamil chemosensitization to QN rather than of QN resistance [143]. Direct transport of QN by PfCRT remains unproven, although the drug can inhibit CQ export by PfCRT in a heterologous system [121]; alterations in pH homeostasis might rather promote its passive efflux from the vacuole [148].

Additional work on PfCRT's role in QN resistance found that introduction of the Dd2-type CQ-resistant and QN-resistant *PFCRT* allele into the moderately QN-resistant and CQ-sensitive GC03 strain reduced QN resistance, while control GC03 parasites transfected with their cognate allele maintained mild QN resistance [149]; GC03 is a CQ-sensitive strain derived from the Dd2-HB3 cross. This result is consistent with QN resistance necessitating co-selection of Dd2-type loci on chromosomes 6 and 7 [143]. In the absence of a fitness-enhancing region on chromosome 6, Dd2-type PfCRT may exact a fitness cost on the GC03 background; the presence of HB3-type PfMDR1 in GC03 may slightly increase QN resistance in that strain [85, 86]. Parasite multidrug resistance therefore appears contingent on optimized interactions among resistance-determining loci promoting both fitness and drug detoxification. The genetic factors involved will depend a strain's history: for instance, comparison of sequence polymorphisms in transporter genes from 97 strains found co-selection of *PFCRT* and *PFMDR1* in QN resistance for African and S- American strains but not SE Asian. Interestingly, African and S American

QN resistance was promoted by selection of unrelated *PFMDR1* mutations (N86Y in African parasites, and S1034C in S American) [110].

Indeed, the sometimes antagonistic resistance profiles of strains to MFQ and QN may provide a reason for the apparent lack of contribution of *PFMDR1* to Asian-type quinine resistance [110, 143]; but other studies associate sequence polymorphisms or amplification in the gene with resistance to both drugs [81, 83, 84]. A full understanding of QN-resistance acquisition, its possible genetic determinants in different malaria-endemic regions, and the physiological basis of resistance is clearly lacking. As was originally believed for CQ resistance, a change in DV or cytosolic homeostasis may reduce the favorability of QN retention in that compartment [148] or a protein or proteins may cooperate to export QN from its site of action [127, 129]. Recent research suggests that *PFCRT*, *PFMDR1*, and *PFNHE1* all have low predictive power in determining QN resistance, confirming the truly multifactorial nature of the trait and suggesting that the phenotype might arise through several as yet uncharacterized pathways [150, 151]. QN is also likely to be mono- rather than di-protonated at DV pH, such that direct transport of the protonated compound might be unnecessary for resistance acquisition in the event of homeostatic alterations.

Diversity in Antimalarial Resistance Genotypes

Within the last sixty to seventy years an arsenal of chemotherapeutics has been deployed against parasites, with widespread antimalarial usage effecting selective sweeps of resistance determinants. Study of field strains collected in areas with differing historical antimalarial treatment policy as well as progeny of genetic crosses performed in the laboratory can elucidate factors involved in establishment of multidrug resistance,

both indirectly, in fitness enhancement, and as direct mediators of drug transport or sequestration.

In an effort to identify selection signatures in parasite populations, 189 strains from Melanesia, SE Asia, S America, and Africa were investigated for polymorphism and found to possess strong inter-population differences, clustering by regional or continental origin [78]. Highly recombinogenic loci, including that surrounding *PFCRT*, were conserved across isolates of disparate provenance; although *PFCRT* itself was stable, enhanced genetic variability could be seen within several hundred kb due to the rapid recent spread of CQ-resistant alleles. African parasites exhibited more sequence diversity at *PFCRT* than other populations (possibly due to higher recombination rates). Other areas under positive selection included that containing *PFAMA-1*, a parasite adhesin involved in erythrocyte binding during invasion and likely under strong host selection, and a locus on chromosome 13 in the neighborhood of a putative QN-resistance determinant [110]. In addition, African and Asian populations strongly diverged at *PFMDR1*, perhaps indicating the effects of region-specific differences in antimalarial chemotherapy [78].

185 of the 189 strains were examined for drug-resistance phenotypes associating with specific genotypes. Isolates both resistant and sensitive to CQ and S/P were detected; all strains were sensitive to dihydroartemisinin (DHA), although African and Cambodian parasites were relatively more resistant to the compound. Positive correlations were noted for DHA and MFQ EC_{50}s, implying a common genetic response to the compounds (perhaps mutation at *PFMDR1*) [78].

Regionally dissimilar drug-resistance phenotypes are heavily dependent on antimalarial treatment policy and parasite endemicity and transmission. While incomplete fixation of CQ resistance owes something to loss of the SE-Asian *PFCRT* allele (which detracts from parasite fitness) after cessation of CQ pressure [3, 139, 140], variability seen in S/P resistance profiles is likely due to the stepwise nature of S/P resistance acquisition, which has led to selective sweeps for some DHFR/ DHPS variants, with others arising independently numerous times [89, 91]. Constraints on multidrug-resistance genotypes are also imposed by drugs' target compartments within the cell; for instance, *PFMDR1* deamplification (which transports solute into the DV) occurs under 4-aminoquinoline selection, but locus expansion is often seen after MFQ and HF application [94, 96, 152]. MFQ and DHA resistance (r^2=0.78) [78] are frequently correlated, suggesting that sequestration in the DV might be an adaptive response to each compound [78, 81, 83]. In contrast, resistance to AQ and CQ arises from mutations in the same gene (*PFCRT*), and presumably through similar mechanisms, but only a weak positive correlation was observed (r^2=0.52) [78]. This may reflect low coincidence of drug application or necessity of specialized polymorphisms in *PFCRT* for resistance to each drug; AQ is credited with selection of the SVMNT S-American type PfCRT isoform, which confers a lower level of CQ-resistance than the SE-Asian CVIET mutations resulting from CQ exposure (Table 1) [104]. The AQ-resistant *PFCRT* allele is stable in the absence of 4-aminoquinoline pressure and is of increasing importance due to AQ's inclusion in ACT [10].

Characterization of drug-resistance factors

PfMDR1. A number of studies have attempted to define associations between polymorphisms in *PFMDR1*, an ATP-binding cassette (ABC) family member homologous to the mammalian multidrug resistance transporter Pgh-1 [138], and resistance to numerous antimalarial drugs, including both quinolines and ART derivatives. Five polymorphisms are found in drug-resistant *P. falciparum* strains (Table 2), at positions 86, 184, 1034, 1042, and 1246 [136]; amplification is sometimes observed during selection of antimalarial resistance [94, 96, 152], and expression level knockdown increases sensitivity to drug [84]. Interestingly, amino acids 1034 and 1042 are both within TM domain 11, which forms an amphipathic helix with amino acid sidechains oriented toward the hydrophilic center; position 1246 is within the protein's second nucleotide binding domain [85].

Both allelic exchange and study of recombinant progeny of a genetic cross have been used to characterize the gene. When D10 and 7G8 *PFMDR1* were replaced with each other's, as well as their own, cognate, allele (NYSND and NDCDY, respectively; see Table 2), QN resistance was increased but parasites sensitized to MFQ and HF congruent with expression of 7G8-*PFMDR1*; expression of D10-*PFMDR1* in the 7G8 background also reduced CQ resistance in that strain in the absence of changes to *PFCRT* [81]. These findings are consistent with a role for S-American type *PFMDR1* in modulating CQ resistance [104], and imply functional differences in substrate transport among differing forms of PfMDR1. Such differences have been borne out by examination of 3D7 x HB3 cross progeny (Table 2) [82] as well as strains engineered to

express *PFMDR1* alleles with varying SNPs [83], in which the N1042D mutation reduces QN sensitivity in parallel with increased MFQ and HF sensitivity.

Model substrate transport. The first attempt at characterizing PfMDR1's ability to transport antimalarial drugs was published in 1994 and involved the expression of both mutant (S1034C/ N1042D) and wildtype versions of the gene in CHO cells, with mutant protein lessening accumulation of CQ [153]. Two more recent studies have also explored the modulation by sequence polymorphisms of PfMDR1's transport capacity. First, Fluo-4, which fluoresces when cleaved by an esterase, was used as a PfMDR1 substrate and its import into the DVs of strains with differing antimalarial-drug resistance profiles (and *PFMDR1* SNPs) assayed. While Dd2 (YYSND) could transport Fluo-4, HB3 and 7G8 (NFSDD and NFCDY, respectively) could not, suggesting that N1042D impeded substrate recognition or transport; these results were confirmed in several strains created by allelic exchange. Fluo-4 transport by PfMDR1 was also investigated in sixteen progeny of the Dd2 x HB3 cross; fluorescence was only found in strains maintaining Dd2 PfMDR1 and correlated with protein expression level (Table 2) [85]. Interestingly, the Dd2-type *PFMDR1* allele was initially associated with CQ resistance [136]; possibly Y86 prevents CQ binding and transport but not that of other substrates.

Expression in *Xenopus*. In a separate study, the contribution of specific amino acids to drug transport by PfMDR1 was investigated in the context of its heterologous expression in *X. laevis* oocytes (Table 2) [86]. 7G8, Dd2, HB3, and D10 alleles were microinjected into oocytes and found to orient with their ATP-binding domains in the cytoplasm (such that transport was outward-directed). Accumulation of substrates was similar in the presence and absence of PfMDR1; all PfMDR1 variants could transport the known Pgh-1

substrate vinblastine in the absence of inhibition, and QN as well as a Pgh-1 inhibitor competed with vinblastine for export, suggesting a common binding site in the protein. The capacity of the different PfMDR1 isoforms for antimalarial transport was also examined: while the isoform from the CQ-sensitive strain D10 transported CQ, none of the other variants was able to, including that from CQ-sensitive HB3 (Table 2) [86]; this is consistent with HB3-PfMDR1's observed modulation of drug-resistance phenotypes [104, 131, 143]. Likewise only D10-PfMDR1 transported QN, to which its parent strain is sensitive. Presumably the N1042D mutation in 7G8 and HB3 versions of PfMDR1 precluded their transport of CQ and QN (although each could transport HF); N86Y in Dd2 might prevent its recognition of CQ and QN, though it too transports HF [86].

Expression in yeast. Expression of *PFMDR1* in *Pichia pastoris* has also shed some light on function. Recoded protein was found to localize to the PM; substrate transport was dependent on Mg^{2+} and ATP and inhibited by concanamycin, as expected for an ABC transporter. Different isoforms of the protein, while comparably expressed, appeared to exhibit dissimilar levels of ATP hydrolysis for a model substrate, with Dd2 (YYSND) of enhanced, and 7G8 (NFCDY) of reduced, activity compared to 3D7 (NYSND). ATPase activity was induced by mefloquine (very slightly) or by quinine, while a significant reduction was seen for all isoforms in the presence of CQ. This suggests that sequence polymorphisms modulate PfMDR1's transport capacity and that prior selections with multiple antimalarials determine whether an allele in a given strain is more competent to sequester substrate within the DV or to exhibit reduced ATPase activity, as would be expected to augment resistance to a compound effective against DV processes [154].

PfCRT. Even after a decade of concerted effort to determine PfCRT's native function, the path it takes to drug resistance, and the manner in which the parasite reconciles these opposing objectives, we lack fundamental insights into these processes. Ultimately both a crystal structure and extensive information regarding the constraints imposed on PfCRT's mutation will be essential to effective drug design for malaria control. In the meantime, protein expression in numerous heterologous systems has brought us closer to an objective understanding of PfCRT's contribution to 4-aminoquinoline resistance.

Expression in *Dictyostelium*. SE-Asian type and wildtype PFCRT were transfected into *Dictyostelium discoideum*, along with a revertant allele containing compensatory mutations but maintaining K76 and a gain-of-function allele with K76T in otherwise wildtype sequence (Table 1). *Dictyostelium* encodes three CRT orthologues, and expression of the *P. falciparum* native sequence of the gene was feasible in this system (without extensive recoding) due to comparably high AT content. *PFCRT* was expressed and found to localize to acidic vesicles in the cytoplasm contingent on replacement of the N-terminal region (anterior to the first TM domain) with sequence from an endogenous CRT. While CQ accumulation was similar for all transfectant strains at 4 °C, at 20 °C both CQ-resistant and gain-of-function alleles demonstrated reduced accumulation of CQ; the gain-of-function isoform extruded 20-40% less CQ than the true CQ-resistant variant. CQ resistance was verapamil reversible. PfCRT's interaction with QN and its diastereomer QD was also ascertained; these phenotypes proved to be more complex, as untransfected cells did not accumulate either drug, in contrast to cells transfected with the wildtype *PFCRT* allele. Although expression of K76I PfCRT in *P. falciparum* confers QN hypersensitivity [118, 127, 129], this variant was only moderately less competent to

transport the drug than SE-Asian type PfCRT; only SE-Asian type PfCRT could transport QD, which likewise did not accumulate within untransfected cells [155].

Validation of *Dictyostelium* as a model system for PfCRT study is presented by the observed absence of QN hypersensitivity with K76I-PfCRT expression, which may indicate that QN actually inhibits this isoform's native function; indeed, although CQ and QN are normally antagonistic in CQ-resistant strains, in the K76I-PfCRT strain they are strongly synergistic, perhaps through QN-mediated occlusion of PfCRT's binding site both for its native substrate and for CQ (see figure 5 in [127]). Verapamil uncharacteristically increases K76I's resistance to QN (but not to CQ) [129], likely interacting with the substrate-binding site in such a way as to restore transport of the native substrate. Likewise, restoration of positive charge in TM domains 1 or 9 augments QN resistance [127], perhaps by reducing favorability of interaction between PfCRT and QN to permit efficacious substrate transport by the former; QN removal from the DV could be effected by PfCRT or another factor.

Expression in *Xenopus*. Two independent studies have expressed *PFCRT* in *Xenopus laevis* oocytes. An initial study expressed the CQ-sensitive HB3 allele by microinjection and used electrophysiology to compare transfected oocytes with untransfected controls. HB3 production resulted in higher resting pH and lower membrane potential; the PfCRT-dependent effect on pH could be reversed by oocyte incubation with Na^+/H^+-exchanger inhibitors and was attributed to PfCRT activation of endogenous *X. laevis* transporters. Little could be inferred about PfCRT function in the absence of CQ-resistant PfCRT expression, and protein localized to multiple regions of the cell, perhaps confounding interpretation [156].

Work in *Xenopus* by another group has provided great insight into PfCRT's native role and that in drug resistance. CQ-sensitive, CQ-resistant, CQ-resistant revertant (K76 with compensatory mutations), CQ-sensitive gain-of-function, and CQ-resistant loss-of-function (T76K/S163R) alleles of *PFCRT* were codon-optimized and –harmonized for expression in *Xenopus* and putative endosomal-/ lysosomal-targeting motifs removed (Table 1); protein localized exclusively to the oocyte plasma membrane. CQ-resistant variants were found to transport up to tenfold more protonated CQ into the interior of the oocyte than CQ-sensitive in the absence of alterations to membrane potential or cytosolic pH; CQ-resistant loss-of-function alleles with positive charge restored in TM domain 1 or 4 (T76K, S163R) lost CQ transport capacity. In addition, the presence of the K76T mutation on an otherwise wildtype background did not permit CQ transport, suggesting that compensatory mutations contribute to the CQ-resistance phenotype.

CQ transport by PfCRT was dependent on pH and maintenance of oocyte membrane potential and increased with extracellular acidity, but was inhibited by the chemosensitizer verapamil, quinine, amodiaquine, and unlabelled CQ. Peptides (PfCRT's likely native substrate) were also examined for their ability to inhibit PfCRT-mediated CQ transport; while some were shown to reduce CQ transport by CQ-resistant PfCRT dose-dependently, peptide transport itself was not probed, so that although significant evidence now supports the direct movement of CQ by resistant variants of PfCRT we still cannot with certainty name PfCRT's native substrate [121].

Expression in yeast. Extensively recoded CQ-resistant and CQ-sensitive alleles of PfCRT have, in addition, been expressed both in *Pichia pastoris* and in *Saccharomyces cerevisiae*, where they localized to the plasma membrane. Preparation of inside-out

vesicles from *Pichia* membranes loaded with PfCRT showed that while acridine orange fluorescence remained high for control or HB3-expressing vesicles, those containing Dd2 lost fluorescence. This result was interpreted as reflecting acidification of the Dd2-loaded vesicles, possibly by some form of cross-talk between the yeast plasma-membrane ATPase (Pma1p), which was found to be incorporated into the vesicles, and PfCRT. Pma1p should transport protons into the vesicular lumen in this scenario, while PfCRT should rather export substrate from the vesicles. It is possible (perhaps more probable) that loss of fluorescence was due to Dd2-mediated export of protonated acridine orange, which is similar in structure to quinoline antimalarials; the effect was inhibited by verapamil, consistent with native PfCRT-mediated CQ transport [157].

Further work by this group essayed to quantify CQ binding to *Pichia* membranes containing PfCRT. Although a slight difference in affinity was noted, favoring the association of CQ with Dd2-PfCRT over that with HB3-type, the measurement was of CQ's interaction with total protein in the membrane fraction, and therefore these results and their physiological implications are difficult to interpret. QN and CQ transport in inside-out vesicles loaded with CQ-resistant or CQ-sensitive PfCRT or control vesicles was also assessed, but results were inconclusive [158].

One additional study was performed in *Pichia*, this one expressing independently recoded K76, K76T, and K76I alleles of PfCRT reconstituted into proteoliposomes. In making proteoliposomes, it is impossible to control protein topology, but uptake of CQ into liposomes loaded with each PfCRT allele was measured and was found to be greater for CQ-resistant PfCRT. Addition of a protonophore abrogated CQ retention [159].

Unfortunately, internal and external pH are not recorded, but this work implies functional differences in transport capacity for CQ-sensitive and CQ-resistant PfCRT variants.

PvCRT. CQ resistance is increasing in *P. vivax* malaria in the absence of mutations in *PVCRT-o* (orthologous to *PFCRT*). Determination of the sequence for several resistant and sensitive isolates found nonsynonymous mutations that did not associate strongly with resistance [75]. In a more recent comparison of isolates from Thailand, where sensitivity predominates, to those from heavily resistant Indonesia, a codon-sized insertion in Thai samples appeared to associate with CQ sensitivity, but ultimately this mutation was unlinked to sequence changes in *PVMDR1* or to CQ resistance [27]. This has led researchers to surmise that CQ resistance might arise either independent of PvCRT or via changes in its expression level rather than sequence; the latter model was tested by transfection of *PVCRT* into *P. falciparum* parasites and into *Dictyostelium* [160].

PVCRT-o was integrated into CQ-sensitive 3D7 *P. falciparum* parasites at the *HSP86* locus in tandem in each of two transfections (with promoters of differing strengths); negative-control transfectants expressed luciferase. Transcription of host *PFCRT* was shown to decrease with increasing expression of *PVCRT-o*, but western blotting and immunofluorescence localized both gene products to the DV membrane. CQ resistance was conferred dose-dependently by expression of *PVCRT-o*, such that gene expression from the weaker promoter led to a subtler rise than that from the stronger, which enhanced resistance more than twofold. In conjunction, *PVCRT-o* (wildtype and K76T, which is not observed in CQ-resistant *P. vivax* parasites) and *PFCRT* (CQ-resistant and CQ-sensitive alleles) were transfected into *Dictyostelium discoideum*, where

expression of either allele of *PVCRT-o* was found to reduce CQ accumulation in acidic vesicles to approximately the same extent as Dd2 CQ-resistant PfCRT, while HB3 CQ-sensitive PfCRT retained the compound [160]. These studies are perhaps indicative of enhanced functionality of wildtype *PVCRT-o* over that of *PFCRT*, and further investigation into *P. vivax*'s evolution of CQ resistance is clearly warranted. Questions have arisen as to CQ's mode of action in *P. vivax*, since the DV acidifies and begins hemoglobin catabolism later in the *P. vivax* IDC than is observed in *P. falciparum* parasites [43].

Antimalarials in clinical development

A number of novel antimalarial formulations are currently under review at different stages of clinical or pre-clinical testing, including new ART partner drugs. Eurartesim and Pyramax, containing ART derivatives and piperaquine or pyronaridine, respectively, are in registration prior to distribution [99] (more information at http://www.mmv.org/research-development/science-portfolio). These drugs are quinoline analogues developed in China and each retains potency against multidrug-resistant parasites [4, 99]. Additionally, CQ in combination with azithromycin is under investigation for antimalarial chemotherapy of expectant mothers [4, 99], and a synthetic ozonide has entered Phase III clinical trials in co-formulation with piperaquine [4, 99, 161]. New 4-aminoquinoline derivatives such as ferroquine (Phase II trials), isoquine, and AQ-13 (both Phase I) are also being explored, as is the 8-aminoquinoline tafenoquine (Phase I), which, with faster hypnozoite clearance and lessened hemolytic activity relative to primaquine, is intended as its replacement [4, 76, 99].

Current strategies in drug design

Recent work to expand our armamentarium of antimalarials has focused on redesigning compounds directed against known target proteins, identification of novel chemical scaffolds by mining of large libraries, and chemical genomics approaches. Although more work is necessary to provide efficacious alternatives to the current repertoire of available drugs, these efforts have identified new drug chemotypes and a possible family of parasite targets.

Heme-binding antimalarials. Parasite-encoded drug targets are impelled to mutate under selective pressure, and in the case of antimalarials such S/P, single substitutions can impart some measure of resistance, engendering rapid loss of efficacy. In contrast, quinoline antimalarials impact a process believed to occur passively at the physiological pH of the DV. Because heme and the β-hematin dimer sequestered into hemozoin are host-derived and hemoglobin constitutes an essential nutrient supply for the parasite, it can neither cease hemoglobin ingestion nor mutate heme detoxification to escape drug treatment. *PFCRT* mutation represents one of several escape routes from quinoline stress [104, 131, 143], as is evidenced by the alternate pathways to CQ resistance adopted by other Plasmodia [3, 27, 160].

Alterations to CQ's sidechain allow easy bypass of resistance, validating β-hematin crystallization as worthy of further study [111-113]. This was confirmed by recent work designing acridone-based heme-binding antimalarial drugs including inherent chemosensitization activity; these drugs can be used in parallel with quinolines. One such compound, 3-chloro-6-(2-diethylaminoethoxy)-10-(2-diethylaminoethyl)-acridone (T3.5), exhibited good antimalarial activity against CQ-resistant and CQ-

sensitive parasites and was in addition synergistic with quinolines such as CQ, AQ, and QN against Dd2 and 7G8 CQ-resistant parasites, although its activity antagonized MFQ. In combination with CQ, it proved additive against D6 CQ-sensitive *P. falciparum* parasites, perhaps due to a lack of chemosensitization in those strains. Furthermore, its ability to chemosensitize parasites to QN and CQ was similar in both S-American type (7G8) and SE-Asian type (Dd2) parasites, in contrast to verapamil's lower effectiveness against S-American type mutant PfCRT [77, 130].

While verapamil also displays varying interactions with PfCRT position 76 charge-loss mutants, T3.5 was able to potentiate response to QN and CQ in congenic strains with wildtype K76 PfCRT sequence or K76T, K76N, or K76I substitutions. Remarkably, while verapamil in fact enhanced the apparent resistance of the K76I strain to QN [77, 129], T3.5 reduced it fourfold. *In vitro*, T3.5 reduced β-hematin incorporation into heme to $1/10^{th}$ to $1/4^{th}$ of control levels in D6 CQ-sensitive and Dd2 CQ-resistant parasites, respectively [77]. Acridone-derived drugs therefore may present viable alternatives or supplements to the 4-aminoquinoline drugs to which resistance has arisen in much of the world, as they chemosensitize resistant parasite strains to these drugs as well as possessing considerable antimalarial activity in and of themselves.

Large-scale screens. Additional work in novel drug discovery has focused on large-scale screening efforts, which can identify both new classes of drug targets and new scaffolds active against those in existence. GlaxoSmithKline's 2-million compound library has been screened for antimalarial efficacy against 3D7 CQ-sensitive and Dd2 CQ-resistant parasites, with approximately 8000 compounds inhibiting replication in both strains, including members of most classes of antimalarials currently in use. Protein kinase

inhibitors, which have not been utilized against malaria parasites, comprised a significant proportion of previously uncharacterized inhibitors [162]. Further, a library of nearly 400,000 chemicals was examined for activity against the CQ-sensitive 3D7 and CQ-resistant K1 *P. falciparum* strains; more than 500 were effective against both parasite strains but demonstrated low toxicity toward mammalian cell lines. Compounds with synergy toward current classes of antimalarials were identified within this set, and nearly 60 displayed similar activity against multiple strains with widely divergent drug resistance phenotypes [163]. A separate screen of 12000 compounds categorized spiroindolones as growth-inhibitory against drug-sensitive and –resistant *P. falciparum* strains at low nanomolar levels, as well as against *P. vivax* parasites; the lead compound in the series had minimal toxicity in rats and could easily clear *P. berghei* parasites in a murine model. Resistance was selected to the compound over a four-month period of drug application to the multidrug-resistant Dd2 *P. falciparum* laboratory strain and was demonstrated to occur by mutation and/or amplification of *PFATP4*, a gene with homology to the *Saccharomyces PMR1* Ca^{2+}/Mn^{2+} transporter. Resistance to antimalarials with disparate mechanisms of action, such as mefloquine or artemisin, did not increase in spiroindolone-resistant strains [164, 165], suggesting that this class of chemotherapeutics could be adapted to combination therapy. These efforts have pinpointed new chemical structures that may provide starting-points for synthesis of novel antimalarials.

Chemical genomics. Chemical genomics has also been used to isolate potential new antimalarial compounds and their cellular targets. The LOPAC collection of 1300 confirmed bioactive drugs was screened against CQ-resistant (GB4, Dd2, W2, and 7G8)

and CQ-sensitive (D10, HB3, 3D7) *P. falciparum* strains, to detect 155 compounds potent against all strains, 25 of which inhibited growth below 2 μM. Among this subset were known antimalarials, including quinine and quinacrine (which has weak antimalarial activity).

Four of the assayed strains are parents of genetic crosses (3D7 x HB3, Dd2 x HB3, and GB4 x 7G8). Compounds with pairwise differences in growth inhibition of fivefold or more between parents of a cross were used to identify genetic loci segregating with resistance. GB4 was tenfold less sensitive to a serotonin receptor antagonist (dihydroergotamine methanesulfonate) than 7G8; an examination of 32 recombinant GB4-7G8 progeny implicated two loci in resistance, one surrounding *PFMDR1* on chromosome 5 (comprising 34 genes), and a second, less strongly associated, on chromosome 12. PfMDR1's effect on resistance to the compound was determined in allelic-exchange strains expressing *PFMDR1* variants on isogenic backgrounds; 7G8-type PfMDR1 or mutation in residues 1034, 1042, or 1246 reduced parasite sensitivity to dihydroergotamine methanesulfonate [166]. This compound may exert its antimalarial effect in the cytoplasm, as the mutations described in 7G8 have been suggested to reduce *PFMDR1* transport capacity [85, 86]. Pairwise differences in response to an antifolate were also identified and found to correlate with inheritance of the genetic region surrounding *PFDHFR* [166]. This approach represents an efficacious means to identifying the gene product(s) impacted by antimalarial treatment and may provide a system for evaluating proposed combination therapies.

Conclusions

Malaria continues to present many challenges. Despite a century and more of study, identification and functional characterization of resistance determinants is still incomplete, and reduced efficacy of most antimalarial chemotherapeutics exacts a severe toll from human health and life. Reports of reduced disease incidence or even elimination in the field, and the influx of funding into treatment and prevention initiatives under the aegis of the WHO and with support from the Bill and Melinda Gates Foundation and the Medicines for Malaria Venture, inspire the hope that the spectre of disease can be alleviated, ending the thrall in which we and our ancestors have lived for millions of years.

Nevertheless, disease extirpation will require intense and concerted effort from governmental, non-governmental, academic, and industrial organizations—in the design of novel, efficacious antimalarials, with activity against both blood and gametocyte stages (as well as *P. vivax* and *P. ovale* hypnozoites); in development of effective and safe new insecticides for IRS and bed-net impregnation, and creation of vector-control strategies tailored to account for differences in vector feeding and resting behavior; and in renewed attempts to produce vaccines protective against blood-stage parasites and/ or obstructing genesis of vector stages. Moreover, adequate infrastructure is necessary to ensure appropriate and timely delivery of reagents, and for strict surveillance regarding implementation of, and vector or parasite resistance acquisition to, control measures, as well as in prevention of counterfeiting. The task is herculean, but will bring untold benefits to those at risk of disease.

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	Amino acid identity at polymorphic positions									
Strain	72	74	75	76	163	220	271	326	356	371
106/1	C	I	E	K	S	S	E	S	I	I
3D7	C	M	N	K	S	A	Q	N	I	R
D10	C	M	N	K	S	A	Q	N	I	R
HB3	C	M	N	K	S	A	Q	N	I	R
CQS_K76T	C	M	N	T	S	A	Q	N	I	R
CQR_S163R	C	I	E	T	R	S	E	S	T	I
CQR_T76K	C	I	E	K	S	S	E	S	T	I
GB4	C	I	E	T	S	S	E	S	T	I
Dd2	C	I	E	T	S	S	E	S	T	I
7G8	S	M	N	T	S	S	Q	D	L	R
K1H6/2	C	I	E	T	S	S	E	S	I	I

Table 1. Nonsynonymous polymorphisms resulting in amino acid changes in the PfCRT chloroquine resistance transporter. Lab cultivars 3D7, D10, and HB3 have fully wildtype sequence and remain chloroquine-sensitive; however, 106/1 possesses all compensatory mutations characteristic of a chloroquine-resistant variant but the necessary K76T mutation, yet remains entirely sensitive to drug (Fidock et al, 2000). GB4 (a West African strain) and Dd2 (a SE Asian strain) have identical polymorphisms, consistent with the sweep of chloroquine resistance from SE Asia. Resistance arose independently in S America, possibly due to prior selection with the related 4-aminoquinoline amodiaquine, and thus 7G8's complement of polymorphisms is slightly different. CQS_K76T, CQR_S163R, and CQS_T76K could not transport chloroquine upon their heterologous expression in *X. laevis* (Martin et al, 2009).

Strain	Amino acid identity at polymorphic positions				
	86	184	1034	1042	1246
106/1	?	?	S	N	D
3D7	N	Y	S	N	D
D6	N	Y	S	N	D
D10	N	Y	S	N	D
HB3	N	F	S	D	D
K1	Y	Y	S	N	D
Dd2	Y	Y	S	N	D
GB4	Y	F	S	N	D
7G8	N	F	C	D	Y

Table 2. Nonsynonymous polymorphisms resulting in amino acid changes in the PfMDR1 multidrug resistance protein. Lab cultivars 3D7, D6, and D10 are considered wildtype and maintain functional protein. The HB3 N1042D mutation is believed to reduce its capacity for substrate transport, as are the S1034C, N1042D, and D1246Y mutations in 7G8 (Sidhu et al, 2005; Rohrbach et al, 2006). The N86Y mutation found in K1, Dd2, and GB4 is often associated with reduced chloroquine sensitivity (Foote et al, 1990).

Chapter 2: Chemical-genetic and transcriptional profiling of yeast response to the antimalarial drug chloroquine

Abstract

Pathogenic resistance to chemotherapeutics can be conferred by point polymorphisms in numerous transporters and/ or biosynthetic enzymes. In the parasite *Plasmodium falciparum*, causative agent of the deadliest form of human malaria, resistance to the former frontline antimalarial chloroquine arises from selection of mutations in the *P. falciparum* chloroquine resistance transporter (*PFCRT*), but other *Plasmodium* parasites exhibit independent modes of chloroquine-resistance acquisition. *Plasmodium*'s genetic intractability precludes comprehensive *in vivo* examination of the mechanics of chloroquine resistance. We have expressed *PFCRT* in *S. cerevisiae* for investigation of the genetic pathways implicated in chloroquine resistance via synthetic genetic analysis in the presence and absence of chloroquine and PfCRT; we have also investigated yeast's short-term transcriptional response to chloroquine treatment. We find that chloroquine impairs yeast vacuolar homeostasis, protein trafficking, and ability to determine nutritional repletion, and that expression of chloroquine-resistant *PFCRT* can partially suppress these defects.

Introduction

Malaria is a parasitic, vector-borne disease responsible for approximately half a billion incidents and a million deaths annually; any of five *Plasmodium* species can infect humans [1, 2]. Sub-Saharan Africa is most significantly impacted, with malaria reaching hyperendemicity in many regions with great detriment to health and economic development [3-5]. Disease symptoms and pathology stem from the asexual stages, during which parasites synchronously colonize red blood cells to undergo 48-hour cycles of differentiation and proliferation followed by egress and reinvasion [2, 3]. This intraerythrocytic developmental cycle (IDC) is the target of antimalarial chemotherapeutics. Although substantial inroads on disease containment have been made through public health initiatives, resistance has emerged to most available drugs and is near universal for chloroquine, formerly the frontline antimalarial of choice due to its ease of synthesis and low human toxicity and cost [4, 6].

Chloroquine is a 4-aminoquinoline and a lipophilic weak base, freely membrane-permeant when uncharged, but protonated and impermeant under acidic conditions. During the IDC *Plasmodium* parasites uptake and catabolize large quantities of host hemoglobin to obtain amino acids, a process that liberates large quantities of the reactive heme moiety [7]. Hemoglobin degradation occurs in the parasite's digestive vacuole (DV), an acidic hydrolytic compartment analogous to the mammalian lysosome, in which chloroquine can bioaccumulate to millimolar concentrations to interact with, and prevent detoxification of, heme to cause parasite death. Although heme remains a druggable antimalarial target and is the focus of continued research [8-10], chloroquine resistance

was discovered at several independent foci in SE Asia, Melanesia, and S America by the late 1950s and has spread worldwide [11].

