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# Functional analysis of epigenetic regulation of tandem *RhopH1/clag* genes reveals a role in *Plasmodium falciparum* growth

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## Summary

The *Plasmodium* RhopH complex is a high molecular weight antigenic complex consisting of three subunits – RhopH1/clag, RhopH2 and RhopH3 – located in the rhoptry secretory organelles of the invasive merozoite. In *Plasmodium falciparum* RhopH1/clag is encoded by one of five *clag* genes. Two highly similar paralogous genes, *clag 3.1* and *clag 3.2*, are mutually exclusively expressed. Here we show clonal switching from the *clag 3.2* to the *clag 3.1* paralogue *in vitro*. Chromatin immunoprecitation studies suggest that silencing of either *clag 3* paralogue is associated with the enrichment of specific histone modifications associated with heterochromatin. We were able to disrupt the *clag 3.2* gene, with a drug cassette inserted into the *clag 3.2* locus being readily silenced in a position-dependent and sequence-independent manner. Activation of this drug cassette by drug selection results in parasites with the *clag 3.1* locus silenced and lack full-length *clag 3.1* or *3.2* transcripts. These *clag 3*-null parasites demonstrate a significant growth inhibition compared with wild-type parasites, providing the first genetic evidence for a role for these proteins in efficient parasite proliferation. Epigenetic regulation of these chromosomally proximal members of a multigene family provides a mechanism for both immune evasion and functional diversification.

## Introduction

The apical end of the invasive merozoite form of *Plasmodium falciparum* parasites contains organelles – micronemes, rhoptries and dense granules – critical for erythrocyte invasion and the subsequent establishment of infection within the host red blood cell (Cowman and Crabb, 2006). Proteins contained in these organelles are often encoded by multigene families, including the *PfRh* and *PfEBA* families, which are rich in polymorphisms and/or exhibit variant expression (Duraisingh *et al.*, 2008). As such proteins mediate processes crucial to parasite survival and growth, a better understanding of the regulation of their expression is a high priority.

The RhopH complex, a high molecular weight complex expressed at the schizont stage in developing merozoites, is comprised of three distinct subunits – RhopH1/clag, RhopH2 and RhopH3 – in a 1:1:1 ratio (Holder *et al.*, 1985; Brown and Coppel, 1991; Sam-Yellowe and Perkins, 1991; Kaneko *et al.*, 2001; 2005; Shirano *et al.*, 2001). It localizes to the rhoptry



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body and is transferred, to some extent, to the developing early ring stages in the subsequent cycle (Ling *et al.*, 2004). It is proposed to have a role in the invasion process and/or the subsequent remodelling of the host erythrocyte, although a direct demonstration of the function(s) of this unique complex has remained elusive. Additionally, RhopH can bind erythrocytes and antibodies against the complex partially block invasion, strengthening the argument for a role in invasion (Sam-Yellowe and Perkins, 1991; Doury *et al.*, 1994; Wang *et al.*, 2006) and making it an attractive asexual stage vaccine candidate.

The inability to genetically disrupt the RhopH2 and RhopH3 subunits, each encoded by a single gene, implies that the presence of this intact complex is essential for parasite viability in the asexual cycle (Cowman and Crabb, 2006; data not shown). The RhopH1 subunit is encoded by five distinct *RhopH1/clag* genes; three of which – *clag 2, clag 3.1* and *clag 9* – have been shown by experiments with specific antibodies to complex with RhopH2 and RhopH3 (Kaneko *et al.*, 2005). It is presumed by sequence homology that the other two *RhopH1/clag* family members; *clag 3.2* and *clag 8*; also complex with RhopH2 and RhopH3, giving rise to five distinct RhopH complexes. The function of this expansion is unclear.

Different parasite clones variantly express different subsets of the *RhopH1/clag* genes (Cortes *et al.*, 2007). Expression of the *clag 2* gene varied among subclones and the *clag 3.1* and *clag 3.2* genes appeared mutually exclusively expressed. By analogy with the regulation of the *var* gene family of virulence genes in *P. falciparum*, epigenetic phenomena may have a role in regulating the variant expression of the *RhopH1*/*clag* genes.

Here, we report our analyses of the dynamic expression of the *RhopH1/clag 3* genes. We find that *clag 3.1* and *clag 3.2* are expressed in a mutually exclusive and dynamic fashion. Enrichment of specific histone marks suggests that silencing of an individual *clag 3* gene is heterochromatin-mediated. A drug cassette inserted into the *clag 3.2* locus, disrupting the gene, is readily silenced, demonstrating a positional effect that is sequence-independent for silencing at this locus. Drug selection results in activation of the *clag 3.2* locus and silencing of the *clag 3.1* locus, and results in parasites in which there are no detectable full-length *clag 3* transcripts. Although this line demonstrates no observable changes in utilization of alternative invasion pathways, silencing of both *clag 3* genes does appear to confer a significant growth disadvantage on these parasites, genetically demonstrating the first functional role for this gene family in efficient parasite growth.

## Results

### Variant expression in transcript levels of *RhopH1/clag* genes in 3D7 clones

Two of the genes encoding RhopH1 – *clag 3.1* (PFC0120w) and *clag 3.2* (PFC0110w) – lie in a tandem array in the sub-telomeric region of chromosome 3, separated by a *var* pseudogene (Fig. 1A). The two *clag* 3 genes are highly homologous to one another (97% in 3D7), have a high abundance of single nucleotide polymorphisms between strains and are mutually exclusively expressed (Cortes *et al.*, 2007; Volkman *et al.*, 2007). In order to more thoroughly characterize this variant expression in a well-studied and sequenced parasite isolate, we used the 3D7 strain for further experiments on expression and dynamics.

We developed a quantitative PCR-RFLP assay to distinguish transcript levels expressed between the two paralogues in 3D7. Following the amplification of sequences from both genes with conserved primers, digestion with restriction enzymes can distinguish the two paralogues (Figs 1B and S1). Four lines with the 3D7 genetic background were assessed for expression of *clag 3.1* and *clag 3.2*. Interestingly, we found that all of the *in vitro* cultured lines expressed *clag 3.1*, although only 3D7(KW) expressed significant levels of *clag 3.2*

(Fig. S2), suggesting that there may be a preference for *clag 3.1* expression in *in vitro* culture in this genetic background. From a bulk culture of 3D7(KW), we generated four clones. These were assayed for *clag 3.1* and *clag 3.2* expression using the PCR-RFLP assay. Clones 1 and 2 demonstrated that only *clag 3.1* was expressed in clone 1 and only *clag 3.2* was expressed in clone 2 (Fig. 1B). Clone 1 is further referred to as clone 3.1+ (3.1+, 3.2− expression) whereas clone 2 is referred to as clone 3.2+ (3.1−, 3.2+ expression). The same assay on gDNA revealed that the genetic locus appears intact in each of these clones.

We further confirmed these findings with qRT-PCR experiments that revealed that the levels of individual *clag 3.1 and 3.2* genes do vary considerably between four clones of 3D7(KW). The levels of *RhopH2* expression and total *RhopH1/clag* gene expression do not vary significantly, while *RhopH3* demonstrated a twofold increase in clone 2 (Fig. 1C). *Clag 3.2*, however, exhibited an approximately 80-fold difference in expression levels between clones 1, 3 and 4, compared with clone 2. The relative contribution of each gene to total *RhopH1/clag* expression in each clone was also variable, with the largest changes resulting from the contribution of *clag 3.2* transcripts (Fig. 1D).

## Clonally variant expression of *RhopH1/clag* genes in 3D7 clones

To determine whether the level of *clag 3* expression was dynamic and moreover, if the mutually exclusive expression of an individual *clag 3* gene was reversible, clone 3.1+ and clone 3.2+ were continuously cultured for approximately 60 life cycles (4 months) after clone recovery. Expression of *clag 3.1* and *clag 3.2* was measured in schizonts after 15 and 60 life cycles by PCR-RFLP analysis. Clone 3.1+ exhibited no switching and continued to only express *clag 3.1* throughout the entire time-course. Clone 3.2+ switched to a parasite population expressing both *clag 3.2* and *clag 3.1*, with an increasing proportion expressing *clag 3.1* over time (Fig. 2A). Therefore, *clag 3* expression is facultative and these results suggest there is a preference for *clag 3.1* expression in *in vitro* culture in the 3D7 genetic background, either because it offers some growth advantage or exists as a default expression state. In contrast, we found that *clag 3.2* is preferentially transcribed in the parasite strain T9/94 (Fig. S3).

To investigate a role for the mutually exclusive expression of *clag 3.1* or *clag 3.2* in the utilization of different erythrocyte receptors (alternative invasion pathways) for invasion, invasion assays were carried out on clone 3.1+ and clone 3.2+. These assays revealed that clone 3.1+ and clone 3.2+ have the same invasion pathway profile (Fig. 2B).

