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Direct evidence for the adaptive role of copy number variation on antifolate susceptibility in *Plasmodium falciparum*

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Summary

Resistance to antimalarials targeting the folate pathway is widespread. GTP-cyclohydrolase (*gch1*), the first enzyme in this pathway, exhibits extensive copy number variation (CNV) in parasite isolates from areas with a history of longstanding antifolate use. Increased CN of *gch1* is associated with a greater number of point mutations in enzymes targeted by the antifolates, pyrimethamine and sulfadoxine. While these observations suggest that increases in *gch1* CN are an adaptation to drug pressure, changes in CN have not been experimentally demonstrated to directly alter drug susceptibility. To determine if changes in *gch1* expression alone modify pyrimethamine sensitivity, we manipulated *gch1* CN in several parasite lines to test the effect on drug sensitivity. We report that increases in *gch1* CN alter pyrimethamine resistance in most parasites lines. However we find evidence of a detrimental effect of very high levels of *gch1* overexpression in parasite lines with high endogenous levels of *gch1* expression, revealing the importance of maintaining balance in the folate pathway and implicating changes in *gch1* expression in preserving proper metabolic flux. This work expands our understanding of parasite adaptation to drug pressure and provides a possible mechanism for how specific mutations become fixed within parasite populations.

Keywords

malaria; drug resistance; *dhfr*; *Plasmodium falciparum*; Folate; Copy Number Variation

Introduction

The rapid emergence of resistance to antimicrobials poses an increasingly difficult problem for the treatment of all infectious diseases. In the case of malaria, the spread of chloroquine resistance led to a dramatic increase in malaria related morbidity and mortality, and the subsequent widespread use of alternative antimalarial compounds such as the antifolate compounds, pyrimethamine and sulfadoxine (Wellems, 2002; Laufer & Plowe, 2004). Increased use was followed by increased resistance rates and the subsequent discontinuation of antifolates as first line treatments for malaria (Sibley *et al.*, 2001). Now in the face of

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potential resistance to artemesinin based combination therapies, studies of the development of resistance and how resistant alleles are sustained in a population are needed.

Sulfadoxine and pyrimethamine, combined as the antimalarial Fansidar, target two enzymes in the folate pathway, dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), and are competitive inhibitors of the enzymes' natural substrates (7,8-dihydrofolate and PABA, shown in Fig. 1). Accumulation of point mutations within the target enzymes provides parasites with increased levels of resistance (Peterson et al., 1988; Triglia & Cowman, 1994). Many studies have documented the strong association between resistance to antifolates and point mutations in the target genes, however, the role of secondary and compensatory mechanisms that contribute to the spread and stability of resistant parasites within a population is less well understood.

Improved techniques that accurately assess copy number variation (CNV) have highlighted the role that gene amplification plays in the development of resistance to various antimalarials and altering invasion phenotypes (Anderson *et al.*, 2009, Eastman *et al.*, 2011, Triglia *et al.*, 2005, Van Tyne *et al.*, 2011) and CNV is now seen as a major contributor to genetic and phenotypic variation in *P. falciparum* (Estivill & Armengol, 2007). Multiple studies have observed CNV of *GTP cyclohydrolase* (*gch1*) (PF3D7_1224000), a gene which encodes the enzyme that catalyzes the first step in the folate biosynthesis pathway (Fig. 1) (Kidgell *et al.*, 2006; Nair *et al.*, 2008; Jiang *et al.*, 2008; Ribacke *et al.*, 2007). The relationship between *gch1* CNV and resistance associated point mutations was explored in detail by comparing the parasite population genetics of two countries with contrasting histories of antifolate drug pressure (Nair et al., 2008). In Thailand, where antifolates were used as a first line treatment from 1970-1980, *dhps* and *dhfr* mutations are highly prevalent and 72% of Thai parasites were found to carry more than one copy of *gch1*. In contrast, in Laos where antifolates were rarely used until 2006, low frequencies of *dhps* and *dhfr* mutations were identified and almost all parasites studied (98%) carried only one copy of *gch1*. Additionally, parasites harboring the *dhfr* 164L mutation, which confers a high level of pyrimethamine resistance, had a statistically significant higher *gch1* copy number than parasites carrying the wildtype *dhfr* 164I allele.

The stability of *gch1* amplification, even with reduced drug pressure, contrasts with other amplified regions, such as *P. falciparum multidrug resistance protein 1* (*Pfmdr1*), where an increase in copy number is disfavored due to the fitness cost associated with gene amplification (Preechapornkul et al., 2009; Nair et al., 2008). This persistence of increased *gch1* CN along with the positive association of the *dhfr* 164L allele lends additional support to the idea that *gch1* may compensate for a fixed mutation in the genome, be it the *dhfr* 164L mutation or some mutation at another unidentified genetic locus. While these studies linked *gch1* CNV to local patterns of antifolate selection pressure and provided population-level evidence that increased *gch1* CN are a result of positive selection, no study has demonstrated the direct adaptive role of *gch1* CN on drug sensitivity.

It has been hypothesized that overexpression of *gch1* increases the metabolic flux through the *de novo* folate pathway thereby compensating for mutated, less efficient DHPS and DHFR enzymes (Kidgell et al., 2006; Nair et al., 2008). To directly test the hypothesis that *gch1* CNV contributes to pyrimethamine drug resistance we overexpressed *gch1* in different genetic backgrounds and assessed resistance phenotypes. Our results demonstrate that increases in *gch1* CN and expression alter resistance phenotypes differently in diverse parasite genetic backgrounds. We found that in parasites that express low levels of endogenous *gch1*, increases in *gch1* expression result in a significant increase in antifolate resistance. However in parasite lines that have high endogenous expression levels of *gch1*, further increases were not beneficial and could even be detrimental to parasite growth.

These studies highlight how *gch1* expression, *dhps* and *dhfr* mutations all contribute to the establishment of a drug resistant parasite population by creating a balanced flux through the folate pathway enabling drug resistance while maintaining parasite fitness.

Results

Establishment of baseline levels of gch1 copy number and levels of expression

Alterations in gene copy number are often associated with changes in levels of gene expression (Gonzales *et al.*, 2008; Nair et al., 2008). To obtain baselines for *gch1* levels for the parasite isolates used in this study, late stage parasites were collected and DNA and RNA were harvested. *gch1* copy numbers and expression levels were analyzed by quantitative PCR (Q-PCR). In general, the more genomic copies of *gch1*, the higher the expression levels observed (Fig. 2). However, in this analysis of 5 isolates there was not a strict linear relationship between copy number and expression indicating that expression is not necessarily simply a reflection of gene dosage. With the exception of 3D7, parasites with mutations in *dhfr* have increased copy number and expression of *gch1* compared to our wild type line D6, an observation that corroborates previous studies reporting associations between *dhfr* mutations and amplification of the *gch1* locus (Kidgell et al., 2006; Nair et al., 2008).