While the mechanics of chloroquine resistance are not fully understood, the phenomenon has been extensively studied in *P. falciparum*, predominant cause of African malaria and most lethal to humans of the Plasmodia. In this parasite, trait acquisition is concomitant with selection of mutations in the DV-localized *P. falciparum* chloroquine resistance transporter (PfCRT), which loses a positive charge in chloroquine-resistant parasite isolates to incur the capacity for extrusion of charged chloroquine from the organelle [12]. Although it has been postulated that mutant PfCRT transports protons, favoring chloroquine's passive leak from the compartment, it now appears likely that charged chloroquine is a substrate for resistant PfCRT isoforms [13-16]. Several lines of evidence support direct interaction of drug with PfCRT, including (i) bypass of resistance by chloroquine derivatives [9, 10, 17, 18], (ii) chemosensitization to drug by verapamil contingent on inheritance of the chloroquine-resistant *PFCRT* genetic locus [19], (iii) characteristic patterns of cross-resistance observed to structurally related compounds [20, 21], and (iv) loss of resistance accompanied by sequence alterations in *PFCRT* after selection by antimalarials [22-24].

Orthologues of *PFCRT* are found in *Plasmodium* species, *Dictyostelium*, and *Arabidopsis*, but none has been crystallized and the gene belongs to a novel family [25-27]. It appears essential in *Plasmodium* parasites, where it is believed to shuttle small hemoglobin-derived peptides to the cytoplasm using the energy of the DV's H^+ gradient [28]. While mutations in PfCRT confer resistance to multiple quinoline antimalarial chemotherapeutics in addition to chloroquine, recent evidence suggests modulation of

this resistance both by sequence polymorphisms external to *PFCRT* and through changes in gene expression [12, 22-24, 29-31]. Moreover, both degree and stability of chloroquine resistance are variable and influenced by parasites' selection history and prior exposure to antimalarial drugs [21, 32], and chloroquine resistance is increasing in prevalence in *P. vivax*, another significant cause of human malaria, apparently in the absence of mutations in *PVCRT-o* [33].

Comprehensive analysis of PfCRT's role in multidrug resistance and the potential multifactorial nature of input to chloroquine resistance are each clearly warranted, and could inform design of new antimalarial chemotherapeutics and drug administration paradigms. However, current strategies for identifying multidrug-resistance determinants are limited by the parasite's genetic intractability (including the dearth of forward-genetic techniques and difficulty of reverse genetics), high complement of uncharacterized ORFs, and the unwieldy nature of culture-based antimalarial selections requiring starting populations of 10^{10} parasites or more [22, 23, 34]. *PFCRT*'s essentiality also hampers its study *in situ*. Largescale attempts to identify resistance factors have focused on genome-wide association studies among highly outbred field isolates from diverse geographic areas [21, 32, 35] or progeny of genetic crosses between lab cultivars [19, 29, 31]. In the former case chromosomal regions encoding dozens of genes segregate with resistance phenotypes; in the latter, significant contributions from known multidrug-resistance determinants, such as the *Plasmodium* homologue of Pgh-1, often obscure the roles of alternate loci. Moreover, these loci often enhance fitness of resistant strains rather than directly participating in resistance, such that there is no single resistance strategy adopted by parasites [19, 31].

Heterologous study of PfCRT would permit elucidation of its contribution to antimalarial resistance, and to this end it has been expressed in *Xenopus*, *Dictyostelium*, and yeast for assay of antimalarial transport [16, 26, 36-39]. Neither *Xenopus* nor *Dictyostelium* is amenable to genetics, but the existence of high-throughput technologies for chemical- and synthetic-genetic screening suggests that *Saccharomyces* could be uniquely suited to investigate resistance in the presence and absence of PfCRT; these techniques could be utilized to identify the set of mutants most refractory or susceptible to the compound and elucidate any potential modulation by PfCRT of that phenotype. A genome-wide chemical-genetic profiling study has been undertaken with the related antimalarial quinine and identified a specific impairment by that drug of yeast tryptophan uptake, proposed to cause some side-effects seen in humans [40]. Yeast's transcriptional response to chloroquine has been previously assessed [41] and a candidate-based approach employed to investigate the role of ABC transporters in chloroquine resistance [42], but to our knowledge no systematic screen of chloroquine's genetic interactions has been performed; however, application of the drug to treatment of lupus and potentially cancer, in addition to malaria, provides a rationale for such work. Synthetic-genetic screens have successfully identified yeast genetic pathways interacting with heterologous factors [43], implying potential for characterization of PfCRT.

We have opted to study chloroquine's effects on cellular physiology and *PFCRT*'s modulation thereof by chemical- and synthetic-genetic analysis in *S. cerevisiae*, as well as investigation of chloroquine's alteration of the yeast transcriptional program (Figure 1). We find that chloroquine disrupts yeast vacuolar homeostasis with pleiotropic

effects. This work should inform the multitude of paths available for parasite acquisition of resistance in addition to expanding our understanding of PfCRT's transport capacity.

Results

To understand the genetic pathways and physiological processes upon which chloroquine impinges in the eukaryotic cell, we screened the *Saccharomyces* haploid collections of approximately 6000 nonessential-gene deletions and essential-gene hypomorphic alleles (DAmP collection) [44-46] for chloroquine-mediated colony size changes. Previous work in yeast has found that wildtype cells are highly resistant to the drug and can grow normally in concentrations as high as 200 mM [42], perhaps due to drug exclusion by the cell wall, as spheroplasted cells are known to respond to 200μM (compared with 200 nM for *Plasmodium* parasites) [12, 24, 47]. We therefore reasoned that treatment with millimolar concentrations of drug would identify classes of mutants with dysregulated drug response.

Chloroquine chemical-genetic interactions. Strains were robotically transferred onto medium containing 0, 50, 100, or 200 mM chloroquine and supplemented with dextrose or galactose; three independent iterates of the screen were performed on dextrose medium and two on galactose. Colony size was determined after two days' growth at 30°C, and results sorted according to fold size-change and subjected to hierarchical clustering [48]. 167 strains, comprising 2.8% of the library, were identified in at least two of three dextrose screens as reduced in size by 1.5-fold or more on 200 mM chloroquine, and on galactose, growth of 235 strains (3.9%) was diminished at least as much in duplicate experiments. 129 strains qualify as chloroquine-sensitive on either carbon source (Table 1; complete datasets for these screens are also found in Supplementary Tables 1a-e).

Although chloroquine resistance, defined as growth enhancement of at least 1.5-fold at 200 mM chloroquine, was conferred by deletions in several hundred genes, only two (of 92) mutant strains fit the criteria for chloroquine resistance in replicate screens on dextrose, and 50 (of 339) on galactose (Figure 2; Supplementary Table 1).

We categorized mutations altering yeast response to drug by FunCat analysis [49] and manual curation. Chloroquine resistance could be conferred by deletions in some protein trafficking or folding genes, or by loss of transcription, RNA processing, or translation machinery (data not shown; Supplementary Table 1). In chemical-genetic profiling, contingent on a drug's mechanism of action its target's absence may engender resistance, with sensitivity stemming from disruption of pathways buffering drug effects on the cell [50, 51]. As chloroquine affects the *Plasmodium* parasite's capacity for heme sequestration rather than inhibiting an enzymatic process conserved in yeast [11, 52], yeast resistance (as in *Plasmodium* species) is unlikely to occur via a straightforward cohort of factors. The strains qualifying as resistant in this study possess low median colony size (data not shown). The identification of such chloroquine-refractory strains presumably results from errors in colony size measurement and normalization as well as suppression of growth defects by relative nutrient enrichment proximal to small colonies, and chloroquine-resistant strains were therefore not characterized further.

Chloroquine sensitivity was incurred by loss of function in the cell wall integrity pathway and deletion of components of the vacuolar H^+-ATPase (v-ATPase), amino-acid or lipid/ sterol biosynthetic pathways, or factors involved in vesicular protein trafficking, as well as by deficiency in central-dogma related processes such as DNA replication or repair, transcription, and translation. Additionally, strains with defects in energy

production or storage displayed drug sensitivity (Table 1 and Supplementary Table 1; Figure 2). Previous chemical genetic screens have established a subset of deletion mutants sensitive to drugs with disparate modes of action [50, 53, 54]. We compared chloroquine-sensitive strains to 121 multidrug-sensitive mutants identified in parallel screens of 82 chemical compounds [53]. In replicate screens on dextrose, 14 (of 167) chloroquine-sensitive strains were also multidrug-sensitive; 6 chloroquine-sensitive strains were multidrug-resistant (one strain, *tup1*, was both multidrug-sensitive and –resistant). On galactose, 19 multidrug-sensitive strains (of 235 chloroquine-sensitive strains; 7 of these strains were multidrug-resistant) were also chloroquine-sensitive, suggesting that chloroquine-inflicted distress is specific (Supplementary Table 1).

Because chloroquine sensitivity could be engendered by disruptions to the cell wall or vacuole and/or pH-sensitive processes such as vesicle-mediated protein trafficking, we theorized that drug treatment perturbed pH homeostasis, and that its accumulation in acidic compartments was toxic. Chloroquine's site of action in the malaria parasite is the acidic digestive vacuole, a catabolic compartment that replenishes amino acid stores by degradation of host-derived proteins [55, 56]. Drug entry into the cell is passive, but the digestive vacuole's low pH promotes sidechain protonation and drug retention, culminating in accumulation to millimolar concentration [8]. Likewise, chloroquine enters the yeast cell by diffusion but accumulates in acidic organelles; in the yeast vacuole it can increase compartmental pH by 1-2 units [57]. Genetic lesions affecting cell wall composition might augment the intracellular concentration of drug, while vacuolar nutrient storage and protein shipment among cellular compartments

require a pH differential likely dissipated or reduced by chloroquine. We examined our datasets to find evidence for this model.

Chloroquine sensitivity is conferred by compromised cell wall integrity. Cell wall composition is influenced by activation of the cell wall integrity (CWI) pathway, a MAP kinase cascade activated by external stresses and culminating in the transcriptional activation of cell wall genes [58-61]. Deletion of upstream or downstream components of the pathway including plasma membrane sensors (*SLG1*, *MID2*), kinases (*ACK1*, *BCK1*/*2*, and *SLT2*), and the transcription factor encoded by *RLM1* resulted in chloroquine sensitivity. Loss of cell wall biosynthetic machinery (*KRE6*, *KRE9*, *KRE11*, *LAS21*) or a factor coupling cell wall integrity to the cell cycle (*SMI1*) also reduced chloroquine-tolerance, suggesting that wildtype yeast's ability to modify the cell wall is fundamental to drug escape (Table 1 and Supplementary Table 1; Figure 3). In agreement with this model, we detect induction of several cell wall integrity transcriptional targets by chloroquine treatment in wildtype yeast (discussed below).

Deficient organelle acidification confers chloroquine sensitivity. Cellular pH homeostasis is effected by proton and counterion transport via dedicated pumps and channels [62-64]. The v-ATPase drives proton influx into the *trans*-Golgi, endosome, and vacuole to maintain acidity in protein trafficking compartments and ensure appropriate and timely vacuolar delivery of hydrolases. Deletion in its components results in the *vma* phenotype, characterized by a neutral vacuole with reduced hydrolytic and ion-storage capacity; cells are sensitive to growth at pH 7.5 or in the presence of high calcium but can sustain growth and residual vacuolar function under acidic conditions [62, 63]: our assays were performed at pH 4, which should well support the cell in the absence of chloroquine.

However, we identified a dose-dependent response to chloroquine exposure in strains lacking numerous subunits both of the V_1 ATP-hydrolysis domain (*TFP1*, *VMA2*, *VMA5*, *VMA7*, *VMA8*, *VMA10*) and the V_0 proton pore (*CUP5*, *PPA1*, *TFP3*, *VMA6*, *VMA9*); loss of v-ATPase trafficking and assembly factors (*VMA21*, *VPH2*, *RAV1, RAV2*) was also detrimental to growth (Table 1 and Supplementary Table 1; Figure 4). This suggests that chloroquine impairs vacuolar homeostasis through disruption of the pH gradient.

Recent work has determined that v-ATPase assembly and function require ergosterol and sphingolipids at the vacuolar membrane [65, 66]. Deletions in ergosterol biosynthetic genes (*ERG2*, *ERG3*, *ERG4*, *ERG13*, *ERG24*) resulted in chloroquine sensitivity, but interpretation of these results is confounded by increased or unchanged size on chloroquine of other ergosterol auxotrophs (data not shown; Supplementary Table 1). Ergosterol auxotrophy is associated with impaired response to a number of drugs and may represent a chloroquine-nonspecific result potentially stemming from increased membrane fluidity or cellular permeability [50, 53, 54]. Deletions in *SUR4* and *FEN1*, encoding enzymes responsible for sphingolipid chain extension to 26 carbons, have previously been reported to reduce v-ATPase function and promote disassembly [65], but neither strain is strongly affected by chloroquine treatment (data not shown). Ergosterol and sphingolipid depletion may therefore result in subtle v-ATPase defects not detrimental to yeast growth on chloroquine.

Chloroquine hampers yeast protein trafficking. The requirement of a functional v-ATPase for effective intracellular protein trafficking, endocytosis, and exocytosis is well documented [57, 62, 63, 67], and strains lacking genes involved in these processes form the largest group impacted by chloroquine. Entry into the secretory pathway involves

protein import into, and folding and modification within, the endoplasmic reticulum (ER), followed by vesicular transport to the Golgi and further modification in that compartment before sorting to a range of destinations [45, 68, 69]. In yeast, protein departure from the *trans*-Golgi network can be exocytic or vacuole-directed by either of two routes, while separate machinery governs protein transport from cytoplasm to vacuole [62, 63, 69, 70]. Deletion of ER and Golgi glycosylation (*ANP1*, *ALG8*, *CAX4*, *MNN10*, *OST6*, *OCH1*) or lipid anchor (*GPI11*) machinery causes chloroquine sensitivity, as do loss of the clathrin heavy and light chains, a clathrin adapter (*APL2*), components of the GET complex, ESCRT, CORVET and HOPS, the vacuolar fusion machinery, the retromer, and the GARP and TRAPP tethering complexes, which mediate both anterograde and retrograde transport at multiple secretory organelles (Table 1 and Supplementary Table 1). This suggests a general impact on secretory function by abrogation of organellar pH gradients. Consistent with previous observations [71], chloroquine suppresses growth defects due to endosomal hyperacidification in the class E *vps* mutant *nhx1*, but this is not seen for other Class E mutants, including members of the ESCRT complex (involved in endosomal maturation [72]; data not shown and Figure 5). Chloroquine does not, however, impact autophagy deficient strains, although it inhibits the process in mammalian cells [73]: while loss of integrity in the complex II phosphatidylinositol 3-kinase involved in protein routing at the endosome (and comprising *VPS15*, *34*, *30*, and *38*) is strongly affected by chloroquine treatment, loss of *ATG14*, which associates with *VPS15*, *30*, and *34* to form the complex I autophagy associated kinase, does not influence colony size (Table 1, Supplementary Table 1, and data not shown).

Chloroquine impairs vacuolar homeostasis. Along with its roles in protein trafficking or degradation and maintenance of cellular pH homeostasis, the yeast vacuole functions as a storage repository for cellular nitrogen, phosphorus, and cation cofactors or toxins [74]. Nitrogen is retained in the form of basic or neutral amino acids imported H^+-dependently by antiporters encoded by *AVT* and *VBA* genes [75, 76], and nitrogen starvation represses the activity of the TORC1 complex, a positive regulator of growth and the cell cycle, initiating a transcriptional program that enhances expression of nonspecific amino-acid permeases exported to the plasma membrane [77-84].

We anticipated that chloroquine treatment might induce nitrogen starvation through impaired vacuolar uptake of amino acids, and detected strikingly diminished growth with increased chloroquine concentration for arginine or lysine auxotrophs (*ARG1*, *ARG3*, *ARG4*, *ARG5,6*, *ARG8*, *ORT1*; *LYS1*, *LYS2*, *LYS4*, *LYS5*, *LYS9*; Figure 5). Loss of *PTR3*, a component of the SPS plasma membrane amino acid sensor implicated in storage of basic amino acids in the vacuole as well as control of amino acid permease gene expression [85], also led to chloroquine sensitivity (data not shown). While deletion of aromatic amino acid biosynthetic machinery or the *GCN4*-encoded transcription factor regulating general amino acid control could reduce chloroquine tolerance, this effect was not reproducible and may reflect strain multidrug sensitivity rather than a chloroquine-specific effect (Supplementary Table 1) [54, 86].

Phosphorus constitutes an essential energy source for most organisms, and is found in the vacuole as polyphosphate concatemers [87]. The vacuolar transport chaperone (VTC) complex conjugates ATP-derived phosphates together both for energy supply and as a sink for cationic cofactors and heavy metals [87, 88]. Phosphate uptake

requires vacuolar acidification, as low vacuolar polyphosphate has been reported in *vma2* and *vma4* mutants [87, 89]; polyphosphate-deficient strains upregulate transcription of iron-starvation genes likely as a result of reduced ion content [90]. Concatemers are mobilized by *PHM*-encoded phosphatases and transport proteins [87]. PHO pathway, *vtc*, or *phm* mutants do not exhibit substantial chloroquine sensitivity, and comparison of chloroquine-sensitive strains to a set of 255 with altered polyphosphate storage [91] found overlap of only 10-15% (Supplementary Table 3). However, chloroquine chemical-genetic profiling was performed at high inorganic phosphate concentration (400 mM), which might have suppressed growth effects from low vacuolar polyphosphate by increasing cellular access to phosphorus.

We also sought to determine the effect of chloroquine treatment on calcium and iron homeostasis. Calcium ions are required as cofactors in vesicular transport, protein folding, mitotic entry, and assembly of the actin cytoskeleton [92, 93], and are uptaken into the vacuole by the Vcx1p Ca^{2+}/H^{+} antiporter [62, 63]. In the absence of *VCX1*, calcineurin is constitutively activated and upregulates transcription of the *PMR1*- and *PMC1*-encoded calcium-dependent ATPases to sequester the ion in the Golgi and vacuole [62, 82, 92]. Chloroquine, in increasing vacuolar pH, likely also diminishes Vcx1p function. In support of this model we found that loss of the genes encoding the *CNB1* calcineurin regulatory subunit, *PMR1*, or *HUR1*—a dubious ORF overlapping *PMR1*—sensitized yeast to chloroquine (Supplementary Table 1). Additionally, calcium depletion from the ER can activate the unfolded protein response (UPR) and a calcineurin-mediated stress response requiring the Cch1p-Mid1p plasma-membrane

calcium channel [93]; we identified *cch1* and *mid1* as chloroquine-sensitive (Table 1). These effects suggest some hindrance by chloroquine of calcium homeostasis.

During iron starvation, expression of high- and low-specificity permeases is induced by the Aft1p transcription factor, and iron actively uptaken at the plasma membrane for use in iron-sulfur complex formation, oxidative phosphorylation, and heme or fatty acid biosynthesis [94-96]. The iron regulon reportedly also maintains zinc homeostasis [97]. Redundancy among iron transporters at both plasma and vacuolar membranes obscured chloroquine-mediated growth defects in single-gene deletions, but drug treatment likely affects cellular iron homeostasis, as *aft1* eliminates growth on chloroquine (data not shown). Additionally, in a separate deletion library constructed as a control for synthetic genetic analysis (discussed below; Supplementary Table 2), we identified chloroquine sensitivity in strains lacking *FET3*, *FTR1*, *CCC2*, or *GEF1*, respectively encoding the subunits of a high-affinity iron permease, its chaperone, and the Golgi chloride channel necessary for permease sorting. Chloroquine thus appears to alter partitioning of calcium, iron, zinc, and potassium ions through its effect on vacuolar pH. While wildtype yeast are well able to tolerate high drug concentrations, secretory pathway and cation homeostasis mutants demonstrate significant compound sensitivity.

Chloroquine affects transcriptional control. The final category of mutants affected by chloroquine included those deleted for central-dogma related genes (Table 1 and Supplementary Table 1; Figure 2). It has been posited that chloroquine is an intercalator [98, 99], and we accordingly find genetic interactions between chloroquine and DNA replication and repair machinery (*orc3*, *mcm7*, *mgs1*). Impingement of the drug on transcription, RNA processing and nuclear export, translation, or cell cycle entry and

progression might result from off-target effects [50, 53, 54, 100]; however, recent studies postulate regulation of protein trafficking and degradation or vacuolar biogenesis at the level of DNA replication and repair [101], transcription [102], or protein folding [103], suggesting the existence of cellular feedback loops among compartments that chloroquine might instead disturb. We detect interactions between chloroquine and INO80 (*rvb1*, *taf14*) or RSC (*npl6*) chromatin-remodeling factors, the Sin3-Rpd3 histone deacetylase ensemble (*sin3*, *sap30*, *ume6*: galactose medium), transcription effectors (*bdf1*, *ctk2*, *ctk3*, *rpb3*, *tfg1*, *rrn11*), RNA processing (*pop2*, *pno1*, *prp22*, *prp24*, *prp39*, *rnt1*, *rpp1*, *rrp46*, *sas10*) or export (*mex67*) components, and cytosolic (*krs1*, *rpl27a*, *rrs1*, *tef4*) or mitochondrial (*mrpl35*, *yml6*) translation machinery (Table 1; Supplementary Table 1). Interestingly, RSC integrates proliferative cues and nutrient stress signals at the level of transcription [104]. It is not unreasonable to surmise that impaired vacuolar homeostasis, by influencing protein trafficking as well as perception of nutritional state, might contribute to chloroquine sensitivity of strains lacking factors implicated in essential cellular processes detailed here.

Chloroquine-mediated transcriptional alterations. Chemical-genetic analysis illustrates the cumulative effects exerted by chloroquine on the cell over time. We therefore sought to complement that picture with an understanding of acute chloroquine-induced transcriptional changes, especially with regard to drug effect on the cell wall and ion stress responses. While studies in *P. falciparum* identify few alterations in gene expression after brief chloroquine exposure, the transcriptional platform is highly invariant [105-107] and long-term drug selection results in substantially altered gene expression [30]. We treated wildtype yeast cultures with 0, 25, 50, or 100 mM

chloroquine, sampling at the commencement of our timecourse and after 30, 60, or 120 minutes. Samples were hybridized against a pooled reference on spotted arrays and scanned oligonucleotide intensities processed in Genepix 6.0 and Nomad (http://ucsf-nomad.sourceforge.net), normalized to gene expression at the initial timepoint, ranked by fold transcriptional induction or repression, and subjected to hierarchical clustering [48].

We determined the set of genes induced or repressed through time at all concentrations of chloroquine concentration. Expression of 65 genes was enhanced (relative to t_0) more than twofold at 30, 60, or 120 minutes' chloroquine exposure for each concentration of drug, while 17 genes' transcription was comparably diminished in at least two chloroquine exposure conditions and timepoints (Tables 2a,b and Supplementary Table 4a-f; Figure 6a). Gene ontological analysis GoStat [108] identified positive regulation by chloroquine of processes such as carbohydrate storage, uptake, and catabolism: transcription of hexose transporters (*HXT3*, *4*, *6*, *7*, *9*, *15*), and glycolytic (*ADH1*, *ENO2*, *GPM2*, *HXK1*) and glycogen-synthetic (*GSY1*, *PGM2*) or –metabolic (*GLK1*) enzymes was augmented, as was that of factors involved in galactose utilization (*GAL1*, *GAL2*, *GAL10*; Figure 6b). Transcription of the metallothionein-encoding genes *CUP1-1* and *CUP1-2* was increased by chloroquine treatment; their known stimuli encompass both ion stress and glucose deprivation. Ribosomal gene transcripts were repressed by chloroquine (*RPS14A*, *RPS14B*, *RPS16B*, *RPS17B*, *RPS23A*), as were those of three plasma-membrane transporters: the methionine permease *MUP1*, the fatty acid transporter *HNM1*, and the glycerol transporter *STL1* (Figure 6c).

Transcriptional induction of genes implicated in carbohydrate transport and energy production is characteristic of the diauxic shift, in which yeast transition from

dextrose consumption (fermentation) to respiration [109]; however, at no time during pre-growth or experimentation were cultures nutrient-starved, and their optical density was never greater than OD_{600}=0.5. Moreover, not only was the entire timecourse carried out within the doubling time (approximately 130 minutes) for cultures in galactose-supplemented synthetic medium, but Krebs cycle or mitochondrial electron transport chain enzymes were not induced.

Chloroquine induction of iron starvation. In *Saccharomyces*, low-level (3-5 mM) chloroquine treatment elicits significant transcriptional upregulation of iron transporter genes (*ARN2*, *ENB1*, *SIT1*) suggestive of chloroquine-specific iron depletion [41]. Although iron transporters are not induced under our experimental conditions, the geneset we detect is consistent with the yeast transcriptional response to iron starvation, in which gene expression transitions away from mitochondrial processes (many involving proteins with Fe-S clusters), energy is generated glycolytically, and glycogen is actively stored [94, 95]. Accordingly, we compared our gene expression data to published iron-starvation datasets [94]. We find induction or diminished repression of many positively regulated transcripts over time with increasing concentration of chloroquine (including the *ARN1* and *ARN2* plasma membrane iron importers), while the reverse is true of negatively regulated transcripts (involved in respiration or heme biosynthesis and including *QCR7*, *QCR8*, and *HAP4*). However, high expression of certain genes is not fully consistent with iron starvation (*QCR6*, *KGD1*, *CCP1*) [94], perhaps due to difference in chloroquine concentration or time of sampling between this and previous work (Table 2 and Supplementary Table 5) [58]. Similar results were observed from comparison of our data to a set of Crz1p-regulated transcripts expressed under calcium stress (Supplementary

Table 5) [110], suggesting that chloroquine stress exhibits many hallmarks of iron or calcium stress in addition to possessing unique attributes.

Expression of cell wall integrity transcripts. Our detection of chemical-genetic interactions between chloroquine and cell wall integrity mutants led us to investigate drug impact on the set of genes controlled by the cell wall integrity pathway. Transcriptional targets appear to vary with cell wall stressor [58, 59, 111], and we therefore examined the expression of transcripts under direct regulation by the Rlm1p transcription factor, as well as that of the *FKS2*-encoded β(1,3)glucan synthase [58]. Pathway activation is incomplete, as several transcripts expressed under CWI activation are repressed or minimally induced (*DFG5*, *PIR1*, *YKL161C*, *YLR194C*) and expression of at least two CWI-downregulated transcripts increases (*CTT1*, *SPS100*); however, chloroquine treatment generally appears to induce CWI transcripts (Supplementary Table 5, Supplementary Figure 1). Interestingly, transcription of *FIT2*, a cell wall protein implicated in uptake of iron-bound siderophores, is repressed by the CWI pathway [58, 96]; interaction between iron-starvation and CWI pathways may explain the incomplete induction of the iron regulon in our data.

Unfolded protein response. The detrimental effect of chloroquine treatment on secretory pathway mutants led us to query a role for the drug in UPR induction. In addition to external stimuli such as loss of protein-folding capacity or deficient protein glycosylation, mutation of genes involved in protein modification or trafficking, vacuolar homeostasis, and lipid or sterol biosynthesis can stimulate the UPR, in which accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER) triggers an expansion in organelle volume concomitant with transcriptional induction of secretory pathway and

ER-homeostatic genes [103, 112]. Expression of UPR-regulated genes is mediated by the Hac1p transcription factor [113]; we find moderate transcriptional induction of *HAC1* mRNA with chloroquine treatment (1.9-fold after 30 minutes treatment with 100 mM chloroquine; 2.9- and 2.7-fold at 60 and 120 minutes, respectively). Additionally, we compared chloroquine-mediated gene induction to a published dataset in which protein folding was inhibited by addition of dithiothreitol or tunicamycin to yeast cultures, resulting in induction of 381 transcripts [112]. We find subtly enhanced transcription of a subset of UPR-regulated genes, chiefly those encoding cell wall proteins and factors involved in protein transport or fatty acid or sterol biosynthesis (Supplementary Table 5). Chloroquine's effect on protein folding and ER homeostasis appears mild, but expression of chaperone genes (encoded by *CPR7, FMO1, HSP26, HSP78, HSP104, SSC1, SSE2, TCP1*) is upregulated by treatment with higher concentrations of the drug, perhaps indicating reduction in protein folding capacity stemming from lessened secretory pathway flow (Supplementary Table 5).

Modulation by PfCRT of chloroquine chemical-genetic interactions. The *Plasmodium falciparum* chloroquine resistance transporter (*PFCRT*) was identified in 2000 as an essential gene that can mutate to engender chloroquine resistance in the malaria parasite [12]. It encodes a 10-pass transmembrane protein sorted to the parasite digestive vacuole, a hydrolytic compartment analogous to the yeast vacuole or mammalian lysosome [12, 28]. While genetic intractability of *P. falciparum* parasites and the gene's essentiality have hindered its characterization *in situ*, it has been recoded for expression and studied ectopically in several systems, including *Saccharomyces* [16, 26, 36-39]. We obtained chloroquine-resistant and –sensitive gene variants recoded for yeast expression and

integrated them at the *ADE2* locus under control of the inducible *gal1-10* promoter; expression of a correctly sized protein was confirmed by western blotting (data not shown). N- or C-terminally GFP-tagged PfCRT localized to the yeast vacuole membrane (Supplementary Figure 2): co-localization of chloroquine and its resistance determinant validate the yeast system for genetic study of resistance to the drug.

We created three synthetic genetic array (SGA) libraries for study of PfCRT's capacity for modulation of yeast genetic interactions with chloroquine [114-116]. Two libraries, comprising both deletion and DAmP collections [45, 46], were engineered to express either chloroquine-sensitive (HB3) or chloroquine-resistant (Dd2) PfCRT (from the *ADE2* locus, under control of *Pgal1-10*); additionally, a congenic library did not express *PFCRT*, but instead replaced *ADE2* with the *URA3* cassette for uracil prototrophy used to select for *PFCRT* integration. Each library was sporulated in triplicate and the three copies passaged separately before examination of response to chloroquine (Supplementary Figure 3). Two independently sporulated replicates of each library were transferred onto medium containing 0, 25, 50, or 100 mM chloroquine, with galactose as carbon source for *PFCRT* induction. Strains were imaged after 48 hours' growth at 30°C; colony sizes at each concentration of drug were adjusted relative to size at 0 mM chloroquine and the dataset subjected sorted and subjected to hierarchical clustering [48].

Chemical-genetic screening in the absence of PfCRT. A preliminary investigation of yeast response to chloroquine in the SGA background and without *PFCRT* expression identified 126 strains with growth reduced by 1.5-fold or more at 100 mM chloroquine; 41 were detected in both libraries, including five strains previously denoted multidrug-sensitive (Table 3 and Supplementary Table 2a-f) [53]. Comparison of chloroquine

sensitivity in screens of SGA libraries to our chemical-genetic profiling of the deletion and DAmP collections found coincidence of 29 strains (22.4%; 17 in both libraries) with the set of deletions conferring chloroquine sensitivity on both dextrose and galactose, and 47 strains (20%; 23 in both experimental replicates) with the dataset comprising strains sensitized to chloroquine on galactose (data not shown). This minor overlap does not appear to result from differences in chloroquine treatment conditions (i.e., maximal concentration 100 *v*. 200 mM drug; data not shown), but instead is probably a function of genetic disparities between the BY4741 library subjected to chloroquine chemical-genetic analysis and the extensively modified MAT α query strain mated into that background for SGA library construction. Deletions in genes involved in protein trafficking or modification, ion homeostasis, lipid or sterol biosynthesis, and the cell wall integrity pathway conferred chloroquine sensitivity, in agreement with our chemical-genetic profiling, as did loss of numerous factors involved in transcription or translation. While strains lacking amino acid or polyamine biosynthetic genes (*ARO2*, *ARO7*, *SPE2*, *SPE3*) or a transcription factor regulating amino acid synthesis (*BAS1*) were chloroquine-sensitive, those auxotrophic for arginine or lysine were absent from the library, likely due to synthetic lethality with the *can1* or *lyp1* mutations (encoding arginine and lysine permeases, respectively) introduced into the library via the query strain (Table 3 and data not shown).

Chemical-genetic screening with PfCRT. We next investigated PfCRT's ability to influence chloroquine-mediated genetic interactions. Although we have not directly examined PfCRT's topology in *Saccharomyces*, a recent study in *P. falciparum* verified that its N and C termini are cytosolic [117]. Our detection of full-length protein, tagged

with GFP or the HIS epitope, in yeast, suggests preservation of orientation, as loss of signal from the C-terminal HIS tag would be expected if the termini were exposed to vacuolar hydrolases, while the acidic lumen of the vacuole would likely quench GFP fluorescence. Expression of functional, full-length, chloroquine-resistant *PFCRT* should therefore reduce the apparent accumulation of drug in the yeast vacuole.

The HB3 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) alleles of *PFCRT* differ at eight residues and their presence in *P. falciparum* parasites elicits chloroquine EC_{50} values of approximately 20 and 200 nM *in vitro*, respectively [12, 24]. We assessed their ability to modulate yeast response to chloroquine by screening at 0, 25, 50, or 100 mM drug; *PFCRT* expression was induced with galactose. Screens of two replicate, independently sporulated, libraries were performed in parallel with the no-PfCRT SGA profiling described above. Scrutiny of differences in growth at 100 mM chloroquine relative to that in its absence identified 55 Dd2-expressing, and 107 HB3-expressing, strains as reduced in size more than 1.5-fold in duplicate libraries (Tables 4, 5; complete list of data in Supplementary Table 2). 5 strains that were chloroquine-sensitive despite Dd2-PfCRT expression were also multidrug-sensitive, and three multidrug-resistant, while 10 or 6 sensitive strains expressing HB3-PfCRT were multidrug-sensitive or –resistant, respectively (Supplementary Table 2) [53]. Resistance to chloroquine was enhanced by HB3 expression in 41 strains, 13 in both libraries, and by Dd2 expression in 53 strains (22 in replicate libraries; Figure 8 and Tables 7, 8). Of these, three HB3-expressing chloroquine-resistant strains were multidrug-sensitive; one (*erg6*) was both multidrug-sensitive and –resistant. Dd2 expression conferred chloroquine resistance on seven multidrug-sensitive strains and the *erg6* strain [53].