## Enrichments of histone modifications are associated with the expression and silencing of *clag 3* paralogues

Our data on the expression of *clag 3* genes – namely, that expression is clonally variant, reversible and not associated with changes in DNA sequence – are hallmarks of epigenetic transcriptional regulation. Enrichments of different histone modifications are associated with variant expression of the *var* gene family. Specifically, the ‘active’ marks of H3K4me2, H3K4me3 and H3K9ac are increased in the upstream region of an active *var*. The mark of heterochromatin-mediated silencing – H3K9me3 – is increased upstream and throughout the coding region of silenced *var* genes (Chookajorn *et al.*, 2007; 2008; Lopez-Rubio *et al.*, 2007a).

To investigate how changes in expression over the relatively small chromosomal distance of the *clag 3* locus are associated with alterations in the landscape of histone modifications, we performed targeted chromatin immunoprecipitation (ChIP) assays on clones 3.1+ and 3.2+. Fourteen qPCR primer sets were used, spanning the 24 kb region containing both *clag 3* genes and the intervening *varC* pseudogene; primer sets were more concentrated in the

promoter regions, where many histone marks are typically most concentrated and genomic sequences were least conserved between these paralogues (Fig. 3A).

Immunoprecipitation experiments revealed that levels of H3K9me3 increased twofold to fivefold within a wide region upstream of *clag 3.2* and throughout its coding region when it is silenced. Silent *clag 3.1* is associated with significant, yet smaller region of H3K9me3 enrichment (Fig. 3B). When *clag 3.1* or *clag 3.2* were active, H3K4me3 and H3K9ac were indeed enriched upstream of the active gene (maximum enrichment fivefold and twofold respectively) (Fig. 3C and D). This enrichment but not the absolute levels of these histone marks was found to be associated with transcriptional activity. Interestingly, no significant enrichments of the 'poised' histone modification – H3K4me2 – were found in the regions upstream of either *clag 3.1* or *3.2* (Fig. 3E). With this one exception, regulation of expression of the *clag 3* gene family involves the same histone modifications as regulation of the *var* gene family, suggesting that this is heterochromatin-mediated (Fig. 3F). Interestingly, the pattern of H3K9me3 enrichment at the *var* pseudogene is similar to that of *clag 3.2*; to which it is in closer proximity on the chromosome.

## The *clag 3.2* gene is readily disrupted in 3D7

In order to investigate the effect of genetically disrupting a *clag 3* gene on the tightly co-ordinated expression of these paralogues, a construct consisting of the first ~ 1.4 kb of the coding region of the *clag 3* genes (91% homology to *clag 3.1*, 100% homology to *clag 3.2*) was used to target these genes for disruption by single cross-over homologous recombination (Fig. 4A). Integrants were recovered and the disruption of *clag 3.2* was confirmed by Southern blot (Fig. 4B). This parasite line will be hereafter referred to as *clag 3.2ins*. A second, independent transfection and integration event also yielded parasites in which *clag 3.2* had been disrupted (data not shown), raising the possibility that either (i) the homology of the targeting fragment for *clag 3.1* was not sufficient for recombination into this locus, or (ii) it is unfavourable to genetically disrupt *clag 3.1*, as it appears to be the predominant *clag 3* gene expressed in our *in vitro* 3D7 cultures. In the latter scenario, any integration events obtained in *clag 3.1* would be out-competed by the *clag 3.2* integrants and never recovered. We created a construct for the direct disruption of *clag 3.1* by either single or double cross-over recombination strategies using flanks that correspond to *clag 3.1* sequences. We obtained episomal transfectants but were unsuccessful in generating *clag 3.1* disruptants following multiple rounds of cycling the parasite on and off drug, and negative selection with ganciclovir, in two independent transfectants (data not shown).

## Position-dependent silencing and reactivation of a drug-resistance marker inserted at the *clag 3.2* locus

Parasite clones of *clag 3.2ins* were generated by limiting dilution. After the approximately 6 week period of cloning and culture *in vitro* in the absence of drug selection, *clag 3.2ins* clones were placed on 2.5 nM WR99210 (WR) (Fig. 5A). Strikingly, 100% of the clones tested were highly WR sensitive (<0.1% parasitaemia one full cycle after drug treatment), suggesting that the *hDHFR* drug cassette had become silenced. Clone 5 (further denoted as *clag 3.2ins cl5*) was chosen for more in depth analysis (Fig. 5A). After applying drug pressure for 7 days on *clag 3.2ins cl5*, parasites were recovered, indicating that the silencing was reversible. WR sensitive (*clag 3.2ins cl5 WR*−) and resistant (*clag 3.2 ins cl5 WR*+) parasites were maintained in parallel, with or without drug pressure, and Southern blots of these two lines confirmed that differences in WR sensitivity were not due to gross genetic changes or rearrangements (Fig. 5B). Hypoxanthine incorporation growth assays indicated that while *clag 3.2ins cl5 WR*+ parasites have an IC50 for WR99210 of 0.066 μM, *3.2ins cl5* WR- parasites were highly sensitive to every concentration of WR99210 tested (Fig. 5C). Quantitative RT-PCR with primers specific for *hDHFR* demonstrated that *clag 3.2ins cl5*

*WR*+ parasites expressed 109-fold more *hDHFR* transcripts than *clag 3.2ins cl5 WR*− parasites (Fig. 5D). Taken together, these results indicate that there is a reversible, positional effect on a drug cassette inserted into *clag 3.2*, which silences it in the absence of drug pressure, presumably by a mechanism similar to that which silences *clag 3.2* in *clag 3.1*+ clones.

## Activation of the inserted drug cassette in the *clag 3.2* locus results in loss of *clag 3.1* and *clag 3.2* expression

Using our PCR-RFLP assay to look at full-length *clag 3* transcripts, we determined that *clag 3.2ins cl5 WR*− expressed only *clag 3.1* transcripts, which was expected as the *clag 3.2* coding region had been disrupted. Surprisingly, in *clag 3.2ins cl5 WR*+ parasites, we were unable to detect any *clag 3* transcripts by PCR-RFLP (Fig. 6A). Further investigation with qRT-PCR revealed that the truncated 5′ UTR of *clag 3.2* was activated following WR selection, resulting in the production of partial transcripts. In parasites in which the drug cassette is actively transcribed, there is an approximately 18-fold increase in transcripts from the 5′ UTR of *clag 3.2* (Fig. 6B), indicating that activation of the *hDHFR* directly downstream influences the transcriptional state of the *clag 3.2* promoter. This suggests that activation of the *clag 3.2* locus does effect expression of *clag 3.1* in a mutually exclusive manner. As a result, activation of the truncated *clag 3.2* promoter in *clag 3.2ins cl5 WR*+ parasites prevents the expression of *clag 3.1*. The lack of a full-length *clag 3.2* transcript in these parasites results in what is effectively a *clag 3.1/clag 3.2*- null line (Fig. 6C). The effect of activation of the *clag 3.2* locus on the expression levels of the other *RhopH1/clag* genes was examined. qRT-PCR revealed that, compared with the parental strain 3D7, *clag 3.2* locus activation had no significant effect on *clag* 2, *clag 8* and *clag 9* transcripts in *clag 3.2ins cl5 WR*− and *clag 3.2ins cl5 WR*+ (Fig. 6B). *RhopH2* and *RhopH3* transcription was also unchanged (Fig. S4A). Immunoprecipitation of the RhopH complex with an anti-RhopH2 antibody pulled down all three members of the complex in both *clag 3.2ins cl5 WR* − and *clag 3.2ins cl5 WR*+ (Fig. S4B). Interestingly, a band corresponding to Clag 3.1 appears to be absent in *clag 3.2ins cl5 WR*+ but is present in *clag 3.2ins cl5 WR*−.

## Silencing of both *clag 3* genes alters parasite growth efficiency but does not change alternative invasion pathway utilization

To investigate a functional role for the *clag 3* genes, we performed standard invasion pathway assays on *clag 3.2ins cl5 WR*+ (expressing no *clag 3*) versus *clag 3.2ins cl5 WR*− (expressing *clag 3.1*). These parasites demonstrate no differences in alternative invasion pathway utilization despite the lack of expression of both *clag 3* genes (Fig. 7A). To investigate any changes in growth efficiency, *clag 3.2ins cl5 WR*+ parasites were mixed in an approximately 1:1 ratio with 3D7-1A parasites (expressing clag 3.1), which contain the *hDHFR* gene downstream of the wild-type *PfRh2b* gene (PfRh2b/WT, which we have termed 1A) (Desimone *et al.*, 2009). These mixed cultures were maintained in culture on 2.5 nM WR for 21 life cycles (6 weeks). Genomic DNA was prepared at the 2 and 6 week time points, and Southern blotted using a probe against the *hDHFR* gene. The relative densities of the bands reflect the relative proportion of the strains at each time point. In two independent trials, the *clag 3.2ins cl5 WR*+:3D7-1A ratio decreased 43.9% and 60.7% over 6 weeks, corresponding to a 2.09% and 2.89% slower growth rate of *clag 3.2ins cl5 WR*+ parasites (Fig. 7B). These results indicate that *clag 3* expression is necessary for optimal parasite growth and is the first genetic demonstration of a functional role for the RhopH complex in growth efficiency.