Direct manipulation of gch1 *CN and expression levels in cultured parasites*

To mimic *gch1* CNV seen in the field, we set out to alter *gch1* expression in cultured parasites and examine its effect on pyrimethamine drug resistance using a regulatable transgene expression system (Epp et al., 2008). By altering the concentration of blasticidin used for selection, this system allowed us to modulate copy number and expression of *gch1* in various genetic backgrounds. Since blasticidin pressure can have off-target effects including altered membrane permeability (Hill et al., 2007), control parasites were transfected with a plasmid expressing *Renilla* luciferase, which does not interfere with folate metabolism. Changes in pyrimethamine sensitivity as a result of *gch1* overexpression were then determined. Chloroquine sensitivity assays were also performed on all transfected lines as an additional control to ensure that our genetic manipulations specifically affected antifolate resistance and were not leading to a generalized multi-drug resistant phenotype. As expected chloroquine sensitivity did not change with *gch1* overexpression in any of the lines tested (Supplementary Fig 1).

gch1 *overexpression in wild type parasites*

As mentioned, *gch1* overexpression appears to be strongly associated with *dhfr* genotype. We were therefore interested in how parasites with different *dhfr* alleles respond to increasing *gch1* expression. D6 is a West African parasite isolate that has only one genomic copy of *gch1* and relatively low endogenous RNA levels (Fig. 2). In addition, D6 has no mutations in *dhps* and *dhfr*, making this parasite highly sensitive to antifolates. The *gch1* expressing plasmid was stably transfected into D6 and selected under 5 and 20 μg ml^{-1} blasticidin, thus creating parasite lines carrying increasing plasmid copy numbers. Q-PCR analysis of gDNA confirmed that there was a step-wise increase in copy numbers for both *gch1* and *blasticidin-s-deaminase* (*bsd*) (Fig. 3A). RNA expression levels of *gch1* showed an initial 48-fold increase (compared to untransfected parasites) when parasites were grown under 5 ug ml^{-1} blasticidin pressure and an additional rise to 66-fold increased expression with higher blasticidin concentrations (Fig. 3B). D6 parasites overexpressing *gch1* became more pyrimethamine resistant as illustrated by the right shift in the dose response curve and the higher IC_{50} value compared to the control lines (Fig. 3C, D). This demonstrates for the first time that increasing *gch1* expression levels alone alters pyrimethamine IC_{50} values in cultured parasites. To determine if the effect of increased *gch1* expression was specific to

pyrimethamine, we also performed assays with proguanil, another DHFR inhibitor, and found similar decreases in drug sensitivity (Supplementary Fig. 2). In both cases, though the changes in IC_{50s} were small compared to the fold increase in *gch1* expression, the higher IC_{50} associated with increased *gch1* expression was highly reproducible, statistically significant and the assays were performed with independent transfections and dose titrations. In contrast, assays with artemisinin and chloroquine showed no shifts in IC_{50} values (Fig. 4C, D), confirming that the effect is specific to the folate pathway. As an additional control, we removed blasticidin pressure prior to repeating our drug assay. In the absence of continued blasticidin selection, parasites rapidly shed episomal plasmids, thus losing the source of increased *gch1* expression. After one week grown in the absence of blasticidin, the parasite's IC_{50} reverted back to the level of our control *Renilla* line (Fig. 4A), thus demonstrating that our genetic manipulations only affected the folate pathway and that the parasites did not harbor any additional off target mutations that might contribute to a generalized increased drug resistance phenotype. These data strongly suggest that the IC_{50} shift was only attributable to changes in *gch1*.

gch1 overexpression in parasites with low level pyrimethamine resistance

We next examined the Honduran parasite line, HB3. HB3 has a greater *gch1* CN and higher expression than D6 and carries one mutation in *dhfr* (S108N, Fig. 2) which confers low level resistance to pyrimethamine and is generally thought to be the first mutation acquired in *dhfr* in response to exposure to DHFR inhibitors. Like D6, transfected HB3 parasites increased *gch1* copy number and expression levels in response to increased blasticidin pressure (Fig. 5A, B). However, there was less of an increase in *gch1* transcript levels in HB3 than in D6 (9-fold in HB3 GCH(20)). Overexpression of *gch1* in HB3 parasites under high but not low blasticidin pressure caused a subtle but statistically significant decrease in pyrimethamine susceptibility compared to the *Renilla* control (Fig. 5C, D) reflective of a modest increase in *gch1* expression. Our inability to amplify *gch1* CN and expression to the same degree in HB3 as we did in D6 appears to be a limitation of our system. Overexpression of *Renilla* was similarly limited to less than 10-fold in HB3 (Supplementary Fig. 3), suggesting that the limited increases in *gch1* expression levels were secondary to the parasite's response to blasticidin rather than a direct effect of increased *gch1*.

gch1 overexpression in parasites with low level sulfadoxine resistance

To examine the role of *dhps* mutations, we next looked at changes in 3D7 drug sensitivity resulting from *gch1* overexpression. 3D7, however, has an unusual set of mutations within the folate pathway. In general, mutations in *dhfr* are thought to precede mutations in *dhps,* and high baseline *gch1* expression is more often associated with resistant *dhfr* and *dhps* haplotypes, as previously discussed. 3D7 harbors one mutation in *dhps,* providing it with a low level of resistance to sulfadoxine, a wildtype *dhfr,* and a high baseline *gch1* CN and expression (Fig. 2). However, we do not expect sulfadoxine resistance to change with *gch1* overexpression as this drug competes with PABA, which is not an intermediate of the folate pathway. While selection of transfected lines resulted in up to a 30-fold increase in *gch1* expression levels (Fig. 6A, B), unlike the shift observed in D6, there was no increase in pyrimethamine resistance in these parasites, but rather a slight but statistically significant increased sensitivity to pyrimethamine with *gch1* overexpression (Fig. 6C, D).

Highly resistant dhfr *alleles and* gch1 *overexpression*

In parasites isolated from various geographical regions, increasing numbers of mutations in *dhfr* are associated with higher levels of antifolate resistance (Fig. 2). For example, the hyper-resistant parasites Dd2 and V1/S have been shown to have three and four *dhfr* mutations respectively, three mutations in *dhps* as well as high levels of *gch1* expression (Fig. 2). Unfortunately, our overexpression system was not compatible with parasite lines

Dd2 and V1/S. Transfected lines showed no significant increase in *gch1* expression (Supplementary Fig. 4). This trend was observed in our *Renilla* controls as well, indicating that failure to overexpress our construct was due to the parasites' response to blasticidin and not a response to *gch1* overexpression as was seen to a limited extent in HB3 (Supplementary Fig. 3).

Microarray studies have shown that expression levels can differ between clones of the same genetic background (Rovira-Graells *et al.*, 2012). We observed such variation in both Dd2 and V1/S when we examined clonal lines that had been maintained in different laboratories. Therefore, although we were unable to manipulate *gch1* CN, we were able to utilize spontaneous alterations in *gch1* expression to investigate changes in pyrimethamine sensitivity. We analyzed two V1/S parasite lines and determined that while they had similar *gch1* CN (Fig. 2A), they displayed substantially different *gch1* expression levels (Fig. 7A). We confirmed that these lines were derived from the same original clone using Southern analysis of genomic DNA. A probe to the conserved region of the hypervariable *var* gene family yielded identical hybridization patterns, thus confirming that the parasites are isogenic. In addition, both lines carried the same four *dhfr* mutations (Supplementary Fig. 5). These parasite lines therefore enabled us to examine parasites with the same genetic background and *dhfr* haplotype yet with significantly different *gch1* expression levels. They also allowed us to examine changes in *gch1* expression in the setting of a quad mutant *dhfr*. Drug sensitivity assays confirmed that they have drastically dissimilar pyrimethamine IC_{50} values (Fig.7B, C); the higher *gch1* expressing clone, V1/S B displayed an IC_{50} that was nearly twice that of its sibling clone V1/S A, further implicating *gch1* overexpression as a modulator of pyrimethamine resistance.

Direct manipulation of both dhfr *haplotype and* gch1 *expression*

To further explore the effects of *dhfr* alleles and *gch1* expression on pyrimethamine resistance, we introduced a quad mutant *dhfr* (N51I, C59R, S108N, I164L) into D6 and 3D7 parasites in combination with increased *gch1* expression. We created parasites with a plasmid expressing the quad mutant *dhfr* allele maintained as an episome rather than by allelic exchange. This method is relatively rapid and avoids the significant population "bottle neck" resulting from selection for genomic integration, and the resulting "pseudo-diploid" lines are therefore less likely to have acquired other compensatory mutations that would have the potential to alleviate the fitness cost associated with the mutant *dhfr*. In the presence of increasing amounts of pyrimethamine, the mutant DHFR will become dominant due to the inhibitory effect of the drug on the wildtype enzyme. D6 DHFR (4M) and 3D7 DHFR (4M) are parasite lines that harbor an endogenous wild type *dhfr*, and an episomally expressed quad mutant *dhfr* along with episomally expressed *gch1* or *Renilla*. Our transgenic lines were created using blasticidin and neomycin as selectable markers and thus the drug sensitivity assays were the first time they were exposed to pyrimethamine and forced to rely on the quad mutant DHFR.