PfCRT-mediated chloroquine sensitivity. We identified ten strains that were chloroquine-sensitive in the presence or absence of PfCRT (Figure 7a; Tables 3-5). The majority of these strains were deficient in biosynthetic pathways (*aro1*, *bas1*, *opi3*, *psd1*, *spe3*, *thi3*), though two lacked cell wall integrity genes (*bck1*, *slt2*). An additional thirty mutants were chloroquine-sensitive irrespective of *PFCRT* allele expressed, including strains lacking regulators of potassium transport (*sat4*, *hal5*) or components of the EGO complex (*gtr1*, *slm4*). The latter integrates vacuolar nitrogen-repletion signals with trafficking of the amino acid permease Gap1p to promote its residence at the plasma membrane under conditions of amino acid starvation in addition to controlling TORC1 nutrient-sensor complex signaling [118, 119]. *rts1,* a deletion in a regulatory subunit of protein phosphatase 2A, is also chloroquine-sensitive in the presence of PfCRT; Rts1p couples cell size to the decision to proliferate and is a downstream effector of TORC1 [120].

Loss of protein-sorting or –transport factors constituted the primary reason for chloroquine-sensitivity in the control SGA library (Figure 7b); however, strains expressing *PFCRT* demonstrated considerable genetic interaction with components of the transcription, RNA processing or export, and translation machinery, with the combined groups comprising approximately 30% of chloroquine-sensitive strains (Figure 7b, Tables 4 and 5). In previous studies such interactions have been proposed to be nonspecific [50, 51, 53], and given PfCRT's predicted role in amino acid or peptide transport [16, 28] its influence on these operations is likely indirect. Additionally, the wildtype HB3 isoform of PfCRT demonstrated aggravating interactions with protein-trafficking mutants on chloroquine medium, but in the presence of the Dd2 chloroquine-resistant protein variant, the drug more often impaired growth of strains wanting biosynthetic machinery.

PfCRT-mediated chloroquine resistance. Chloroquine resistance, specifically that engendered by expression of *PFCRT*, may give us insight into PfCRT's range of substrates and the modulation of their transport capacity by sequence polymorphisms in Dd2 or other gain-of-function alleles. Chloroquine resistance was conferred on control strains by loss of genes participating in a multitude of biological functions; among these strains only two were detected in parallel screens (*kcs1* and *pdx3*, deleted for a vacuolar biogenesis and a fatty acid biosynthetic factor, respectively; Figure 8 and Supplementary Table 2; note that *pdx3* confers chloroquine resistance in all libraries regardless of PfCRT presence or absence). In contrast, strains resistant to the drug through *PFCRT* expression exhibited a disparate signature, with enhanced growth for osmotic-stress (*hog1*, *pbs2*, *pmp3*) or protein-trafficking (*vps5*, *gsf2*) mutants. Chloroquine-sensitive PfCRT also suppressed the growth phenotype of a Mediator-Complex deficient strain (*med1*), a chaperone mutant (*yme1*), and a strain lacking a TORC1 regulatory protein involved in repressing proliferation under conditions of amino acid starvation (*npr3*).

Although chloroquine-resistant PfCRT benefited a second Mediator deletion (*ssn8*), its primary role was to enhance growth in anterograde- (*apm1*, *cdc50*) or retrograde-trafficking mutants between *trans*-Golgi network (TGN) and endosome (*arl1*, *pep8*, *ric1*, *vps35*, *vps51*), as well as in other secretory pathway genes (*bts1*, *vps13*, *vps63*, *vam10*; Supplementary Table 2). Dd2 expression additionally suppressed the growth defect of several other strains defective in trafficking to or from the endosome, though the strains themselves were present in only one of the two libraries assayed (*ypt6*, *vps17*, *arl3*, *vps30*; Supplementary Table 2). We confirmed this effect by serial dilution (Figure 9, Supplementary Figure 4, and data not shown); it lends support to PfCRT

functionality in yeast, as protein transport is likely promoted in these mutants by Dd2-mediated extrusion of chloroquine from acidifying secretory pathway compartments. Appropriate anterograde and retrograde sorting from the endosome (to the vacuole or to the TGN) is dependent on the presence of pH microdomains in that compartment likely abolished by weak-base trapping [71], and the presence of Dd2, in creating an exit route for chloroquine, presumably restores this capacity; this effect is not seen for protein transport between other secretory pathway organelles (Supplementary Figure 4).

Discussion

We have characterized yeast response to the antimalarial drug chloroquine, a lipophilic weak base, in the presence and absence of the *P. falciparum* chloroquine resistance transporter, a protein in the digestive vacuole membrane of malaria parasites that can mutate to transport diprotonated, membrane-impermeant chloroquine down its concentration gradient from the digestive vacuole and thence the cell [12, 16]. In contrast to the recent chemical-genetic profiling of yeast interactions with the related antimalarial quinine, which found that yeast growth was impacted by inhibition of Tat2p-mediated tryptophan uptake [40], chloroquine's principal effect on yeast is through disruption of vacuolar homeostasis. The yeast vacuole coordinates secretory and endocytic pathway functions in addition to maintaining intracellular nitrogen, phosphate, and ion stores [62, 63, 67, 90, 121], and accordingly we find that chloroquine reduces or abrogates growth of strains deficient in protein-trafficking and amino-acid biosynthetic machinery, unable to respond to calcium stress, or lacking high-affinity iron permeases. Chloroquine tolerance is also reduced in strains with cell wall or vacuolar acidification defects or deleted for components of DNA replication and repair, transcription, or translation complexes; these

chemical-genetic interactions were apparent irrespective of carbon source. Galactose served instead to enhance the number and degree of chemical-genetic interactions observed on dextrose medium, likely due to its inefficiency in stabilizing the v-ATPase complex, which is relatively more dissociated in dextrose-depleted cells [62, 63]. Reduction in the capacity for vacuolar acidification presumably prolongs the time to recovery from defects in pH homeostasis to exacerbate the effects of drug.

Recent work has demonstrated genetic interactions between protein transport through the secretory pathway and the yeast DNA repair [101], protein folding [103], or chromatin remodeling [70, 102] apparatus, in accord with our findings regarding chloroquine's impact on the vacuole. Moreover, we detected transcriptional effects consistent with the calcium stress response and iron, though not nitrogen or phosphorus, starvation, as well as a partial induction by chloroquine of the unfolded protein response, which may stem either from calcium dysregulation or protein trafficking defects [93, 103]. Our observation of the strong growth-inhibitory effect of chloroquine on mutants with dysfunctional cell wall integrity pathway signaling or alkalinized vacuoles suggests the cell wall as a major means of chloroquine exclusion from yeast. That subverted, chloroquine is free to partition into the vacuole, perturbing pH homeostasis with pleiotropic consequences, and wildtype yeast correspondingly appear to activate the transcriptional program of the cell wall integrity pathway to increase their refractoriness to the compound (Figure 10).

We investigated PfCRT's capacity for modulation of yeast chemical-genetic interactions with chloroquine. In the absence of *PFCRT* expression, libraries created for synthetic genetic analysis [45, 46, 101, 114-116, 122] demonstrated differing interactions

with the drug than did the BY4741 deletion and DAmP collections. While a larger proportion of interactions were seen with genes involved in biosynthesis, principally that of basic amino acids (arginine and lysine), in our initial screen, the *can1* and *lyp1* mutations present in the SGA mater strain [116] appear to have led to synthetic lethality of not only biosynthetic genes but also components of the v-ATPase with our SGA-mater derived query strain, and these mutants did not survive library construction. Since basic amino acids are stored in the vacuole [86], loss of competence for storage (through vacuole alkalinization), uptake, or biosynthesis will strongly diminish fitness. Other approaches to chemical-genetic profiling utilize diploid heterozygous (essential-gene) or homozygous (non-essential gene) knockout libraries, and SLAM or dSLAM (synthetic lethal by microarray/ diploid SLAM) approaches [54, 100] might facilitate investigation of chemical-genetic interactions with biosynthetic genes.

PfCRT expression substantially influences yeast genetic interactions with chloroquine: the chloroquine-sensitive HB3 allele of the gene more than doubles the number of mutant strains sensitive to the drug, and chloroquine-resistant Dd2 PfCRT does not fully compensate. We have observed that wildtype yeast expressing either allele of *PFCRT* are less robust on control or chloroquine-containing medium than those without (data not shown); PfCRT's localization at the vacuole may permit its transport of a substrate whose repartitioning is detrimental to yeast growth. While chloroquine resistance among strains lacking PfCRT is conferred by loss of gene products of several disparate functions and is not reproducible, expression of the gene, especially the chloroquine-resistant allele, is beneficial to protein trafficking machinery (Figure 10); chloroquine-resistant PfCRT seemingly also ameliorates defects in transcription or RNA

processing and export. Genetic coupling between central-dogma related processes and protein transport [101, 102] may effect this suppression. Protein sorting and localization are frequently associated with multidrug-sensitivity or –resistance [50, 53, 54], but Dd2's alleviating interactions with factors mediating anterograde and retrograde transport from the endosome in particular lead us to suppose that extrusion of chloroquine from these compartments or from the vacuole as Dd2-PfCRT passes through the secretory pathway is the principal means of mitigating these defects. We do not observe alleviating genetic interactions of Dd2 with the ESCRT complex (for endosomal maturation) [72, 123, 124]; in fact, these strains are also lost during library construction, presumably also due to synthetic lethality with the SGA mater strain. In this instance strains deficient in ESCRT (Class E *vps* mutants) possess abnormally large, hyperacidic endosomes but are unable to acidify their vacuoles [70]. The loss of these mutants from our library is also consistent with amino acid uptake and storage defects and reinforces our identification of the basic amino acid biosynthetic machinery in our preliminary screen. Sensitivity to drug in chemical-genetic profiling results from loss-of-function of factors buffering its effects [50, 53], and consequently, we identify chloroquine sensitivity in strains with either a vacuolar acidification defect or an amino-acid biosynthetic one; growth is unsustainable when amino acid permeases are also absent.

Chloroquine resistance in malaria parasites is now pervasive in *P. falciparum* and *P. vivax*, respectively the causes of most malaria-related mortality and morbidity worldwide. *P. falciparum*'s range is limited to the tropics, but *P. vivax* inflicts a significant impact on temperate regions, and immunity to this latter parasite is rare [5]. Additionally, the mechanism of chloroquine-resistance acquisition in vivax parasites

remains unidentified and does not appear to necessitate mutations in *PVCRT*, although increased functionality or substrate interaction may be a feature of the wildtype protein [33, 125]. Contributions to *P. falciparum* chloroquine resistance from genetic loci external to *PFCRT* are also noted in some strains and seem to depend on prior selection history with other antimalarial drugs [29, 31]. Interestingly, both resistance phenotypes and those indicative of parasite tolerance to drug (ability to recrudesce after treatment with a dose lethal to sensitive parasites) are detected in strains expressing the same *PFCRT* allele [126]. In conjunction with the status quo of resistance or reduced sensitivity to all antimalarials currently in use [6], these observations are disheartening and indicate the pressing need for further scrutiny of both the range of parasite adaptations to drug selection pressure and PfCRT's capacity for substrate transport as a means of bettering drug co-administration and design strategies.

A significant aim of our work was therefore the creation of a heterologous system permissive for the study of PfCRT, a member of a novel family of membrane transporters conserved in plants and the slime mold *Dictyostelium discoideum* [25-27]. Its function in *Plasmodium* is thought to be export of small peptides from the digestive vacuole, although the substrates of related transporters in *Arabidopsis* include glutathione-synthesis intermediates [16, 27]. Study in malaria parasites has been infeasible due to its essentiality and parasites' genetic intractability, and consequently characterization of protein expressed ectopically in *Dictyostelium*, *Xenopus*, and yeast has been attempted [16, 26, 36-39]. While antimalarial-drug transport has been demonstrated repeatedly and substrate preference is under elucidation, in the absence of both the latter and a protein crystal structure the design of efficacious new antimalarials is crippled. Heme, the target

of quinoline antimalarials, is the focus of continued research, as its uptake from the host (as part of hemoglobin) is essential to parasite survival but not subject to mutation by parasites to evade selection pressure [9-11, 52]. Novel bifunctional chemotherapeutics that bind heme while blocking transport through drug-resistant PfCRT are a promising strategy for bypassing parasite resistance [9], but at present there is no way of inferring parasites' potential for escaping this additional class of antimalarials. Yeast's amenability to genetic manipulation thus presents it as an attractive model system for devising assays to study transport of current and future antimalarial chemotherapeutics by a range of PfCRT variants, not only to understand constraints imposed on the mutation of this determinant of antimalarial drug resistance, but to facilitate design of novel drugs and development of effective co-administration strategies.

Materials and Methods

Yeast strains. The *Saccharomyces cerevisiae* deletion and DAmP collections in strain BY4741 (marked with the *KANMX6* cassette) [44-46] were the gift of D Cameron and JS Weissman, UCSF; the SGA mater strain (MAT α, *can1*Δ*::STE2pr-SpHIS5 lyp1*Δ*::STE3pr-LEU2 LYS2 his3*Δ*1 leu2*Δ*0 MET15 ura3*Δ*0*) [116] was the gift of DK Breslow and JS Weissman, UCSF.

Software for data analysis. Software for colony size determination (HT-Colony-Measurer, EMAP Toolbox) [127] was obtained from SR Collins and JS Weissman, UCSF.

Constructs and vectors. *PFCRT* (Dd2 and HB3 alleles) recoded for expression in yeast and subcloned into pRS423 [128] under control of *pGal1-10* (pRS423-*PFCRT*) was the gift of F Hays and R Stroud, UCSF. The construct was epitope-tagged with FLAG (5')

and 10x HIS (3'). Additional vectors used in this work were the gift of T Matsuguchi and EH Blackburn (pRS315) [128] or DK Breslow and JS Weissman (pFA6a-3HA-*KANMX6* [129]; pAG60 [130]; pFA6a-*NATMX4_pTEF2*_eGFP_Adh1-tm-AG [131]), UCSF.

pRS315-*PFCRT* was subcloned from pRS423-*PFCRT* by amplification of the *PFCRT* cassette using a *HindIII*-marked primer directed against the *pGal1-10* promoter (GATCaagcttTAGTACGGATTAGAAGCCGC) and a modified M13F primer containing a *NotI* restriction site (GATgcggccgcTAAAACGACGGCCAGTG); the digested PCR product of approximately 2200 bp was introduced into *HindIII*/*NotI* sites in pRS315 [128]. The M13F primer annealed approximately 300 nt downstream of PfCRT's terminal codon; this vector-derived region was used as terminator sequence in subsequent manipulations.

N- and C-terminally GFP-tagged *PFCRT* constructs were subcloned into pRS315 [128]. Briefly, the *PFCRT* cassette was amplified from pRS315-*PFCRT* such that either promoter and *PFCRT* were encoded on one fragment and terminator on the other (C-terminal tagging), or with promoter comprising one fragment and *PFCRT* and terminator the other (N-terminal tagging). eGFP was amplified from pFA6a-*NATMX4_pTEF2*_eGFP_Adh1-tm-AG [131]. PCR fragments were purified, digested, and repurified (Wizard SV, Promega) before subcloning into the *HindIII*/*NotI* sites of pRS315 [128].

Constructs were introduced into BY4741 yeast by plasmid transformation [132] and selection on SD-URA before microscopy. *PFCRT* expression was confirmed by western blotting.

Antibodies. α-HIS antibody for PfCRT detection (HRP-conjugated) was purchased from Qiagen.

SGA query strains. Query strains for SGA libraries were constructed from the SGA mater strain (see above) [116] by integration of *URA3* or *PFCRT-URA3* into the *ADE2* locus by standard PCR-based techniques [132, 133]. Integration was confirmed by PCR and *PFCRT* expression by western blotting.

To integrate *URA3*, the cassette was amplified from pAG60 [130] with the following primers, which contain sequence proximal to F1 and R1 sites within the *URA3* cassette:

Inside_F1_ADE2 ATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACAA TTGGGACGTATAATTAAGGCGCGCCAGATCT

Inside_R1_ADE2 TTACTTGTTTTCTAGATAAGCTTCGTAACCGACAGTTTC TAACTTTTGTGTAAACTGGATGGCGGCGTTAG

PFCRT (Dd2 and HB3 alleles, with promoter and terminator sequences) was amplified from pRS315-PFCRT with the pGal1 primer (as above) and the pfcrt_term_R primer (AGCGCGCGTAATACGACTCACT).

URA3 was amplified with pGal1_overlap_inside_F1

(CACGGAGGAGAGTCTTCCTTCGGAGGGCTGTCACCCGCTCGGCGGCTTCTAA TCCGTACTAATTAAGGCGCGCCAGATCT)

and Inside_R1 (TAAACTGGATGGCGGCGTTAG) primers.

These PCR fragments were purified (Wizard SV kit, Promega) and fused head-to-head by overlap extension PCR with the Inside_R1_ADE2 primer and the pfcrt_term_R_ADE2 primer

(ATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACAATTGGGACGT ATAGCGCGCGTAATACGACTCACT).

Yeast membrane purification for western blotting, α-HIS HRP-conjugated Ab. Two days before a protein prep, a single colony of *PFCRT*-transformed yeast (expressed from *Pgal1-10*; transformed on a CEN-ARS plasmid [128] or integrated into the *ADE2* locus using standard PCR-based techniques) [132, 133] was inoculated into minimal medium with 2% raffinose, 0.05% dextrose, and the appropriate amino acid dropout mixture and incubated at 30°C. After overnight growth cultures were diluted back in the same medium, and at mid-logarithmic phase further diluted into minimal medium supplemented with 2% galactose for a 16-hour *PFCRT* induction. After induction, cultures were centrifuged for ten minutes at 3500 rpm (Eppendorf) and washed in 1 mL lysis buffer (50 mM Tris pH 7.4, 10% glycerol, 150 mM NaCl, 2 mM PMSF, 1 mM EDTA, and protease inhibitor tablet (Roche)) prior to pelleting for 10 minutes at 5000 g (Eppendorf). Pellets were resuspended in 200 µL lysis buffer and vortexed with glass beads (Sigma) for half an hour at 4°C. Lysed cells were placed on ice for 2 minutes before lysate collection by brief centrifugation, and cell debris cleared from the lysate by an additional centrifugation step of ten minutes at 8000 g (performed at 4°C). Supernatant was then removed to an ultracentrifuge tube (Beckman Coulter) and samples balanced by addition of cold lysis buffer; membrane fractions were precipitated by centrifugation at 100, 000 g for 1 hour (Beckman Coulter), after which pellets were decanted and resuspended in membrane storage buffer (Tris pH 6.8, 20% glycerol, 500 mM NaCl, 2 mM PMSF). Protein concentration was determined by BCA assay (Pierce) and fractions stored at -80°C prior to protein electrophoresis and western blotting.

α-HIS western blotting. 1-5 µg protein from purified membrane fractions was resuspended in 2x SDS loading buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 200

mM DTT, and bromophenol blue) and subjected to protein electrophoresis (4-12% gradient gel, Invitrogen) in MOPS buffer at constant voltage (110-120 V). When the dye front had cleared the gel, samples were wet-transferred onto nitrocellulose in Tris-Glycine buffer (constant voltage) for 2 hours before membrane blocking and immunoblotting with α-HIS HRP-conjugated primary antibody (Qiagen) according to the manufacturer's instructions.

Microscopy. Expression and localization of GFP-tagged PfCRT was confirmed by microscopy. Briefly, a single colony of each strain was inoculated into synthetic medium (YNB) supplemented with 2% raffinose and 0.05% glucose and complete amino acids, containing 400 mM H_3PO_4 (to pH 4 with KOH). After overnight culture at 30°C, cells were diluted back in the same medium until they had achieved logarithmic growth, then harvested and resuspended in synthetic medium as above, but containing 2% galactose. *PFCRT* expression was induced for five hours.

FM4-64 staining. After four hours' induction, cells were harvested and labelled with FM4-64 (Invitrogen) according to the 30°C method of Vida and Emr [134]. Washes and chase were performed with YEP-gal (2%) to continue *PFCRT* induction. Chase length varied between 60 and 90 minutes.

DAPI staining. After full induction, cells were permeabilized by fixation in a final concentration of 50% ethanol and stained with DAPI (Invitrogen) as described (http://www.invitrogen.com/site/us/en/home/References/protocols/cell-and-tissue-analysis/dapi-protocol/dapi-nucleic-acid-stain.html).

Imaging. Cells were imaged on a Nikon 6D High-throughput fluorescence microscope (Nikon Imaging Center, UCSF).

Chemical genetics and synthetic genetic analysis. All library manipulation was undertaken via Singer RoToR (Somerset, UK); colonies were arrayed in 384 (two replicates of chemical-genetic screen) or 1536 format on Singer trays (PlusPlates; Singer, UK) and transferred among plates by short-pin RePads (Singer, UK).

Library construction for SGA. Query strains for SGA library creation were generated by standard PCR-based integration [132, 133] into the *ADE2* locus of the *URA3* cassette [130] or a head-to-head fusion of *URA3* and *PFCRT* driven by the *Gal1-10* promoter into the SGA mater strain (see Reagents and Strains). Library construction was as previously described [45, 46, 116], with the following modifications: query strains were marked with *URA3*, and consequently diploids were selected with G418 and synthetic medium lacking uracil and supplemented with monosodium glutamic acid, while doubly mutant strains were selected with G418 and an amino-acid dropout mixture lacking histidine, lysine, arginine, and uracil.

SGA reagents. All synthetic media contained 400 mM H_3PO_4, equimolar to an aqueous solution of 200 mM chloroquine diphosphate (Sigma) and adjusted to pH 4 by addition of 5N KOH. Agar-based medium for synthetic-genetic screens contained 2% galactose, SC amino acids (lacking histidine, lysine, arginine, and uracil (2 g/L)), and 0, 25, 50, or 100 mM chloroquine diphosphate. Agar-based medium for chemical-genetic screens contained either 2% dextrose or 2% galactose, CSM complete amino acids (Sunrise Science), and 0, 50, 100, or 200 mM chloroquine diphosphate. YNB and agar were autoclaved and combined with a filter-sterilized solution of galactose, amino acids, and H_3PO_4 or chloroquine (25, 50, 100, or 200 mM CQ) and H_3PO_4 (0 mM CQ) before

dispensation into Singer trays (47 mL per tray). Plates were dried overnight at room temperature before screening.

Chemical-genetic and SGA screening. Source plates for screening were created by library transfer onto YPAD medium containing 100 mg/L G418 (Invitrogen) and two days' growth at 30°C. For chemical genetic and SGA screens, cells were transferred from source plates onto chloroquine medium in triplicate; for SGA, two independently sporulated replicates of each library were screened in parallel. After two days' growth at 30°C, plates were imaged and colony size determined by HT-Colony-Measurer software [127]. Colony sizes were normalized to median colony size in each plate (rawN) or with a separate correction for position within a plate (rawN2) via the E-MAP Toolbox collection of MATLAB scripts [127].

Chemical-genetic and SGA analysis. For each deletion or DAmP strain, colony size at the highest concentration of chloroquine was adjusted with respect to colony size in drug absence and data $\log_2$-transformed and clustered by Euclidean distance in Cluster 3.0 before visualization in Java TreeView [48]. Additionally, all datasets were sorted by magnitude of colony size-change (size at highest concentration of chloroquine relative to that at 0); colony size alterations of 1.5-fold or greater were considered noteworthy. Gene ontology analysis was performed on datasets comprising strains with size alterations above the 1.5-fold cutoff via FunCat (http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html [49]).

Microarray expression analysis. All experiments were conducted on wildtype BY4741 yeast (MAT **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* [133]). Strains were cultured in synthetic medium (YNB) containing 400 mM H_3PO_4, equimolar to the concentration of phosphoric

acid in a 200 mM aqueous solution of chloroquine diphosphate (Sigma) and adjusted to pH 4 with KOH.

Briefly, a single yeast colony was inoculated into synthetic medium containing 2% dextrose and supplemented with complete amino acids for overnight growth at 30 °C; this culture was diluted into the same medium until mid-log phase (OD_{600}=0.5) was achieved. Culture absorbance was determined and cells diluted into synthetic medium, containing 2% raffinose, 0.05% dextrose, and complete amino acids, for derepression of galactose-inducible genes during overnight growth (up to 16 hours), at 30°C, with shaking. At mid-log phase (OD_{600}=0.4), cells were collected by centrifugation at room temperature for 10 minutes at 3500 rpm (Sorvall RC-3b), culture supernatants decanted, and pellets resuspended in synthetic medium containing 2% galactose, supplemented with complete amino acids, for shaking at 30°C for 4 hours. Cells were collected (as above) and resuspended in synthetic medium with 2% galactose, complete amino acids, and 0, 25, 50, or 100 mM chloroquine.

Chloroquine perturbation. Cells were harvested (0 mM chloroquine) upon commencing the experiment and thereafter (0, 25, 50, or 100 mM chloroquine) at 15, 30, 60, and 120 minutes. Samples were collected by filtration onto nitrocellulose membranes (Whatman) and immediately flash-frozen and stored at -80°C before RNA extraction.

Sample preparation. Total RNA was extracted by the hot acid phenol method. Selection of polyadenylated RNA was performed by adsorption to oligo-dT cellulose (Ambion). For each sample, 2 µg polyadenylated RNA was reverse-transcribed to cDNA containing amino-allyl dUTP (Ambion) using SuperScript II reverse trancriptase (Invitrogen). Chloroquine-treated samples were coupled to Cy5 and reference pool samples (reverse-

transcribed from pooled mRNA) to Cy3 (GE Healthcare) and competitively hybridized against the Operon yeast genome oligo set on printed microarrays.

Microarrays were scanned on a GenePix 4000A scanner and images analyzed in GenePix 3.0 software. Data were stored and normalized in the NOMAD database (http://ucsf-nomad.sourceforge.net). Gene expression data were normalized to the t_0 sample, $\log_2$-transformed, median-centered, and clustered by Pearson correlation in Cluster 3.0; clustered arrays were visualized in Java TreeView [48].

Additionally, data were sorted by fold gene induction or repression with respect to the level of transcript in the t_0 sample. Datasets compiled from transcripts with changes in gene expression of 1.5-fold or more or 2-fold or more were subjected to gene ontology analysis, performed via GoStat (http://gostat.wehi.edu.au [108]), with the following parameters: minimal length of considered GO paths = 3, maximal p-value = 0.01, cluster GOs = -1, correct for multiple testing = none.

Serial dilution (frogging) assays. Strains were recovered from SGA libraries or reconstructed by transformation into BY4741 using standard PCR-based methods [132, 133]. All synthetic culture medium was adjusted to contain 400 mM H_3PO_4, pH 4, equimolar to a 200 mM solution of chloroquine diphosphate (Sigma).

No PfCRT. One day prior to the assay, a single colony from each strain was inoculated into 3 mL synthetic medium supplemented with appropriate amino acids and 2% dextrose. After overnight growth at 30°C, cultures were diluted back in the same medium and replaced at 30°C for 4 hours to achieve log phase. Culture ODs were then determined and cultures diluted into 1.25x S (YNB) in a 96-well block with a capacity of 2 mL per

well such that the most concentrated spot in a dilution series would comprise 15000 cfu, according to the following calculations:

A culture at OD_{600}=1 was assumed to have 3E7 haploid cells per mL

The volume of each spot transferred by frogger was assumed to be 3 μL

From 15000 cfu, cultures were serially diluted 7 times further in 3.3x steps, such that the least-concentrated spot would contain approximately 5 cfu. After dilution, 150 μL was removed from each well and pipetted into a 96-well plate. Cells were resuspended and transferred onto assay medium (YPAD or synthetic medium containing 2% dextrose and different concentrations of chloroquine) with a 96-pin frogger. When spots were dry, the plates were placed at 30°C for 48 hours.

PfCRT. Assays were performed as above with the following modifications:

Strains were inoculated into medium containing 2% raffinose and 0.05% dextrose as carbon source; after overnight growth, the culture was diluted back into medium containing 2% galactose and replaced at 30°C for five hours' induction; diluted strains were frogged onto assay medium containing 2% galactose and different concentrations of chloroquine.

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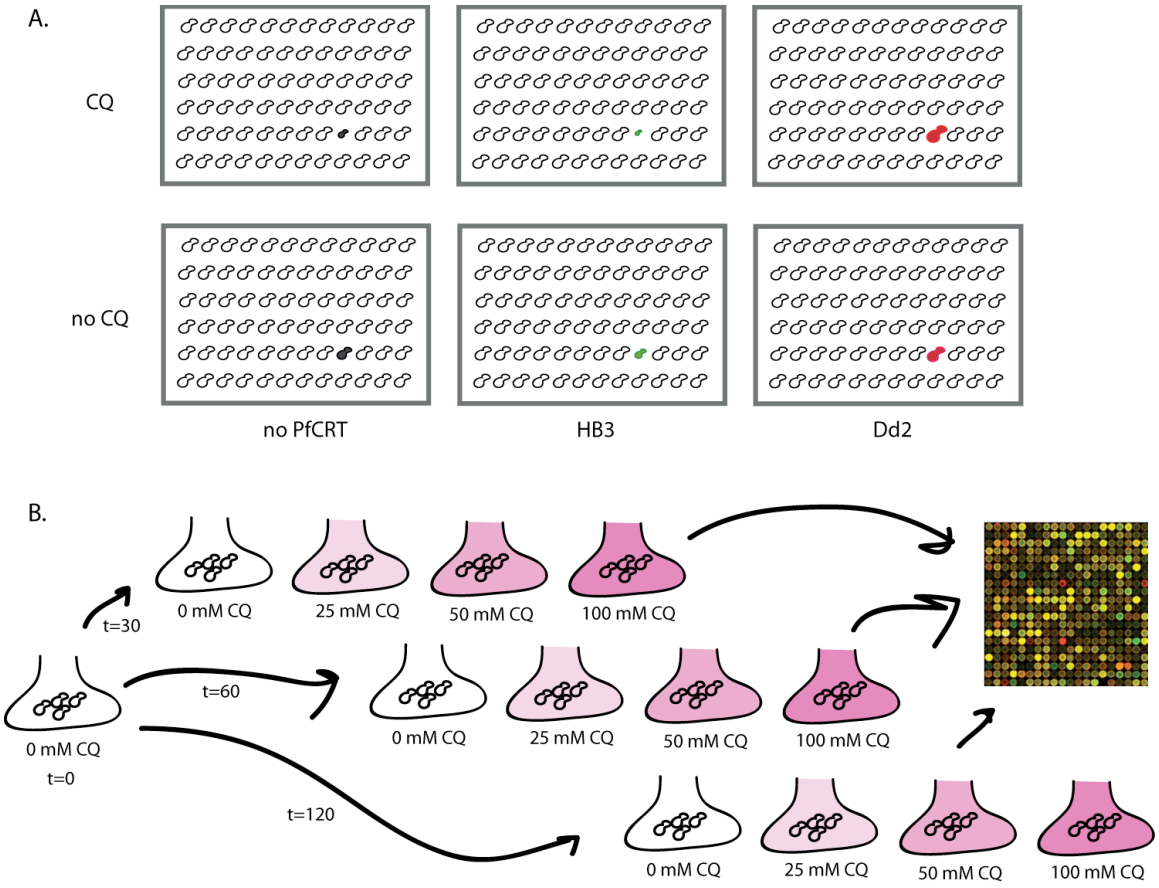


Figure 1. Summary of experimental design.
A. Chemical-genetic profiling of yeast response to chloroquine in the presence and absence of PfCRT explores the capacity of chloroquine-resistant and –sensitive isoforms to modulate the set of mutants with altered response to drug.
B. Characterization of chloroquine-dependent gene induction and repression garners an insight into the short-term effects that the drug inflicts on the cell.