## Discussion

In this study we demonstrate that dynamic switching between two paralogous genes, *clag 3.1* and *clag 3.2*, is controlled by epigenetic regulation. The *RhopH1/clag* family has expanded in *P. falciparum* to contain a maximum of five genes (Kaneko *et al.*, 2005). The duplication of *clag 3.1* and *clag 3.2* occurred before the divergence of the primate malarias, as *P. reichenowi* possesses both paralogues. We favour a model in which both *clag 3.1* and *clag 3.2* perform alternative functions in parasite growth, or the same function with different efficiency. Epigenetic silencing of each paralogue facilitates this functional diversification, while also providing a mechanism for immune evasion (Fig. 8).

The chromosomal organization of *clag 3.1* and *clag 3.2* in a tandem array is reminiscent of the organization of many variantly expressed *var* genes in the genome of *P. falciparum*. However, when compared with the ~ 60 *var* genes per genome, this small number seems restricted in facilitating immune evasion. Additionally, the other two subunits of the complex are only encoded by one gene and therefore may be constantly exposed to the host immune system. Furthermore, a single merozoite contains RhopH complexes with multiple types of RhopH1/clag (Kaneko *et al.*, 2005). However, in addition to the expansion in gene number and the variant expression of individual members, RhopH1/clag diversity in *P. falciparum* is further increased by a high frequency of single nucleotide polymorphisms (Volkman *et al.*, 2007; Iriko *et al.*, 2008), and through deletion of one of the paralogues (Chung *et al.*, 2007), making a role for this multi-gene family in immune evasion more compelling.

Variant expression of *RhopH1/clag* genes in 3D7 clones has previously been described (Cortes *et al.*, 2007), and our studies confirm that *clag 3.1* and *clag 3.2* exhibit mutually exclusive expression in recently cloned parasites. In a previous report, clones only expressing *clag 3.1* or *clag 3.2* were maintained in continuous culture and exhibited no switching (Cortes *et al.*, 2007). However, our clone 3.2+, but not clone 3.1+, exhibited switching over 4 months and expressed an increasing proportion of *clag 3.1* over time. Interestingly, we found that parasites of the 3D7 genetic background from multiple sources all expressed *clag 3.1*, but only one expressed *clag 3.2*. This may suggest a growth advantage in routine *in vitro* culture for parasites expressing *clag 3.1*. However, this may be specific to the 3D7 genetic background, as we found that the parasite line T9/94 expresses only *clag 3.2*. The facultative nature of switching between the *clag 3* paralogues suggests the presence of only epigenetic regulation; however, it is formally possible that reversible genetic changes may contribute to variant expression.

Our ChIP studies across the region of chromosome 3 containing both *clag 3* genes demonstrate that epigenetic mechanisms underlie the variant expression of *RhopH1/clag* genes. H3K4me3 and H3K9ac, marks of active transcription, are enriched upstream of a *clag 3* gene when it is expressed, while H3K9me3, a mark of heterochromatin, is enriched upstream and through the coding region of a *clag 3* gene when it is silenced. These patterns of histone modification enrichments are similar to those previously correlated with distinct *var* gene expression states, and suggest that *clag 3* gene silencing is heterochromatin-mediated. Evidence that these same histone modifications are associated with variant *RhopH1/clag* expression establishes that the targets of these modifications are diverse, involving gene families expressed in distinct parts of the life cycle, *var* genes at the ring stage versus *clag 3* genes in schizonts, and with different roles in the parasite. Histone modifications have also been shown to be associated with the expression of the *PfRh4* invasion gene (Jiang *et al.*, 2010). Considering both our 3D7 expression and ChIP data, we propose in our model that 3D7 parasites express *clag 3.1* as a ‘default’ expression state *in vitro*, and that the *clag 3.2* remains heterochromatic and transcriptionally silenced.

Following stochastic switching between the paralogues, with a transition to a euchromatic state at this locus and cross-talk to the *clag 3.1* locus, host pressure can select for the expression of *clag 3.2* (Fig. 8).

We have examined the regulation of the *clag 3* genes more closely in a line with a drug cassette inserted into the *clag 3.2* gene. Routine culturing off drug resulted in the dramatic silencing of the drug cassette over the course of 6–8 weeks, suggesting a strong pressure for *clag 3.1* to be on and *clag 3.2* to be off *in vitro*, despite *clag 3.2* activation during drug selection for the integration event. The silenced drug cassette can be reversibly activated with the addition of drug, and its activation results in the co-ordinate activation of the previously silenced *clag 3.2* promoter. This result demonstrates a sequence-independent, position-dependent effect at this locus.

The wild-type *clag 3* loci exhibit mutually exclusive expression and, strikingly, maintain this expression pattern even in our *clag 3.2ins* line. Cross-talk occurs between the *clag 3* genes to achieve this mutually exclusive expression, despite the disruption of the clag 3.2 coding sequence and an additional ~ 25 kb of intervening sequence between the two genes. The potential role of the *var* pseudogene in the mutually exclusive expression of the clag 3 genes, perhaps by providing genetic elements that mediate silencing will require further investigation.

Maintenance of mutually exclusive *clag 3* expression in our *clag 3.2ins* line led to parasites with no full-length *clag 3* transcripts, as the inserted drug cassette interrupted full-length transcription of the *clag 3.2* locus and activation of this cassette silenced transcription of *clag 3.1*. This was therefore the equivalent of a *clag 3* double knockout parasite line, allowing for the functional analysis of the *clag 3* genes. Both of the other RhopH subunits – RhopH2 and RhopH3 – appear to be essential. In contrast, it seems very likely that other members of the *RhopH1/clag* gene family, specifically the more related type A members, *clag 2* and *clag 8*, have some degree of functional redundancy with RhopH1/clag 3.

Although we demonstrate the first functional role for a subset of *RhopH1/clag* genes and, thus, the RhopH complex in optimal parasite growth, the more specific function of these genes still remains unknown. It appears that the clag 3 proteins confer some advantage to parasite survival, a feature that may be more pronounced *in vivo*. The exact role and function of the RhopH complex remains unclear. However, the presence of this complex of proteins in the rhoptry bulb, and on the surface of the merozoite (Singh *et al.*, 2010), implicates the complex in erythrocyte invasion. A function for these proteins in invasion is further supported by data demonstrating that the RhopH complex can bind to red blood cells and antibodies targeted against it can partially block invasion (Sam-Yellowe and Perkins, 1991; Doury *et al.*, 1994). However, the role of other rhoptry bulb proteins, both in *Plasmodium* and in the closely related apicomplexan parasite *Toxoplasma gondii*, have indicated these proteins may function in tight-junction formation (facilitating entry into cells) as well as the generation and maintenance of a parasitophorous vacuole (PV). Interestingly, the RhopH1 subunits have a domain homologous to RON2, a protein that has been shown to complex with AMA-1 and localize to the tight junction during invasion (Tonkin *et al.*, 2009). Despite this homology, however, it has been previously reported that the RhopH complex does not appear to interact with RON2 and their functions are not related (Cao *et al.*, 2009). A second possibility is that the expansion of the *RhopH1/clag* family in *P. falciparum* could provide functional diversification for the transport of different proteins. The inability of *clag 3*-null parasites to efficiently traffic a subset of proteins may underlie the competitive disadvantage we have observed. Interestingly, members of the LMW and RhopH complex are also expressed outside of the erythrocytic life cycle, including the liver stages, where RAP1 has also been shown to localize to the PV. Thereby,

these proteins may have a role in adapting host cells in multiple stages of the parasite's life cycle (Tufet-Bayona *et al.*, 2009).

In conclusion, the *clag 3* locus represents a paradigm for the epigenetic regulation of virulence genes in *P. falciparum*. The mutually exclusive expression of *clag 3.1* and *clag 3.2* is all the more remarkable considering their proximity on the chromosome. The ability to switch between the expression of paralogous proteins provides a mechanism for the parasite to link antigenic variation and functional diversification.

## Experimental procedures

### Maintenance of parasite cultures

The 3D7 (KW) strain of *P. falciparum* was kindly provided by Dr Kim Williamson (Loyola University, Chicago) and was maintained in *in vitro* culture as previously described (Trager and Jensen, 1976). Clones were generated from bulk culture by limiting dilution.

### Quantitative real-time PCR analysis

To assess *RhopH1/clag* expression, cDNAs were prepared essentially as previously described (Frank *et al.*, 2007). Briefly, mid-schizont stage RNA from 3D7 parasites (corresponding to peak *clag* expression) was extracted from saponin-lysed parasites using TriZOL Reagent (Invitrogen). RNA was subsequently purified on PureLink RNA mini columns (Invitrogen), treated with DNase I (Invitrogen) and then reverse-transcribed using the Superscript II reverse transcriptase kit (Invitrogen).

Quantitative RT-PCR was carried out on synthesized cDNA using ITAQ SYBR SUPERMIX (Bio-Rad) in an ABI Prism thermocycler, as described previously (Dzikowski *et al.*, 2006). Primers used in this assay are described in Table S1, and reaction conditions were as follows: 95°C for 15 s, 50°C for 20 s and 60°C for 30 s, repeated for 40 cycles. Fold changes in *clag* gene expression was calculated using the relative standard curves for each primer set generated from serial dilutions of genomic DNA. The highly expressed schizont stage gene *PfAMA-1* was used to control for parasite stage as well as amount of cDNA used in the assay.