The dose response curves to pyrimethamine revealed complex interactions between different DHFR alleles and levels of GCH1 expression. As expected, *gch1* overexpression in D6 resulted in an increase in resistance, however as drug pressure was increased the advantage of *gch1* overexpression diminished to the point where the two curves meet (Figure 8A). It is also notable that there was a decline in survival in D6 DHFR(4M) *Renilla* (20) when compared to D6 DHFR(4M) GCH (20) at doses of pyrimethamine below the IC_{50} of the quad mutant DHFR. This suggests a growth disadvantage when D6 parasites are forced to depend on the quad mutant *dhfr* in the absence of increased *gch1* expression. For 3D7, in the presence of the quad mutant *dhfr,* increased *gch1* expression resulted in a significant increase in pyrimethamine resistance at pyrimethamine concentrations below the IC_{50} (Figure 8B). However at concentrations above the IC_{50}, 3D7 DHFR(4M) GCH(20) parasites

became significantly more sensitive to pyrimethamine than the control line as was seen in the previous 3D7 experiments expressing only increased *gch1*.

Discussion

Our data provide the first direct evidence of the adaptive role of *gch1* and is consistent with *gch1* amplification serving as a compensatory genetic alteration in parasites with resistance mutations in *dhfr* or *dhps*. Antifolate resistance in *P. falciparum* has been noted to occur in a stepwise fashion, with each point mutation in the target genes contributing to a greater level of resistance. We have presented direct evidence that in addition to point mutations, CNV with subsequent increased expression of the enzyme responsible for the first step in the folate pathway alters parasite susceptibility to pyrimethamine. The diversity of responses observed in different genetic backgrounds reveals the interesting and complex biology surrounding the many factors contributing to antifolate resistance in this important human pathogen.

Increased gch1 levels have a variable effect on pyrimethamine IC_{50} values

Across different *dhfr* haplotypes in three different parasite lines, D6, HB3, and V1/S, increased *gch1* expression resulted in a shift in IC_{50} to a more pyrimethamine resistant phenotype. This is consistent with the model that increased flux through the folate pathway leads to more intermediate products that compete with pyrimethamine for binding to their target enzymes and thus a more resistant parasite. 3D7 was the outlier with a mild, though significant shift to increased sensitivity with increased *gch1* expression. This may reflect the unusual makeup of the folate pathway of 3D7, which displays very high levels of endogenous *gch1* expression and thus any benefit from increased activity at this step in the pathway is potentially saturated. 3D7 also harbors a mutation in *dhps* (Fig. 2), conferring low level resistance to sulfadoxine and potentially altering flux through the pathway (Fig. 1).

There is a balance of flux through the folate pathway that when disturbed can be detrimental to parasite survival

As mentioned above, increases in *gch1* expression did not result in increased pyrimethamine resistance in 3D7. Further, increased *gch1* expression was even detrimental to parasite growth in our 3D7 DHFR(4M)GCH(20) parasites when challenged with high levels of pyrimethamine (Fig. 8B). This demonstrates that there is a limit to the benefit of increased GTP-CH activity. In wildtype parasites, it is assumed that the first step catalyzed by GTP-CH is rate limiting as has been experimentally validated in other organisms (Hossain *et al.*, 2004). However, when parasites acquire mutations in the enzymes downstream of GTP-CH, or in the presence of inhibitors like pyrimethamine, these enzymatic steps are slowed and could become rate limiting. Increased input into the folate pathway with amplified *gch1* and resultant accumulation in metabolic intermediates can contribute to antifolate resistance by competing with drugs for binding to enzymes. However it is also possible that excess accumulation of pathway intermediates could be deleterious, especially in the setting of a marked decrease in efficiency downstream of GTP-CH.

Higher levels of certain metabolites may either be directly toxic or may inhibit other essential enzymes, with both possibilities resulting in decreased parasite viability. This second scenario is termed the “domino effect” and describes a situation in which one drug (*e.g.* pyrimethamine) inhibits a known enzyme (*e.g.* DHFR*)* and causes accumulation of a substrate (*e.g.* DHF) which will then inhibit another enzyme. This phenomenon has been observed in *E. coli* where DHFR inhibition by trimethoprim results in a buildup of DHF, which in turn inhibits folylpoly-γ- glutamate synthetase, another enzyme in folate metabolism (Kwon *et al.*, 2008). It is conceivable that these deleterious effects will be

magnified when upstream reactions generate excess metabolites as is the case with *gch1* overexpression. In other words, parasite survival in the presence of pyrimethamine can be seen as a balance between acquiring the necessary folate for survival and minimizing the harmful effects of metabolite buildup that might accumulate as a result of *gch1* overexpression.

Plasticity of copy number

In other organisms gene duplications and amplifications are known to be unstable and more frequent, compared to point mutations, and this is likely also the case for *P. falciparum*. It is well described that CNV occurs in the field as well as *in vitro* under selection conditions, usually with amplification breakpoints in monomeric tracts of A or T base pairs (Anderson *et al.*, 2009). For *gch1*, amplifications have only been found in field isolates and have not been observed in parasites maintained *in vitro* under low doses of pyrimethamine. In contrast, *dhfr* amplification has only been found in parasites grown *in vitro*, while in circulating parasites point mutations rather than CNV are detected, indicating a difference in selective pressure under culture and field conditions. It is also worth noting that most field studies that have identified CNV in parasite populations have not directly examined expression levels. In both the laboratory isolates studied here and in our genetically manipulated lines there was not a linear relationship between *gch1* copy number and mRNA expression. This additional complexity should be taken into account in future studies of CNV variation and parasite phenotypes.

Implications for antifolate development

While changes in IC_{50} like those reported here are modest, they are likely to be sufficient to facilitate the establishment of resistant alleles of downstream target enzymes within a population of circulating parasites. By compensating for less efficient DHFR (and possibly DHPS) enzymes and thereby maintaining a required balance within the folate pathway, increased *gch1* CN could contribute to fixation of *dhfr* and *dhps* mutations within a geographical region, as has been observed in Thailand. Thus, *gch1* CNV likely contributes to antifolate resistance through subtle changes in parasite fitness rather than directly through drug treatment failure. However, the observation that the compensatory benefit of *gch1* overexpression has a limit and can even be deleterious in the presence of the quad mutant *dhfr* suggests that this could provide an avenue for intervention. Considering that new inhibitors of the quad mutant DHFR are in development, it is important to consider all adaptations the parasite makes to establish and maintain drug resistance, and even to consider targeting GTP-CH itself.

Experimental Procedures

Plasmodium falciparum *culture and transfection*

P. falciparum lines were cultured at 5% hematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate, and 0.1 mg/ml gentamicin. Parasites were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen. V1/S and D6 parasite lines were obtained from MR4. Parasites were transfected by using "DNA loaded" red blood cells as previously described (Deitsch *et al.*, 2001). For stable transfections, parasites were cultured in media containing either 500 $\mu g\ ml^{-1}$ neomycin or the designated concentration of blasticidin.

Plasmid construction

The HBIRH plasmids expressing *blasticidin-s-deaminase* (*bsd)* and *Renilla luciferase* were constructed as described previously (Epp *et al.*, 2008). The plasmid contains a bidirectional

promoter that drives both the selectable marker *bsd* and the gene of interest, *Renilla* or *gch1*. The more blasticidin added to the culture the higher copy numbers and expression levels, thus allowing for manipulation of copy number. The promoter driving *gch1* expression from the plasmid is transcriptionally active late in the cell cycle (Epp et al., 2008), corresponding to the peak expression of endogenous *gch1* (Nirmalan et al., 2002). Construction of a plasmid expressing GTP-CH, was made by PCR amplification of the coding region of *gch1* (PF3D7_1224000) with primers to introduce NotI and SacI cloning sites. Construction of the mutant DHFR plasmid was made by PCR amplification of *dhfr−ts* (PF3D7_0417200) with primers to introduce restriction sites NotI and SacI using V1/S gDNA as template. All primers used for plasmid construction are shown in Supplementary Table 1.

