

# Clonally variant gene families in *Plasmodium falciparum* share a common activation factor

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## Summary

The genome of the malaria parasite *Plasmodium falciparum* contains several multicopy gene families, including *var*, *rifin*, *stevor* and *Pfmc-2TM*. These gene families undergo expression switching and appear to play a role in antigenic variation. It has recently been shown that forcing parasites to express high copy numbers of transcriptionally active, episomal *var* promoters led to gradual downregulation and eventual silencing of the entire *var* gene family, suggesting that a limiting titratable factor plays a role in *var* gene activation. Through similar experiments using *rifin*, *stevor* or *Pfmc-2TM* episomal promoters we show that promoter titration can be used as a general method to downregulate multicopy gene families in *P. falciparum*. Additionally, we show that promoter titration with *var*, *rifin*, *stevor* or *Pfmc-2TM* episomal promoters results in downregulation of expression not only of the family to which the episomal promoter belongs, but also members of the other gene families, suggesting that the *var*-specific titratable factor previously described is shared by all four families. Further, transcriptionally active promoters from different families colocalize within the same subnuclear expression site, indicating that the role that nuclear

architecture plays in *var* gene regulation also likely applies to the other multicopy gene families of *P. falciparum*.

## Introduction

The genome of *Plasmodium falciparum*, the causative agent of the most severe form of human malaria, contains several multicopy gene families including *var*, *rifin*, *stevor* and *Pfmc-2TM* (Dzikowski *et al.*, 2006a). The members of these families are interspersed immediately adjacent to the telomeric repeats found at chromosome ends, as well as within large gene clusters located at several internal chromosomal loci (Kyes *et al.*, 1999). While there are ~60 *var* genes within the haploid parasite genome, the *rifin*, *stevor* and *Pfmc-2TM* gene families contain approximately 150, 35 and 13 members respectively (Gardner *et al.*, 2002). Of these four families, only *var*, which encodes the variable surface antigen called PfEMP1, has a known function.

Upon invasion of human red blood cells, the parasite places PfEMP1 on the surface of the infected cell (iRBC) (Kyes *et al.*, 2001). This protein mediates cytoadherence of the iRBCs to various receptors on the vascular endothelium of the host's circulatory system, removing them from the circulation and avoiding splenic clearance. The resulting vascular obstruction and localized immune response is thought to be responsible for many of the symptoms associated with severe malaria, and PfEMP1 is currently the sole known *Plasmodium* virulence factor (Miller *et al.*, 2002). Due to its exposure on the iRBC surface, infected individuals readily produce antibodies to the form of PfEMP1 expressed by the parasites; however, by limiting expression to a single *var* gene at a time, and by regularly switching between different *var* genes, parasites are able to avoid antibody recognition and maintain a lengthy, persistent infection. This process, called antigenic variation, requires strict mutually exclusive expression within the *var* gene family, a complex mechanism that remains poorly understood.

Like *var* genes, the *rifin*, *stevor* and *Pfmc-2TM* families have recently been shown to undergo expression switching (Kyes *et al.*, 1999; Lavazec *et al.*, 2007; Niang *et al.*, 2009), leading to the suggestion that they may be involved, along with *var* genes, in antigenic variation. The

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proteins encoded by these gene families all belong to the two-transmembrane superfamily and have similar structures (Sam-Yellowe *et al.*, 2004; Lavazec *et al.*, 2006), and have been shown to localize to the RBC surface (Kyes *et al.*, 1999; Lavazec *et al.*, 2006; Niang *et al.*, 2009), suggesting a role in interactions with the extracellular environment. However, despite this evidence, phenotypes associated with changes in expression within these multicopy gene families have been difficult to ascertain and as of now, the RIFINs, STEVORs and PfMC-2TMs have no known function.