The relative expression of *hDHFR* in *clag 3.2ins + WR* versus *clag 3.2ins – WR* was assayed as above using the following primers: hdhfr-F/R 5′-gaatcacccaggccatctta and 5′-atgcctttctcctcctggac. Data were analysed using the ΔCt method and calculated as relative to *Pfactin* expression, which was measured using primers described previously (Stubbs *et al.*, 2005).

### Restriction fragment length polymorphism assay

To determine relative proportions of *clag 3.1* versus *clag 3.2* RNA transcripts, an approximately 700 bp region of the highly homologous 3′ coding region of these genes was amplified from schizont stage cDNA with conserved primers. Primers used are as follows: 3.1/3.2 conserved primer F/R 5′-ggaccacagtttattgccaccatatgcga and 5′-ggaccggcatacatgaatccatt tacgag. Previously published *Pfactin* qRT-PCR primers were used as positive controls (Stubbs *et al.*, 2005). The resulting amplicon was subsequently digested for 2 h at 37°C with either SpeI or HaeII restriction enzymes, which have an additional digestion site in the *clag 3.2* and *clag 3.1* amplicon respectively. In the case of SpeI, complete digestion of the amplicon could be confirmed as there is a conserved restriction site at the amplicon's 3′ end. Digestions were resolved on 2% agarose gels.

### Chromatin immunoprecipitation

For chromatin immunoprecipitation assays, infected red blood cells containing schizont stage parasites were saponinlysed, and resulting parasite pellets were washed with PBS. Parasite pellets were fixed for 10 min in 2.7% formaldehyde after which cross-linking was quenched by the addition 250 mM glycine for 10 min. After washing fixed parasite pellets with PBS, nuclei were extracted by the following two 10 min treatments: (i) buffer A (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) and (ii) buffer B (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Pelleted nuclei were resuspended in immunoprecipitation buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Nadeoxycholate, 5 mM DTT) at a concentration of $10^8$ schizonts per 1 ml. DNA shearing was carried out on 1 ml volumes on a Misonix 3000 sonicator with microtip. Samples were sonicated for 7.5 min in 30 s pulses at power level 3, which resulted in a DNA smear centred at 300 bp. Supernatants were collected by centrifugation and pre-cleared with protein G agarose beads (Upstate 16–201). One-twentieth of each sonication volume was used for an individual IP, and 5 μl of αH3 (06-755, Millipore), αH3K4me3 (ab8580, Abcam), αH3K4me2 (07–030, Millipore), αH3K9me3 (07–442, Millipore) or αH3K9ac (07–352, Millipore) was added per sample. After overnight incubation at 4°C, antibodies were bound with protein G beads for 4 h. Bound beads were subsequently washed as follows: (i) five washes of cold IP buffer, (ii) one wash in LiCl buffer (250 mM LiCl, 10 mM TrisHCl pH 8.0, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxycholate) and (iii) one wash in TE. All washes were performed in a Spin-X column (Costar 8161) to minimize variability. Bound material was eluted from beads with the addition of 100 ml elution buffer (10 mM TrisHCl pH 7.5, 10 mM EDTA, 1% SDS) and heating to 65°C. Recovered material was boiled to reverse cross-linking, purified with the Qiagen PCR clean-up kit and eluted in 150 ml of TE buffer. Quantitative PCR was performed in triplicate with 2 μl of eluted IP material or 2 μl of a 1:100 dilution of input material per reaction on an ABI Prism machine with annealing temperatures of 45°C or 50°C (depending on primer set).

Primers for ChIP were positioned throughout the region of chromosome 3 containing both *clag 3.1* and *clag 3.2*, and are described in Table S2. Previously published GBP130 and PfGAM ChIP primers were used as assay controls (Lopez-Rubio *et al.*, 2007b). Standard curves from gDNA were constructed from all primer pairs and differing primer efficiencies were accounted for in calculating all % input values with the following equations: x = log 100/log (slope of standard curve) and % input = x $\Lambda$ $^{-\Delta Ct}$. Data displayed in Fig. 3 represent % input values normalized to H3 occupancy at each primer position.

### Generation of transgenic parasite line

To generate parasites with *clag 3* gene disruption by insertion, approximately 1.4 kb of the most 5′ end of *clag 3.2* (PFC0110w), with 91% identity to *clag 3.1* (PFC0120w), with the ATG site mutated to AAG was cloned into the pHCD parent vector to derive pHCD-clag (Duraisingh *et al.*, 2002). Transfection and selection for stable single cross-over parasites was carried out by cycling on and off WR99210, as previously described (Fidock and Wellems, 1997). Stable transfectants were cloned by limiting dilution. Chromosomal integration was assessed by Southern blot using standard procedures.

### Assays for drug susceptibility and efficiency of invasion

Sensitivities of *clag 3.2ins cl5WR*− versus *clag 3.2ins cl5WR*+ to WR99210 treatment was determined with standard radiolabelled hypoxanthine incorporation drug assays, as previously described (Duraisingh *et al.*, 2003). The use of alternative invasion pathways were assessed by measuring the efficiency of invasion into erythrocytes treated with different enzymes that remove alternative sets of receptors, using a radiolabelled

hypoxanthine incorporation assay as described previously (Reed *et al.*, 2000). The assay was carried out twice in triplicate. Erythrocytes were treated using the following enzymes: neuraminidase (Calbiochem, 66 mU $ml^{-1}$), high trypsin (Sigma, 1 mg $ml^{-1}$), chymotrypsin (Worthington Bio-chemical, 1 mg $ml^{-1}$), low trypsin (0.66 mg $ml^{-1}$) and chymotrypsin (1 mg $ml^{-1}$)/trypsin (0.66 mg $ml^{-1}$). In both cases, plates were incubated at 37°C for 48 h, at which time tritium-labelled hypoxanthine was added at a final concentration of 0.5 mCi per well. Parasite maturation was permitted for an additional 24 h, after which assays were harvested on glass filter plates and the level of radiolabel incorporation was measured using a scintillation counter.

### Growth competition assays

3D7-1A (Desimone *et al.*, 2009) and *clag 3.2ins cl5 WR*+ were mixed in a 1:1 ratio and continuously cultured on 2.5 nM WR99210. Infected red blood cells were saponin-lysed, and genomic DNA was prepared for the resulting parasite pellets at the following time points: time 0 (day of mixing), time 2 weeks (7 life cycles) and time 6 weeks (21 life cycles). To assess any growth differences, gDNA was digested with BstUI, BanI and PacI enzymes, and a Southern blot was performed using standard procedures. The probe corresponds to the coding region of the *hDHFR* gene, which is contained in both the 3D7-1A and *clag 3.2ins cl5 WR*+ genomes. The relative proportions of 3D7-1A and *clag 3.2ins cl5 WR*+ parasites present at each time point were determined as the relative density of the predicted band (4 kb for *clag 3.2ins cl5 WR*+ and 2.3 kb for 3D7-1A) for each strain by Southern blot. Band density was quantified with ImageJ (NIH) image analysis software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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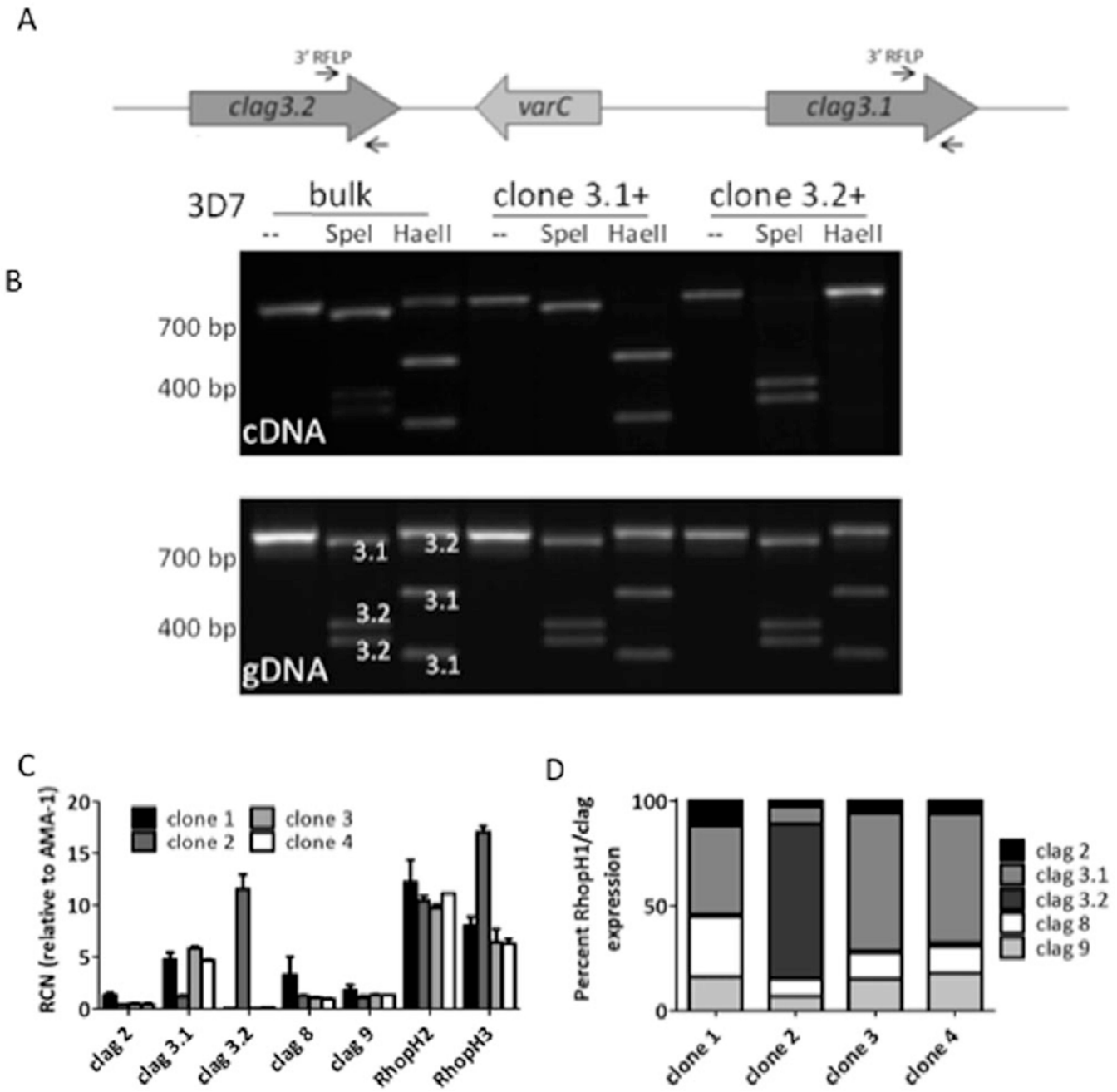