Determination of gch1 *copy number and expression by quantitative PCR*

For all parasites grown under blasticidin pressure, the drug was removed 24-48 hours prior to synchronization to eliminate the effect of blasticidin on sorbitol permeability (Hill *et al.*, 2007). Parasites were synchronized using percoll-sorbitol gradients and purified schizonts were placed back in culture and allowed to mature to the late trophozoite stage. DNA was isolated by phenol:chloroform extraction and RNA isolation was performed using the TRIZOL LS Reagent (Invitrogen) as described previously(Epp et al., 2008) cDNA was synthesized from 2 μg total RNA in a reaction volume of 50 μl. For each cDNA synthesis reaction, a control reaction without reverse transcriptase was made with identical amounts of template and primers. Q-PCR was carried out as follows. All reactions were performed at a final primer concentration of 0.5 μM using Bio-Rad ITAQ SYBR SUPERMIX® in 20 μl reactions on an ABI Prism® 7900HT real-time PCR machine. A validation experiment was performed, as described in the Applied Biosystems User manual, to ensure equal amplification efficiencies of the housekeeping and target gene primer sets. The ΔCT for each individual primer pair was determined by subtracting the CT value of the target gene from the CT value of the control gene, seryl-tRNA synthetase (PF3D7_0717700) (Applied Biosystems, User Bulletin 2). ΔCTs were then converted to relative copy numbers or expression with the formula $2^{\Delta Ct}$. All runs were done in triplicate and results are representative of at least two experiments. Multiple concentrations of template gDNA and cDNA were used in each run and the calculated copy number and expression levels were averaged. Raw ct values are included in Supplementary file 1.

Drug sensitivity assays and IC_{50} analysis

Drug sensitivity assays were performed on cultured parasites using SYBR Green I as described previously (Smilkstein *et al.*, 2004). Prior to the assay, parasite cultures were synchronized using an alanine-HEPES solution (Braun-Breton *et al.*, 1988) to obtain a synchronous culture of ring stage parasites. 100 μl aliquots of parasite culture were distributed into clear 96 well plates to achieve a starting parasitemia of 0.2-0.5% and 2% hematocrit. Stock solutions were prepared at 20 mg ml^{-1} in DMSO for pyrimethamine and 1 mg ml^{-1} in water for chloroquine. Final concentrations for each assay were varied between isolates and ranged from 804 uMto 0.8 nM for pyrimethamine and 1.7 uM to 1.7 nM for chloroquine. Drug dilutions were in complete media and 100 μl of the prepared drug dilution was added for a total volume of 200 μl per well. Edge wells were filled with media only to prevent any scatter in data resulting from disproportionate dehydration near the edge of the plate. Plates were placed in an airtight chamber, flushed with 5% oxygen, 5% carbon dioxide, and 90% nitrogen, and allowed to grow for 72 hours. At the end of the growth period, contents of wells were resuspended and 150 μl of the culture was transferred to a 96 well black plate designed for fluorescent readings. Plates were then placed in the −80°C freezer overnight. After allowing the plates to thaw completely, 100 μl of SYBR Green diluted in lysis buffer (0.2 μl SYBR Green/ml lysis buffer) (Smilkstein et al., 2004) was added to each well and allowed to shake in the dark at room temperature for 1 hour. Plates

were then read using SpectraMax Gemini using an excitation wavelength of 490 nm and 530 nm detection. Data analysis was performed with Graphpad Prism software. Counts were plotted against the logarithm of the drug concentration, normalized, and then curve fitted by nonlinear regression (sigmoidal dose-response/variable slope equation) to yield IC_{50} values. One way ANOVA and unpaired t-tests were performed in Excel combining the data from repeat dose response experiments. All error bars in generated plots show standard deviation. Multiple independent transfections were performed in the D6 line and yielded consistent results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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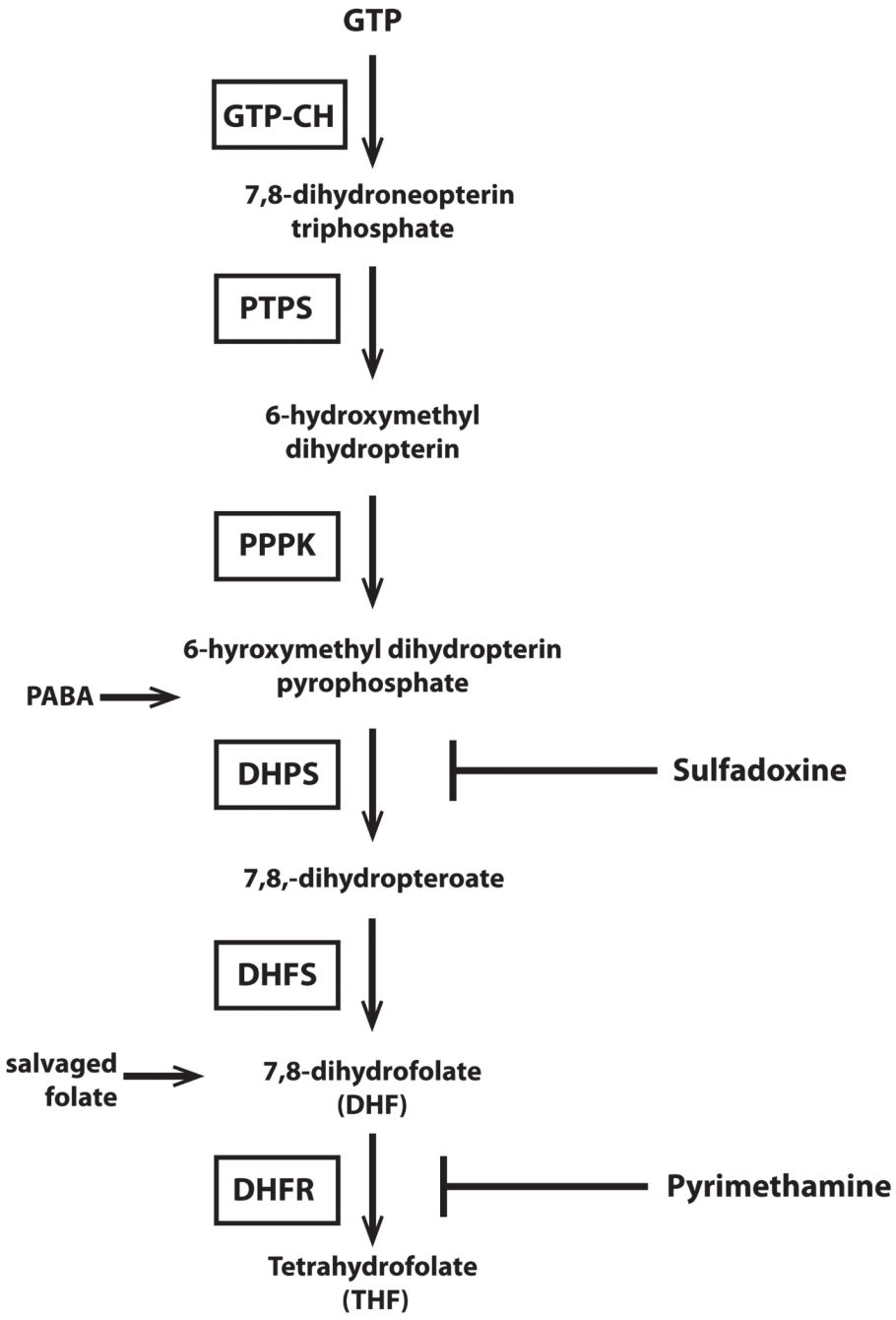
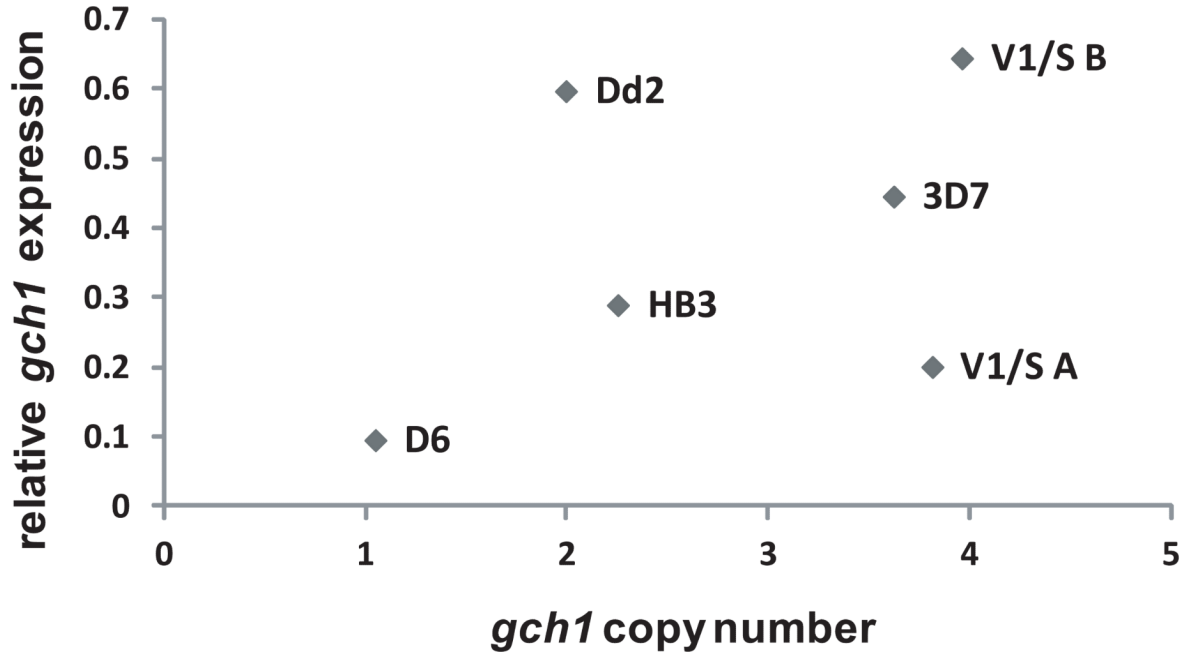