Little is known about the mechanisms that regulate transcription in *P. falciparum*. Initial computational analysis of the *Plasmodium* genome identified few putative specific transcription factors (Gardner *et al.*, 2002) leading to the suggestion that other mechanisms, such as promoter interactions, non-coding RNAs, chromatin modification and subnuclear architecture might play an unusually important role in regulating gene expression (Aravind *et al.*, 2003). Studies of transcriptional regulation of the *var* gene family have identified many components that contribute to activation, silencing and mutually exclusive expression, including aspects of chromatin remodelling (Freitas-Junior *et al.*, 2005; Chookajorn *et al.*, 2007; Lopez-Rubio *et al.*, 2007; 2009), promoter–promoter interactions (Deitsch *et al.*, 2001a; Calderwood *et al.*, 2003; Frank *et al.*, 2006; Dzikowski *et al.*, 2007) and non-coding RNAs (Su *et al.*, 1995; Kyes *et al.*, 2003; Epp *et al.*, 2008a). There is also evidence that subnuclear positioning might be important for *var* gene activation (Duraisingh *et al.*, 2005; Ralph *et al.*, 2005; Voss *et al.*, 2006), and transgenic parasites that have been genetically modified to activate more than one *var* promoter at a time have shown that active *var* promoters colocalize to a specific spot in the periphery of the parasite nucleus (Dzikowski *et al.*, 2007), suggesting the presence of a *var*-specific transcriptionally active site. This type of subnuclear localization has been shown to play a role in the mutually exclusive expression of variant surface glycoprotein (*vsg*) genes in African Trypanosomes (Navarro and Gull, 2001).

Despite the prominent involvement of chromatin modification and nuclear architecture in regulating variant gene expression in *P. falciparum*, it is likely that specific transcription factors also play a prominent role. Basal transcription factors including the TATA-box-binding protein have been identified, suggesting a transcription factor-based regulatory system (Callebaut *et al.*, 2005), and DNA elements from the upstream regulatory regions of various genes display the proper timing of expression when placed on transfected episomal constructs (Dechering *et al.*, 1999; Koning-Ward *et al.*, 1999). Additionally, the complex nature of the *Plasmodium* life cycle and its tightly regulated gene expression profiles suggest a need

for specific transcriptional regulators (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003). Recently, the ApiAP2 family of proteins, possessing weak homology to the plant AP2/ERF transcription factors, have been investigated as potential transcriptional regulators in *P. falciparum* (Balaji *et al.*, 2005). One member of this family (PF14\_0633) has been found by protein-binding microarray and computational analysis to associate with *var* promoters (De Silva *et al.*, 2008). Additionally, DNA binding assays using parasite nuclear extracts have identified protein complexes that bind to specific elements within the regulatory regions upstream of *var* promoters (Voss *et al.*, 2003), and more recent studies have demonstrated the presence of a specific titratable nuclear factor required for *var* gene expression (Dzikowski and Deitsch, 2008). Titration of this factor by the presence of multiple transcriptionally active episomal *var* promoters is capable of repressing transcription of the entire endogenous multicopy *var* gene family.

As *P. falciparum* lacks the canonical RNAi pathway, and disrupting up to 150 genes in a multicopy gene family is not feasible, knockdown of the *rifin*, *stevor* and *Pfmc-2TM* gene families and subsequent phenotypic analysis has been impossible to achieve. Here we show that downregulation of gene expression by promoter titration as originally described by Iyer *et al.* (2007) and subsequently applied to the *var* gene family by Dzikowski and Deitsch (2008) can be used as a general method to downregulate multicopy gene families, including *rifin*, *stevor* and *Pfmc-2TMs*, in *P. falciparum*. Additionally, we show via microarray and qRT-PCR that the *var*, *rifin*, *stevor* and *Pfmc-2TM* gene families share a common titratable factor necessary for expression. Episomal promoters of the four families are reciprocally capable of downregulating the chromosomal members of the other families. The extent of the cross-downregulation is dependent upon the temporal expression patterns of the gene families, with the most similarly expressed gene families showing the greatest reciprocal titration effect. Interestingly, although high copy numbers of episomal *stevor* promoters result in downregulation of their own gene family, they upregulate the *Pfmc-2TM* gene family, suggesting the possibility of functional redundancy and compensation between the proteins encoded by the two families. Finally, through DNA-FISH (fluorescent *in situ* hybridization), we show that a transcriptionally active episomal *rifin* promoter colocalizes with an active *var* promoter, indicating that these gene families utilize the same subnuclear expression site.