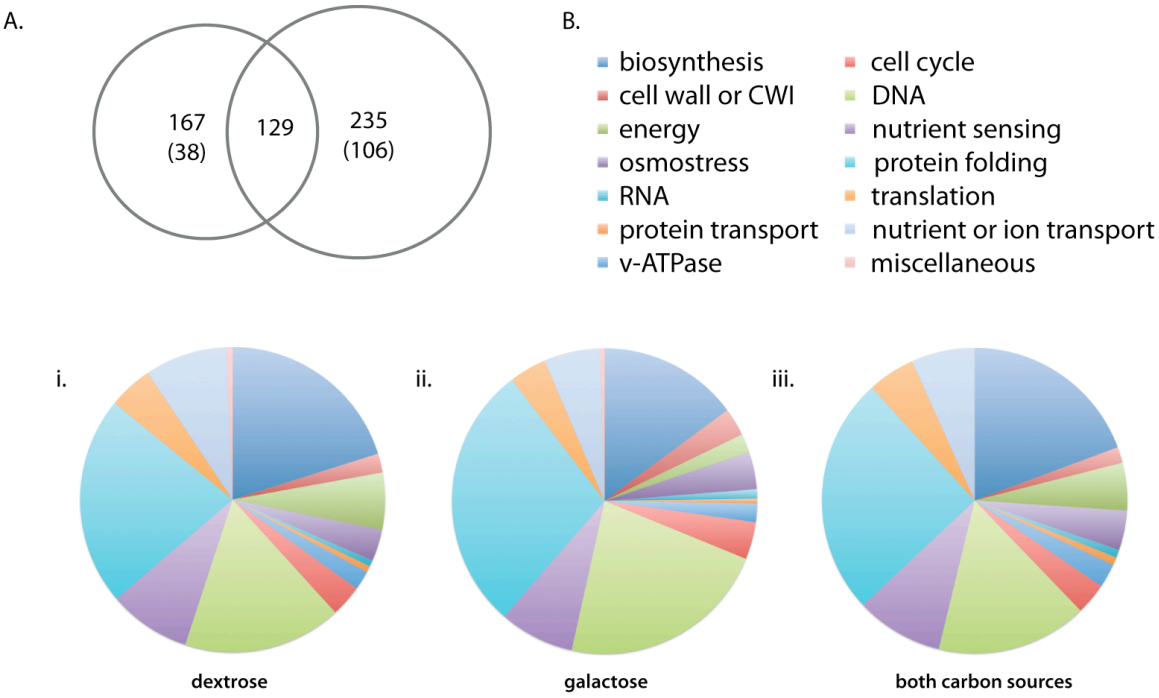


Figure 2. Chloroquine sensitivity.
A. Screening on dextrose identifies 167 strains as chloroquine-sensitive in at least two of three experimental replicates. Screening on galactose identifies 235 strains as chloroquine-sensitive in both experimental replicates (chloroquine-sensitivity calculated as 1.5-fold or greater size change at 200 mM chloroquine relative to size at 0). 129 strains are chloroquine-resistant on both carbon sources.
B. Similar proportions of deletion strains qualify as chloroquine-sensitive on either carbon-source. Strains lacking components of biosynthetic pathways, transcription associated genes, or protein-trafficking factors constitute the majority of chloroquine-sensitive mutants. Miscellaneous: protein modification, protein degradation.
Not shown: dubious or uncharacterized ORFs (see Table 1, Supp. Table 2).
i. dextrose medium; ii. galactose medium; iii. both

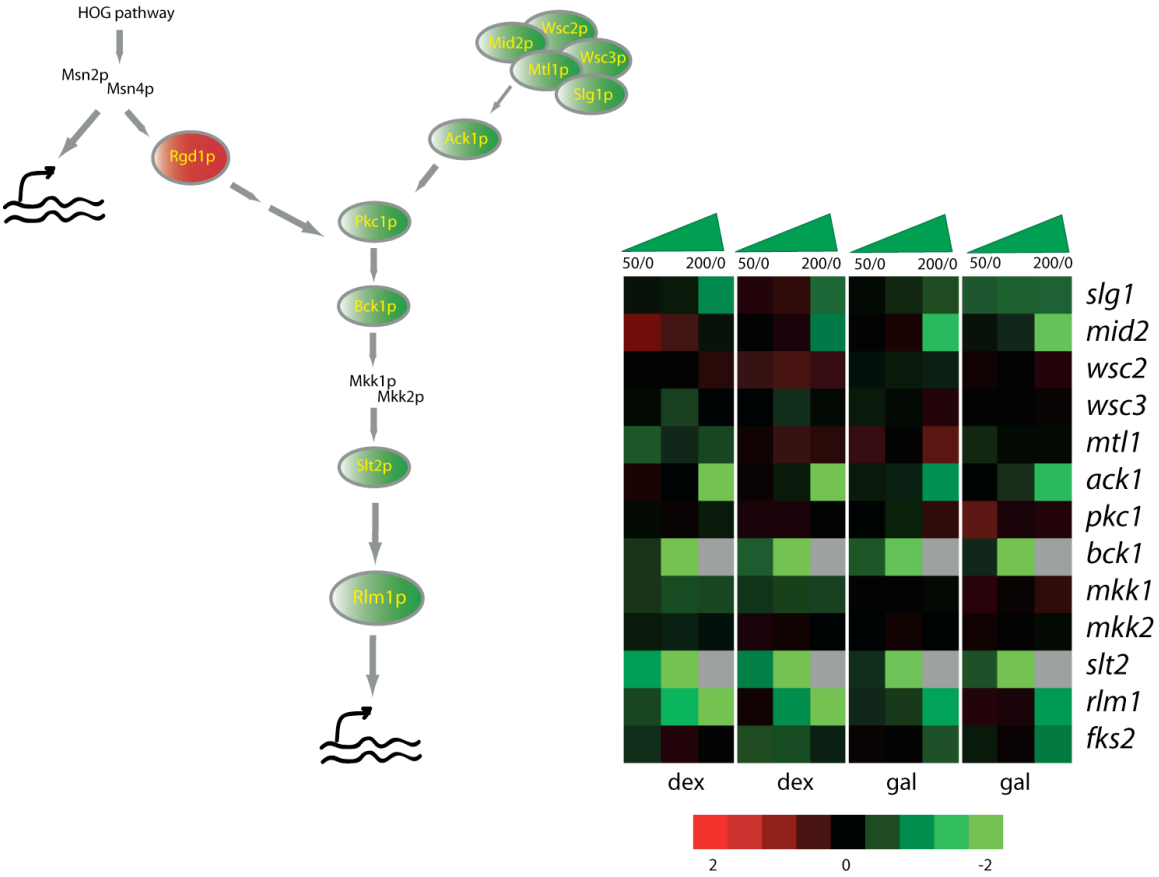


Figure 3. Cell wall integrity pathway mutants are sensitive to chloroquine stress.
The cell wall integrity (protein kinase C) pathway (cartoon) activates a transcriptional program to alter cell wall composition in response to external stresses. Full activation is dependent on cross-talk from the HOG osmotic-stress response pathway.
Heatmap data are colony sizes from replicate screens normalized by size in the absence of chloroquine.
Mkk1/2 are functionally redundant.

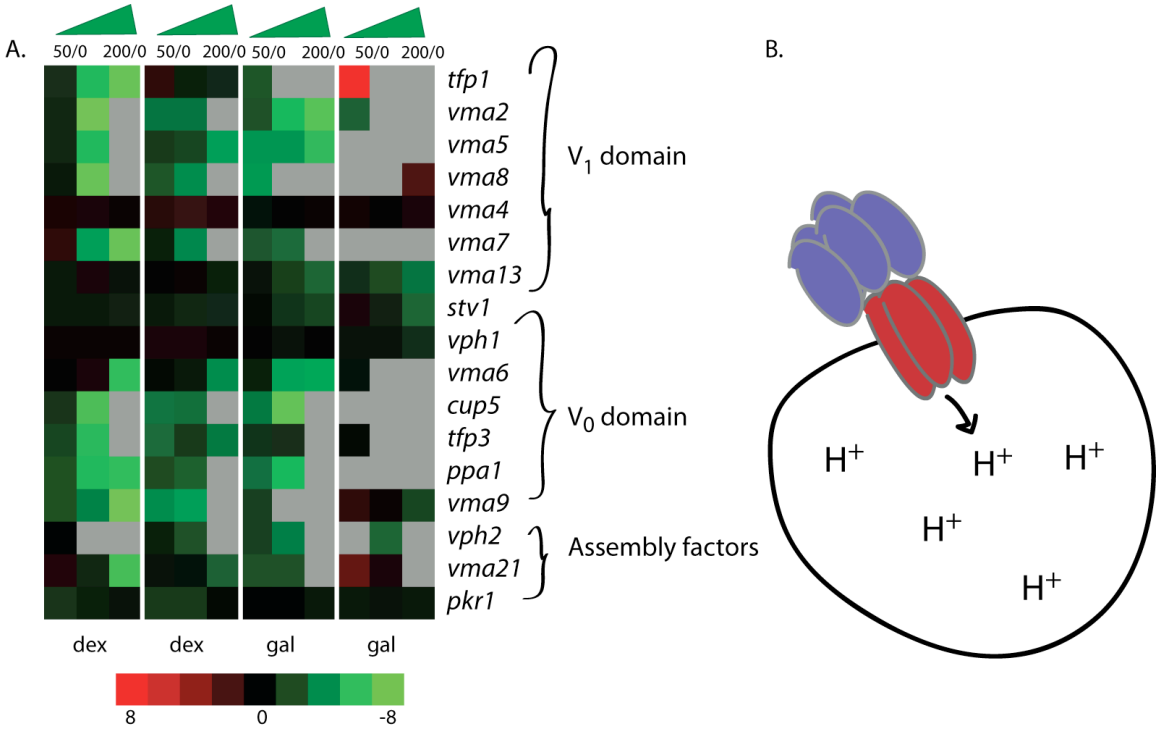


Figure 4. *vma* mutants exhibit pronounced chloroquine sensitivity.
A. Deletion of v-ATPase components or assembly factors renders yeast unable to acidify their vacuoles. These strains demonstrate significant sensitivity to chloroquine treatment (heatmap reflects colony size normalized by that in the absence of drug).
B. Cartoon of the v-ATPase. The V_1 domain (purple) hydrolyzes ATP, while the V_0 domain (red) forms a proton transport channel in the vacuole membrane.

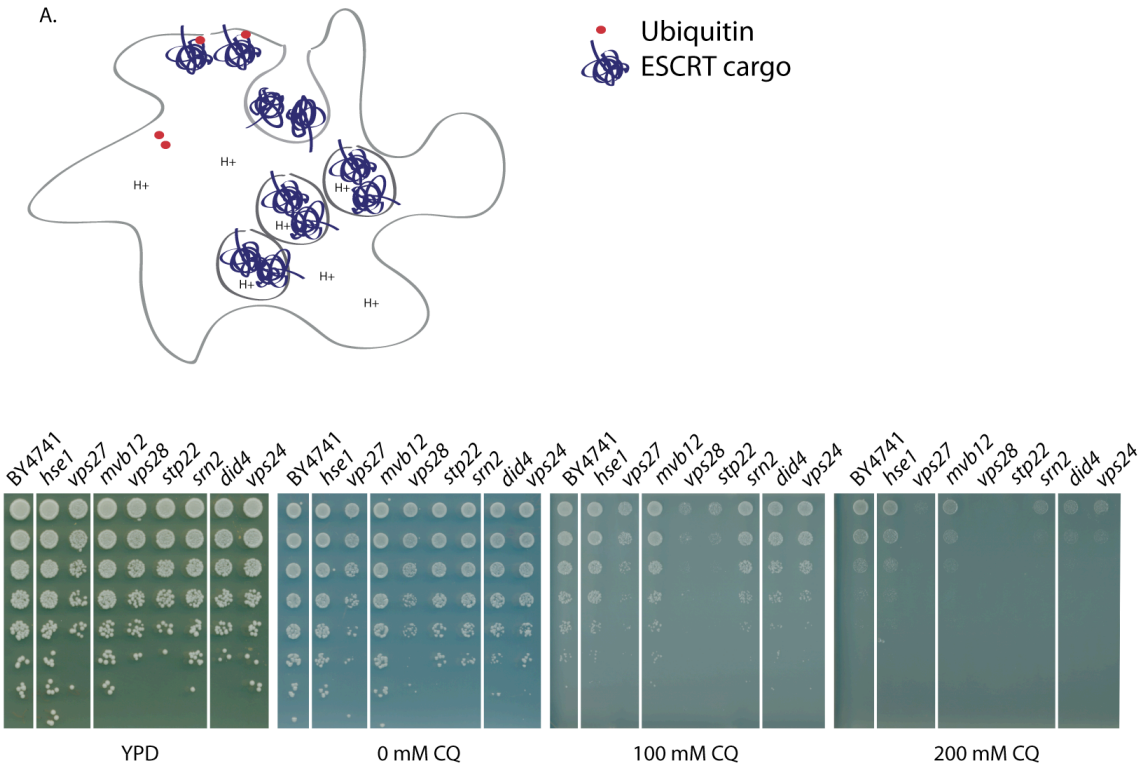
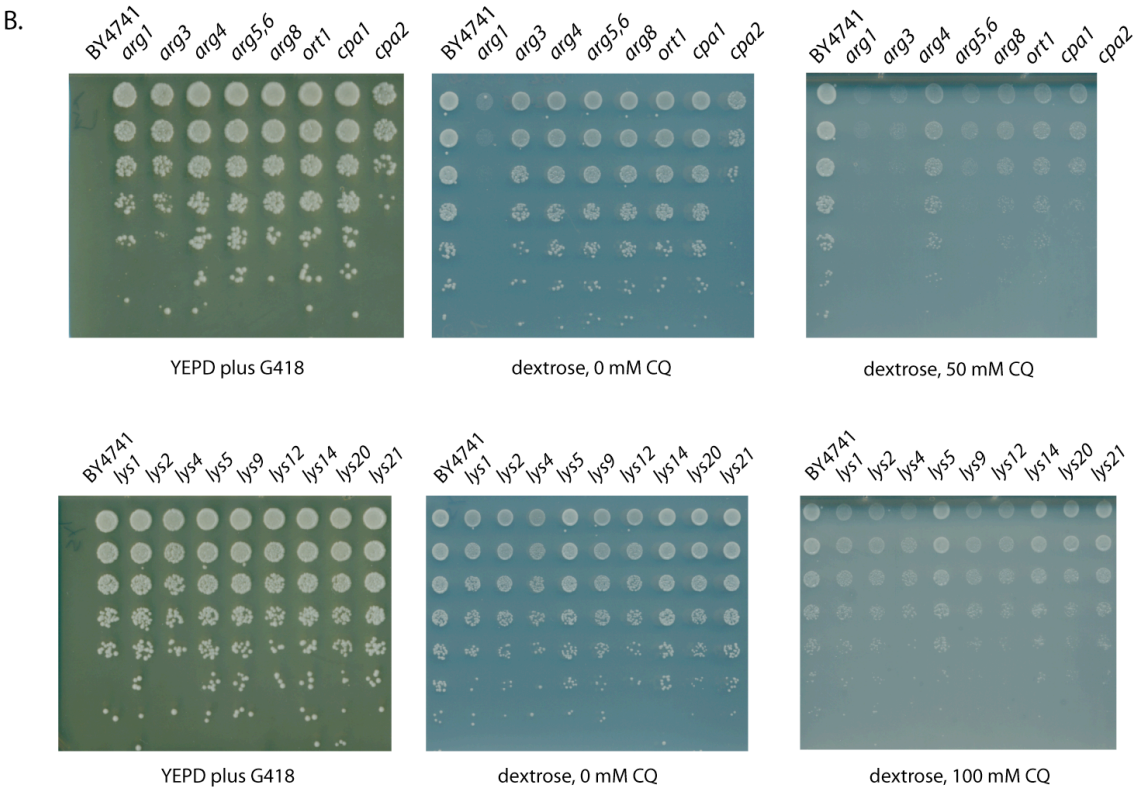
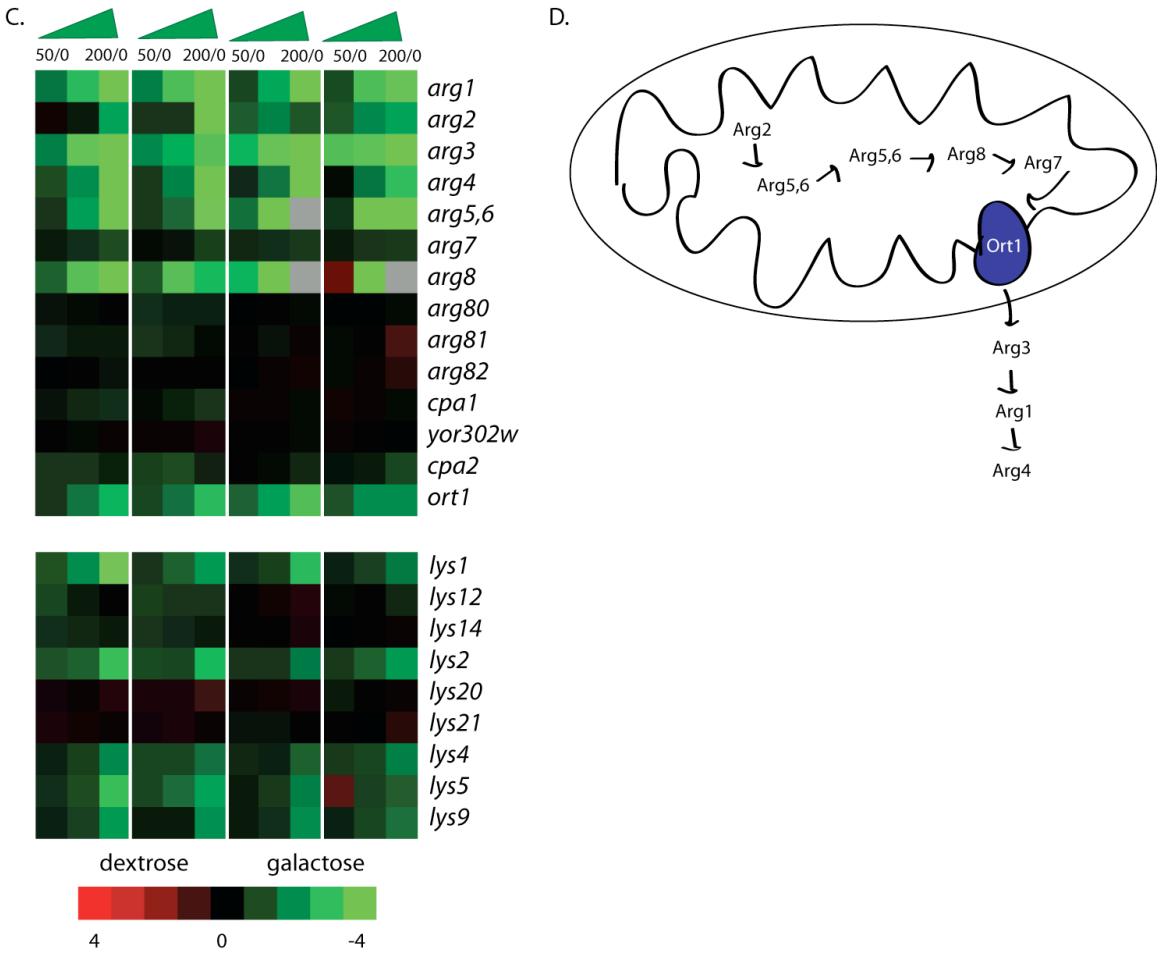


Figure 5. Chloroquine sensitivity in protein-trafficking mutants and arginine and lysine auxotrophs.
A. ESCRT mutants are chloroquine-sensitive. ESCRT, the endosomal sorting complex required for transport, recruits cargo into multivesicular bodies within the endosome. ESCRT-0 (Hse1 and Vps27) interacts with ubiquitylated cargo proteins, while ESCRT-I (Mvb12, Vps28, Stp22, Srn2) and -II (Vps22, Vps25, Vps36) initiate vesiculation; ESCRT-III (Snf7, Vps20, Did4, Vps24) mediates vesicle scission. ESCRT complex members are Class E *vps* mutants with a highly acidic endosome unable to effect anterograde transport. Note that ESCRT-II and ESCRT-III are chloroquine-hypersensitive.



B. Amino-acid biosynthetic deficiencies confer chloroquine sensitivity. Both arginine and lysine are stored in the vacuole and their uptake is H^+-coupled, suggesting that diminution or abrogation by chloroquine of the vacuolar pH gradient is responsible for the growth defect. Note that arginine auxotrophs are more sensitive than lysine in serial dilution assays. The BY4741 control strain was sensitive to G418 (left panel).



C. Chemical-genetic profiles of arginine and lysine auxotrophs. *CPA1* and *YOR302w*/*CPA2* encode biosynthetic factors for the arginine precursor citrulline. *ARG80-82* and *LYS14* encode transcription factors that activate biosynthesis. Lys20/21 are redundant. Heatmap data indicate colony sizes relative to that in the absence of chloroquine.

D. Cartoon of arginine biosynthesis. Ornithine is produced in the mitochondrion and exported to the cytosol for arginine synthesis.

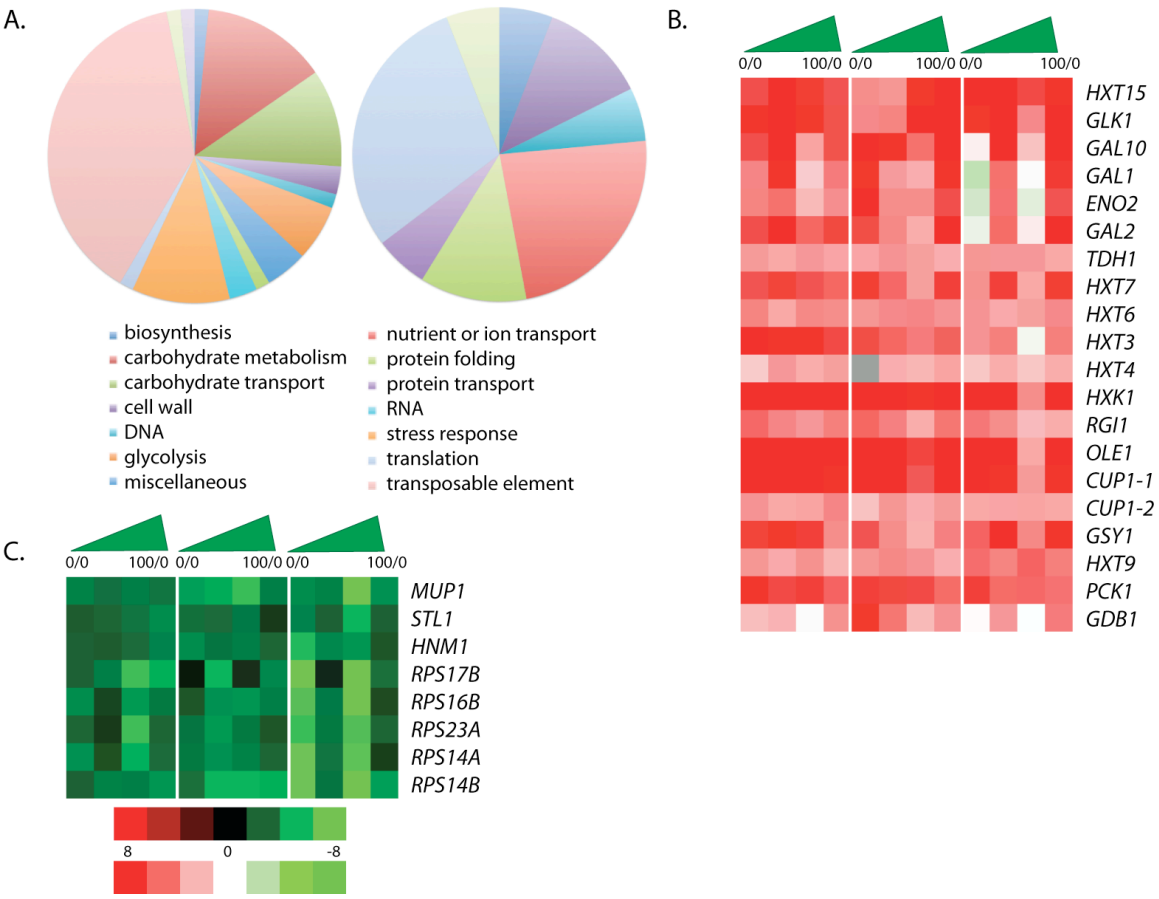


Figure 6. Chloroquine-mediated transcriptional alterations.
A. Left panel: transcripts most induced by chloroquine irrespective of dosage concentration or timepoint. Right panel: transcripts most repressed.
B. Chloroquine treatment upregulates glycolytic genes and carbohydrate transporters, as well as metallothioneins and two transcripts induced under iron deprivation.
C. Chloroquine downregulates ribosomal genes and plasma-membrane nutrient permeases.
Data are normalized with respect to the t=0 sample.

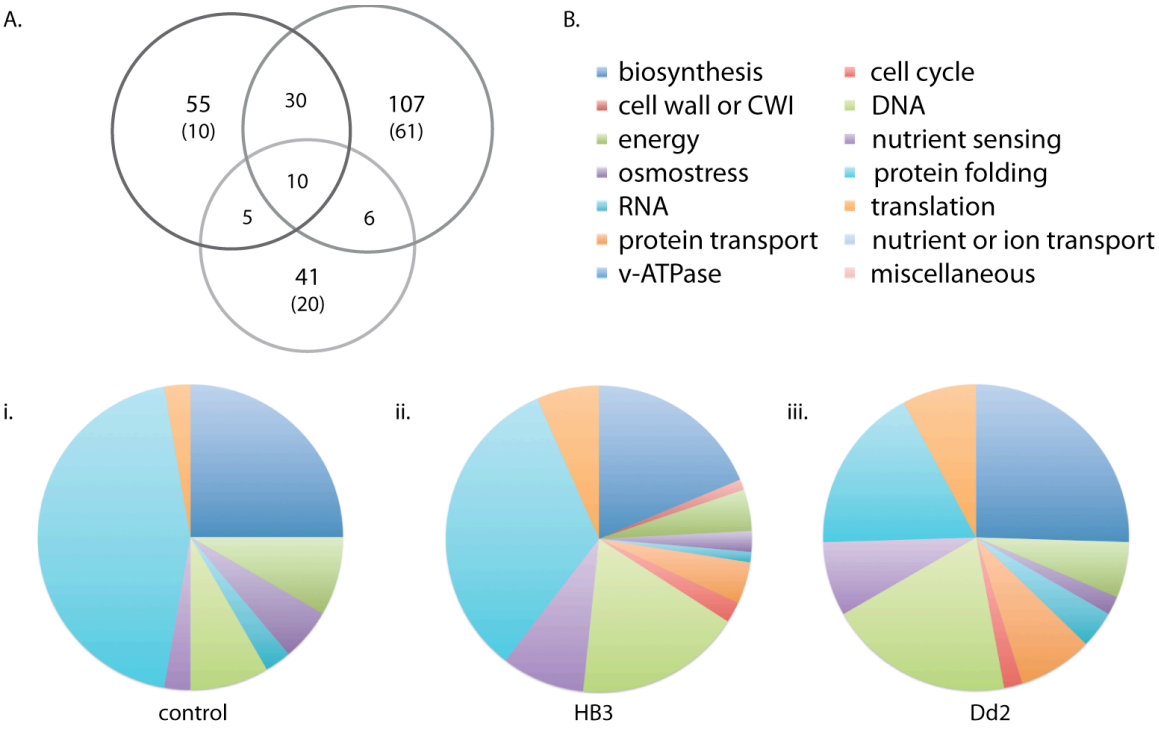


Figure 7. Chloroquine sensitivity in libraries constructed for synthetic genetic analysis.

A. Chloroquine sensitivity in different SGA libraries. 55 strains expressing chloroquine-resistant PfCRT and 107 expressing chloroquine-sensitive PfCRT are chloroquine-sensitive. 41 control strains are chloroquine-sensitive. 40 strains are chloroquine-sensitive irrespective of *PFCRT* allele expressed. 10 strains from all libraries are sensitive to chloroquine.

B. Categories of mutants affected by drug in each library. Protein-transport and biosynthetic defects cause chloroquine sensitivity in all genetic backgrounds. Strains expressing *PFCRT* also demonstrate chemical-genetic interactions with transcription and translation machinery.

i. control library; ii. HB3 (chloroquine-sensitive) library; iii. Dd2 (chloroquine-resistant) library

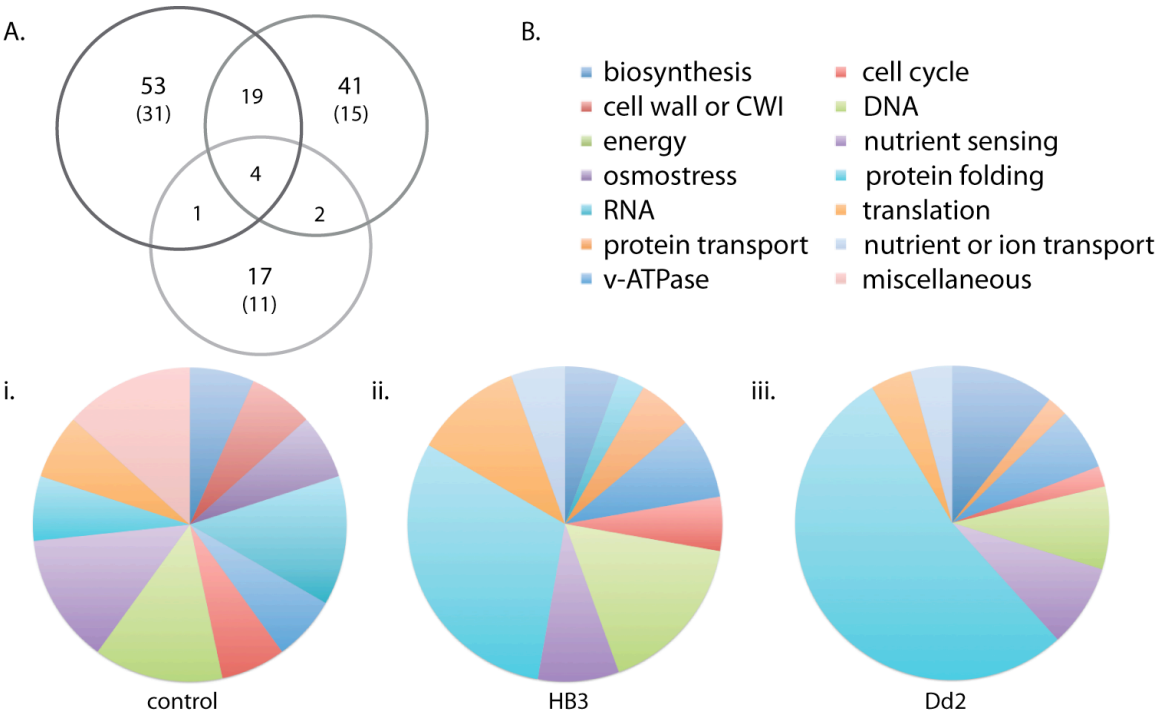


Figure 8. Chloroquine resistance in libraries constructed for synthetic genetic analysis.

A. Chloroquine resistance in different SGA libraries. 53 strains expressing chloroquine-resistant PfCRT and 41 expressing chloroquine-sensitive are refractory to chloroquine, as well as 17 control strains. 22 strains are chloroquine-resistant irrespective of *PFCRT* allele expressed; 4 strains from all libraries are chloroquine-resistant.