Figure 1. The *de novo* folate biosynthesis pathway of *P. falciparum*

GTP-CH is the first enzyme in the folate biosynthesis pathway and has been found to exhibit extensive copy number variation. Enzymes in the folate pathway are boxed and substrates are in plain text. Abbreviations: GTP-cyclohydrolase (GTP-CH), pyruvolytetrahydropterin synthase (PTPS), hydroxymethyldihydropterin pyrophosphokinase (PPPK), dihydropteroate synthase (DHPS), dihydrofolate synthase (DHFS), dihydrofolate reductase (DHFR). Inhibitors of folate biosynthesis are shown at the right of the pathway: sulfadoxine (SDX), pyrimethamine (PYR). *para*-aminobenzoic acid (PABA) enters as a substrate for DHPS. Salvaged folate can also enter the pathway upstream of DHFR.

A



B

Parasite isolate (origin)	*dhfr* point mutations	*dhps* point mutations
D6 (Sierra Leone)	None	None
HB3 (Honduras)	S108N	None
3D7 (The Netherlands)	None	A437G
Dd2 (Indochina)	N51I, C59R, S108N	S436F, A437G, A613S
V1/S (Vietnam)	N51I, C59R, S108N, I164L	S436F, A437G, A613T

Figure 2. Baseline levels of *gch1* copy number and levels of expression for isolates with differing *dhfr* and *dhps* haplotypes

(A) *gch1* copy number and expression levels were measured by Q-PCR and Q-RTPCR and plotted against each other. (B) The table shows the antifolate drug resistance profiles of the different isolates along with the accompanying mutations in *dhfr* and *dhps.* V1/S A and V1/S B are isogenic lines cultured in different laboratories. Point mutations listed are taken from (Peterson et al., 1988).

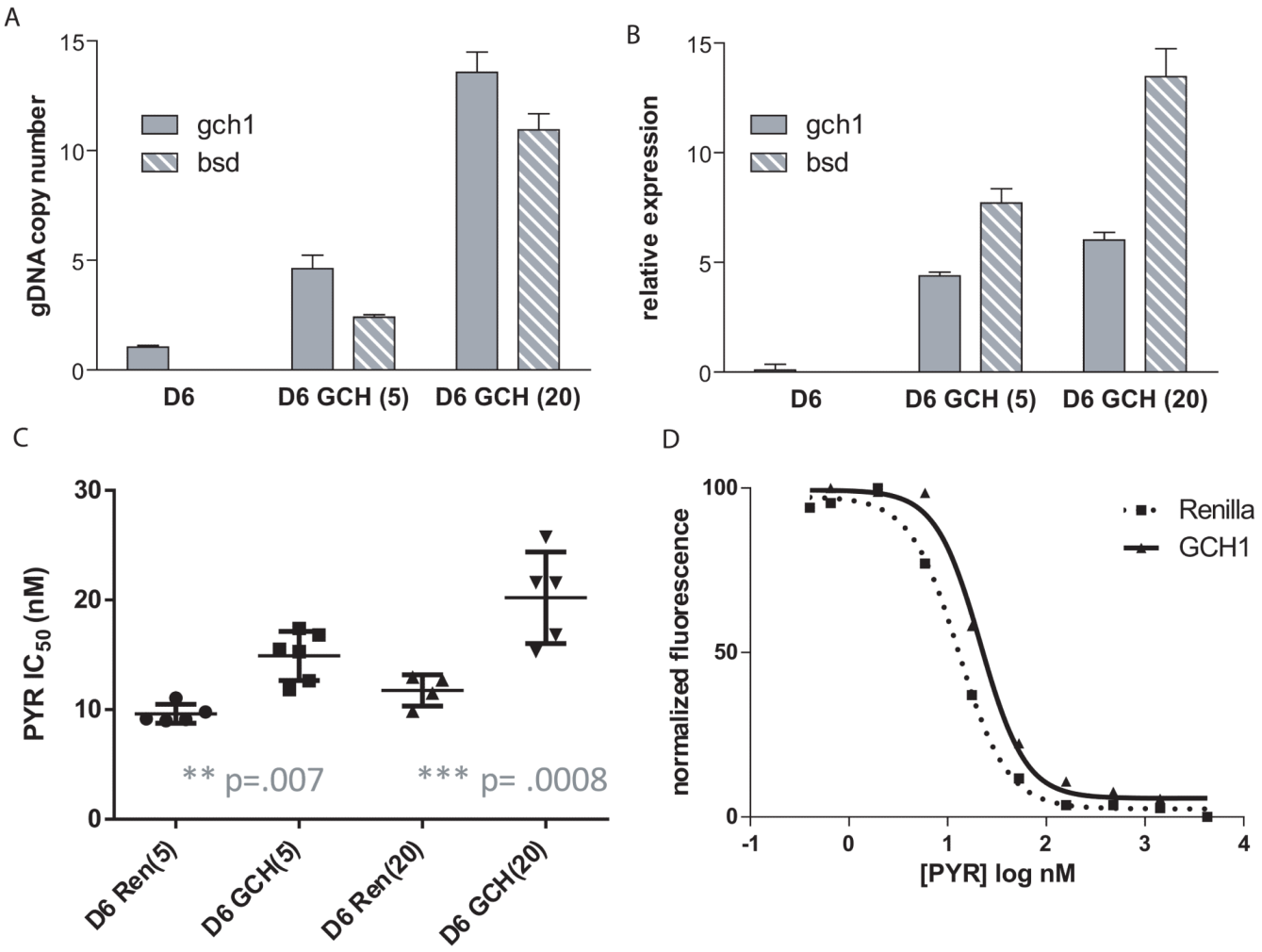


Figure 3. *gch1* amplification in D6 increases pyrimethamine resistance

Bar graphs show *gch1* and *bsd* copy number (A) and expression levels (B) in untransfected parasites (D6), parasites grown under 5 μg ml^{-1} blasticidin, D6 GCH(5), and 20 ug ml^{-1} blasticidin, D6 GCH(20). (C) Pyrimethamine IC_{50} values were calculated for each *gch1* line and compared to parasites transfected with a *Renilla* expressing control plasmid grown under the same amount of blasticidin. Both D6 GCH(5) and D6 GCH(20) show a statistically significant increase in pyrimethamine IC_{50} values (unpaired t-test, p=.007 and . 0008, respectively, n=4 or greater). (D) The dose response curves for parasite lines grown under 20 μg ml^{-1} blasticidin is graphically depicted and reveals a shift to the right in *gch1* overexpressing parasites. This curve is representative of multiple experiments.