**Fig. 1. Measurement of variant expression of *clag 3* genes and other genes encoding members of the RhopH complex in 3D7 clones**

A. Schematic of the locus, depicting the chromosomal organization of *clag 3.1*, *clag 3.2* and the *varC* pseudogene. Region of primer binding for the PCR-RFLP quantitative assay is indicated by arrows.

B. PCR-RFLP analysis examining full-length *clag 3* expression in bulk and clonal 3D7 parasites. Top panel represents experiment on schizont stage cDNA; bottom panel was performed on gDNA to confirm that, in each, the genomic locus is intact. Samples were either left undigested (−) or digested with SpeI or HaeII, as indicated.

C. qRT-PCR on *RhopH* genes to examine absolute levels of expression of each *RhopH1/clag* gene, RhopH2 and RhopH3 in four 3D7 clones. While *RhopH2* and total *RhopH1/clag*

expression is similar between clones, the level of *clag 3.2* transcripts varies dramatically. Assays were performed on schizont stage cDNA and normalized to the expression of the schizont stage gene *PfAMA-1* using a relative standard curve approach. RCN, relative copy number to *PfAMA-1*. Error bars denote standard deviation of reactions performed in triplicate.

D. Graph representing relative contributions of each gene to total *RhopH1/clag* expression in 3D7 clones. The per cent contribution of each *RhopH1/clag* gene varies between clones, with the greatest variability in *clag 3.1* and *clag 3.2* expression.

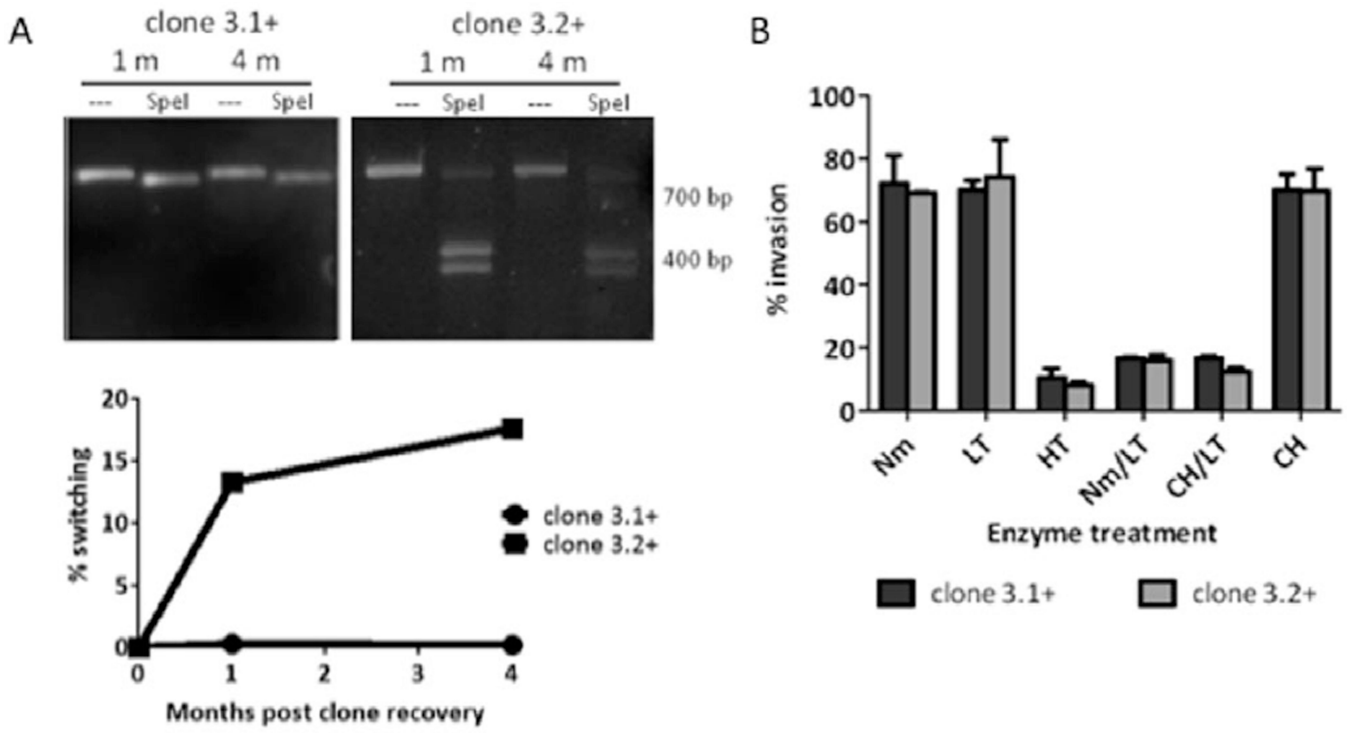


**Fig. 2. Dynamic expression of *clag 3* genes in continuous *in vitro* culture**

A. PCR-RFLP analysis on clonal 3D7 parasites carried in continuous culture to determine any switching at 1 month and 4 months after clone recovery. Clone 3.1+ does not exhibit any switching away from mutually exclusive *clag 3.1* over the 4 month time period whereas clone 3.2+ switches to express more *clag 3.1* over time. Samples were either left undigested (−) or digested with SpeI, as indicated. Quantification of *clag 3* switching in clone 3.1+ and clone 3.2+ is indicated. Data for start of the parasite culture (day 0) for clone 3.1+ and clone 3.2+ are shown in Fig. 1B. Clone 3.1+ does not exhibit any switching; clone 3.2+ exhibits 22.5% switching to expression of *clag 3.1*. Per cent switching was calculated as follows: [(intensity of band(s) for 'OFF' *clag 3* gene)/(total intensity of bands for ALL *clag 3* expression)] × 100. Switching was normalized for any background by making this measurement and applying the above equation at time = 0, and subtracting this value from any subsequent measurements for each clone. Density of bands from gel eletrophoresis was determined with ImageJ software.

B. Invasion profile of clone 3.1+ and clone 3.2+ parasites. Parasites with mutually exclusive *clag 3.1* or *clag 3.2* expression do not differentially utilize invasion pathways. Error bars denote standard deviation of triplicates. Graph represents one representative experiment from two biological replicates. Nm, neuraminidase treatment; LT, low trypsin treatment; HT, high trypsin treatment; CH, chymotrypsin treatment.