B. Categories of mutants affected by drug in each library. Expression of either *PFCRT* allele suppresses protein trafficking defects, although this effect is more evident in the Dd2 chloroquine-resistant background.

i. control library; ii. HB3 (chloroquine-sensitive) library; iii. Dd2 (chloroquine-resistant) library

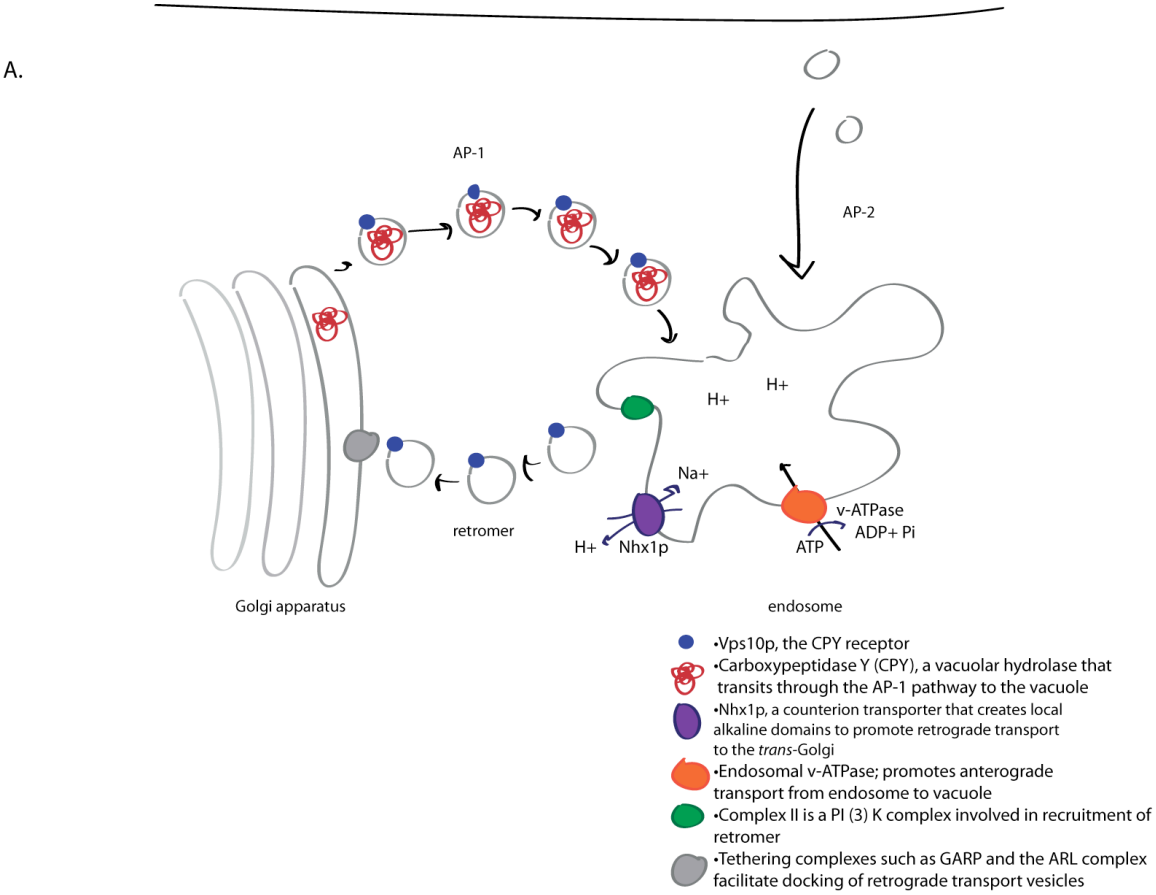
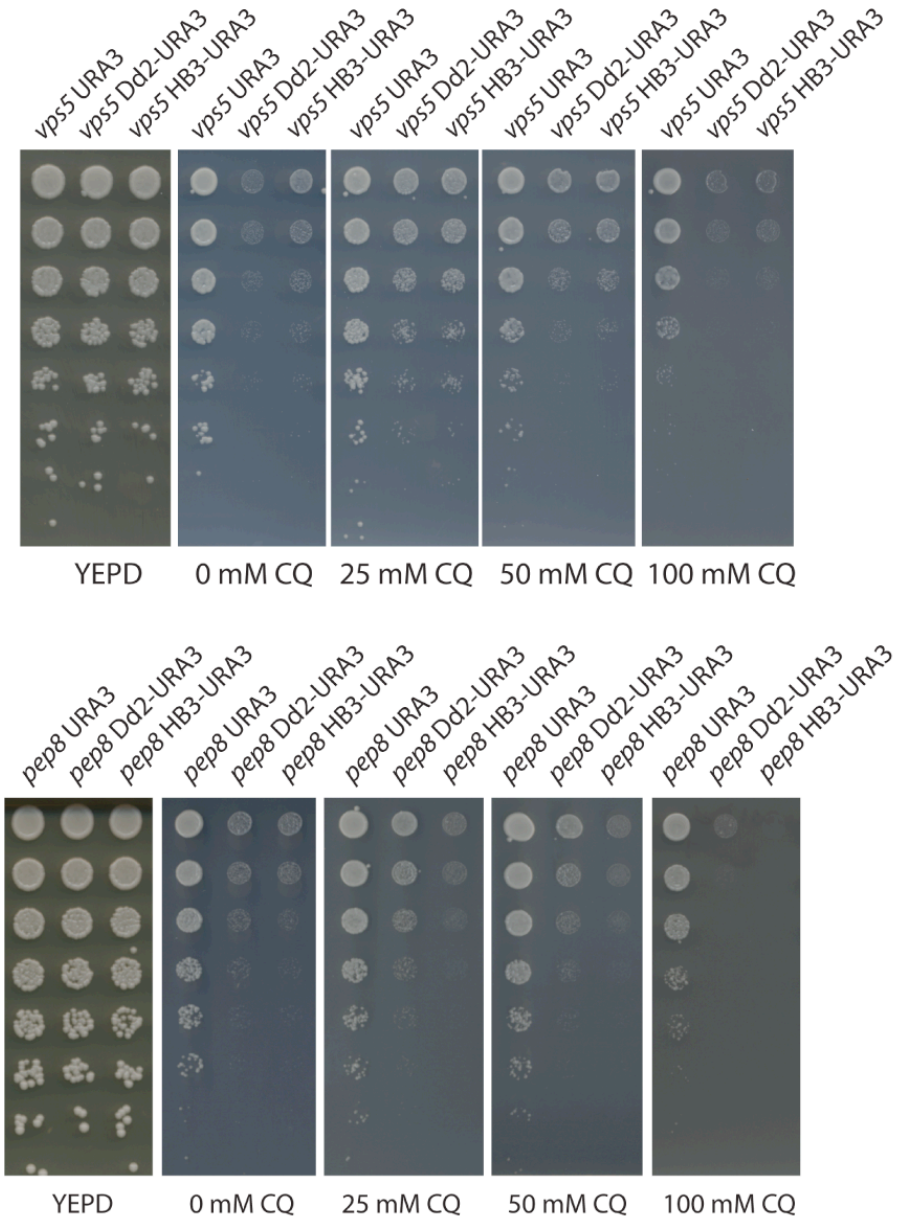
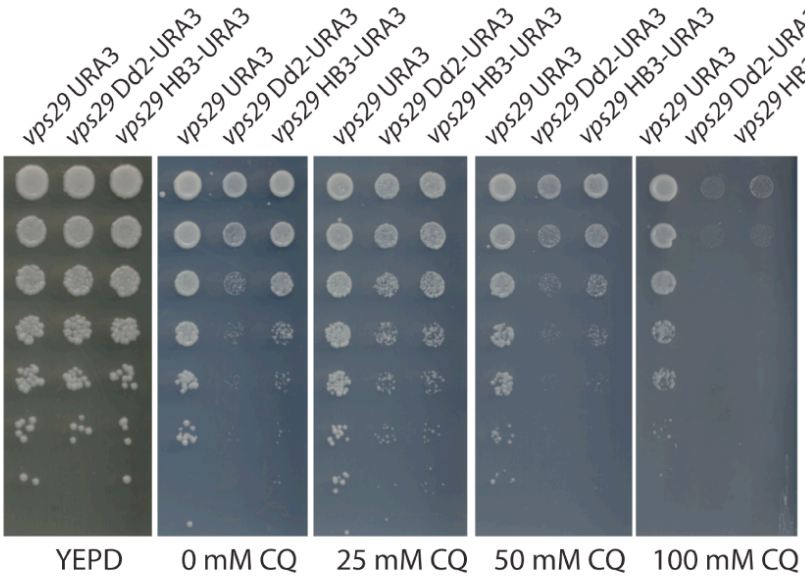


Figure 9. Anterograde and retrograde transport from the endosome.
A. Cartoon of transport. Drs2p and Cdc50p participate in vesicle formation to target proteins to the endosome; retromer (Vps5, 17, 29, 35; Pep8), complex II (Vps30), tethering complexes (Arl1, 3; Vps51), and the small GTPase Ypt6 (and its GEF Ric1) recycle adapters. Maintenance of a pH gradient is essential for appropriate trafficking, and Dd2's suppressive effect probably works through its extrusion of chloroquine to restore endosomal pH microdomains.



B. Chloroquine-resistant PfCRT suppresses the growth defect of retromer-deficient strains. Note that growth of Dd2-expressing mutants is augmented by chloroquine, suggesting a specific interaction between protein and substrate. Vps5 (and Vps17, not in library) recruit retromer cargo; Pep8 coordinates cargo recruitment with the coat proteins (Vps29 and Vps35).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vps29 HB3-URA3
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YEPD
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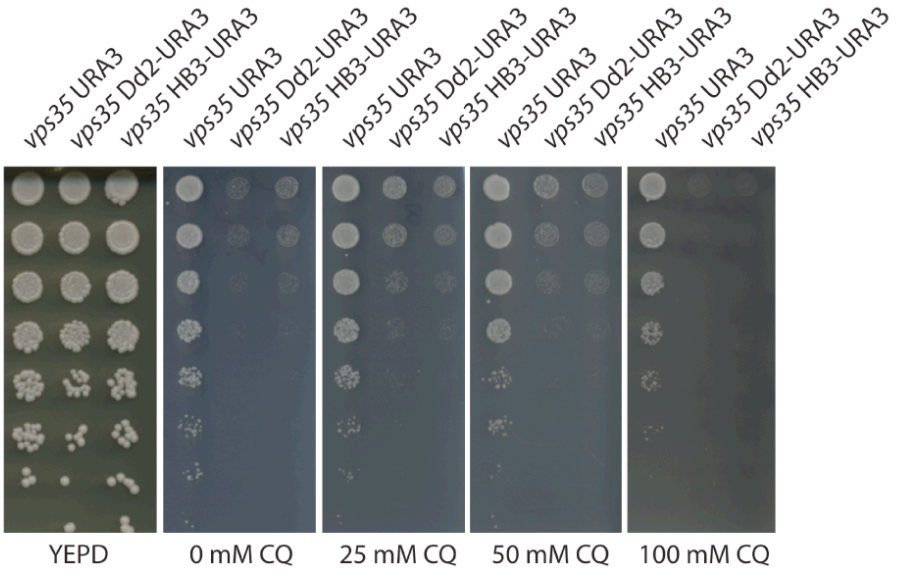
25 mM CQ
50 mM CQ
100 mM CQ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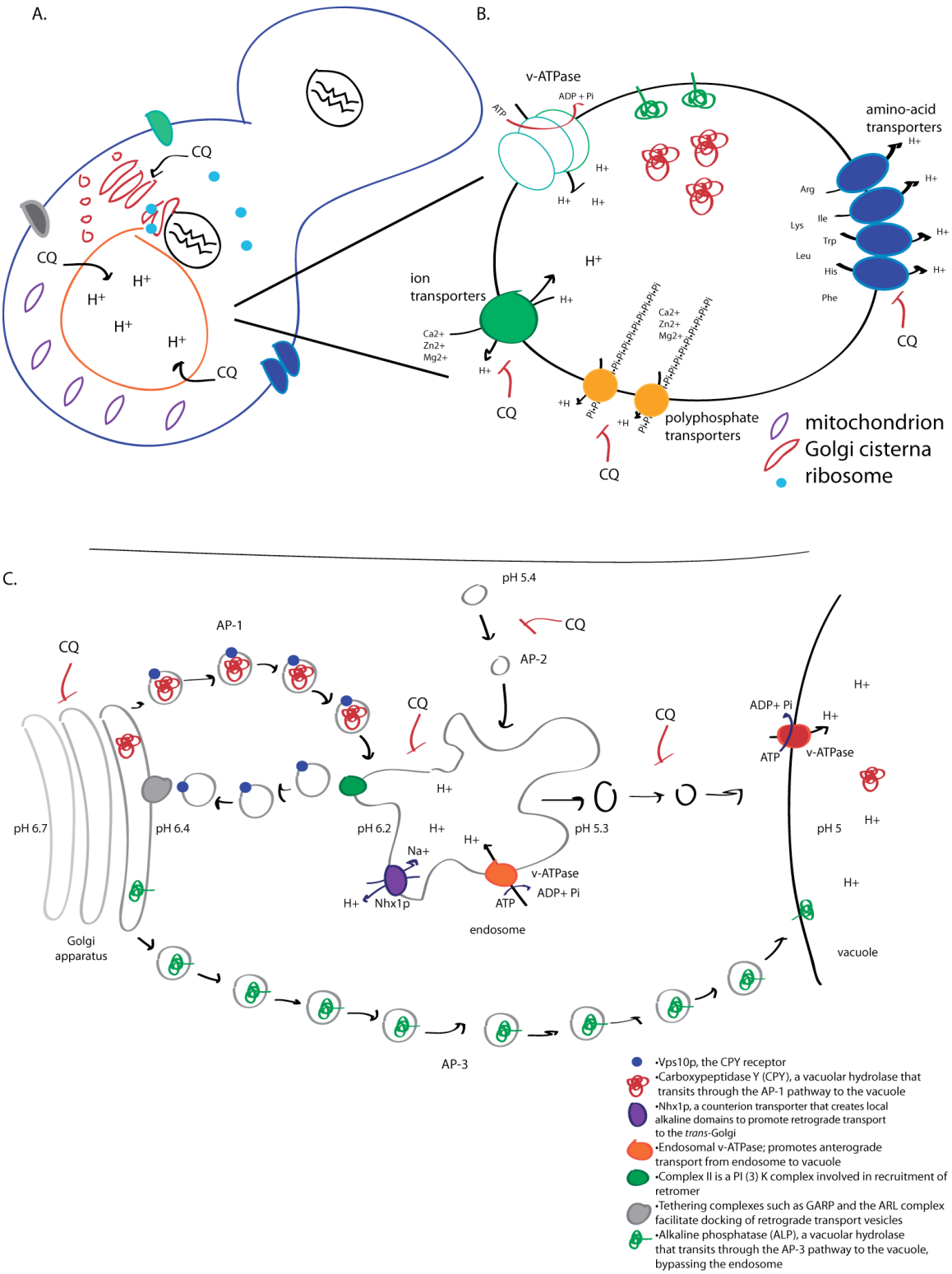


Figure 10. Yeast response to chloroquine.
A. The yeast cell wall responds to extracellular chloroquine by activating the cell wall integrity pathway (note membrane sensors). Intracellular drug diffuses into acidic compartments, including the vacuole, where it becomes membrane-impermeant through diprotonation.

B. The vacuole is both a hydrolytic and a storage compartment and maintains cellular nitrogen, phosphate, and ion reserves. Uptake is dependent on acidification (v-ATPase), and chloroquine partitioning into the compartment likely impairs both uptake and mobilization of nutrient and ion stores, resulting in activation of stress responses. Protein degradation can reportedly occur, albeit at a reduced rate, irrespective of vacuolar acidification.
C. Chloroquine diffusion into and retention in acidic secretory compartments such as the Golgi, endosome, and vacuole impairs protein progression through the secretory pathway through perturbation of pH homeostasis. Protein trafficking via the endosome (i.e., through AP-1 or AP-2 pathways) is chloroquine-sensitive. Suppression by chloroquine-resistant PfCRT of the chloroquine-mediated trafficking defect seemingly occurs preferentially at the endosome, as protein endocytosis from the cell surface, as well as both anterograde and retrograde transport from *trans*-Golgi network to endosome, are rescued by expression of Dd2-PfCRT. In contrast, transport from endoplasmic reticulum to Golgi or from *trans*-Golgi to vacuole via the AP-3 pathway cannot be rescued by PfCRT expression. .

Table 1. Strains exhibiting chloroquine-sensitivity on dextrose and galactose.

Systematic name	ORF name	Cell process	Fold Change, Screen 1 (dex)	Fold Change, Screen 2 (dex)	Fold Change, Screen 1 (gal)	Fold Change, Screen 2 (gal)
YJL088W	ARG3	biosynthesis (amino acid)	0.09	0.30	0.15	0.14
YER069W	ARG5-6	biosynthesis (amino acid)	0.09	0.25	0.00	0.22
YOL058W	ARG1	biosynthesis (amino acid)	0.15	0.18	0.18	0.27
YOL140W	ARG8	biosynthesis (amino acid)	0.08	0.39	0.00	0.00
YHR018C	ARG4	biosynthesis (amino acid)	0.08	0.16	0.13	0.35
YGL154C	LYS5	biosynthesis (amino acid)	0.35	0.43	0.55	0.66
YBR115C	LYS2	biosynthesis (amino acid)	0.34	0.39	0.56	0.46
YIR034C	LYS1	biosynthesis (amino acid)	0.26	0.46	0.36	0.56
YNR050C	LYS9	biosynthesis (amino acid)	0.46	0.49	0.51	0.60
YJL071W	ARG2	biosynthesis (amino acid)	0.44	0.25	0.67	0.43
YOR130C	ORT1	biosynthesis (amino acid)	0.40	0.37	0.31	0.50
YDR234W	LYS4	biosynthesis (amino acid)	0.52	0.59	0.65	0.54
YER086W	ILV1	biosynthesis (amino acid)	0.34	0.62	0.24	0.44
YGL012W	ERG4	biosynthesis (ergosterol)	0.08	0.00	0.12	0.23
YLR056W	ERG3	biosynthesis (ergosterol)	0.47	0.49	0.22	0.18
YER014W	HEM14	biosynthesis (heme)	0.51	0.41	0.49	0.26
YBR029C	CDS1	biosynthesis (lipid)	0.23	0.31	0.33	0.34
YJR073C	OPI3	biosynthesis (lipid)	0.42	0.49	0.42	0.35
YEL029C	BUD16	biosynthesis (other)	0.32	0.36	0.46	0.54
YBL033C	RIB1	biosynthesis (other)	0.69	0.51	0.57	0.41
YML022W	APT1	biosynthesis (purine)	0.37	0.47	0.60	0.64
YDR226W	ADK1	biosynthesis (purine)	0.63	0.59	0.41	0.30
YJR057W	CDC8	biosynthesis (pyrimidine)	0.49	0.25	0.40	0.37
YAL047C	SPC72	cell cycle	0.54	0.32	0.50	0.31
YML064C	TEM1	cell cycle	0.30	0.22	0.15	0.25
YJL174W	KRE9	cell wall	0.08	0.18	0.31	0.42
YGR229C	SMI1	cell wall	0.90	0.61	0.59	0.38
YPR159W	KRE6	cell wall	0.46	0.37	0.41	0.29
YEL004W	YEA4	cell wall	0.34	0.50	0.44	0.68
YHR030C	SLT2	cell wall integrity	0.00	0.00	0.00	0.00
YJL095W	BCK1	cell wall integrity	0.00	0.00	0.00	0.00
YKL049C	CSE4	DNA	0.23	0.24	0.21	0.61
YBR202W	MCM7	DNA	0.37	0.32	0.00	0.29
YLL004W	ORC3	DNA	0.21	0.23	0.62	0.53
YNL218W	MGS1	DNA	0.31	0.48	0.38	0.62
YDL074C	BRE1	DNA	0.77	0.67	0.63	0.59
YER068C-A	YER068C-A	dubious	0.09	0.24	0.00	0.18
YBR190W	YBR190W	dubious, overlaps RPL21A	0.25	0.12	0.19	0.29
YPL238C	YPL238C	dubious, overlaps SUI3	0.53	0.41	0.21	0.17
YGL168W	HUR1	dubious/ overlaps PMR1	0.30	0.30	0.32	0.32
YKL118W	YKL118W	dubious/ overlaps VPH2	0.00	0.00	0.00	0.00
YLR087C	CSF1	energy	0.58	0.67	0.52	0.55
YFR029W	PTR3	nutrient sensing	0.12	0.26	0.38	0.56
YPR160W	GPH1	osmostress	0.72	0.57	0.46	0.28

YLR336C	SGD1	osmostress	0.14	0.00	0.00	0.00
YDL006W	PTC1	osmostress	0.73	0.64	0.62	0.53
YGR285C	ZUO1	protein folding	0.09	0.32	0.13	0.19
YDR212W	TCP1	protein folding	0.65	0.54	0.61	0.40
YER017C	AFG3	protein folding	0.00	0.00	0.00	0.00
YNL007C	SIS1	protein folding	0.34	0.45	0.15	0.17
YDR200C	VPS64	protein transport	0.54	0.60	0.66	0.42
YKR001C	VPS1	protein transport	0.54	0.77	0.00	0.09
YKL212W	SAC1	protein transport	0.12	0.32	0.00	0.00
YKL135C	APL2	protein transport (clathrin AP-1 adapter)	0.49	0.38	0.35	0.40
YGL206C	CHC1	protein transport (clathrin)	0.67	0.36	0.32	0.00
YBR097W	VPS15	protein transport (complex II)	0.00	0.00	0.00	0.00
YDR495C	VPS3	protein transport (CORVET)	0.14	0.00	0.16	0.00
YOR089C	VPS21	protein transport (CORVET)	0.58	0.65	0.62	0.66
YML097C	VPS9	protein transport (CORVET)	0.52	0.61	0.34	0.17
YBL007C	SLA1	protein transport (endocytosis)	0.53	0.44	0.36	0.34
YGL095C	VPS45	protein transport (endosome)	0.00	0.18	0.00	0.00
YMR077C	VPS20	protein transport (ESCRT)	0.65	0.66	0.19	0.60
YJR102C	VPS25	protein transport (ESCRT)	0.67	0.45	0.25	0.00
YPL065W	VPS28	protein transport (ESCRT)	0.53	0.50	0.23	0.25
YCL008C	STP22	protein transport (ESCRT)	0.66	0.80	0.31	0.60
YLR025W	SNF7	protein transport (ESCRT)	0.64	0.44	0.21	0.36
YPL002C	SNF8	protein transport (ESCRT)	0.85	0.65	0.29	0.26
YKL002W	DID4	protein transport (ESCRT)	0.85	0.84	0.32	0.22
YLR330W	CHS5	protein transport (exomer)	0.24	0.51	0.62	0.54
YDR027C	VPS54	protein transport (GARP)	0.66	0.50	0.00	0.00
YDR484W	VPS52	protein transport (GARP)	0.46	0.54	0.00	0.00
YEL036C	ANP1	protein transport (Golgi alpha 1,6 mannosyltransferase)	0.49	0.23	0.00	0.27
YDR245W	MNN10	protein transport (Golgi alpha 1,6 mannosyltransferase)	0.77	0.43	0.38	0.34
YJL062W	LAS21	protein transport (GPI anchor synthesis)	0.56	0.50	0.30	0.26
YDR080W	VPS41	protein transport (HOPS)	0.57	0.83	0.32	0.45
YGR036C	CAX4	protein transport (N-linked glycosylation)	0.63	0.54	0.58	0.35
YML019W	OST6	protein transport (N-linked glycosylation)	0.65	0.66	0.47	0.50
YOR069W	VPS5	protein transport (retromer)	0.76	0.59	0.53	0.36
YGR166W	KRE11	protein transport (TRAPP)	0.62	0.47	0.51	0.52

YGL212W	VAM7	protein transport (vacuolar fusion)	0.19	0.43	0.00	0.00
YML043C	RRN11	RNA	0.47	0.44	0.24	0.29
YLR399C	BDF1	RNA	0.94	0.46	0.39	0.21
YDR028C	REG1	RNA	0.00	0.00	0.20	0.00
YIL021W	RPB3	RNA	0.12	0.00	0.00	0.00
YDR190C	RVB1	RNA	0.10	0.19	0.29	0.34
YPL129W	TAF14	RNA	0.19	0.16	0.25	0.00
YJL006C	CTK2	RNA	1.12	0.66	0.20	0.00
YML112W	CTK3	RNA	0.99	0.61	0.20	0.25
YGR186W	TFG1	RNA	0.35	0.37	0.49	0.45
YMR091C	NPL6	RNA	0.85	0.64	0.64	0.49
YKL054C	DEF1	RNA	0.33	0.28	0.19	0.00
YER013W	PRP22	RNA processing and export	0.19	0.18	0.44	0.45
YMR268C	PRP24	RNA processing and export	0.21	0.30	0.14	0.63
YGR095C	RRP46	RNA processing and export	0.22	0.36	0.39	0.55
YML046W	PRP39	RNA processing and export	0.66	0.59	0.50	0.36
YMR239C	RNT1	RNA processing and export	0.52	0.65	0.31	0.32
YPL169C	MEX67	RNA processing and export	0.51	0.44	0.12	0.00
YNR052C	POP2	RNA processing and export	0.86	0.67	0.64	0.30
YHR062C	RPP1	RNA processing and export	0.65	0.55	0.25	0.25
YLR244C	MAP1	translation	0.48	0.28	0.40	0.45
YOR145C	PNO1	translation	0.34	0.50	0.15	0.29
YDL153C	SAS10	translation	0.22	0.19	0.00	0.00
YDR037W	KRS1	translation	0.09	0.18	0.00	0.49
YDR341C	RRS1	translation	0.15	0.23	0.18	0.64
YHR010W	RPL27A	translation	0.14	0.35	0.12	0.00
YML025C	YML6	translation	0.37	0.24	0.56	0.66
YDR322W	MRPL35	translation	0.50	0.70	0.26	0.18
YKL081W	TEF4	translation	0.38	0.57	0.33	0.39
YMR116C	ASC1	translation	0.66	0.26	0.31	0.20
YGR083C	GCD2	translation	0.34	0.33	0.17	0.24
YGR217W	CCH1	transport (calcium)	0.16	0.21	0.30	0.24
YNL291C	MID1	transport (calcium)	0.00	0.21	0.25	0.38
YJL129C	TRK1	transport (potassium)	0.15	0.20	0.00	0.18
YER060W-A	FCY22	transport (purine)	0.56	0.56	0.64	0.64
YOR306C	MCH5	transport (riboflavin)	0.28	0.43	0.14	0.16
YBR021W	FUR4	transport (uracil)	0.41	0.45	0.47	0.67
YDL160C-A	YDL160C-A	unknown	0.26	0.38	0.42	0.56
YJR005C-A	YJR005C-A	unknown	0.32	0.41	0.51	0.58
YKL096C-B	YKL096C-B	unknown	0.53	0.44	0.41	0.35
YIR042C	YIR042C	unknown	0.75	0.67	0.46	0.22
YGR196C	FYV8	unknown	0.38	0.28	0.12	0.34
YDL185W	TFP1	v-ATPase	0.14	0.78	0.00	0.00

YBR127C	VMA2	v-ATPase	0.00	0.00	0.16	0.00
YHR039C-B	VMA10	v-ATPase	0.00	0.00	0.00	0.00
YPL234C	TFP3	v-ATPase	0.00	0.42	0.00	0.00
YKL119C	VPH2	v-ATPase	0.00	0.00	0.00	0.00
YGR105W	VMA21	v-ATPase	0.19	0.51	0.00	0.00
YLR447C	VMA6	v-ATPase	0.21	0.35	0.27	0.00
YCL005W-A	VMA9	v-ATPase	0.13	0.00	0.00	0.62

BOLD: multidrug-sensitive; <u>Underlined</u>: multidrug-resistant (Parsons et al. 2006)

Table 2a. Transcripts induced by chloroquine treatment.

Systematic name	Gene name	Cell process	Fold induction, 25 mM	Fold induction, 50 mM	Fold induction, 100 mM
YGL055W	OLE1	biosynthesis	8.09	3.03	7.58
YBR019C	GAL10	carbohydrate metabolism	3.65	0.74	4.86
YBR020W	GAL1	carbohydrate metabolism	3.83	0.96	6.29
YCL040w	GLK1	carbohydrate metabolism	5.86	2.29	6.15
YFR017C	YFR017C	carbohydrate metabolism	2.79	2.57	2.72
YFR053C	HXK1	carbohydrate metabolism	3.32	0.88	3.37
YMR105C	PGM2	carbohydrate metabolism	5.64	4.73	6.30
YMR303C	ADH2	carbohydrate metabolism	2.71	1.78	3.28
YPR184W	GDB1	carbohydrate metabolism	2.67	2.65	2.32
YKR097W	PCK1	carbohydrate metabolism	3.19	4.27	3.30
YDL245C	HXT15	carbohydrate transport	2.16	1.74	2.16
YDR342C	HXT7	carbohydrate transport	7.03	2.59	6.92
YDR343C	HXT6	carbohydrate transport	11.10	2.29	8.45
YDR345C	HXT3	carbohydrate transport	17.76	2.94	11.96
YHR092C	HXT4	carbohydrate transport	2.91	1.90	2.16
YLR081w	GAL2	carbohydrate transport	4.01	1.19	11.55
YJL219W	HXT9	carbohydrate transport	2.34	2.37	2.30
YBR067C	TIP1	cell wall	3.32	2.27	3.80
YNL160W	YGP1	cell wall	3.97	2.02	6.22
YLR085C	ARP6	DNA	2.04	1.91	2.97
YDR344C	YDR344C	dubious	3.98	3.57	3.49
YGR008C	STF2	energy	3.84	4.71	4.44
YDL021W	GPM2	glycolysis	2.11	2.15	2.05
YHR174W	ENO2	glycolysis	11.06	1.78	8.18
YJL052W	TDH1	glycolysis	2.66	0.99	3.43
YOL086C	ADH1	glycolysis	4.46	0.97	4.03
YDL038c	PRM7	pheromone; regulated by Gcn4	2.13	3.75	2.62
YCL057w	PRD1	protein degradation	3.12	2.57	3.35
YHR176W	FMO1	protein folding	2.61	1.99	2.23
YDL070w	BDF2	RNA	2.03	3.40	2.44
YLR228C	ECM22	RNA	2.50	2.08	4.01
YLR083c	EMP70	stress response	2.16	1.29	4.84
YCR021c	HSP30	stress response	3.48	3.59	2.46
YER067w	RGI1	stress response	3.99	4.05	3.70
YFR015C	GSY1	stress response	2.23	2.49	3.02
YHR053C	CUP1-1	stress response	9.62	5.36	7.14
YHR055C	CUP1-2	stress response	9.58	2.96	8.30

YMR251W-A	HOR7	stress response	18.24	3.04	11.87
YLR327C	TMA10	translation	7.00	5.70	7.64
YAR010C	YAR010C	transposable element	2.17	2.75	3.83
YBL005W-B	YBL005W-B	transposable element	2.72	1.23	4.18
YDR098C-B	YDR098C-B	transposable element	2.40	1.69	3.87
YDR261C-C	YDR261C-C	transposable element	2.65	2.70	5.19
YDR316W-B	YDR316W-B	transposable element	2.55	1.65	4.50
YDR365W-A	YDR365W-A	transposable element	3.21	2.96	5.86
YER160c	YER160C	transposable element	2.48	1.76	5.16
YFL002W-A	YFL002W-A	transposable element	2.82	1.76	4.01
YGR038C-B	YGR038C-B	transposable element	2.41	1.59	4.57
YGR161C-C	YGR161C-C	transposable element	2.66	2.62	5.29
YHR214C-B	YHR214C-B	transposable element	2.67	1.46	4.15
YJR026W	YJR026W	transposable element	3.39	1.97	4.49
YJR028W	YJR028W	transposable element	2.93	1.48	4.25
YLR157C-A	YLR157C-A	transposable element	3.07	2.73	7.97
YLR227W-A	YLR227W-A	transposable element	4.29	2.01	6.95
YLR410W-B	YLR410W-B	transposable element	2.34	2.10	4.49
YML039W	YML039W	transposable element	2.30	1.55	4.22
YMR045C	YMR045C	transposable element	2.40	1.66	3.55
YMR050C	YMR050C	transposable element	2.26	1.71	4.44
YOL103W-A	YOL103W-A	transposable element	2.40	3.22	5.38
YOR142W-B	YOR142W-B	transposable element	2.10	2.31	5.08
YOR343C-B	YOR343C-B	transposable element	2.14	1.28	3.74
YPL257W-A	YPL257W-A	transposable element	4.31	2.85	6.38
YPR137C-A	YPR137C-A	transposable element	3.69	2.34	5.95
YPR158W-A	YPR158W-A	transposable element	2.53	2.78	6.16
YPL186C	UIP4	unknown	2.66	4.82	4.62

Samples are induced at least twofold at two or more of three datapoints.
Data shown are for gene expression at 120 minutes relative to that in the initial experimental sample.

Table 2b. Transcripts repressed by chloroquine treatment.

Systematic name	Gene name	Cell process	Fold induction, 25 mM	Fold induction, 50 mM	Fold induction, 100 mM
YER062c	HOR2	biosynthesis	0.57	0.41	0.48
YKL096W	CWP1	cell wall	0.70	0.16	0.30
YLL064C	PAU18	cell wall	0.68	0.40	0.36
YBR088C	POL30	DNA	0.47	0.38	0.50
YDR250C	YDR250C	dubious	0.64	0.58	0.48
YDR536W	STL1	nutrient or ion transport	0.39	0.11	0.34
YGL077C	HNM1	nutrient or ion transport	0.51	0.25	0.51
YGR055W	MUP1	nutrient or ion transport	0.36	0.33	0.56
YJL193W	YJL193W	nutrient or ion transport	0.50	0.33	0.36
YDL093W	PMT5	protein modification	0.45	0.07	0.49
YNL038W	GPI15	protein modification	0.65	0.43	0.75
YNL036W	NCE103	protein transport	0.67	0.05	0.89
YCR031c	RPS14A	translation	0.78	0.06	0.46
YDL083C	RPS16B	translation	0.41	0.10	0.60
YDR447C	RPS17B	translation	0.42	0.18	0.49
YGR118W	RPS23A	translation	0.44	0.16	0.65
YJL191W	RPS14B	translation	0.43	0.09	0.30

BOLD: transcript repressed at all three timepoints, all concentrations of chloroquine. Other samples are repressed at least twofold (relative to t=0) at two of three timepoints.
Data shown are for gene expression at 120 minutes relative to that in the initial experimental sample.

Table 3. Chloroquine-sensitivity in SGA control library strains.

Systematic name	ORF name	Cell process	Fold Change, Library 1	Fold Change, Library 2
YDR127W	ARO1	biosynthesis (amino acid)	0.38	0.52
YGL148W	ARO2	biosynthesis (amino acid)	0.46	0.60
YPR060C	ARO7	biosynthesis (amino acid)	0.44	0.67
YLR056W	ERG3	biosynthesis (ergosterol)	0.17	0.25
YJR073C	OPI3	biosynthesis (lipid)	0.34	0.45
YNL169C	PSD1	biosynthesis (lipid)	0.39	0.42
YDL080C	THI3	biosynthesis (other)	0.55	0.53
YPR069C	SPE3	biosynthesis (polyamine)	0.28	0.45
YKR099W	BAS1	biosynthesis (purine, histidine)	0.36	0.66
YBR260C	RGD1	cell wall integrity	0.55	0.62
YJL095W	BCK1	cell wall integrity	0.51	0.53
YHR030C	SLT2	cell wall integrity	0.41	0.58
YGL058W	RAD6	DNA	0.67	0.37
YML023C	NSE5	DNA	0.67	0.68
YDR269C	YDR269C	dubious	0.65	0.66
YNL170W	YNL170W	dubious	0.27	0.44
YDR271C	YDR271C	dubious/ overlaps CCC2	0.66	0.68
YJL175W	YJL175W	dubious/ overlaps SWI3	0.39	0.44
YLR038C	COX12	energy	0.54	0.52
YOR122C	PFY1	protein transport	0.58	0.57
YKL212W	SAC1	protein transport	0.59	0.44
YKR001C	VPS1	protein transport	0.17	0.24
YML097C	VPS9	protein transport (CORVET)	0.54	0.66
YDR495C	VPS3	protein transport (CORVET)	0.24	0.41
YBL007C	SLA1	protein transport (endocytosis)	0.53	0.56
YJL029C	VPS53	protein transport (GARP)	0.36	0.53
YDR027C	VPS54	protein transport (GARP)	0.18	0.29
YDR484W	VPS52	protein transport (GARP)	0.20	0.28
YDR245W	MNN10	protein transport (Golgi alpha 1,6 mannosyltransferase)	0.58	0.58
YJR040W	GEF1	protein transport (Golgi chloride channel)	0.59	0.44
YGL084C	GUP1	protein transport (GPI anchor biosynthesis)	0.61	0.63
YDR080W	VPS41	protein transport (HOPS)	0.31	0.66
YGR036C	CAX4	protein transport (N-linked glycosylation)	0.50	0.42
YGL212W	VAM7	protein transport (vacuolar biogenesis)	0.45	0.62
YDR045C	RPC11	RNA	0.63	0.60
YHL025W	SNF6	RNA	0.42	0.62
YOR290C	SNF2	RNA	0.32	0.39
YKL081W	TEF4	translation	0.43	0.40
YDR270W	CCC2	transport (copper)	0.66	0.67
YER145C	FTR1	transport (iron)	0.64	0.58
YGR196C	FYV8	unknown	0.54	0.52

BOLD: multidrug-sensitive; <u>Underlined</u>: multidrug-resistant (Parsons et al. 2006)

Table 4. Chloroquine-sensitivity in SGA HB3 (chloroquine-sensitive) PfCRT library strains.