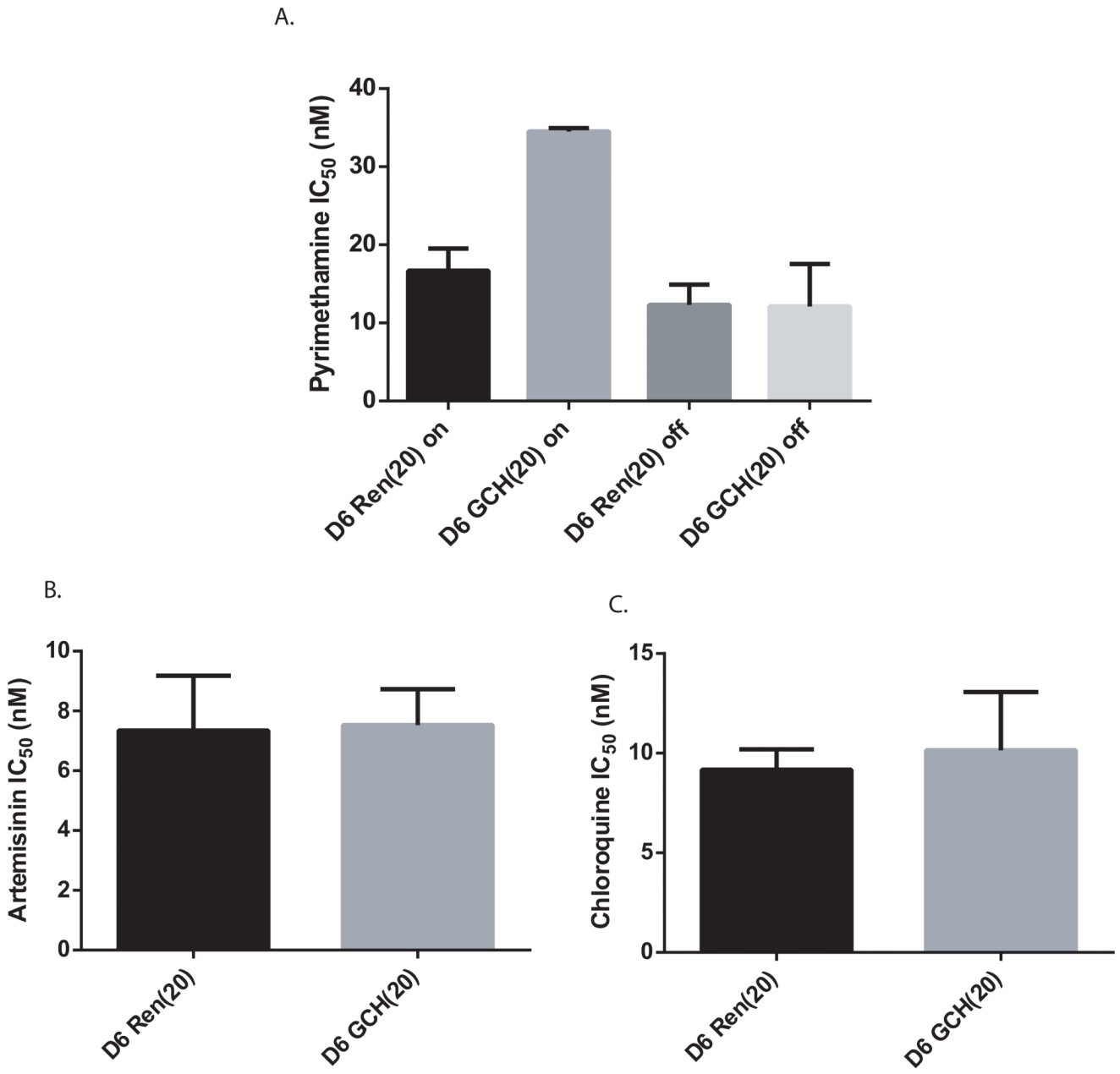


Figure 4. Continuous *gch1* overexpression is required for increased pyrimethamine resistance and does not affect sensitivity to artemisinin or chloroquine

(A) IC_{50} values are shown for parasites grown under continuous blasticidin pressure (on) and those removed from blasticidin pressure for one week prior to performing the drug assay (off). Similar IC_{50} values are observed for Ren(20)on and GCH(20)off, supporting the conclusion that overexpression of *gch1* is the factor leading to increased drug resistance seen in D6 GCH(20)on when compared to D6 Ren(20)on. Chloroquine and artemisinin IC_{50}s do not change with *gch1* overexpression (B,C).