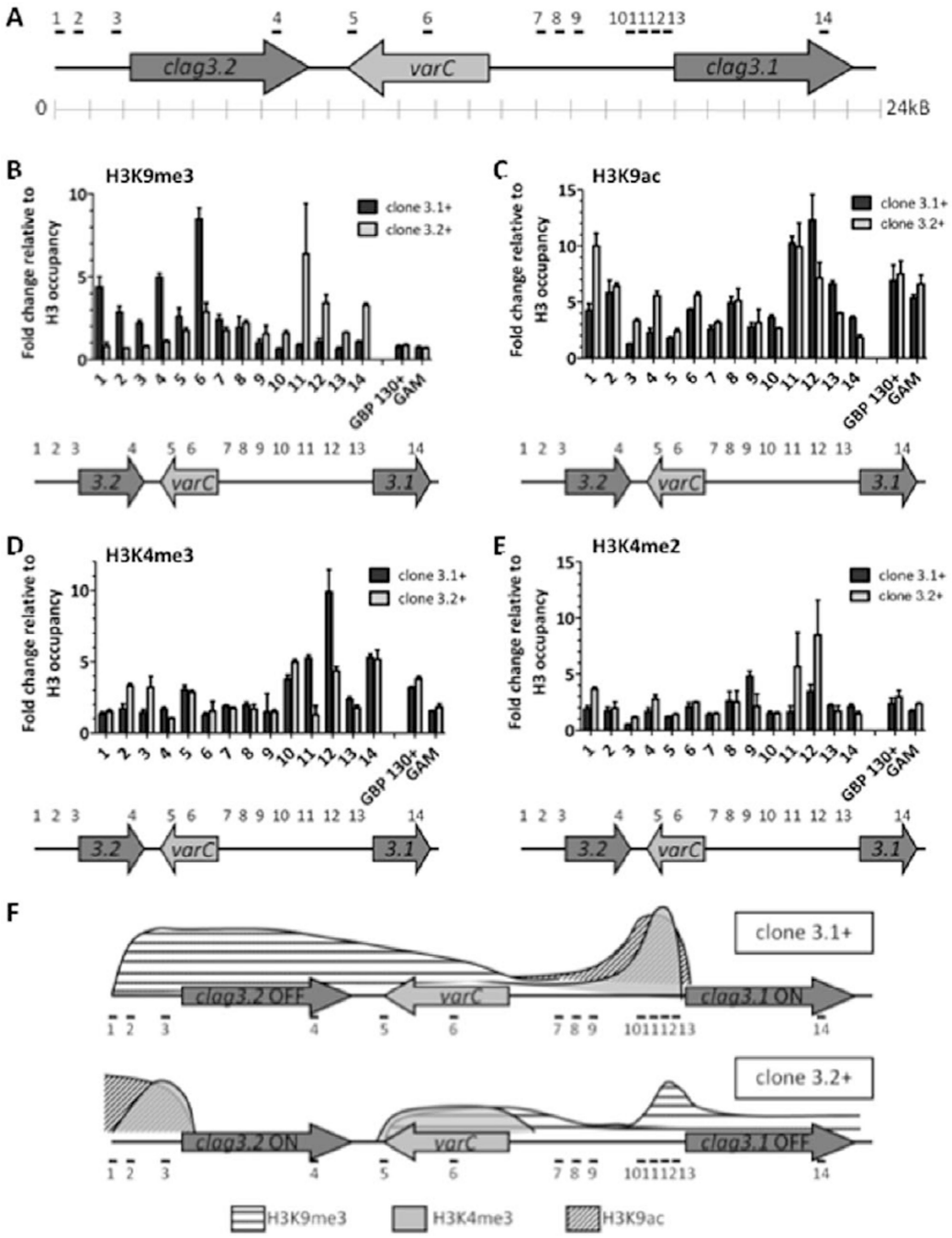


**Fig. 3. Enrichments of histone modifications are associated with the expression and silencing of *clag 3* paralogues**

A. Diagram of the 24 kb region of chromosome 3 containing both *clag 3* genes assayed for histone modifications in ChIP experiments. Positions of ChIP qPCR primers are denoted with black lines and numbers above the schematic.

B. Distribution of H3K9 trimethylation along the *clag 3* gene-containing region in clone 3.1+ (dark bars) and clone 3.2+ schizont stage parasites (light bars). In all ChIP experiments, % input is normalized for each primer set relative to H3 occupancy. Error bars denote standard deviation from three biological replicates.

C. Distribution of H3K9 acetylation along the *clag 3* gene-containing region in clone 3.1+ (dark bars) and clone 3.2+ schizont stage parasites (light bars).
D. Distribution of H3K4 trimethylation along the *clag 3* gene-containing region in clone 3.1+ (dark bars) and clone 3.2+ schizont stage parasites (light bars).
E. Distribution of H3K4 dimethylation along the *clag 3* gene-containing region in clone 3.1+ (dark grey bars) and clone 3.2+ schizont stage parasites (light grey bars).
F. Schematic representation of histone H3 marks linked to silent or active *clag 3* gene expression. Histone modifications throughout the region of chromosome 3 containing *clag 3* genes are shown for the *clag 3.1*+ */clag 3.2*− state as well as the *clag 3.1*-/*clag 3.2*+ state. H3K9me3 is associated with a wide region upstream and throughout the coding region of *clag 3.2* when it is silenced, whereas silenced *clag 3.1* is associated with a smaller region of H3K9me3 enrichment. Histone H3 modifications at lysine 4 and 9 linked to active *clag 3* expression (H3K4me3 and H3K9ac) are enriched upstream of the *clag 3.1* and *clag 3.2* genes when they are active.

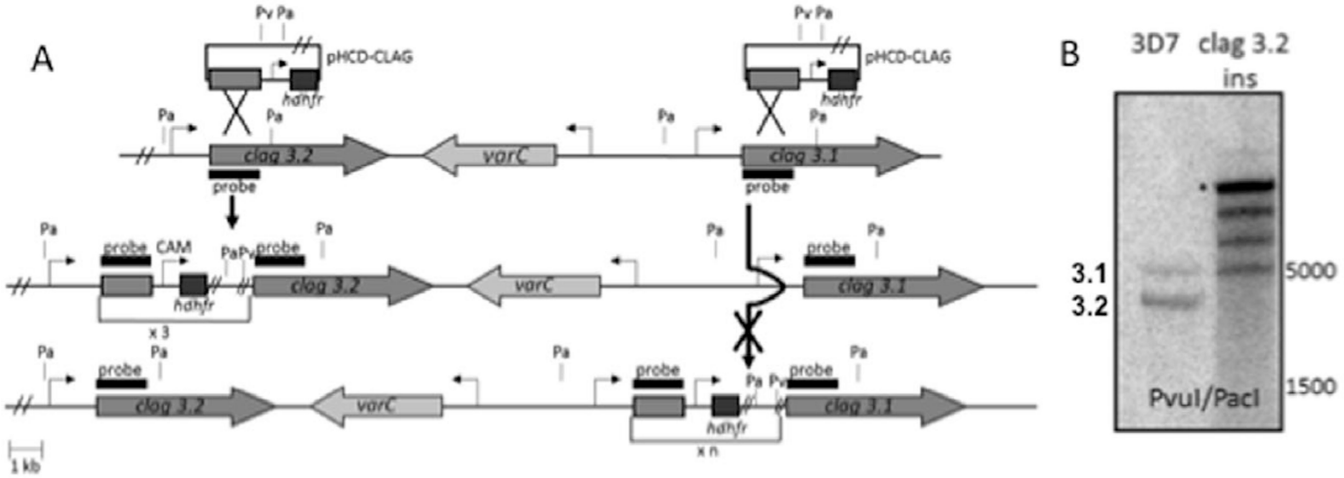


**Fig. 4. An inserted drug cassette in the *clag 3.2* locus is reversibly silenced**

A. Schematic of potential *clag 3.1 and clag 3.2* disruption by single cross-over homologous recombination. Small black lines denote relevant enzyme cutting sites for Southern blot analysis, with Pv and Pa representing PvuI and PacI respectively. Thick arrow indicates the successful disruption of the *clag 3.2* locus. Thick arrow with a cross indicates the inability to directly disrupt the *clag 3.1* locus.

B. Southern blot analysis of *clag 3.2ins. Clag 3.2* has been disrupted, and *clag 3.1* remains intact. Asterisk denotes episomal band, present after cloning, which indicates the episome has integrated multiple copies into the *clag 3.*2 locus.