Systematic name	ORF name	Cell process	Fold Change, Library 1	Fold Change, Library 2
YEL046C	GLY1	biosynthesis (amino acid)	0.41	0.64
YDR127W	ARO1	biosynthesis (amino acid)	0.49	0.54
YGL148W	ARO2	biosynthesis (amino acid)	0.43	0.47
YBR248C	HIS7	biosynthesis (amino acid)	0.67	0.63
YLR056W	ERG3	biosynthesis (ergosterol)	0.23	0.45
YGR157W	CHO2	biosynthesis (lipid)	0.45	0.59
YNL169C	PSD1	biosynthesis (lipid)	0.46	0.47
YKL192C	ACP1	biosynthesis (lipid)	0.55	0.33
YJR073C	OPI3	biosynthesis (lipid)	0.13	0.26
YLR372W	SUR4	biosynthesis (lipid)	0.34	0.25
YKR072C	SIS2	biosynthesis (other)	0.61	0.65
YOR209C	NPT1	biosynthesis (other)	0.63	0.60
YDL080C	THI3	biosynthesis (other)	0.35	0.38
YOL052C	SPE2	biosynthesis (polyamine)	0.33	0.35
YPR069C	SPE3	biosynthesis (polyamine)	0.39	0.41
YKR099W	BAS1	biosynthesis (purine, histidine)	0.49	0.44
YML022W	APT1	biosynthesis (purine)	0.25	0.29
YDR532C	YDR532C	cell cycle	0.19	0.49
YOR008C	SLG1	cell wall integrity	0.58	0.56
YJL095W	BCK1	cell wall integrity	0.23	0.32
YHR030C	SLT2	cell wall integrity	0.22	0.20
YAL053W	FLC2	cell wall integrity	0.53	0.58
YGR180C	RNR4	DNA	0.30	0.40
YMR072W	ABF2	DNA	0.49	0.19
YGR237C	YGR237C	dubious	0.07	0.08
YEL045C	YEL045C	dubious	0.53	0.68
YLR402W	YLR402W	dubious	0.55	0.59
YOL150C	YOL150C	dubious	0.66	0.63
YNL170W	YNL170W	dubious	0.41	0.41
YOR135C	IRC14	dubious	0.30	0.65
YGL024W	YGL024W	dubious/ overlaps PGD1	0.22	0.25
YNL114C	YNL114C	dubious/ overlaps RPC19/ RNA pol	0.46	0.64
YKL083W	YKL083W	dubious/ overlaps RRP14	0.45	0.54
YLR317W	YLR317W	dubious/ overlaps TAD3	0.40	0.58
YLR338W	OPI9	dubious/ overlaps VRP1	0.39	0.39
YPL262W	FUM1	energy	0.38	0.20
YHR205W	SCH9	nutrient sensing	0.61	0.47
YOR014W	RTS1	nutrient sensing	0.17	0.19
YBR077C	SLM4	nutrient sensing (EGO complex)	0.35	0.40
YML121W	GTR1	nutrient sensing (EGO complex)	0.17	0.42
YJL008C	CCT8	protein folding	0.64	0.50
YOR265W	RBL2	protein folding	0.24	0.23
YBL017C	PEP1	protein transport	0.62	0.54
YDR372C	VPS74	protein transport	0.59	0.59

YKL212W	SAC1	protein transport	0.66	0.48
YGL023C	PIB2	protein transport	0.18	0.16
YCR032W	BPH1	protein transport	0.61	0.66
YBL058W	SHP1	protein transport	0.39	0.57
YOR070C	GYP1	protein transport	0.52	0.46
YPL145C	KES1	protein transport	0.42	0.52
YOL018C	TLG2	protein transport	0.30	0.31
YGR057C	LST7	protein transport	0.10	0.15
YNL238W	KEX2	protein transport	0.53	0.19
YBR164C	ARL1	protein transport (ARL complex)	0.63	0.49
YEL053C	MAK10	protein transport (ARL complex)	0.39	0.45
YPL051W	ARL3	protein transport (ARL complex)	0.42	0.42
YKR019C	IRS4	protein transport (autophagy)	0.47	0.48
YEL013W	VAC8	protein transport (CVT pathway)	0.65	0.49
YDL146W	LDB17	protein transport (endocytosis)	0.46	0.58
YJL204C	RCY1	protein transport (endocytosis)	0.48	0.55
YKR020W	VPS51	protein transport (GARP)	0.43	0.55
YDR245W	MNN10	protein transport (Golgi alpha 1,6 mannosyltransferase)	0.44	0.52
YGL084C	GUP1	protein transport (GPI anchor biosynthesis)	0.55	0.63
YML001W	YPT7	protein transport (HOPS)	0.57	0.26
YDR080W	VPS41	protein transport (HOPS)	0.63	0.51
YDR137W	RGP1	protein transport (retrograde)	0.60	0.66
YLR262C	YPT6	protein transport (retrograde)	0.38	0.65
YKR068C	BET3	protein transport (TRAPP)	0.58	0.57
YBR254C	TRS20	protein transport (TRAPP)	0.48	0.68
YDR108W	GSG1	protein transport (TRAPP)	0.47	0.63
YOR106W	VAM3	protein transport (vacuolar fusion)	0.38	0.50
YGL124C	MON1	protein transport (vacuolar fusion)	0.35	0.28
YBR131W	CCZ1	protein transport (vacuolar fusion)	0.23	0.16
YDR207C	UME6	RNA	0.29	0.46
YDR049W	YDR049W	RNA	0.55	0.49
YMR223W	UBP8	RNA	0.63	0.55
YPR023C	EAF3	RNA	0.61	0.61
YGL019W	CKB1	RNA	0.49	0.66
YCL010C	SGF29	RNA	0.63	0.64
YDL106C	PHO2	RNA	0.54	0.55
YMR172W	HOT1	RNA	0.45	0.53
YHL034C	SBP1	RNA	0.45	0.39
YDR448W	ADA2	RNA	0.62	0.45
YJL006C	CTK2	RNA	0.20	0.41
YJL127C	SPT10	RNA	0.25	0.35
YML112W	CTK3	RNA	0.28	0.24
YKL149C	DBR1	RNA processing and export	0.50	0.65
YGL246C	RAI1	RNA processing and export	0.57	0.66
YBR111W-A	SUS1	RNA processing and export	0.63	0.63
YHR189W	PTH1	translation	0.64	0.47
YDL081C	RPP1A	translation	0.57	0.64
YLR167W	RPS31	translation	0.53	0.62

YDR418W	RPL12B	translation	0.49	0.61
YKL009W	MRT4	translation	0.38	0.59
YPL249C-A	RPL36B	translation	0.50	0.43
YER177W	BMH1	translation	0.45	0.31
YOR293W	RPS10A	translation	0.28	0.39
YPR156C	TPO3	transport (polyamine)	0.64	0.66
YJL165C	HAL5	transport (potassium)	0.09	0.05
YJL129C	TRK1	transport (potassium)	0.27	0.34
YCR008W	SAT4	transport (potassium)	0.20	0.18
YAR042W	SWH1	transport (sterol; endocytosis)	0.58	0.52
YGR196C	FYV8	unknown	0.65	0.58
YGL114W	YGL114W	unknown	0.67	0.66
YKL037W	AIM26	unknown	0.45	0.51
YDR186C	YDR186C	unknown	0.51	0.48
YPL066W	YPL066W	unknown	0.57	0.61

BOLD: multidrug-sensitive; <u>Underlined</u>: multidrug-resistant (Parsons et al. 2006)

Table 5. Chloroquine-sensitivity in SGA Dd2 (chloroquine-resistant) PfCRT library strains.

Systematic name	ORF name	Cell process	Fold Change, Library 1	Fold Change, Library 2
YBR248C	HIS7	biosynthesis (amino acid)	0.58	0.61
YOR241W	MET7	biosynthesis (amino acid)	0.57	0.61
YPR060C	ARO7	biosynthesis (amino acid)	0.62	0.40
YDR127W	ARO1	biosynthesis (amino acid)	0.57	0.60
YJR073C	OPI3	biosynthesis (lipid)	0.50	0.27
YLR372W	SUR4	biosynthesis (lipid)	0.63	0.61
YKL192C	ACP1	biosynthesis (lipid)	0.68	0.62
YNL169C	PSD1	biosynthesis (lipid)	0.59	0.60
YDL080C	THI3	biosynthesis (other)	0.41	0.44
YOL049W	GSH2	biosynthesis (other)	0.62	0.53
YOL052C	SPE2	biosynthesis (polyamine)	0.61	0.32
YPR069C	SPE3	biosynthesis (polyamine)	0.58	0.63
YKR099W	BAS1	biosynthesis (purine, histidine)	0.50	0.59
YJL095W	BCK1	cell wall integrity	0.53	0.48
YBR260C	RGD1	cell wall integrity	0.68	0.45
YHR030C	SLT2	cell wall integrity	0.36	0.44
YMR072W	ABF2	DNA	0.67	0.35
YGR237C	YGR237C	dubious	0.11	0.12
YAR044W	YAR044W	dubious/ overlaps SWH1	0.63	0.60
YLR038C	COX12	energy	0.52	0.65
YPL262W	FUM1	energy	0.29	0.60
YOR014W	RTS1	nutrient sensing	0.61	0.37
YER120W	SCS2	nutrient sensing	0.66	0.57
YML121W	GTR1	nutrient sensing (EGO complex)	0.49	0.55
YBR077C	SLM4	nutrient sensing (EGO complex)	0.63	0.49
YOR265W	RBL2	protein folding	0.65	0.63
YAL026C	DRS2	protein transport	0.25	0.20
YGR057C	LST7	protein transport	0.35	0.35
YGL023C	PIB2	protein transport	0.35	0.50
YBL017C	PEP1	protein transport	0.55	0.50
YPL145C	KES1	protein transport	0.56	0.53
YPR051W	MAK3	protein transport (ARL complex)	0.55	0.59
YBL007C	SLA1	protein transport (endocytosis)	0.37	0.34
YDR080W	VPS41	protein transport (HOPS)	0.37	0.37
YOR106W	VAM3	protein transport (vacuolar fusion)	0.49	0.34
YGL124C	MON1	protein transport (vacuolar fusion)	0.48	0.53
YGR056W	RSC1	RNA	0.41	0.37
YJL006C	CTK2	RNA	0.38	0.54
YML112W	CTK3	RNA	0.46	0.60
YDL106C	PHO2	RNA	0.59	0.57

YDR207C	UME6	RNA	0.54	0.56
YHL034C	SBP1	RNA	0.63	0.59
YJL127C	SPT10	RNA	0.61	0.53
YKL139W	CTK1	RNA	0.54	0.63
YHL025W	SNF6	RNA	0.68	0.47
YGL173C	KEM1	RNA processing and export	0.28	0.42
YER177W	BMH1	translation	0.43	0.50
YHR021C	RPS27B	translation	0.51	0.54
YKL009W	MRT4	translation	0.63	0.46
YLR167W	RPS31	translation	0.38	0.47
YJL165C	HAL5	transport (potassium)	0.07	0.08
YCR008W	SAT4	transport (potassium)	0.32	0.29
YAR042W	SWH1	transport (sterol; endocytosis)	0.59	0.56
YKL037W	AIM26	unknown	0.59	0.56
YGR196C	FYV8	unknown	0.50	0.45

BOLD: multidrug-sensitive; Underlined: multidrug-resistant (Parsons et al. 2006)

Table 6. Chloroquine-resistance in SGA control library strains.

Systematic name	ORF name	Cell process	Fold Change, Library 1	Fold Change, Library 2
YBR035C	PDX3	biosynthesis (lipid)	1.55	4.12
YPL031C	PHO85	cell cycle	1.58	0.00
YGR180C	RNR4	DNA	0.00	1.64
YML090W	YML090W	dubious	0.00	1.55
YJL200C	ACO2	energy	1.70	0.00
YGR183C	QCR9	energy	1.86	0.00
YJL128C	PBS2	osmostress	1.75	0.00
YBR101C	FES1	protein folding	0.00	1.59
YJL046W	AIM22	protein modification	1.54	0.00
YPR131C	NAT3	protein modification	0.00	1.62
YDR017C	KCS1	protein transport	1.79	2.35
YPR070W	MED1	RNA	0.00	1.55
YIL128W	MET18	RNA	0.00	1.67
YOR078W	BUD21	translation	0.00	1.51
YFR001W	LOC1	translation	0.00	1.59
YLR081W	GAL2	transport (galactose)	0.00	1.59

BOLD: multidrug-sensitive; Underlined: multidrug-resistant (Parsons et al. 2006)

Table 7. Chloroquine-resistance in SGA HB3 (chloroquine-sensitive) PfCRT library strains.

Systematic name	ORF name	Cell process	Fold Change, Library 1	Fold Change, Library 2
YBR035C	PDX3	biosynthesis (lipid)	1.53	4.10
YML008C	ERG6	biosynthesis (sterol)	1.98	1.55
YML090W	YML090W	dubious	2.28	2.31
YOL134C	YOL134C	dubious/ overlaps HRT1	0.00	1.53
YJL188C	BUD19	dubious/ overlaps RPL39	0.00	2.69
YML120C	NDI1	energy	1.94	0.00
YIR023W	DAL81	nutrient sensing	0.00	1.62
YHL023C	NPR3	nutrient sensing	1.52	1.52
YDR276C	PMP3	osmostress	1.81	2.01
YJL128C	PBS2	osmostress	2.41	2.15
YLR113W	HOG1	osmostress	2.67	2.40
YMR060C	SAM37	protein folding	0.00	1.70
YPR024W	YME1	protein folding	1.51	1.50
YLL040C	VPS13	protein transport	0.00	1.95
YER151C	UBP3	protein transport	0.00	1.82
YLR370C	ARC18	protein transport	0.00	1.54
YPL069C	BTS1	protein transport	0.00	1.70
YNR051C	BRE5	protein transport	0.00	1.57
YPL259C	APM1	protein transport (clathrin AP-1 adapter)	1.53	0.00
YAL002W	VPS8	protein transport (CORVET)	3.31	2.73
YOR089C	VPS21	protein transport (CORVET)	1.83	0.00
YGL167C	PMR1	protein transport (Golgi ion channel)	1.59	0.00
YOR069W	VPS5	protein transport (retromer)	3.11	3.33
YIL128W	MET18	RNA	0.00	3.34
YHR041C	SRB2	RNA	0.00	1.93
YOR141C	ARP8	RNA	0.00	1.69
YGR063C	SPT4	RNA	0.00	1.60
YGL115W	SNF4	RNA	1.52	0.00
YPR070W	MED1	RNA	1.62	1.68
YGR159C	NSR1	translation	2.27	0.00
YJL189W	RPL39	translation	0.00	1.67
YOR096W	RPS7A	translation	0.00	2.22
YKL137W	CMC1	transport (copper)	1.53	0.00
YML048W	GSF2	transport (galactose)	1.73	2.07
YBR020W	GAL1	transport (galactose)	0.00	1.55
YLR081W	GAL2	transport (galactose)	2.30	1.68
YPL248C	GAL4	transport (galactose)	2.20	0.00
YGL188C-A	YGL188C-A	unknown	1.50	0.00
YMR230W-A	YMR230W-A	unknown	1.70	0.00
YCL005W-A	VMA9	v-ATPase	3.00	0.00
YPR036W	VMA13	v-ATPase	0.00	4.48

BOLD: multidrug-sensitive; Underlined: multidrug-resistant (Parsons et al. 2006)

Table 8. Chloroquine-resistance in SGA Dd2 (chloroquine-resistant) PfCRT library strains.

Systematic name	ORF name	Cell process	Fold Change, Library 1	Fold Change, Library 2
YML008C	ERG6	biosynthesis (ergosterol)	2.30	1.81
YBR026C	ETR1	biosynthesis (lipid)	1.52	0.00
YBR035C	PDX3	biosynthesis (lipid)	3.27	2.53
YER061C	CEM1	biosynthesis (lipid)	1.99	0.00
YDR442W	YDR442W	dubious	1.86	0.00
YLR338W	OPI9	dubious	0.00	1.84
YML090W	YML090W	dubious	1.69	2.45
YLR261C	VPS63	dubious/ overlaps VRP1	3.24	2.43
YPL180W	TCO89	nutrient sensing	1.53	0.00
YDR276C	PMP3	osmostress	1.54	1.55
YJL128C	PBS2	osmostress	2.40	1.96
YLR113W	HOG1	osmostress	2.18	1.60
YPR024W	YME1	protein folding	0.00	1.86
YBR036C	CSG2	protein transport	1.50	0.00
YCR094W	CDC50	protein transport	2.00	1.86
YDR126W	SWF1	protein transport	0.00	2.21
YDR470C	UGO1	protein transport	1.94	0.00
YER151C	UBP3	protein transport	0.00	1.94
YLL039C	UBI4	protein transport	1.64	0.00
YLL040C	VPS13	protein transport	2.97	1.80
YLR337C	VRP1	protein transport	0.00	2.00
YPL069C	BTS1	protein transport	1.81	2.08
YBR164C	ARL1	protein transport (ARL complex)	1.81	2.73
YPL051W	ARL3	protein transport (ARL complex)	1.77	0.00
YPL259C	APM1	protein transport (clathrin AP-1 adapter)	1.88	1.99
YGL223C	COG1	protein transport (COG complex)	0.00	1.52
YPL120W	VPS30	protein transport (complex II)	0.00	1.51
YAL002W	VPS8	protein transport (CORVET)	2.17	2.58
YKR020W	VPS51	protein transport (GARP)	2.12	1.64
YGL167C	PMR1	protein transport (Golgi ion channel)	1.51	0.00
YLR039C	RIC1	protein transport (retrograde)	1.90	1.85
YLR262C	YPT6	protein transport (retrograde)	0.00	1.74
YJL053W	PEP8	protein transport (retromer)	1.83	1.72
YJL154C	VPS35	protein transport (retromer)	1.54	1.70
YOR069W	VPS5	protein transport (retromer)	2.65	3.64
YOR132W	VPS17	protein transport (retromer)	0.00	1.74
YOR068C	VAM10	protein transport (vacuolar fusion)	2.63	1.69
YDR034C	LYS14	RNA	1.58	0.00

YGR063C	SPT4	RNA	1.87	0.00
YGR104C	SRB5	RNA	1.63	0.00
YHR041C	SRB2	RNA	1.52	0.00
YNL025C	SSN8	RNA	2.10	1.59
YHR189W	PTH1	translation	1.96	0.00
YFR001W	LOC1	translation	1.63	0.00
YGR159C	NSR1	translation	1.92	0.00
YMR260C	TIF11	translation	1.62	0.00
YBR020W	GAL1	transport (galactose)	0.00	1.94
YLR081W	GAL2	transport (galactose)	1.92	0.00
YML048W	GSF2	transport (galactose)	2.13	1.55
YGL188C-A	YGL188C-A	unknown	1.52	0.00
YJL197W	UBP12	unknown	0.00	1.59
YCL005W-A	VMA9	v-ATPase	0.00	1.78
YPR036W	VMA13	v-ATPase	2.34	3.64

BOLD: multidrug-sensitive; Underlined: multidrug-resistant (Parsons et al. 2006)

Additional data files:

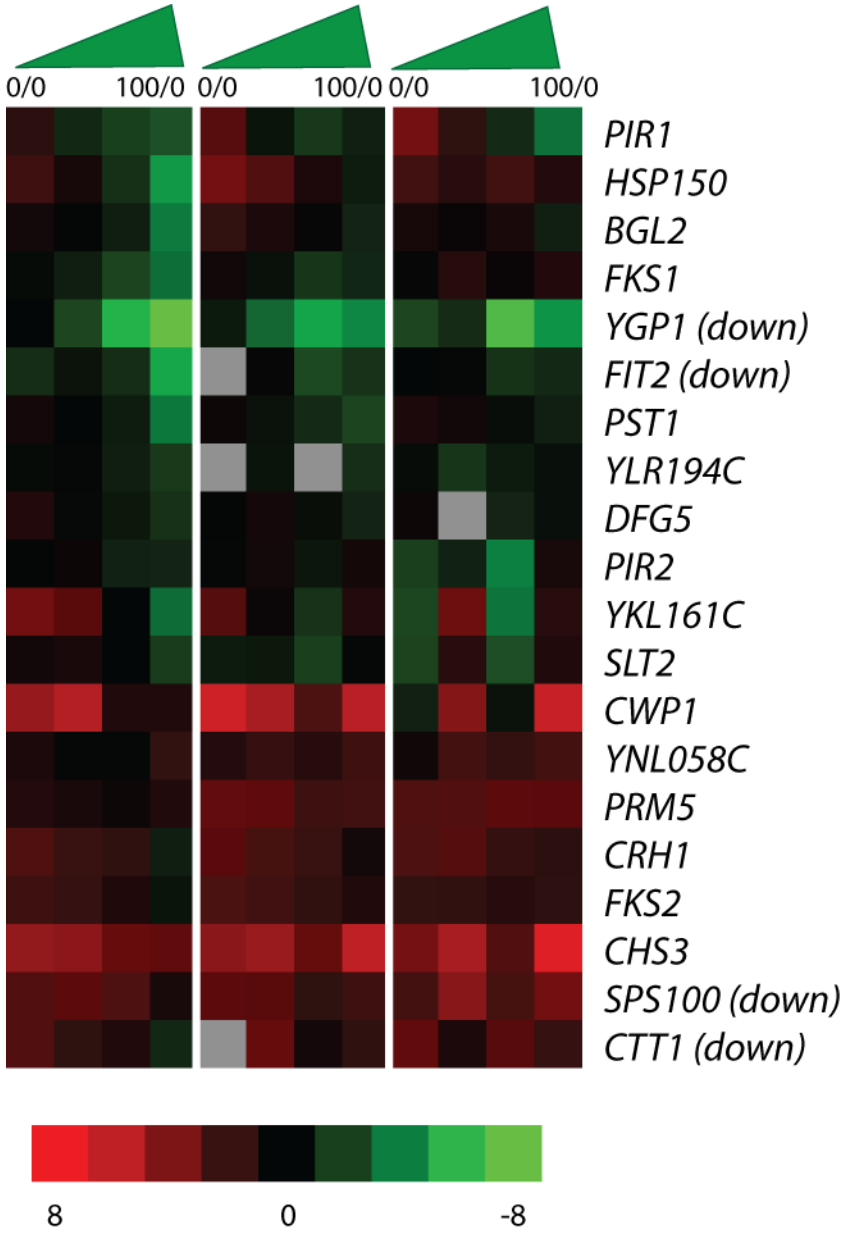
Supplementary Data Tables 1a-e (Excel). Complete list of mutant strains identified by chemical-genetic profiling of the BY4741 deletion and DAmP collections as chloroquine-sensitive or –resistant on dextrose or galactose medium.

Supplementary Data Tables 2a-f (Excel). Complete list of mutant strains identified by chemical-genetic profiling in libraries created for synthetic genetic analysis (SGA) as chloroquine-sensitive or –resistant on galactose medium: no-PfCRT, HB3 chloroquine-sensitive PfCRT, Dd2 chloroquine-resistant PfCRT.

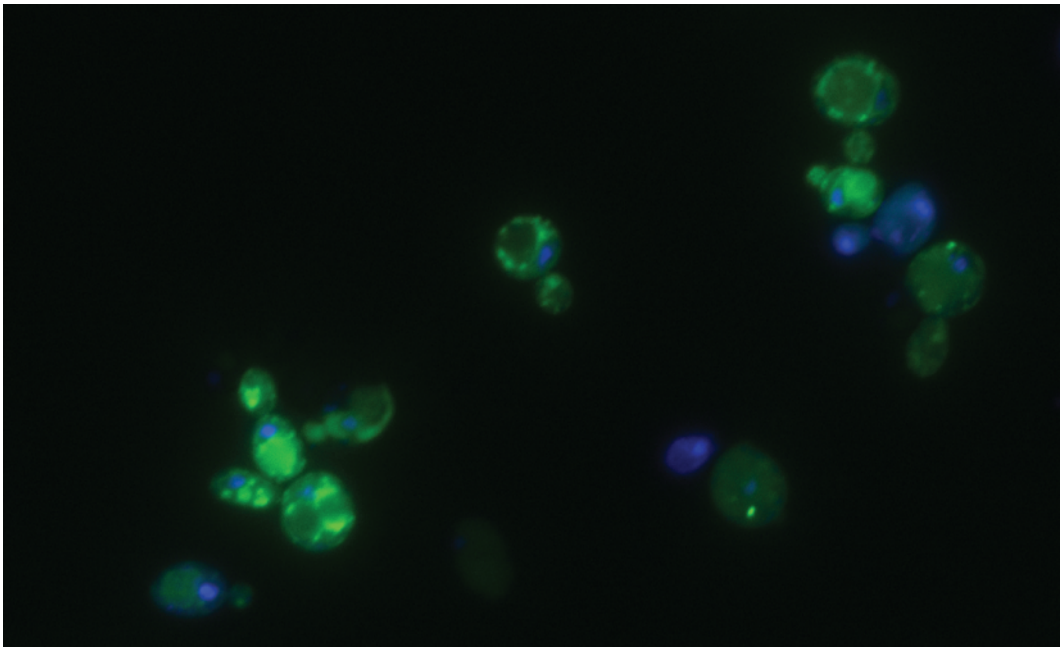
Supplementary Data Tables 3a-c (Excel). Comparison of chemical-genetic profiles to strains deficient in polyphosphate accumulation, strains with altered ion accumulation, and strains hypersensitive to zinc.

Supplementary Data Tables 4a-f (Excel). Complete lists of transcripts induced or repressed by treatment with 25, 50, or 100 mM chloroquine over time.

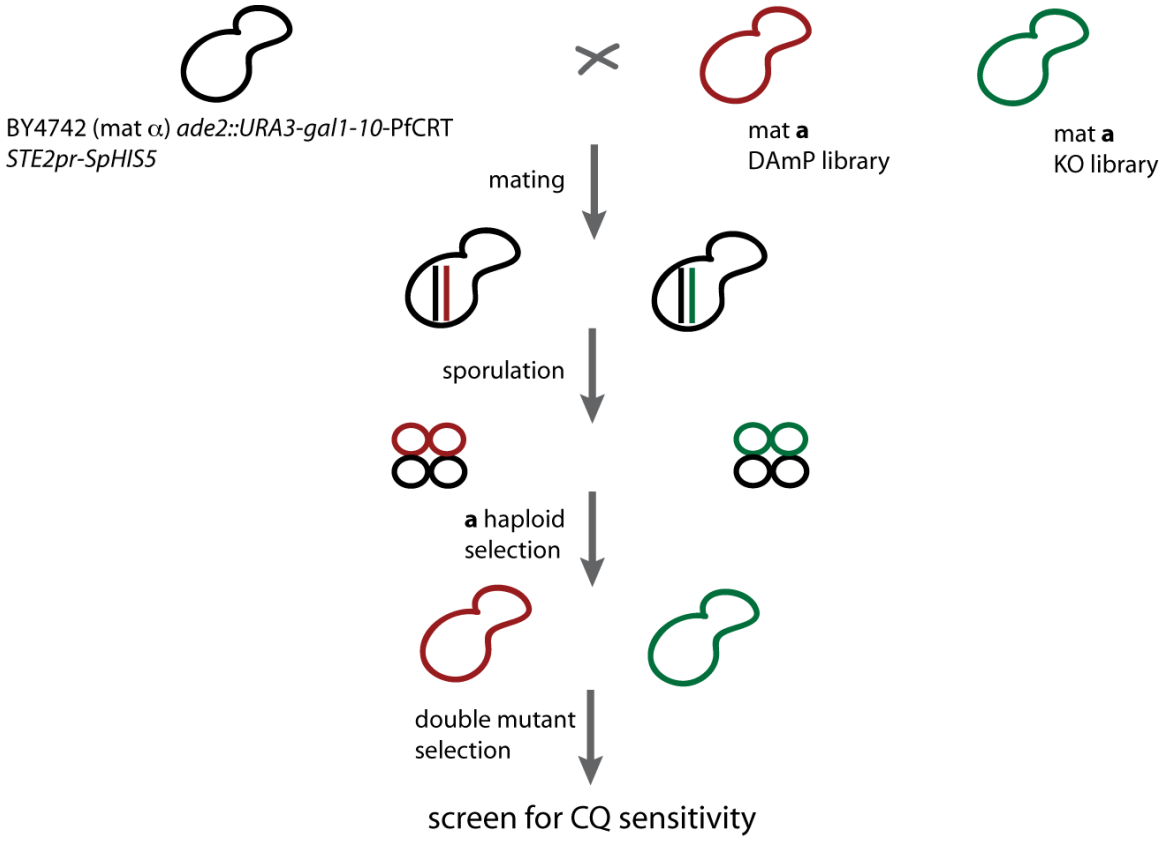
Supplementary Data Tables 5a-j (Excel). Comparison to transcriptional profiling datasets: iron starvation; cell-wall integrity genes; unfolded protein response; alkaline stress; Crz1-responsive transcripts responsive to calcium or sodium stress; zinc stress.



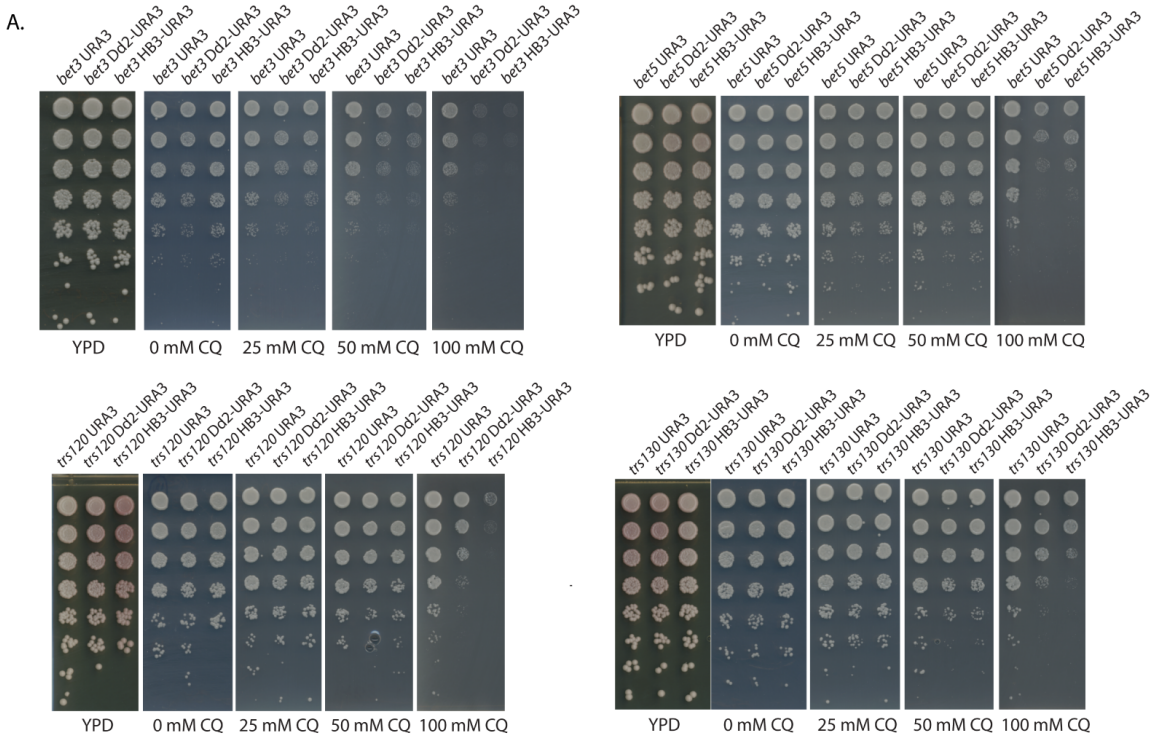
Supplementary Figure 1. Chloroquine-mediated induction of transcripts regulated by the cell wall integrity pathway. Transcripts shown (except for *FKS2*) are targets of the Rlm1p transcription factor.
Data are normalized with respect to the t=0 control sample. (down) indicates a transcript repressed by Rlm1p. Note that most transcripts are either derepressed or induced over time relative to the no-chloroquine control (left column in each panel).



Supplementary Figure 2. PfCRT associates with the yeast vacuole membrane. Cells are co-labeled with DAPI. Juxtanuclear signal, indicative of ER localization, can be detected in some cells. PfCRT is found in punctate spots in the cytoplasm (Golgi apparatus or endosome) or in a circumvacuolar ring. Cultures were induced with galactose for two hours prior to imaging.