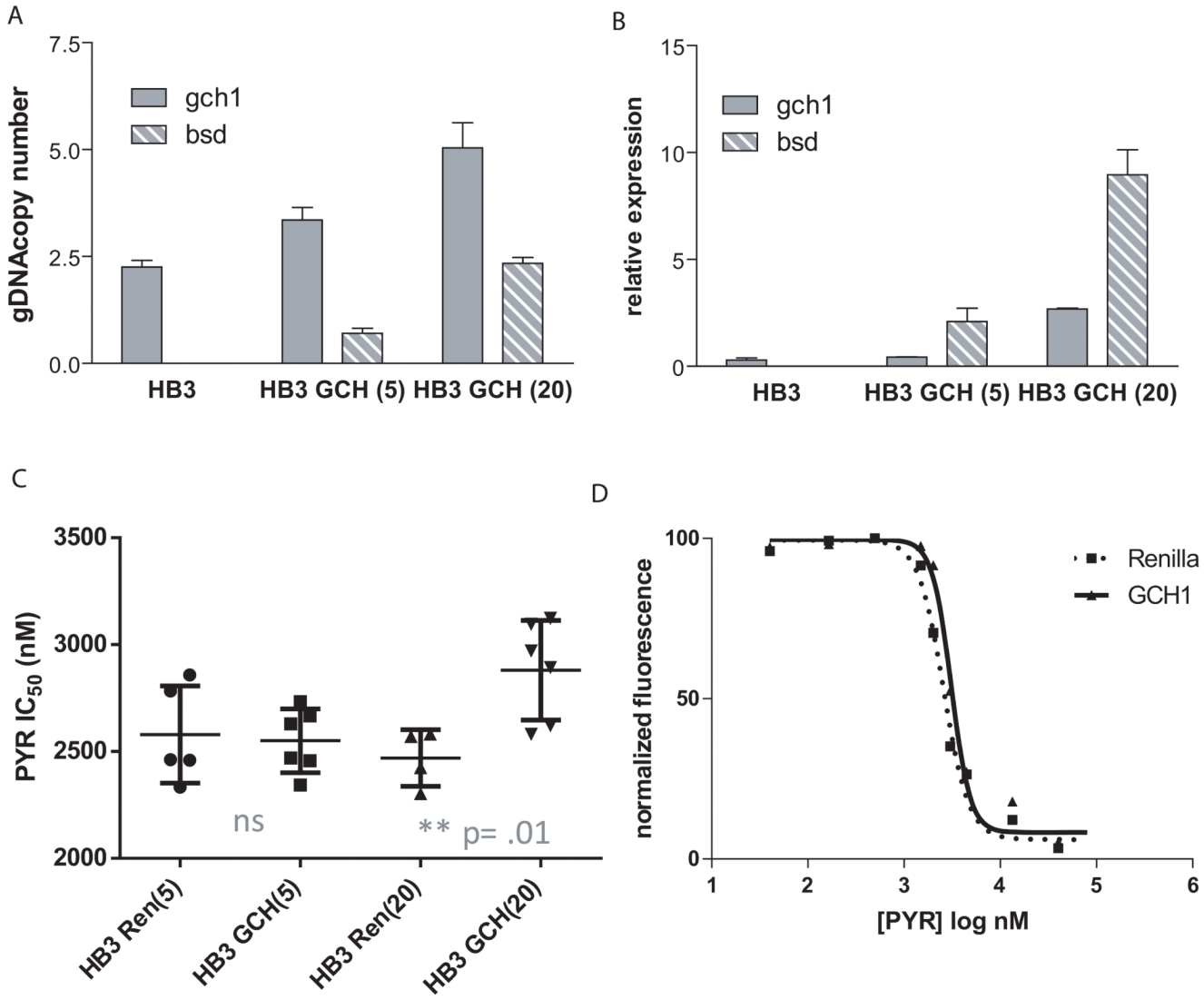


Figure 5. *gch1* amplification in HB3 modestly increases pyrimethamine resistance

Bar graphs show *gch1* and *bsd* copy number (A) and expression levels (B) in untransfected parasites (HB3), parasites grown under 5 μg ml^{-1} blasticidin, HB3 GCH(5), and 20 μg ml^{-1} blasticidin, HB3 GCH(20). (C) Pyrimethamine IC_{50} values were calculated for each *gch1* line and compared to parasites transfected with a *Renilla* expressing control plasmid grown under the same concentration of blasticidin. Statistical analysis revealed a significant increase in pyrimethamine IC_{50} in HB3 GCH(20) but not in HB3 GCH(5) (unpaired t-test, p=.01, n=4 or greater). (D) The dose response curve for parasite lines grown under 20 μg ml^{-1} blasticidin is graphically depicted and reveals a slight shift to the right in *gch1* overexpressing parasites which becomes less pronounced at higher drug concentrations. This curve is representative of multiple experiments.

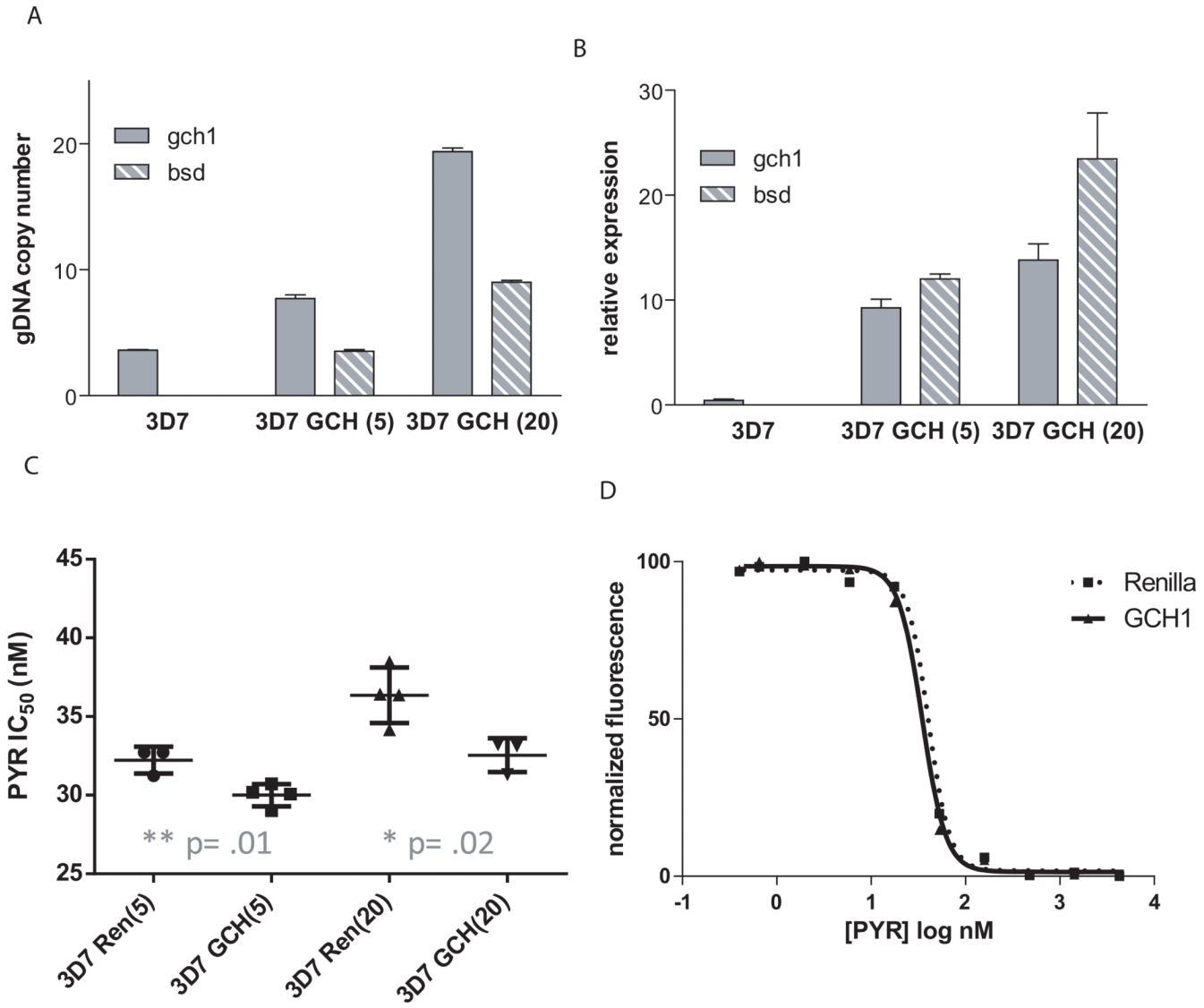


Figure 6. *gch1* amplification in 3D7 fails to increase pyrimethamine resistance

Bar graphs show *gch1* and *bsd* copy number (A) and expression levels (B) in untransfected parasites (3D7), parasites grown under 5 μg ml^{-1} blasticidin, 3D7 GCH(5), and 20 μg ml^{-1} blasticidin, 3D7 GCH(20). (C) Pyrimethamine IC_{50} values were calculated for each *gch1* line and compared to parasites transfected with a *Renilla* expressing control plasmid grown under the same concentration of blasticidin. Statistical analysis revealed a slight but statistically significant increase in pyrimethamine sensitivity for both 3D7 GCH(5) and 3D7 GCH(20) (unpaired t-test, p=.01and .02, respectively, n=4 or greater). (D) The dose response curve for parasite lines grown under 20 μg ml^{-1} blasticidin is graphically depicted and reveals a slight shift towards sensitivity (left shift) in *gch1* overexpressing parasites. This curve is representative of multiple experiments.