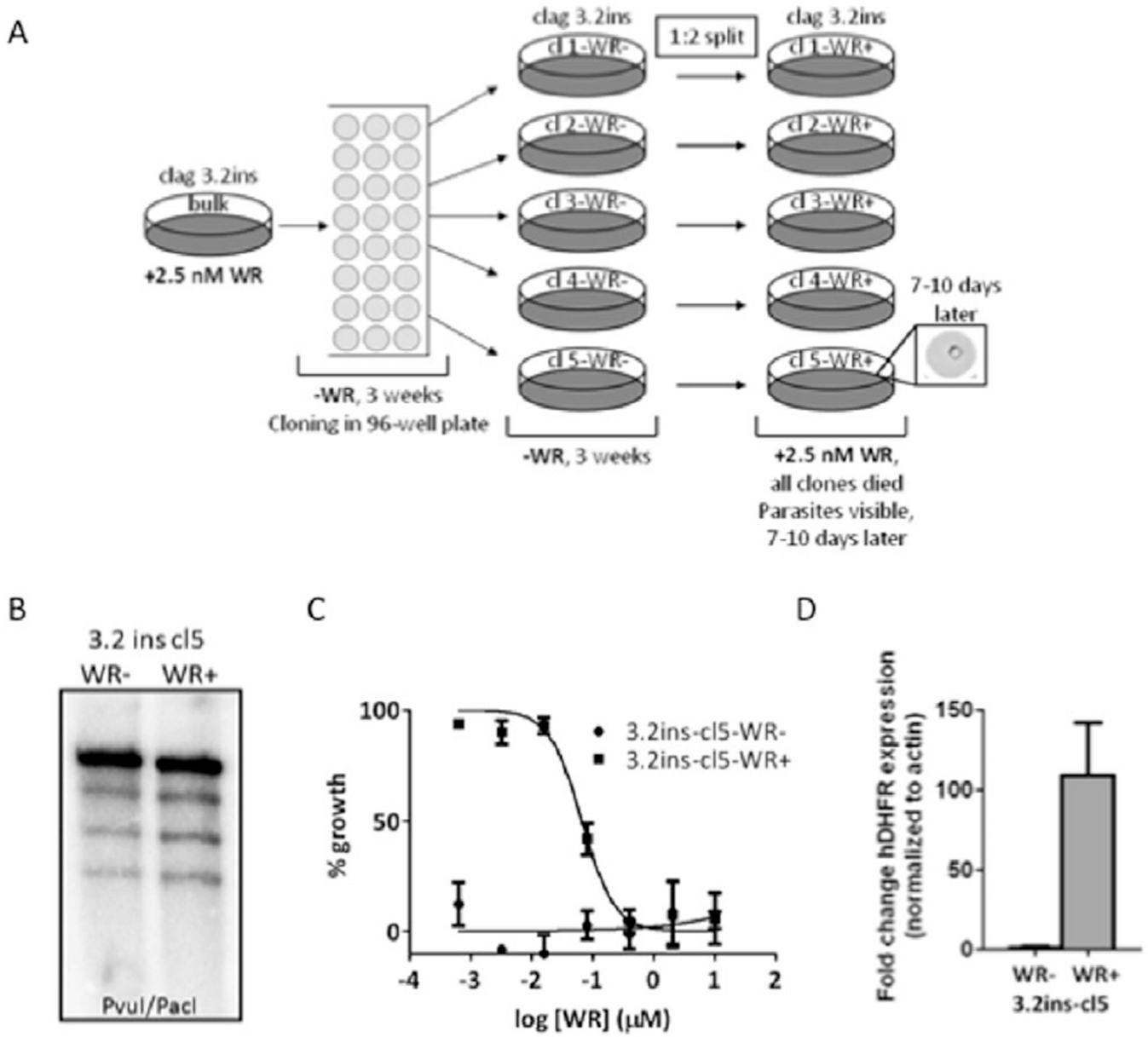


**Fig. 5. Silencing and activation of a transgene marker in the *clag 3.2* locus**

A. Diagram of parasite cloning and the reversible silencing of the inserted *hDHFR* gene.

B. Southern blot analysis of *clag 3.2ins cl5 WR*− and *clag 3.2ins cl5 WR*+ parasites. Both strains still possess a disruption of the *clag 3.2* locus, and no other gross genetic changes to this region of chromosome 3.

C. Hypoxanthine-based survival assay on *clag 3.2ins cl5 WR*− versus *clag 3.2ins cl5 WR*+ parasites. *Clag 3.2ins cl5 WR*− exhibit much greater sensitivity to treatment with WR99210 than parasites recovered after 2.5 nM WR treatment.

D. Measurement of *hDHFR* expression in *clag 3.2ins cl5 WR*− and *clag 3.2ins cl5 WR*+ parasites by qRT-PCR. Assay was performed in triplicate on schizont stage cDNA; *hDHFR* expression was normalized to *Pfactin*. Fold change of *hDHFR* expression is graphed as relative to *clag 3.2ins cl5 WR*− parasites. Error bars represent standard deviation.

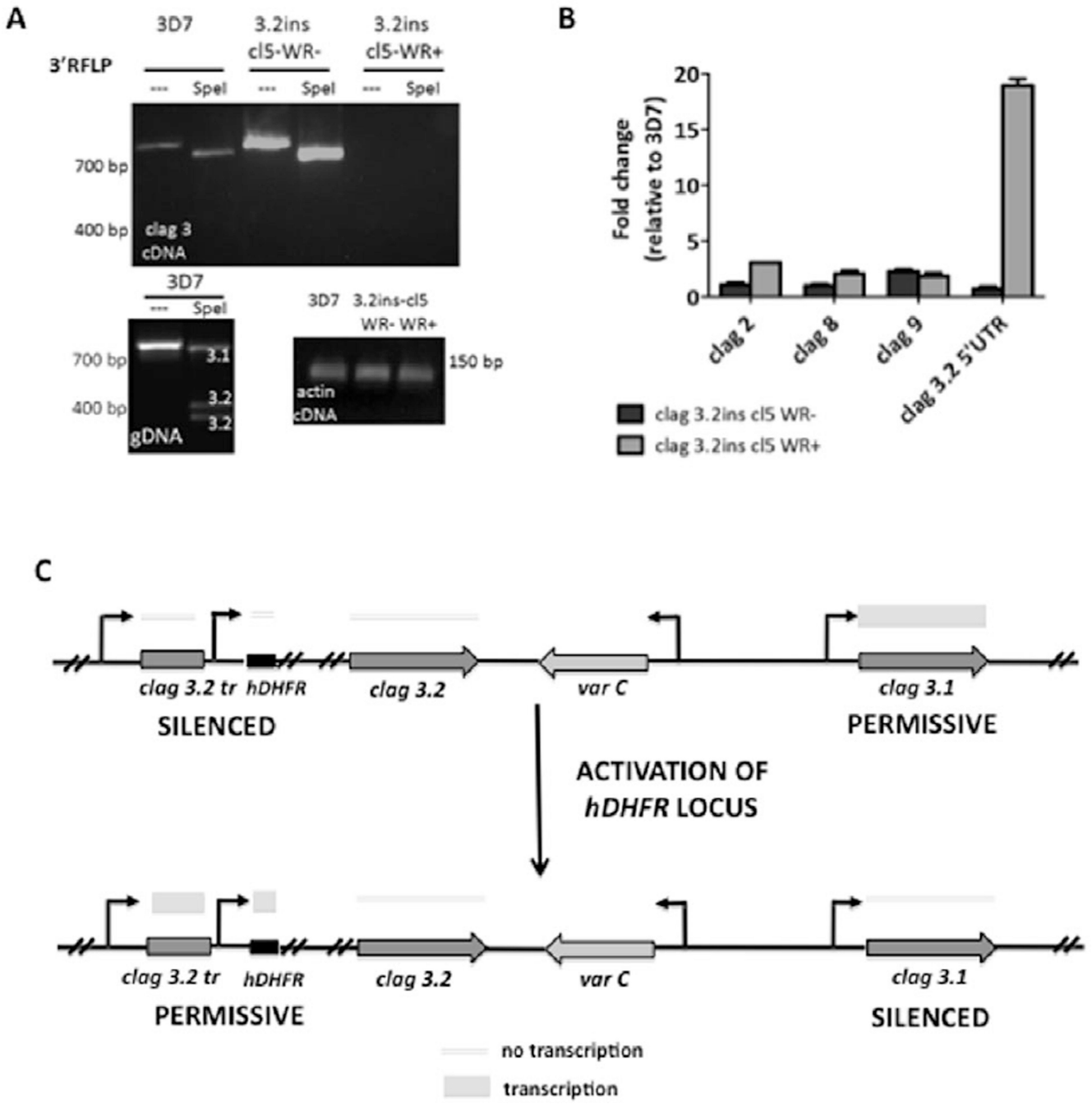


**Fig. 6. Activation of the inserted drug cassette in *clag 3.2* silences expression of both *clag 3* genes and lowers growth efficiency**

A. PCR-RFLP analysis of full-length *clag 3* expression in *clag 3.2ins cl5 WR*− and *clag 3.2ins cl5 WR*+ parasites. The *clag 3.2ins cl5 WR*− parasites express *clag 3.1*, whereas *clag 3.2ins cl5 WR*+ express no *clag 3* transcripts. Assay performed on schizont stage cDNA and gDNA from same strains. Samples were either left undigested (−) or digested with SpeI, as indicated. Actin primers were used as a control for cDNA quality.

B. qRT-PCR on all *RhopH1/clag* genes to determine effect of *clag 3.2* insertion and its activation on gene expression. No significant differences are observed in the transcript levels of other *clag* genes. Transcripts from the 5′ UTR of *clag 3.2* are significantly upregulated in

*clag 3.2ins cl5 WR*+ parasites. Assays were performed on schizont stage cDNA, in technical triplicates and normalized to the expression of the schizont stage gene *PfAMA-1* using a relative standard curve approach. Data are plotted as fold change in expression relative to the 3D7 parental strain. Error bars denote standard deviation.

C. Schematic depicting transcription at *clag 3.1*, *clag 3.2* and the *hDHFR* loci in *clag 3.2ins cl5 WR*− and *clag 3.2ins cl5 WR*+ parasites. Active transcription is indicated as tall boxes, while lack of transcription is indicated as thin lines. Activation of *hDHFR* generates a *clag 3*-null parasite line because no full-length *clag 3* transcripts are generated.