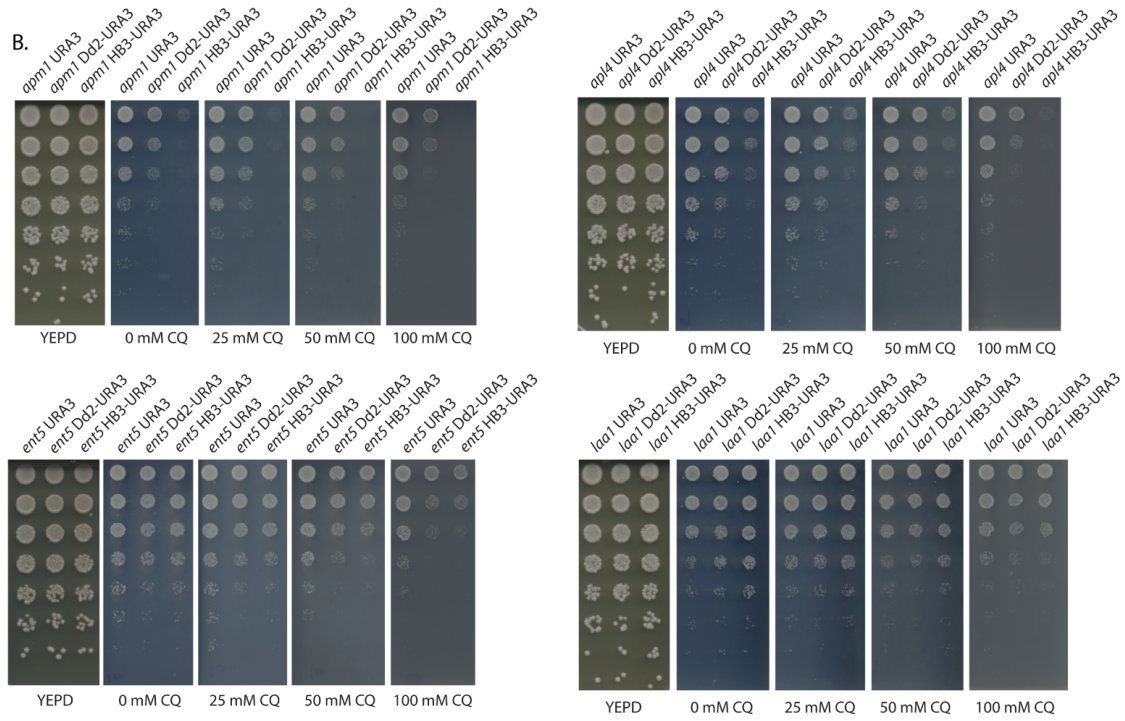


Supplementary Figure 3. Library construction for synthetic genetic analysis of yeast chloroquine sensitivity. Chloroquine-resistant or -sensitive alleles of *PFCRT* are introduced into the SGA mater strain at the *ADE2* locus; a control strain contains only the *URA3* cassette at that locus. Strains are mated to the deletion and DAmP collections; diploids are selected, sporulated, and re-selected for MAT **a** haploids with query mutations (Collins et al., 2010).

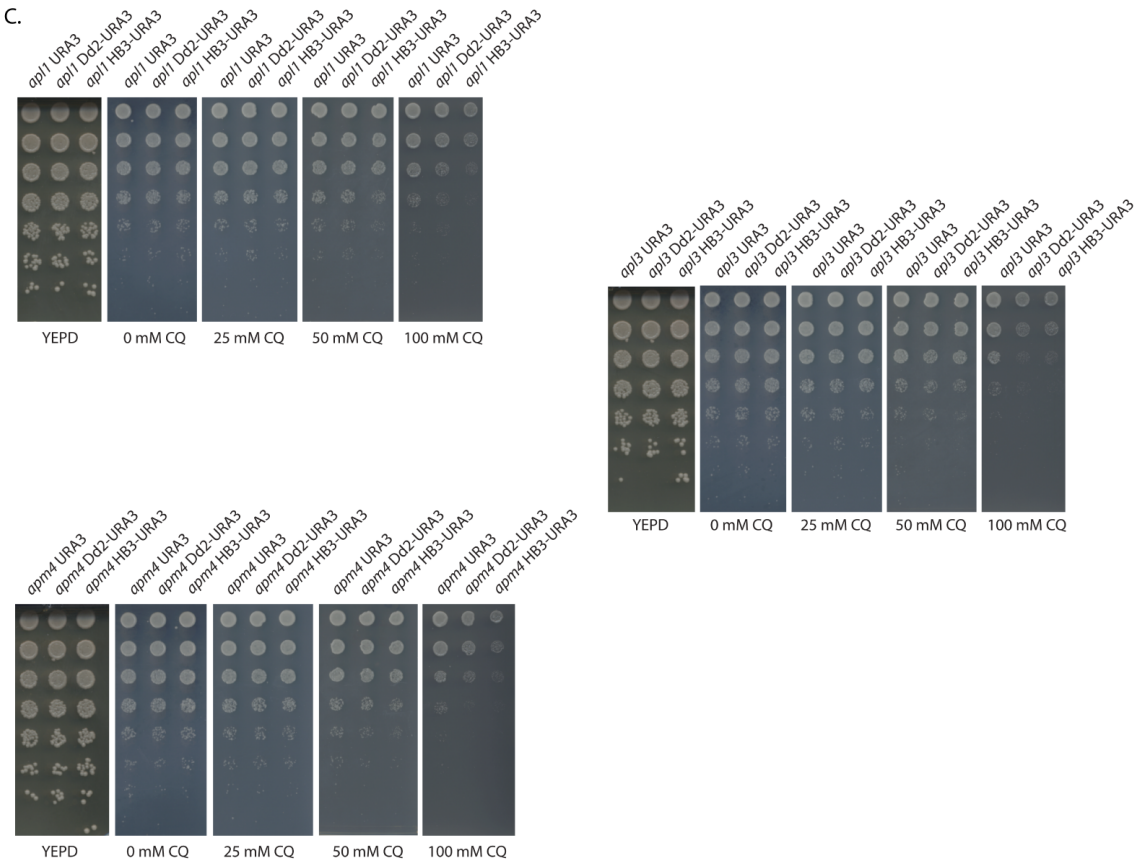


Supplementary Figure 4. Suppression of trafficking defects by chloroquine-resistant PfCRT.

A. TRAPP is a tethering complex that mediates transport among numerous secretory pathways compartments. Bet3 and Bet5 participate in all TRAPP-mediated tethering events, including endoplasmic reticulum to Golgi trafficking. Trs120 and Trs130 are components of TRAPP II, which is required for vesicle departure from the *trans*-Golgi. Note that Dd2 expression suppresses TRAPP II sorting defects, but not those of core TRAPP subunits.



B. The AP-1 clathrin adapter proteins coordinate transport from the *trans*-Golgi network to the vacuole via the endosome. Expression of chloroquine-resistant PfCRT suppresses trafficking defects from loss of adapter subunits. (Not shown: strains mutant for AP-1 subunits encoded by *APS1*, *APL2*.)



C. The AP-2 clathrin adapter subunits coordinate protein transport from the plasma membrane to the endosome. Expression of chloroquine-resistant PfCRT weakly suppresses trafficking defects in AP-2 mutants. (Not shown: subunit encoded by *APS2*.)

Chapter 3: Investigation of chloroquine- and PfCRT-mediated alterations in CPY secretion

Abstract

In the yeast *Saccharomyces cerevisiae*, chloroquine can exacerbate growth defects in strains with impaired protein trafficking, likely due to impedance of pH homeostasis in secretory pathway organelles. Expression of a chloroquine-resistant allele of the *P. falciparum* chloroquine resistance transporter (PfCRT) can suppress these growth phenotypes at the level of trafficking from *trans*-Golgi to endosome, while a chloroquine-sensitive allele cannot. Protein trafficking defects in pre-endosomal stages of the vacuolar-protein sorting pathway are known to result in missorting of the vacuolar hydrolase CPY to the periplasmic space. We investigated the effects of chloroquine on CPY secretion in the presence and absence of PfCRT to determine whether chloroquine-resistant PfCRT restored secretory pathway function in chloroquine-treated protein transport mutants.

Introduction

The yeast vacuole integrates nutrient-storage and hydrolytic functions, serving as a reservoir for phosphate, nitrogen, and cation cofactors such as calcium and iron; a sink for toxins; and a catabolic compartment dedicated to the proteolysis of endocytosed membrane proteins or autophagosomes involved in turnover of unwanted or damaged cytosolic contents. The organelle can also buffer the cell against osmotic changes, increasing or reducing its size by water uptake or loss as a means of rapid adaptation to fluctuations in the extracellular milieu [1-7].

Protein targeting to the vacuole is contingent on the maintenance of a pH gradient through the secretory and endocytic pathways. Progressive acidification from the endoplasmic reticulum (ER), at a pH close to cytosolic, through the Golgi apparatus, in which pH drops rapidly from *cis* to *trans*, to the endosome and finally the vacuole, sustains anterograde transport, and pH in each organelle is tightly regulated by interplay between H^+-ATPases and counterion channels [2-4, 8]. pH homeostasis is required for the appropriate association and dissociation of cargo proteins and their receptors, as well as for directional control of transport [9], and loss of vacuolar H^+-ATPase function impairs vacuolar delivery of certain hydrolases [4].

Vacuolar protein targeting can occur through parallel means. In addition to a cytoplasm-to-vacuole route taken by two soluble vacuolar hydrolases and autophagic bodies [3, 4], proteins in the canonical secretory pathway can be steered toward the vacuole from the *trans*-Golgi network through two independent modalities, one bypassing the endosome entirely. The endosomal route is taken by the proteases carboxypeptidases Y and S (CPY, CPS) and proteinase A, and requires clathrin-coated

vesicles and the AP-1 series of clathrin adapter proteins. Alkaline phosphatase (ALP) is transported to the vacuole directly by means of the AP-3 clathrin-independent adapter complex [10]. Although the same factors coordinate fusion with the vacuole of vesicles originating in or routed via independent cellular domains, separate machinery and sorting signals exist for each transport pathway and have been elucidated through extensive genetic screening [11-14].

Hydrolase progress to the vacuole and the integrity of each trafficking pathway in mutant strains can be inferred from molecular weight, as modifications or processing events generate unique compartment-specific protein isoforms that serve as markers for compromised activity at discrete stages in transport [11]. Although AP-1 and AP-3 pathways can partially compensate for deficiency in each other, their mutation engenders some protein mistargeting, especially with regard to CPY transport along the endosomal route from the *trans*-Golgi network. Cleavage of the CPY signal sequence and minor glycosylation in the ER generates the 67 kDa p1 form of the protein, which is further glycosylated in the Golgi apparatus to produce the 69 kDa p2 CPY, recruited into cargo vesicles at the *trans*-Golgi by the CPY receptor Vps10p. At the endosome Vps10p dissociates from CPY and is trafficked back to the *trans*-Golgi, while CPY enters the vacuole upon endosomal fusion with that compartment. mCPY, the mature, functional vacuolar form of CPY, is a 61 kDa isoform generated by proteolytic processing and is clearly distinguishable from both p1 and p2 forms [4, 10]. Blocked transport of CPY prior to the endosome leads to secretion of p2 CPY, while defects in endosomal maturation are apparent from retention of CPY within that compartment [15].

A screen for chemical-genetic interactions with the lipophilic weak base chloroquine in *Saccharomyces cerevisiae* identified drug-sensitivity in yeast strains lacking protein trafficking factors (Chapter 2). Furthermore, yeast expression of the *P. falciparum* chloroquine resistance transporter (PfCRT), a malaria-parasite transmembrane protein that, in chloroquine-resistant parasite isolates, incurs the capacity to transport protonated chloroquine down its concentration gradient from its site of action in the acidic digestive vacuole [16, 17], could modulate these interactions, with a chloroquine-resistant *PFCRT* allele (Dd2) competent to suppress chloroquine-mediated growth defects, although they were exacerbated by expression of a chloroquine-sensitive (HB3) allele (Chapter 2).

Our findings suggested a model wherein chloroquine, by entering acidic intracellular compartments [6], perturbed pH homeostasis with pleiotropic effects, including disruption of protein trafficking. Suppression of the chloroquine-sensitive growth phenotype by the Dd2 allele appeared to operate primarily at the level of protein transport from *trans*-Golgi network to endosome (Chapter 2), in which latter organelle ion transporters create pH microdomains to manipulate the direction of trafficking, promoting anterograde protein transport through local acidity and retrograde through local alkalinity [18, 19]. A general increase in compartmental pH through the entrance of chloroquine would perturb microdomain formation to stimulate or aggravate trafficking defects. We reasoned that this might be visible at the level of CPY trafficking, and opted to examine its secretion via colony immunoblot in protein-transport mutant strains with robust allele-specific differences in growth, as well as a set of candidate strains mutant in different steps of the secretory pathway.

Results and Discussion

We recovered strains from SGA libraries created to probe yeast genetic interactions with chloroquine and their modulation by PfCRT (Chapter 2). Screening and serial dilution assays suggested that yeast mutant in vacuolar protein trafficking were sensitized to chloroquine and that expression of the Dd2 chloroquine-resistant PfCRT isoform could suppress this effect partially or wholly for some deletion strains, principally those lacking in factors implicated in anterograde and retrograde transport between *trans*-Golgi network and endosome (Chapter 2; Supplementary Figure 1). Loss of factors involved in trafficking in either direction at this step creates a chokepoint due to reduced access to trafficking machinery; this manifests as vacuolar mistargeting of the CPY receptor (Vps10p) and secretion of CPY [10].

We considered it likely that expression of Dd2-PfCRT would permit the extrusion of chloroquine trapped in secretory organelles, thus permitting restoration of pH homeostasis and resumption of trafficking, and that this should reduce secretion of CPY. CPY secretion can be assayed by colony overlay with nitrocellulose membrane and subsequent immunoblotting [11], and we accordingly examined this phenotype in strains deficient in anterograde transport via the AP-1 clathrin-dependent pathway from the *trans*-Golgi to the vacuole via the endosome, through which CPY moves in wildtype cells (Figure 1) [10], the AP-2 clathrin-dependent endocytic pathway, or the AP-3 clathrin-independent protein transport pathway from *trans*-Golgi to vacuole bypassing the endosome (Figure 2) [10, 20]; lacking factors governing parallel retrograde-transport pathways from endosome to *trans*-Golgi (Figures 3 and 4; Ypt6p and ARL complex) [21-23]; or the retromer complex, which serves as a vesicular coat in retrograde transport at

this step (Figure 5) [10]. Additional factors with roles in formation of retrograde transport vesicles (the Complex II PI(3)K, which nucleates retromer coat formation; HOPS and CORVET complexes that promote fusion at vacuole or endosome, respectively) [10] were also assessed for CPY secretion and its suppression by PfCRT.

Anterograde transport: AP-1. AP-1 is a series of adapter proteins (Apl2, Apl4, Apm1, Apm2, Aps1; Laa1 and Ent5 may function as accessory proteins) that recruit cargo, including the CPY receptor, CPY, and other vacuolar hydrolases, into clathrin-coated vesicles forming at the *trans*-Golgi network for anterograde transport to the endosome [10, 24]. Expression of chloroquine-sensitive PfCRT was detrimental to growth on chloroquine of *aps1*, *apm1*, and *apl4* mutant strains (Figure 1a), though suppression of chloroquine-induced CPY missorting was effected by chloroquine-resistant PfCRT in *aps1*, *apm2*, and *ent5* mutants (Figure 1b). Expression of chloroquine-resistant PfCRT therefore does not appear to uniformly facilitate anterograde trafficking in the CPY pathway.

Anterograde transport: AP-2 and Yap1801/2. The AP-2 adapter complex (Apl1, Apl3, Apm4, Aps2) coordinates protein endocytosis and trafficking from plasma membrane to endosome. Cargo is recruited into clathrin-coated vesicles whose assembly is promoted by Yap1801/2 [25]. Chloroquine-sensitive PfCRT aggravated the growth phenotypes on chloroquine of *apl1*, *apl3*, *apm4*, and *aps2,* but not *yap1801/2* mutant strains (Figure 2a and Figure 1a), though little alteration by chloroquine of CPY secretion was detectable in these strains (Figure 2b and Figure 1b), commensurate with any suppressive effect of PfCRT on CPY trafficking acting at the level of *trans*-Golgi to endosome transport.

Anterograde transport: AP-3. AP-3 is an adapter complex that operates clathrin-independently to recruit cargo, such as the vacuolar hydrolase ALP, into vacuole-bound vesicles at the *trans*-Golgi. It traverses a non-endosomal pathway to the vacuole, where it interacts with the HOPS complex (including the Ypt7 small GTPase) and vesicle cargo is subsumed into the larger compartment [10, 26]. PfCRT expression, irrespective of allele, exacerbated the growth phenotype on chloroquine of *apl5*, *apl6*, *apm3*, *aps3*, *yck3*, and *ypt7* strains (Figure 2a, Figure 4a); CPY secretion of *apm3*, *apl6*, and *aps3* strains may actually be augmented by chloroquine, while chloroquine-sensitive PfCRT enhances CPY secretion in the *apl5* strain and chloroquine-resistant in *ypt7* (Figure 2b, Figure 4b). The chloroquine-sensitive growth and CPY secretion phenotypes of these PfCRT-expressing AP-3 mutants are unexpectedly severe, as the AP-1 transport pathway should be fully operational in these strains and assist in CPY's correct localization at the vacuole.

Retrograde transport: Ypt6 small GTPase. Ypt6 orchestrates fusion of endosome-derived vesicles with the *trans*-Golgi network, and is regulated by its *trans*-Golgi localized guanine nucleotide exchange factor (GEF) Ric1/Rgp1 and endosomally localized GTPase activating protein (GAP) Gyp6 [21]; it is assisted in vesicle recognition and tethering by the GARP complex and the SNAREs Tlg1/2 and Vti1 [23, 27]. Gyp6 is postulated to interact with and repress the action of the endosomal Nhx1 Na^+/H^+ ion exchanger to enhance acidity and thus anterograde transport, and Ypt6 to relieve the interaction to direct transport toward the *trans*-Golgi [18, 19]. PfCRT expression diminished growth of *ric1*, *rgp1*, and *tlg2* even in the absence of chloroquine, while chloroquine-sensitive PfCRT suppressed *ypt6* growth on medium with, and chloroquine-resistant with or

without, chloroquine (Figure 3a). Increased CPY secretion on chloroquine was detected for *ypt6*, *ric1*, *rgp1*, and *tlg2* strains expressing either allele of PfCRT; however, PfCRT expression suppressed chloroquine-induced *vti1* CPY secretion (Figure 3b), possibly reflecting the involvement of Vti1 in several stages of protein transport. Nevertheless, these results are inconsistent with expression of PfCRT alleviating chloroquine-mediated defects in retrograde trafficking from the endosome.

Retrograde transport: ARL complex. The ARL complex comprises the Golgi-localized small GTPases Arl1 and Arl3, as well as the Golgi integral membrane protein Sys1, which recruits them to that compartment, and their interacting partner Imh1, which serves as a tethering complex for vesicles trafficked to the *trans*-Golgi. Mon2 is also associated with ARL complex function. Joint deletions in Ypt6 and members of the ARL complex display synthetic lethality [22, 23]. Dd2-PfCRT reduced *arl1* and *arl3* growth in the absence of chloroquine, while either PfCRT variant diminshed *mon2* or *sys1* growth on no-chloroquine medium; although chloroquine did not further alter *arl1* growth, *arl3* growth was abrogated by chloroquine-sensitive PfCRT. In contrast, *imh1* remained unaffected by expression of PfCRT irrespective of the presence of chloroquine (Figure 4a, Figure 5a). CPY secretion was promoted by Dd2-PfCRT on chloroquine medium in *arl1* and *sys1* strains, while either allele of PfCRT enhanced CPY secretion on chloroquine in *arl3* or *mon2* (Figure 4b, Figure 5b). These findings are inconsistent with suppression by Dd2-PfCRT of a defect in retrograde trafficking and confirm our observations with Ypt6 pathway mutants.

Retrograde transport: Retromer. The retromer consists of cargo-interacting (Pep8, Vps29, Vps35) and membrane-deforming factors (Vps5, Vps17) that collaborate in

formation of retrograde transport vesicles responsible for Vps10p recycling from the endosome [25]. Complex II (Vps30, Vps38) is a PI(3)K whose catalytic subunit is Vps34; Vps38 specifies complex localization at the endosome as well as identity, as other subunits of this complex are also found in Complex I, which promotes genesis of autophagosomes. Complex II kinase activity is necessary for Vps5 and Vps17 association with the endosome and thus retromer vesicle nucleation at that compartment [28]. We observed poor growth of PfCRT-expressing retromer mutants in either the presence or absence of chloroquine (Figure 5a); however, lower concentrations of chloroquine enhance growth of Dd2-PfCRT expressing retromer mutants (Chapter 2, and Supplementary Figure 1b). CPY secretion in all retromer mutants was exacerbated by chloroquine, though Dd2-PfCRT mildly suppressed the defect in *pep8*, *vps29*, and *vps35*; instead, HB3-PfCRT appeared to suppress chloroquine-induced secretion in *vps5* (Figure 5b). *vps30* and *vps38* Complex II mutants expressing either allele of PfCRT exhibited poor growth in the presence or absence of chloroquine, and CPY secretion was little altered by drug (Figure 4a, Figure 4b). Dd2-mediated suppression of CPY missorting in retromer mutants is consistent with its restoration of pH homeostasis at the endosome but is difficult to reconcile with the strong CPY sorting phenotype apparent in Ypt6 or ARL mutants.

Anterograde transport: CORVET. We also investigated CPY secretion defects in mutants in the CORVET complex, which fosters the tethering and fusion of early endosomal vesicles during endosomal maturation. Vps8 is an integral subunit of the complex, while Vps21 is a small GTPase involved in vesicular fusion and Vps9 its GEF [29]. *vps9* demonstrates poor growth in the presence of PfCRT on medium lacking chloroquine and

none at all on chloroquine; Dd2-PfCRT mildly enhances *vps21* growth on chloroquine. *vps8* strains expressing PfCRT exhibit a growth defect in the absence of chloroquine that is relieved by PfCRT expression on chloroquine (Figure 4a). Chloroquine mildly enhances CPY secretion in *vps21* and *vps8*, and strongly enhances CPY secretion in *vps9* expressing HB3-PfCRT (Figure 4b). Because these mutants have lost the capacity for endosomal maturation, p2 CPY should be retained in that compartment, and the escalation in its secretion is unexpected.

Conclusions

Our observations of a chloroquine-dependent reduction in growth among vacuolar-protein trafficking mutants and its apparent suppression by chloroquine-resistant PfCRT suggested that the drug might impair pH homeostasis along the secretory pathway, thereby diminishing the efficacy of hydrolase targeting to the vacuole. Because the Dd2 chloroquine-resistant isoform of PfCRT could alleviate growth defects in mutants whose route incorporated the endosome (AP-1, AP-2), but not those that bypassed it (AP-3), we theorized that the Dd2 protein isoform could extrude chloroquine from organelles along its transport route, and that this route encompassed the endosome, to which it restored operation by promoting pH microdomain formation. This therefore suggested a model in which expression of Dd2 PfCRT enhanced vacuolar protein transport and could therefore suppress CPY mistargeting to the periplasmic space. However, colony immunoblot assays were unable to resolve consistent chloroquine-mediated differences among either anterograde- or retrograde-transport mutants.

CPY colony immunoblot assays were likely unable to distinguish Dd2-mediated suppression of CPY missorting for a number of reasons. First, the growth of some

PfCRT-expressing mutants was unequal even in the absence of chloroquine, with one allele promoting growth while the other could not. This may reflect the fact that the strains were recovered from SGA libraries rather than freshly constructed and had accumulated mutations affecting their growth, or may stem from unknown physiological differences among supposedly congenic strains (for instance, relating to PfCRT function). Secondly, these assays are qualitative and cannot detect weak suppressive effects. Previously published protocols call for replica-plating of strains originally spotted onto minimal medium onto the experimental medium of choice, followed by nitrocellulose overlay of the replica plate. However, our experimental design would not permit this, and instead, we began to monitor CPY secretion immediately after culture spotting onto experimental medium. Unfortunately, it was difficult to contrast growth differences after 48 hours with differences in CPY secretion after 6 hours. Thirdly, due to the participation of some factors in more than one stage of transport within the secretory pathway, it was difficult to assign responsibility for CPY secretion phenotype to PfCRT-mediated suppression of particular trafficking defects.

We attempted subcellular fractionation protocols to examine the localization and/or processing of CPY and ALP during timecourses of chloroquine treatment; however, these studies proved less than fruitful. Investigation of PfCRT's modulation of chloroquine-induced alterations in cellular physiology through protein trafficking may not be feasible due to weak suppressive effects, as well as transport factor redundancy or participation in several transport-related processes.

Materials and Methods

Strains were recovered from SGA libraries.

CPY colony immunoblot. Experiments were conducted according to the serial dilution assay protocol (Chapter 2) with the following modifications:

Cultures were diluted such that the most concentrated spot would contain 15,000 to 30,000 cfu and then twice further by 3.3-fold; cells were transferred by frogger onto a number of plates equal to or greater than the number of timepoints. After spots dried (at room temperature), the surface of the plate was overlaid with nitrocellulose membrane (Osmonics) cut to the size of the sampling area. Plates were placed at 30°C for the duration of the timecourse; timepoints were taken at 2, 4, and 6 hours after nitrocellulose overlay. At the time of sampling, each membrane was gently removed from the agar surface and washed in DI water to remove adherent cells, then immediately moved into TBST/ 5% milk for blocking. Plates were replaced at 30°C for two days to allow spot visualization.

Membranes were blocked, with shaking, at room temperature, until 5-30 minutes after the final timepoint, when block was replaced with TBST/5% milk containing α-CPY primary antibody (Abcam) at 1:5000. After incubation (overnight at 4°C), membranes were washed three times for five minutes each with TBST, then incubated with HRP-conjugated goat α-rabbit secondary antibody at 1:5000 (Abcam) in TBST/5% nonfat milk with shaking, at room temperature, for one hour. Membranes were washed three times, for ten minutes, at room temperature, in TBST, before development and chemiluminescence detection (Pierce). Each membrane was exposed to film for 1 minute and 5 minutes.

Detection of CPY subcellular localization upon chloroquine perturbation. Adapted from [30].

Strain creation. *VPS10* or *VPS38* was deleted from strain BY4741 by standard PCR-based integration [31, 32] of the *KANMX6* cassette amplified from pFA6a-3HA-*KANMX6* [33] with the following primers derived from F1 or R1:

VPS10_5'_F1
ATGATATTACTTCATTTTGTCTATTCTCTTTGGGCCTTACTTCTCATTCCcggatcc ccgggttaattaa
VPS10_3'_R1
CTACTGGTTTTCGTTAGATGGCGCTGTAGAATCAGGCCTGTCGATGTTTTgaattc gagctcgtttaaac
VPS38_5'_F1
ATGAAACGGTTCTTACTCAGTCGAAGACAGAGACACCTTCGTATGATTTGcgga tccccgggttaattaa

VPS38_3'_R1
CTATGTTGGACGGTAATTCTCCAGAATCTGTTTAAATTGCAATATATCAGgaatt cgagctcgtttaaac

Sample preparation. BY4741 yeast (MAT **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*; wildtype, *vps10Δ*, or *vps38Δ*) [31] were cultured in synthetic medium containing 400 mM H_3PO_4 (to pH 4 with KOH), equimolar to the phosphoric acid concentration in a 200 mM aqueous solution of chloroquine diphosphate (Sigma), as follows:

A single colony was inoculated into synthetic medium containing 2% raffinose, 0.05% dextrose, and complete amino acids. After overnight growth at 30°C, the culture was diluted into the same medium until it had achieved mid-log phase, when cells were harvested by centrifugation with 5xYP at 3500 rpm for 10 minutes (Sorvall RC-3b); supernatant was decanted and the pellet resuspended in induction medium (as above, with 2% galactose as carbon source). Cultures were induced for five hours, with shaking, at 30°C. Their absorbance was measured and cells collected by centrifugation as above; culture medium was decanted and the cells treated with reduction buffer (100 mM Tris•SO_4, pH 9.4 and 10 mM DTT) and spheroplasted according to the method of Rieder and Emr [30] with zymolyase 100 T (Seikagaku). After wash steps, cells were collected

by centrifugation at 1500g and resuspended to roughly 30 OD U/ mL in galactose-containing medium as above, but supplemented with 1M sorbitol to prevent spheroplast lysis.

CPY trafficking timecourse. CPY's movement through the secretory pathway in the presence and absence of chloroquine was monitored by timecourse. Briefly, the spheroplast slurry was dispensed equally, to a final concentration of 5-10 OD U/ mL, among 9 5-mL aliquots of sorbitol-supplemented medium, 5 without chloroquine and 4 containing 100 mM chloroquine. A stop solution of NaF and NaN_3 was added to one 0 mM chloroquine sample (final concentration of 20 mM each) [30] to define CPY processing at t_0; this sample was immediately placed on ice. All other samples were incubated at 30°C till harvesting at 15, 30, 60, or 120 minutes. Stop solution (as above) was added to each sample immediately upon its collection.

Samples were centrifuged at 1500g (4°C) and an aliquot removed from the supernatant for detection of secreted CPY; the remaining supernatant was decanted and the pellet washed in ice-cold spheroplast buffer B [30]. Following washes, pellets were lysed by douncing in a chilled 7 mL glass dounce (Wheaton) and samples processed to yield an intracellular pellet containing the ER, Golgi, and vacuole [30]. Pellet and supernatant fractions were flash-frozen in liquid N_2 and stored at -80°C until western blotting (see below).

Secreted CPY. Culture supernatants were probed for p2 CPY secretion in the presence or absence of chloroquine. 15 mL culture medium was added to 5 mL 4x SDS-PAGE sample buffer; samples were heated at 95°C for 10 minutes and microfuged at 13000 rpm for 5 minutes before loading of supernatant into a precast Bis-Tris gradient gel (4-12%;

Invitrogen). Samples were run at constant voltage in MOPS buffer for 2 hours or until the dye front had reached the bottom of the gel. Samples were wet-transferred onto nitrocellulose or PVDF at constant voltage. Membranes were blocked at room temperature, with shaking, in TBST/5% nonfat milk and incubated with α-CPY primary Ab (1:5000, Abcam) in blocking solution for 2 hours at room temperature or overnight at 4°C. Membranes were washed 3 times for 5 minutes each, with shaking, in TBST, and incubated for an hour with HRP-conjugated goat α-rabbit secondary antibody (1:20000, Abcam) in blocking solution at room temperature before washing in TBST 3 times for 10 minutes. CPY was detected by chemiluminescence (Pierce).

Intracellular CPY. Pellets containing ER, Golgi, and vacuole (13000g spin) [30] were probed to examine CPY transport and processing. The p1 (ER) form of the hydrolase is glycosylated to a mass of 67 kDa; in the Golgi further glycosylation creates the p2 69 kDa form. mCPY is proteolytically processed in the vacuole to 61 kDa [10]. Protein concentration was first determined by BCA assay (Pierce), after which samples were diluted in 2x SDS-PAGE sample buffer, heated at 65°C for 10 minutes, and centrifuged at top speed for 5 minutes; equivalent amounts of total protein (5-15 μg) were loaded into each lane of a precast Bis-Tris gradient gel (4-12%; Invitrogen). Running conditions, sample transfer conditions, and western blotting conditions were as described above.

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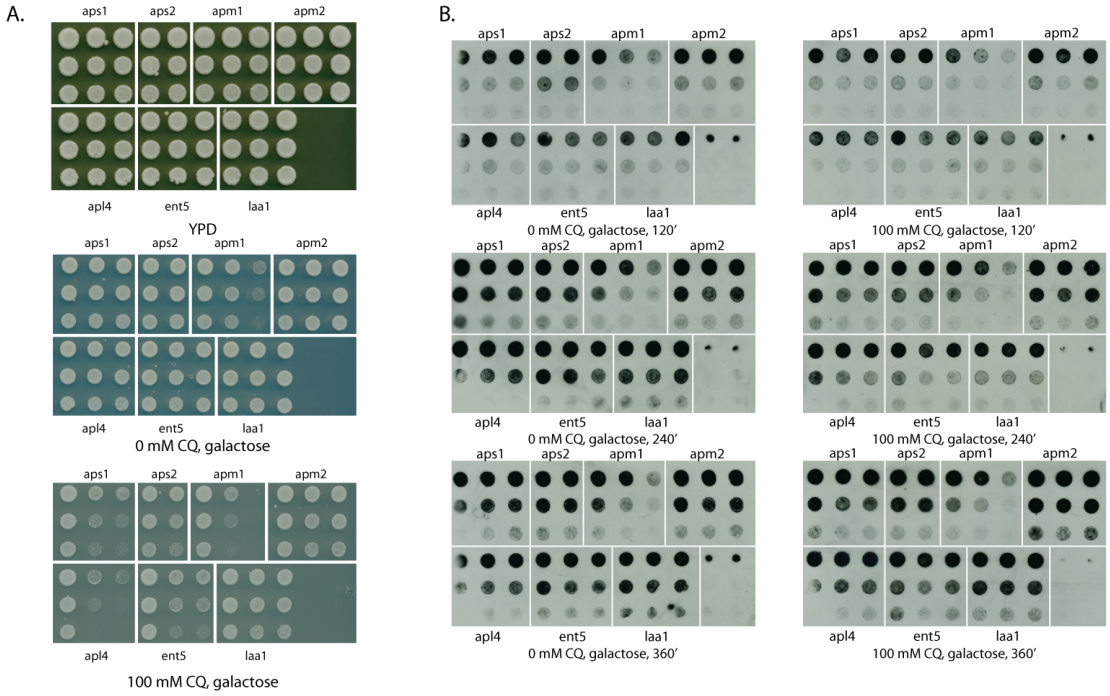


Figure 1. Chloroquine-resistant PfCRT suppresses chloroquine-mediated growth defects in strains lacking AP-1 pathway anterograde-trafficking factors.
A. Serial-dilution assays. Chloroquine exacerbates growth defects in *aps1*, *apm1*, and *apl4* strains expressing chloroquine-sensitive, but not chloroquine-resistant, PfCRT.
B. CPY colony immunoblots. Expression of chloroquine-resistant PfCRT appears to subtly suppress the CPY secretion phenotype of *aps1*, *apm2*, and *ent5* strains in the AP-1 pathway.