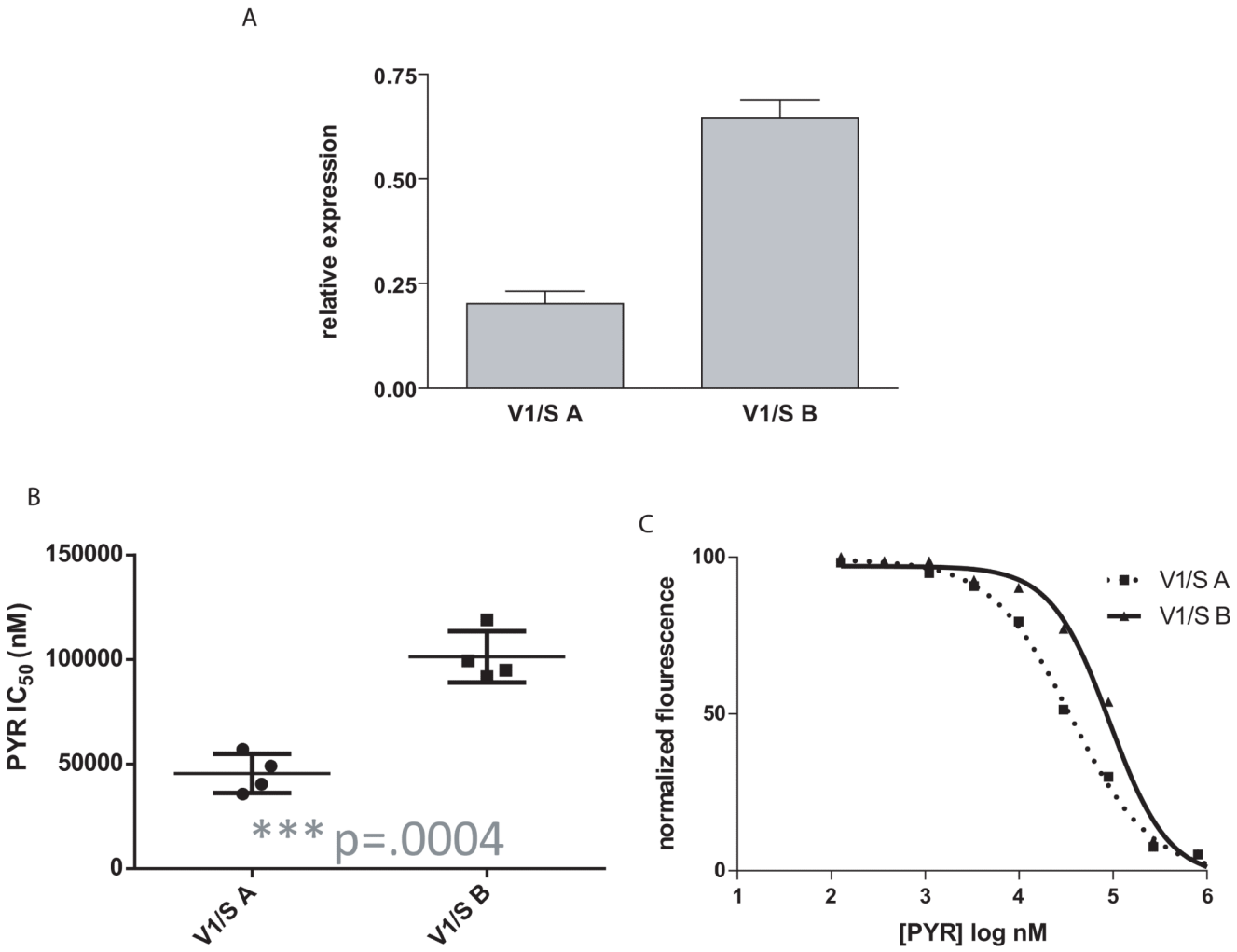


Figure 7. Two V1/S clones have distinct *gch1* expression profiles as well as pyrimethamine IC_{50} values

Q-RTPCR reveals a difference in *gch1* expression levels (A) which follow a similar pattern to pyrimethamine IC_{50} values (B) (unpaired t-test, p=.0004, n=4). Dose response curves show the difference in pyrimethamine resistance between the two lines. This curve is representative of multiple experiments.

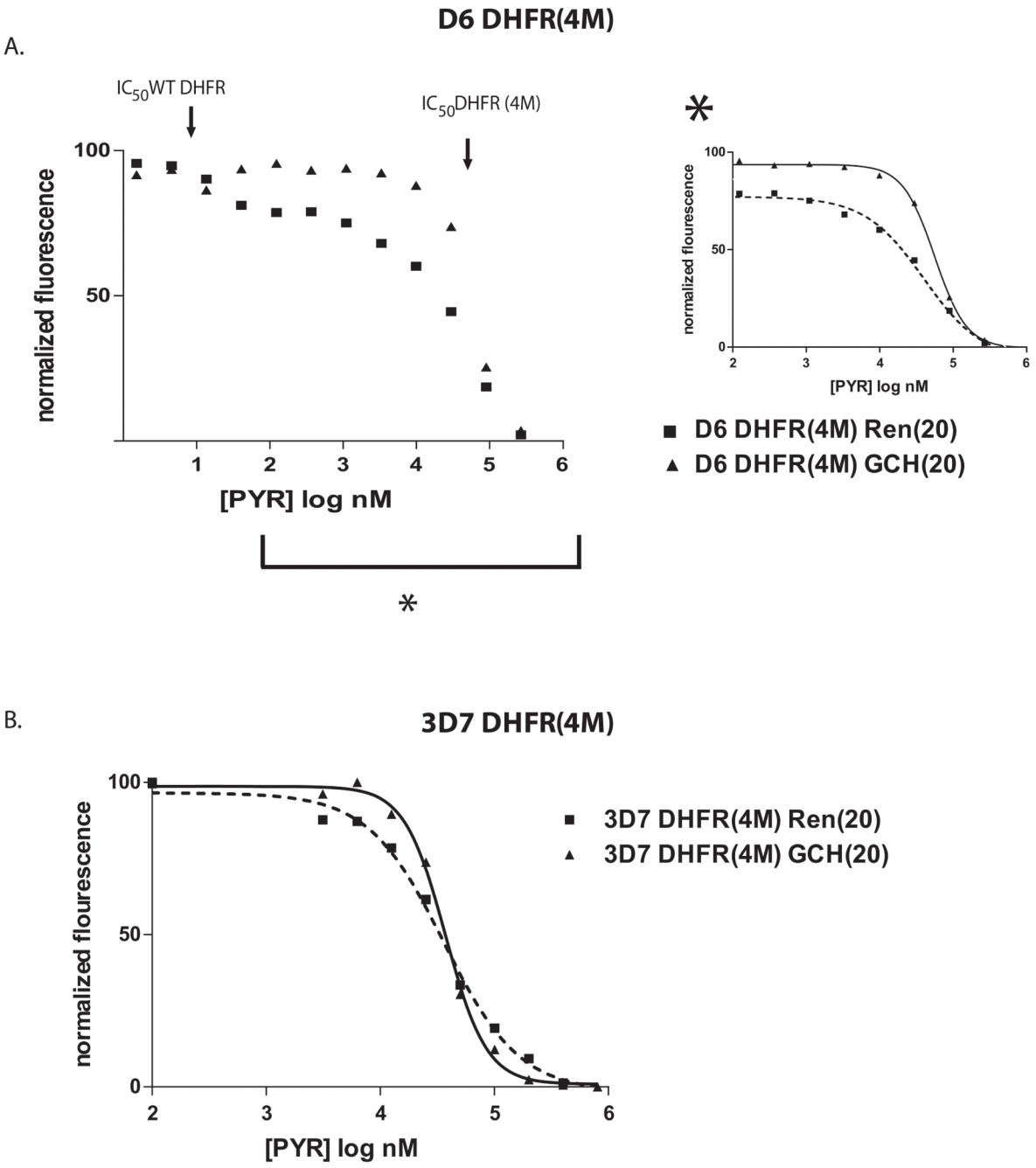


Figure 8. Manipulation of *dhfr* haplotype with increased *gch1*

Dose response curves comparing D6 (A) and 3D7 (B) parasite lines expressing the quad mutant *dhfr* with or without *gch1* overexpression. The curves are representative of multiple experiments and show a complex relationship between *gch1* overexpression and DHFR haplotype. For D6, a bimodal curve was observed in parasites expressing both a wildtype and quad mutant DHFR, each of two distinct slopes roughly correlated with the IC_{50}s of the wildtype and quad mutant DHFRs (shown with arrows above the curve). The curve within the inset to the right (marked with an asterisk) shows the region of the graph depicting the response of parasites reliant on the quad mutant DHFR. Note that the benefit of increased *gch1* expression decreases at higher doses of pyrimethamine. Similarly, the 3D7 DHFR

(4M) data shows a detrimental effect of *gch1* overexpression at high doses of pyrimethamine.