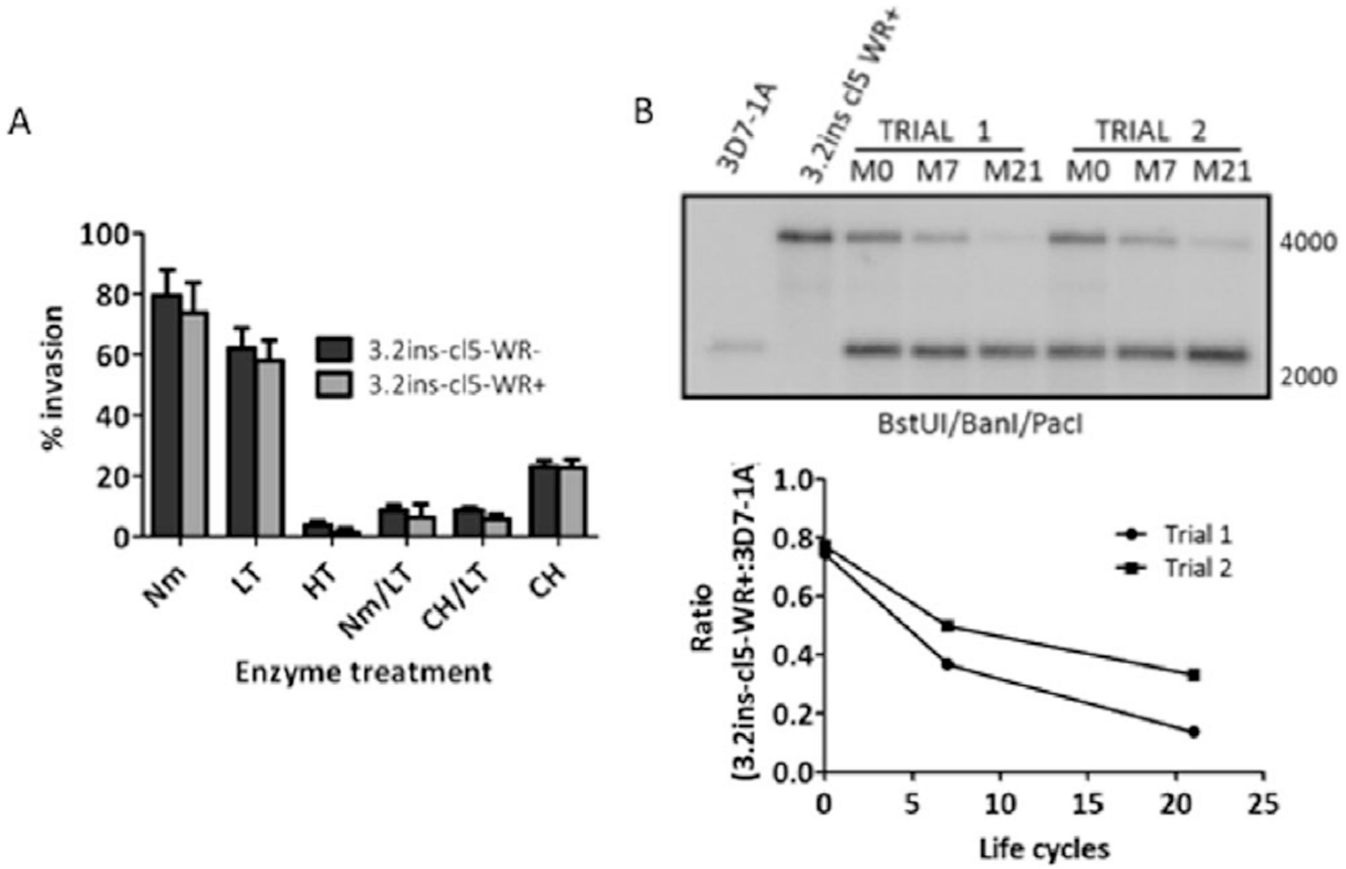


**Fig. 7. Invasion pathways and parasite proliferation in *clag 3* mutant parasite lines**

A. Representative invasion profile of *clag 3.2ins cl5 WR*− and *clag 3.2ins cl5 WR*+ parasites. Parasites with mutually exclusive *clag 3.1* or *clag 3.2* expression do not differentially utilize invasion pathways. Error bars denote standard deviation. Nm, neuraminidase treatment; LT, low trypsin treatment; HT, high trypsin treatment; CH, chymotrypsin treatment.

B. Southern blot of gDNA from a growth competition assay. A probe against *hDHFR* was used to determine relative proportions of each strain in a mixed culture of 3D7-1A parasites (4 kb band), which are *clag 3.1*− expressing, and *clag 3.2ins cl5 WR*+ parasites (2.3 kb band), which have no *clag 3* expression, over time. M0 = time of mixing, M7 = 7 life cycles (2 weeks) after mixing, M21 = 21 life cycles (6 weeks) after mixing. Assay was performed in two independent trials. Quantification of competition assay. Band intensities, representative of parasite number, were quantified using ImageJ software and the *clag 3.2ins cl5 WR*+:3D7-1A ratio was calculated for each trial and time point. *Clag 3.2ins cl5 WR*+ display a growth disadvantage relative to the 3D7-1A strain.

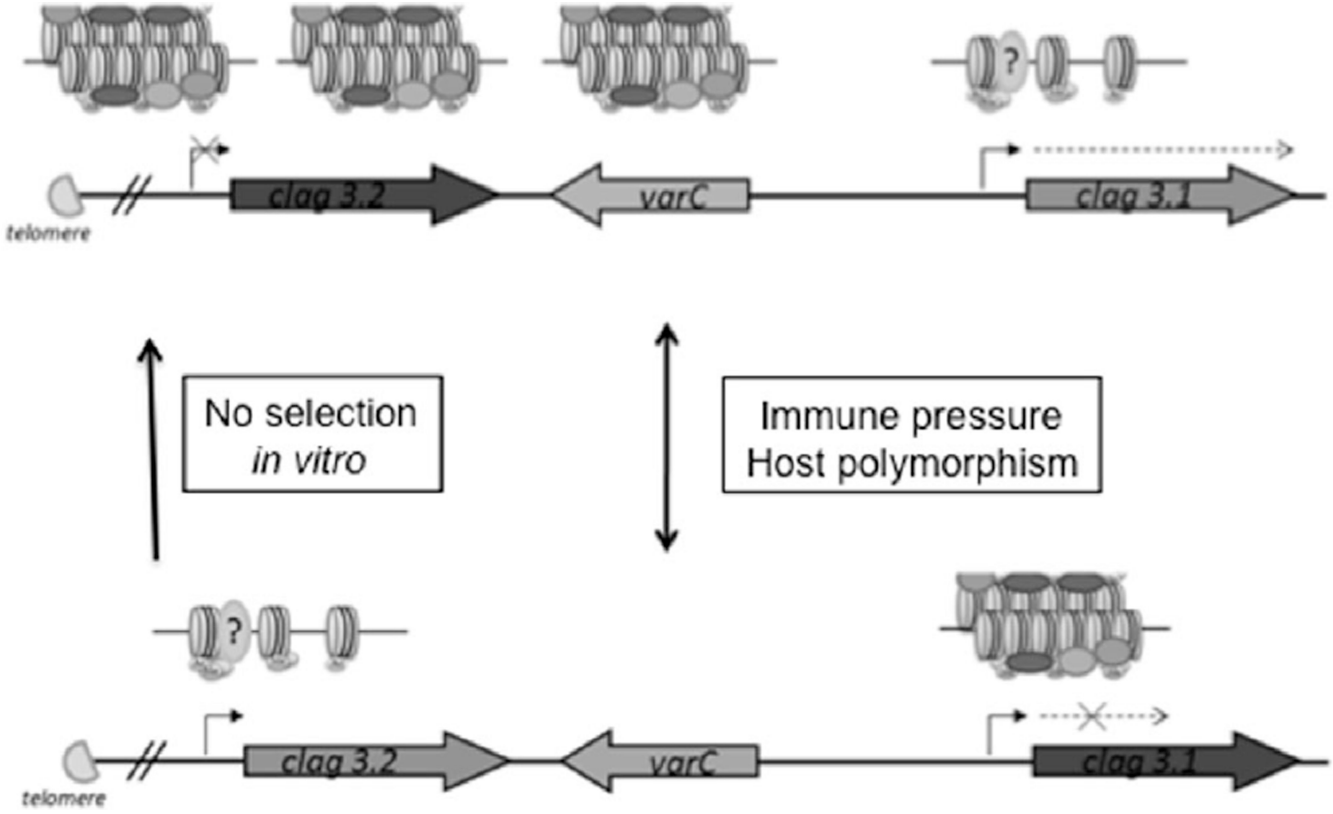


**Fig. 8.**

Model of phenotypic switching between *clag 3* paralogues. Mutually exclusive expression between *clag 3.1* and *clag 3.2* paralogues is heterochromatin-mediated. There is a preference for a particular *clag 3* gene. Selection for parasites expressing the other *clag 3* gene is facilitated either by a humoral response and/or by receptor polymorphism.