Serial dilution plates were grown at 30°C for 48 hours prior to imaging; colony immunoblots represent CPY secretion in two, four, and six hour periods after nitrocellulose overlay.
KEY: AP-1 AP-2 AP-1 AP-1
AP-1 AP-1 AP-1
Strains are arranged as no-PfCRT, Dd2, HB3
(*aps2* Dd2, HB3)

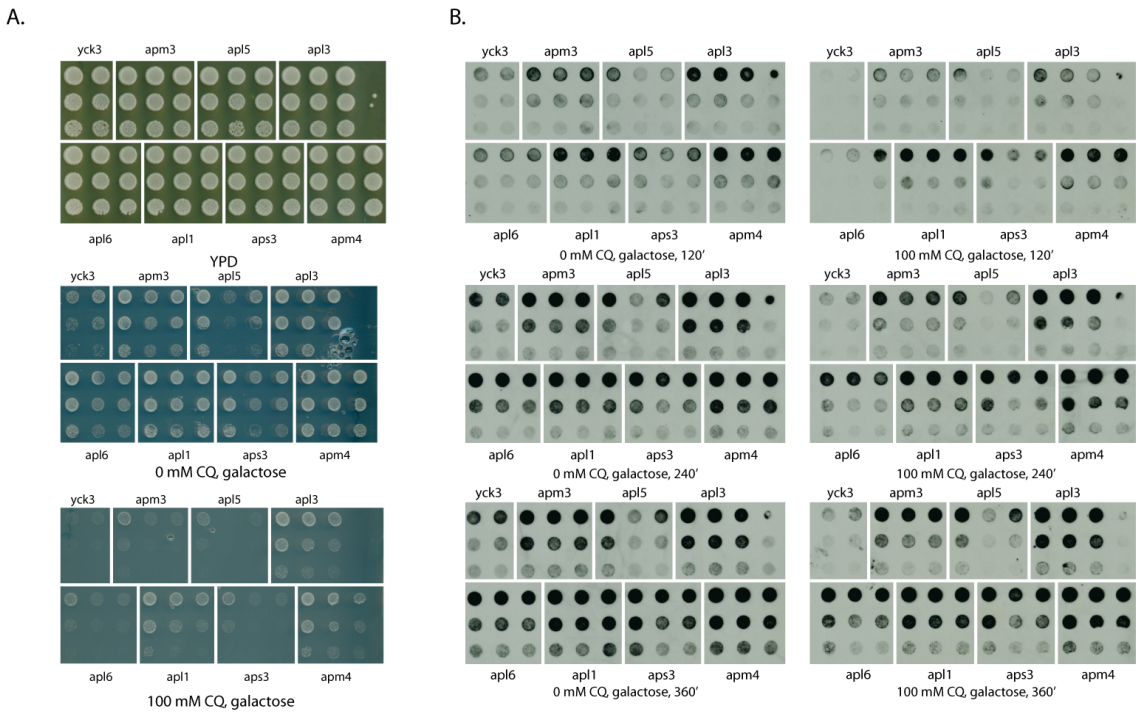


Figure 2. Chloroquine-resistant PfCRT suppresses chloroquine-mediated growth defects in strains lacking AP-2, but not AP-3, pathway anterograde-trafficking factors.

A. Serial dilution assays. Chloroquine exacerbates growth defects in AP-2 deficient strains (*apl1*, *apl3*, *apm4*) expressing chloroquine-sensitive, but not resistant, PfCRT. Expression of PfCRT confers a growth defect on AP-3 deficient strains (*yck3*, *apm3*, *apl5*, *apl6*, *aps3*).

B. CPY colony immunoblots. Chloroquine appears to enhance the CPY secretion phenotype of *apm3*, *apl6*, and *aps3* strains in the AP-3 pathway.

Serial dilution plates were grown at 30°C for 48 hours prior to imaging; colony immunoblots represent CPY secretion in two, four, and six hour periods after nitrocellulose overlay.

KEY: AP-3 AP-3 AP-3 AP-2
AP-3 AP-2 AP-3 AP-2

Strains are arranged as no-PfCRT, Dd2, HB3
(*yck3* Dd2, HB3)

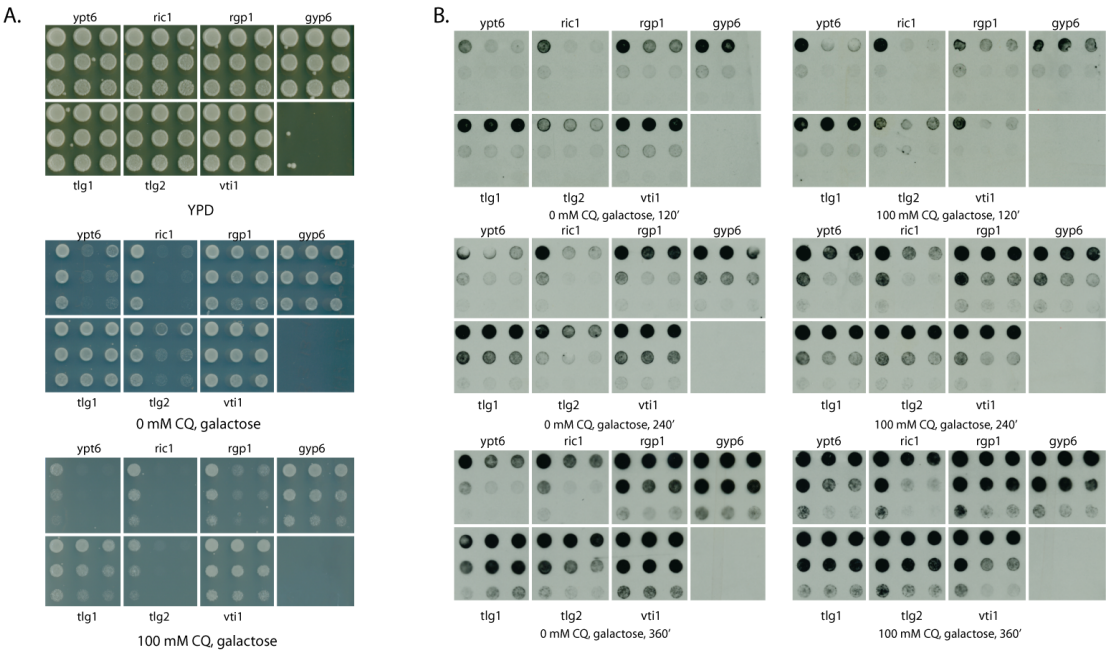


Figure 3. Strains deficient in Ypt6 retrograde-trafficking components exhibit heightened sensitivity to chloroquine.
A. Serial-dilution assays. Chloroquine exacerbates growth defects in *ypt6*, *ric1*, *rgp1*, *tlg2* strains expressing PfCRT. Note that PfCRT expression detracts from *ypt6*, *ric1*, and *tlg2* growth in the absence of chloroquine.
B. CPY colony immunoblots. Expression of PfCRT appears to enhance CPY secretion in *ypt6*, *ric1*, *rgp1*, and *tlg2*, but suppresses secretion of CPY in *vti1*.

Serial dilution plates were grown at 30°C for 48 hours prior to imaging; colony immunoblots represent CPY secretion in two, four, and six hour periods after nitrocellulose overlay.
KEY: RETROGRADE TRANSPORT (endosome to TGN)
RETROGRADE TRANSPORT (endosome to TGN)
Strains are arranged as no-PfCRT, Dd2, HB3

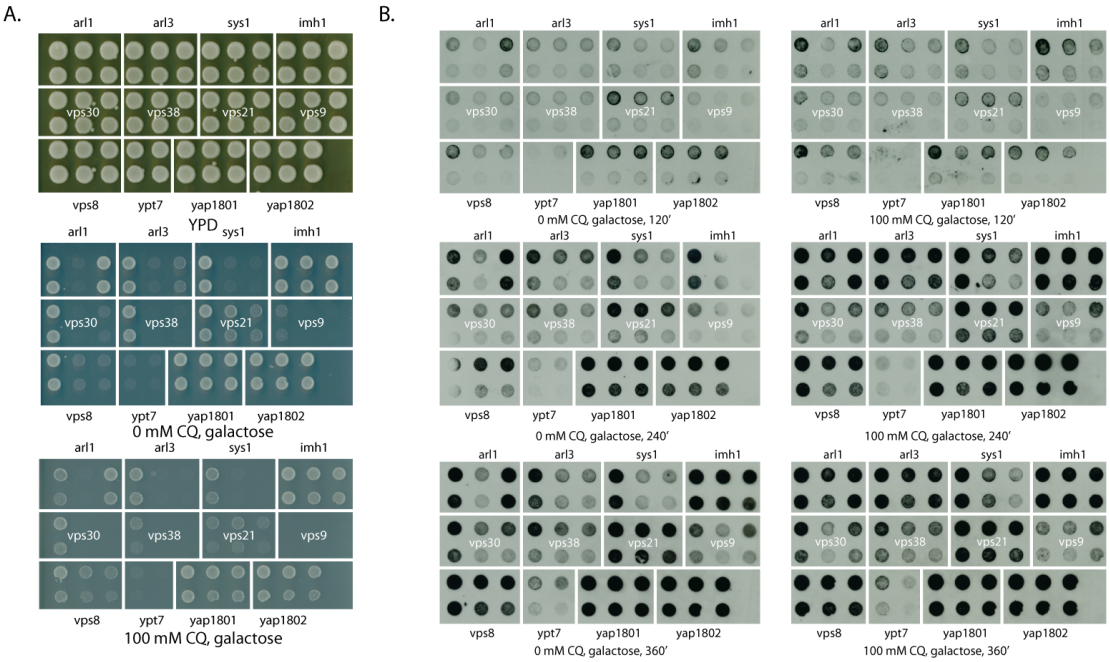


Figure 4. Strains deficient in ARL-complex or complex II retrograde-trafficking components exhibit heightened sensitivity to chloroquine.
A. Serial-dilution assays. Chloroquine exacerbates growth defects in *arl3* and *vps30* strains expressing either allele of PfCRT. Note that *arl1* and *sys1* PfCRT-expressing mutants are sensitized to chloroquine.
B. CPY colony immunoblots. Expression of chloroquine-resistant PfCRT enhances CPY secretion in *arl1* and *sys1* strains; both PfCRT alleles enhance *arl3* CPY secretion. Chloroquine-sensitive PfCRT enhances *vps9* CPY secretion.

Serial dilution plates were grown at 30°C for 48 hours prior to imaging; colony immunoblots represent CPY secretion in two, four, and six hour periods after nitrocellulose overlay.
KEY: ARL COMPLEX (4)
COMPLEX II (2); CORVET (2)
CORVET (1); HOPS (1); endocytosis (2)
Strains are arranged as no-PfCRT, Dd2, HB3
(*ypt7* Dd2, HB3)

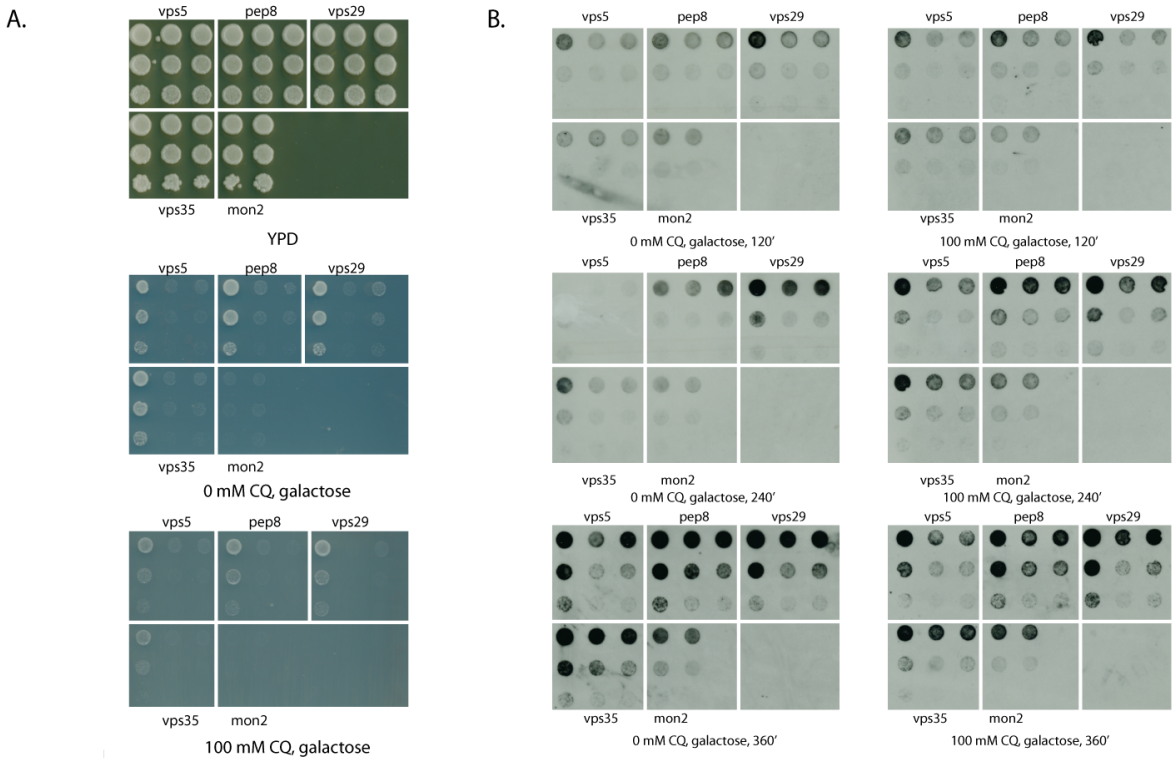
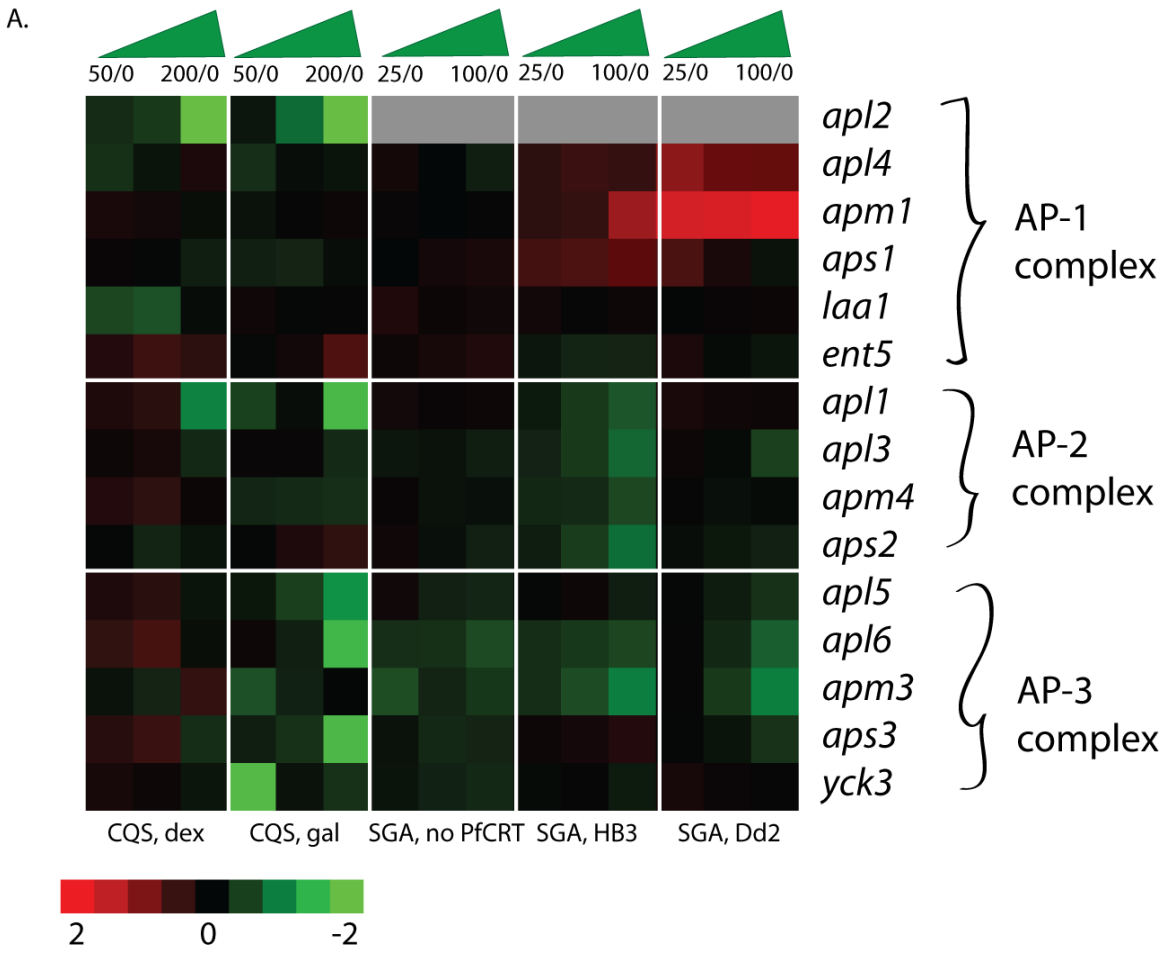


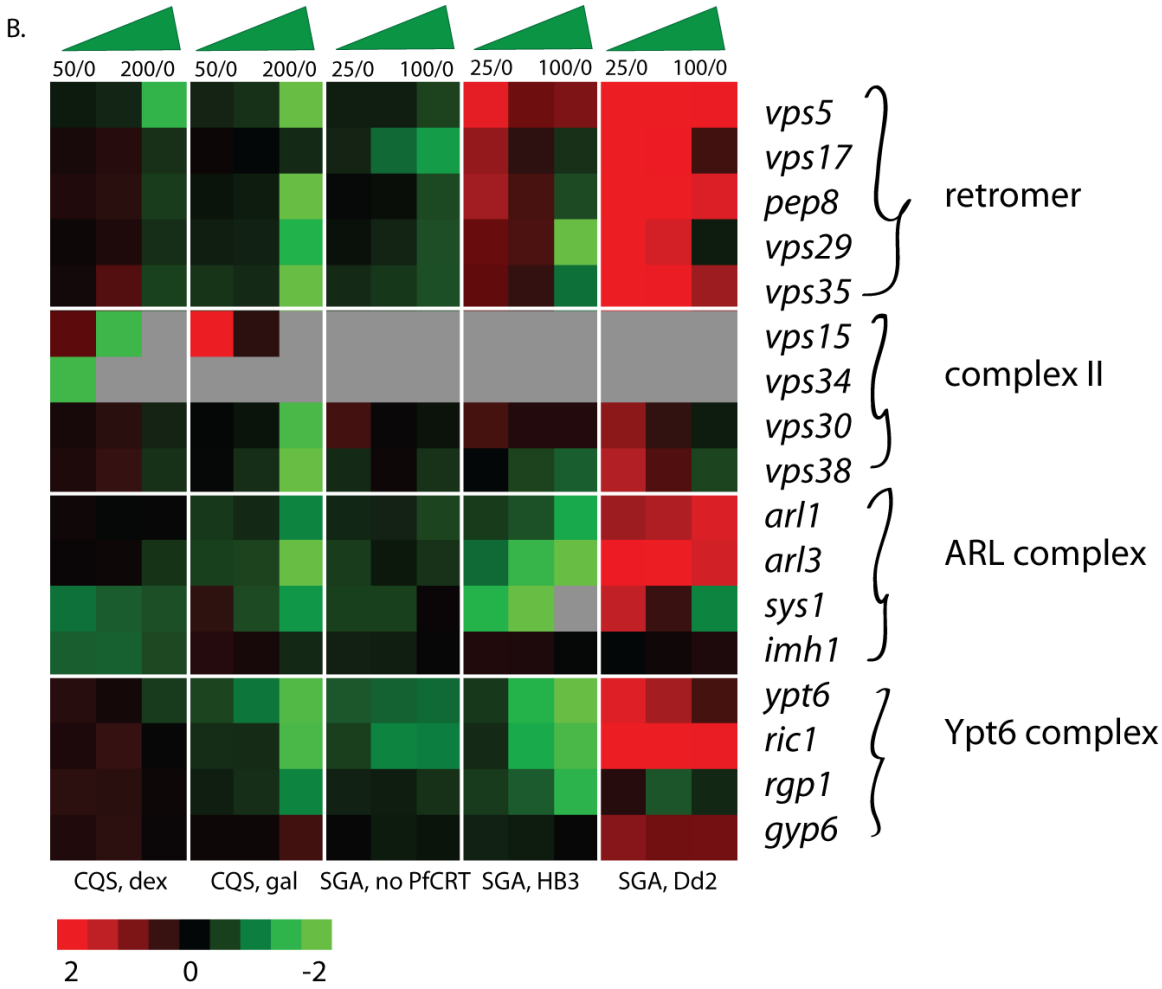
Figure 5. Strains deficient in retromer retrograde-trafficking components exhibit heightened sensitivity to chloroquine.
A. Serial-dilution assays. Chloroquine exacerbates growth defects in *vps5*, *pep8*, *vps29*, *vps35*, and *mon2* strains expressing PfCRT. Note that PfCRT-expressing strains exhibit growth defects in the absence of chloroquine.
B. CPY colony immunoblots. Chloroquine promotes CPY secretion in retromer mutants, although expression of chloroquine-resistant PfCRT appears to suppress the CPY secretion phenotype of *pep8* and *vps35*. Chloroquine-sensitive PfCRT suppresses CPY secretion in *vps5*.

Serial dilution plates were grown at 30°C for 48 hours prior to imaging; colony immunoblots represent CPY secretion in two, four, and six hour periods after nitrocellulose overlay.
KEY: RETROMER (3)
RETROMER (1); ARL COMPLEX (1)
Strains are arranged as no-PfCRT, Dd2, HB3
(*mon2* Dd2, HB3)



Supplementary Figure 1. Exacerbation by chloroquine of protein-trafficking defects and their suppression by PfCRT.
A. Anterograde trafficking defects. Note that
expression of Dd2-PfCRT suppresses defects in the AP-1 endosomal transport route only.

Data are normalized with respect to growth at 0 mM chloroquine.
Left two panels: chemical-genetic profiling of the *S. cerevisiae* deletion and DAmP collections.
Right three panels: chemical-genetic profiling of libraries created for SGA.
Left, no PfCRT; center, chloroquine-sensitive PfCRT; right, chloroquine-resistant



B. Retrograde trafficking defects. Expression of Dd2-PfCRT suppresses growth defects in parallel retrograde transport pathways as well as in the retromer retrograde-transport coat complex.

Data are normalized with respect to growth at 0 mM chloroquine.
Left two panels: chemical-genetic profiling of the *S. cerevisiae* deletion and DAmP collections.
Right three panels: chemical-genetic profiling of libraries created for SGA.
Left, no PfCRT; center, chloroquine-sensitive PfCRT; right, chloroquine-resistant

Chapter 4: Conclusions

Antimalarial drug resistance is a significant problem worldwide, with disease incidence having increased since the failure of the campaign for malaria eradication attempted after the Second World War [1, 2]. The majority of *Plasmodium* species infecting humans are resistant to the former frontline chemotherapeutic chloroquine, and although alternatives to the drug, principally artemisinin derivatives, are widely available, these formulations are expensive and evince reduced efficacy among multidrug-resistant parasites in the field [3, 4].

Mechanisms of action or resistance acquisition by *P. falciparum* parasites remain uncharacterized for many antimalarials, including artemisinin; the arylamino alcohols mefloquine, halofantrine, and lumefantrine; and even quinine, which has been continuously in use for nearly four centuries. However, laboratory based selection studies and characterization of field isolates suggest that the primary mediators of resistance are two proteins localized to the parasite digestive vacuole membrane: the multidrug resistance transporter PfMDR1 (homologous to mammalian P-glycoprotein homologue) and the *P. falciparum* chloroquine resistance transporter (PfCRT), which is implicated in resistance to several antimalarials in addition to that of its namesake, chloroquine [5-12].

PfMDR1's contribution to antimalarial resistance is through drug exclusion from target compartments: loss-of-function mutations in *PFMDR1* that reduce vacuolar antimalarial accumulation have been selected in malaria-endemic locales where drug-treatment regimens historically focused on compounds acting within that compartment (such as S. America), while amplification, which can promote vacuolar sequestration of drugs active in the cytosol, is seen elsewhere (often SE Asia) [11-13]. In contrast, PfCRT exports its substrates from the digestive vacuole, and in drug-resistant parasites gain-of-

function mutations modulate its substrate repertory to include chemotherapeutics [7-9, 14]. PfATP6 (the *P. falciparum* homologue of SERCA) [15], and PfMRP proteins have also been suggested to mediate antimalarial resistance [16], but their associations with resistance to specific chemotherapeutics are unclear [3, 17, 18].

In this work we have characterized yeast response to chloroquine through transcriptional profiling after short-term drug exposure, as well as chemical-genetic screening in the presence and absence of PfCRT, in an effort to identify the cellular processes upon which the drug impinges. Chloroquine's toxicity to *P. falciparum* parasites stems from its interaction with the heme moiety liberated by parasite scavenging and digestion of host hemoglobin. At the acidic pH of the digestive vacuole, the hydrolytic compartment into which hemoglobin is imported for catabolism, the β-hematin dimeric form of heme aggregates into inert crystalline hemozoin in lipid-rich compartmental subdomains ("lipid nanospheres") [19-21]. Chloroquine, by binding β-hematin, prevents this detoxification of heme to cause rampant lipid peroxidation and eventual death from oxidative stress, and mutant PfCRT provides an escape route from the digestive vacuole for diprotonated chloroquine to prevent its lethality [6, 8, 22].

Because PfCRT is not chloroquine's direct target, parasites can presumably adopt a number of strategies to escape drug effects. In fact, only *P. falciparum* appears to achieve chloroquine resistance through mutation of the transporter, with the related human malaria parasite *P. vivax* acquiring the trait in the absence of mutations in *CRT* or *MDR* orthologues [23-25]. Moreover, *P. falciparum* strains resistant to chloroquine or quinine exhibit polymorphisms at numerous genetic loci other than *PFMDR1* or *PFCRT*, though the genes implicated are generally indeterminate or their functions

uncharacterized [5, 26, 27]. Loss of chloroquine-resistant *PFCRT* is also observed in the field subsequent to cessation of chloroquine pressure, and mutant *PFCRT* is therefore believed to exact a fitness cost from parasites that supplementary mutations act to alleviate [28, 29]. Studies such as ours can therefore inform the likely adaptive responses utilized by *P. falciparum* parasites with sequence polymorphisms in *PFCRT* to promote fitness in the face of drug exposure, as well as elucidating the complement of strategies toward resistance adopted by *P. vivax* and other parasites. Because *P. vivax* is not amenable to tissue culture and displays physiological differences relative to *P. falciparum*, the establishment of a model system to investigate its capacity for drug resistance acquisition would provide significant benefits, as the vivax malaria accounts for up to 40% of disease incidence globally per year [30, 31].

In contrast to chloroquine's interaction with digestive-vacuole heme in *P. falciparum*, we find that in *Saccharomyces cerevisiae* the drug principally disrupts pH homeostasis in the yeast vacuole, resulting in genetic interactions with components of the yeast vacuolar H^+-ATPase. In an interesting parallel, early models for *P. falciparum*'s mode of chloroquine resistance acquisition postulated that resistant parasites' digestive vacuole homeostasis was altered to reduce the favorability of chloroquine trapping in the organelle [19, 32]; although this is no longer considered realistic for chloroquine, quinine tolerance in falciparum parasites does appear to operate similarly [10, 33], and vivax malaria's evasion of chloroquine toxicity might likewise involve altered pH homeostasis. Indeed, profiling of the *P. vivax* transcriptome indicates that hemoglobin digestion commences later in the lifecycle than in *P. falciparum* [34], and therefore chloroquine's

injuriousness might stem from obstruction of processes other than hemozoin polymerization in that parasite.

Yeast also exhibit chemical-genetic interactions between chloroquine and the apparatus for protein sorting and transport or for amino acid biosynthesis—the former process being dependent on the existence of a pH gradient along the secretory pathway and the latter likely an effect of the vacuole's maintenance of the cellular supply of nitrogen, chiefly stored as basic amino acids such as arginine or lysine [35-37]. Although *Plasmodium* is auxotrophic for amino acid biosynthesis, amino acid ingestion (in the form of host hemoglobin) is necessary for protein synthesis as well as energy generation [38, 39], and the parasite maintains a complex endocytic system culminating in the digestive vacuole, which contains the hydrolases necessary for hemoglobin catabolism [19]. Chloroquine has been shown by electron microscopy to impair the uptake and fusion of endocytic vesicles with the digestive vacuole, analogous to its effect on yeast protein trafficking [40]. Drug-resistant parasites cultured in the presence of drug display abnormal digestive vacuole morphology [41] also potentially consistent with defects in vesicular fusion with that compartment.

Intracellular protein transport in *Plasmodium* is poorly described, although factors orthologous to the small GTPase encoded by *SAR1*, the TRAPP tethering complex, and the ESCRT multivesicular body maturation machinery are found in *P. falciparum* [39, 42], and parasites also maintain an elaborate exocytic apparatus for host erythrocyte modification [43-45]. To our knowledge the effect of chloroquine on these gene products remains uncharacterized, but it is not unreasonable to suppose an impairment of protein sorting among cellular organelles or to the parasite plasma membrane, parasitophorous

vacuole, or host erythrocyte by disruption of pH homeostasis. In yeast we find that expression of chloroquine-resistant, but not chloroquine-sensitive, *PFCRT* is sufficient to rescue defects in trafficking, principally at the level of anterograde and retrograde protein movement between *trans*-Golgi network and endosome, which is dependent on local pH differentials apparently dissipated by the drug (Chapter 2 and [46]). *P. falciparum* strains resistant to chloroquine and quinine display polymorphisms in numerous transporter genes that may ameliorate drug-mediated dysregulated pH homeostasis [5], although the segregation of mutations in these genes among the progeny of genetic crosses between strains of differing antimalarial resistance phenotypes has seemingly not been investigated.

Yeast's transcriptional response to chloroquine treatment also implicates the vacuole, as it is highly suggestive of ion stress and the yeast vacuole maintains cellular stores of many ions. A previous study found evidence of chloroquine-mediated iron starvation in *Saccharomyces* [47], and our findings are reminiscent of this effect as well as of calcium stress. In *P. falciparum*, short-term treatment with chloroquine impacts the expression levels of few genes, consistent with the relative invariance of the transcriptional program, but interestingly the chloroquine-resistant parasite strain possessing 76I-PfCRT increases expression of the calcium pump encoded by *VCX1* (conserved in yeast), indicative of altered digestive-vacuole homeostasis as a result of long-term chloroquine selection in culture if not short-term drug exposure [48]. Chloroquine selection of *P. falciparum* additionally causes widespread transcriptional changes from loci on each chromosome that have not been parsed at fine resolution [49], but that likely contribute significantly to parasite response to drug. Unfortunately,

understanding of chloroquine-mediated genetic changes is hampered greatly by the large number of uncharacterized ORFs in the *Plasmodium* genome [39]. By analogy to yeast, however, coping strategies for growth in the presence of chloroquine will likely include transcriptional upregulation of ion transporters or the *Plasmodium* vacuolar H^+-ATPase to redress alterations to pH and ion homeostasis, as well as perhaps of digestive vacuole hydrolases and protein transport factors, and with continued work the mechanics of parasite drug-resistance acquisition will be clarified.

We intended this study as a prelude to structure-function analysis for assessment of PfCRT's capacity for interaction with antimalarial chemotherapeutics. Establishment of a heterologous system for the characterization of this determinant of antimalarial multi-drug resistance is warranted for the following reasons: 1) the gene belongs to a novel family, and no crystal structure is currently available for even a related species [50, 51], 2) resistance to several antimalarial drugs is effected or modulated through selection of polymorphic alleles of *PFCRT* [6-10], 3) PfCRT-mediated resistance imposes a fitness cost on parasites [28, 29], suggesting severe constraints on mutation, an understanding of which should inform antimalarial drug design, 4) parasites are refractory to genetic manipulation, making high-throughput parallel culture-based analyses of drug interaction with a panel of modified alleles difficult and time-consuming, and 5) the low rate of resistance acquisition to quinoline antimalarials (1 in 10^{10}) reduces the efficacy of laboratory based selections as a means of investigating the range of alleles that can be selected by drug application [7, 9, 52].

PfCRT has previously been expressed in heterologous systems, including *Dictyostelium discoideum*, both *Saccharomyces cerevisiae* and *Pichia pastoris*, and

Xenopus laevis [22, 53-57]. Although study in *Dictyostelium* and *Xenopus* has enlarged our conception of PfCRT's capacity for drug transport, these systems are less genetically tractable than yeast and lack yeast's complement of tools for rapid high-throughput characterization of drug-protein interactions. The power of yeast genetics suggested the organism as a candidate for the ectopic expression and characterization of *PFCRT* as well as for secondary assays investigating protein competence for interaction with current and novel antimalarial drugs without reference to PfCRT's essential function. Such studies would facilitate identification of chemical formulations to which resistance could easily be acquired by selection of one to two mutations on an already drug-resistant background without lengthy culture-based experimentation. However, though amenable to high-throughput genetic screening, *Saccharomyces* tolerates high concentrations of chloroquine (this work and [58]) and structure-function analysis of PfCRT may require the use of large quantities of drugs, the development of an assay strain sensitized to chloroquine or other antimalarial chemotherapeutics, and/ or the identification of a reporter gene responsive to chloroquine or its secondary effects on yeast. Recent work has miniaturized chemical-genetic screening in yeast [59] and this may prove a workaround to prevent waste of precious novel compounds. Nevertheless, *Saccharomyces*' adaptability to adverse circumstances and high tolerance for chloroquine have hindered our attempts to comprehensively characterize PfCRT in this system, and our apprehension of the protein's essential substrate(s) and potential for mutation remain incomplete. As parasite resistance to antimalarial drugs evolves to encompass recent additions to the chemotherapeutic armamentarium, so too must our understanding of parasite physiology evolve to facilitate malaria eradication.

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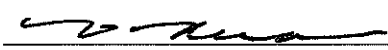

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