## Results

### *Promoter titration can be used to downregulate multicopy gene families in P. falciparum*

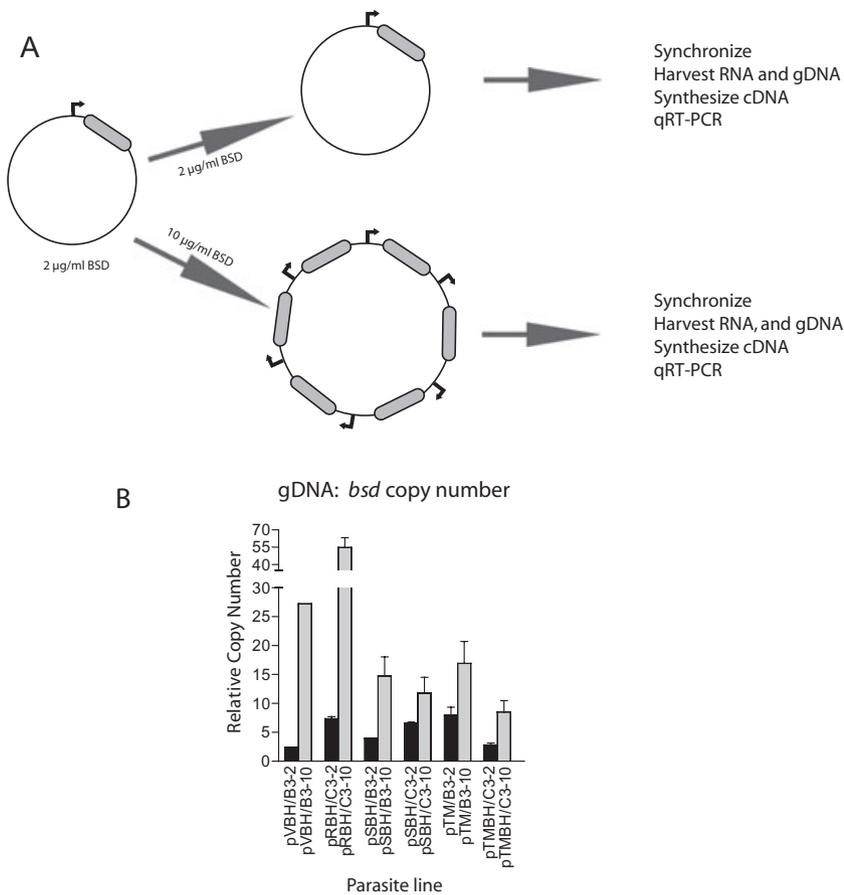
Recently Iyer *et al.* showed that transfection of the rodent parasite *Plasmodium yoelii* with an episomal plasmid car-

rying the upstream regulatory region and promoter of the 235 kDa rhoptry protein encoding gene led to an unexpected downregulation of the endogenous, chromosomal copy of the *py235* gene (Iyer *et al.*, 2007). The authors speculated that this downregulation could be the result of promoter competition for a limiting transcription factor, as has been described in other systems (Schodin *et al.*, 1995). Dzikowski *et al.* subsequently extended this technique to the study of *var* gene expression and showed that by forcing parasites to carry large numbers of transcriptionally active episomal *var* promoters, it is possible to downregulate and ultimately silence all endogenous, chromosomal *var* gene expression (Dzikowski and Deitsch, 2008). This was achieved by transfecting parasites with a plasmid carrying a blasticidin-S-deaminase (*bsd*) cassette driven by a *var* promoter that was made constitutively active by separating it from a regulatory element found in *var* introns. By using the *bsd* selectable marker, it is possible to regulate the copy number of episomally replicating plasmids, with exposure to increasing blasticidin concentrations resulting in larger concatamers (Epp *et al.*, 2008b). This in turn leads to greater levels of repression of the chromosomal genes and suggests the presence of a titratable nuclear factor necessary for endogenous *var* gene expression (Dzikowski and Deitsch, 2008). The ability to knockdown expression of entire multicopy gene families in *P. falciparum* would be a useful tool because current technology for downregulating genes is limited to knockout strategies, which are not applicable to large gene families. We therefore decided to investigate whether promoter titration could be used as a general method for downregulation of multicopy gene families in *P. falciparum*.

The *rifin*, *stevor* and *Pfmc-2TM* multicopy gene families were chosen for further investigation. The *var* promoter in the plasmid pVBH (Dzikowski *et al.*, 2007) was replaced with a representative promoter from each of the three gene families and the resulting constructs were transfected into two recently subcloned lines, C3 and B3, which were derived from the parasite isolate NF54. The C3 parasite line predominantly expresses a single *var* gene (PFD1005c) (Frank *et al.*, 2007) while the B3 parasite line primarily expresses one *stevor* (MAL7P1.227) and one *Pfmc-2TM* (PFA0680c) (Lavazec *et al.*, 2007). Stably transformed parasites were initially selected using  $2 \mu\text{g ml}^{-1}$  blasticidin, a concentration anticipated to select for low plasmid copy numbers. The cultures were then split and one was subjected to treatment with  $10 \mu\text{g ml}^{-1}$  blasticidin for 2 weeks or until normal growth rates were restored. RNA was then harvested from synchronized cultures at 18, 24 or 28 h post invasion (hpi) for investigation into *var*, *stevor* or *Pfmc-2TM* transcript levels respectively, and genomic DNA was collected to determine episomal plasmid copy

numbers (Fig. 1A). qRT-PCR assays were performed on each pair of cDNA samples using gene-specific primer sets to the *var*, *stevor* or *Pfmc-2TM* families. The large size of the *rifin* gene family and our lack of gene specific primers to all 135 members precluded us from assessing expression levels of this family by qRT-PCR. Therefore, analysis of *rifin* gene expression was determined by hybridization to a microarray of *P. falciparum* 3D7-coding regions.

As expected, growth of the transformed parasites lines under high concentrations of blasticidin resulted in a corresponding increase in the copy number of episomal plasmids containing *var*, *rifin*, *stevor* and *Pfmc-2TM* promoters (Fig. 1B). The differences in plasmid copy numbers between the different transgenic lines are most likely due to differences in promoter strengths and thus the number of *bsd* cassettes required to provide sufficient deaminase activity to survive the selection pressure. In order to determine if increasing the number of active episomal *stevor* and *Pfmc-2TM* promoters resulted in downregulation of the endogenous members of these gene families, the B3 transfectants (pSBH/B3 and pTMBH/B3 respectively) grown under low and high doses of blasticidin were assayed by qRT-PCR and the levels of endogenous gene expression compared. In the SBH/B3 line, endogenous *stevor* transcript levels were strongly repressed in the transfected lines, with parasites grown under high doses of blasticidin displaying greater repression than those grown under low doses (Fig. 2A). When the total endogenous *stevor* gene expression levels of the parasites under high doses of blasticidin were compared with that of the untransfected B3 line, a reduction of approximately 95% was observed (Fig. 2B). The relatively strong repression in parasites grown under low levels of blasticidin suggests that the presence of even the small concatamers may be repressing endogenous gene expression to a significant degree. Similarly in the TMBH/B3 line, high copy numbers of *Pfmc-2TM* episomal promoters downregulated total endogenous *Pfmc-2TM* transcript levels by 65% (Fig. 2D). However, unlike *stevor* genes, transfected parasites grown under low doses of blasticidin showed no downregulation as the gene expression levels were comparable with those from untransfected B3 parasites (Fig. 2D). In all cases, levels of repression were more tightly correlated with increases in episomal promoter copy numbers rather than levels of *bsd* transcripts, consistent with the idea that it is the total number of episomal promoters that is important for titration rather than the number of transcripts being produced. Despite the reduced gene expression in the lines grown under high doses of blasticidin, parasite transfected with either plasmid displayed no obvious phenotype outside of the initial delayed growth previously reported (Dzikowski and Deitsch, 2008).



**Fig. 1.** Method of blasticidin titration.

A. Plasmids carrying either a *var* (Dzikowski *et al.*, 2007), *rifin* (PF10\_0398), *stevor* (PFF1550w) or *Pfmc-2TM* (PFF0060w) promoter driving a *bsd* cassette (grey region) were transfected into parasites and selected with  $2 \mu\text{g ml}^{-1}$  BSD. Cultures were then split and one subculture was subjected to higher levels of drug, forcing the parasite to carry higher copy numbers of the episomal promoter. Cultures were then synchronized, RNA extracted, cDNA synthesized, and qRT-PCR performed.

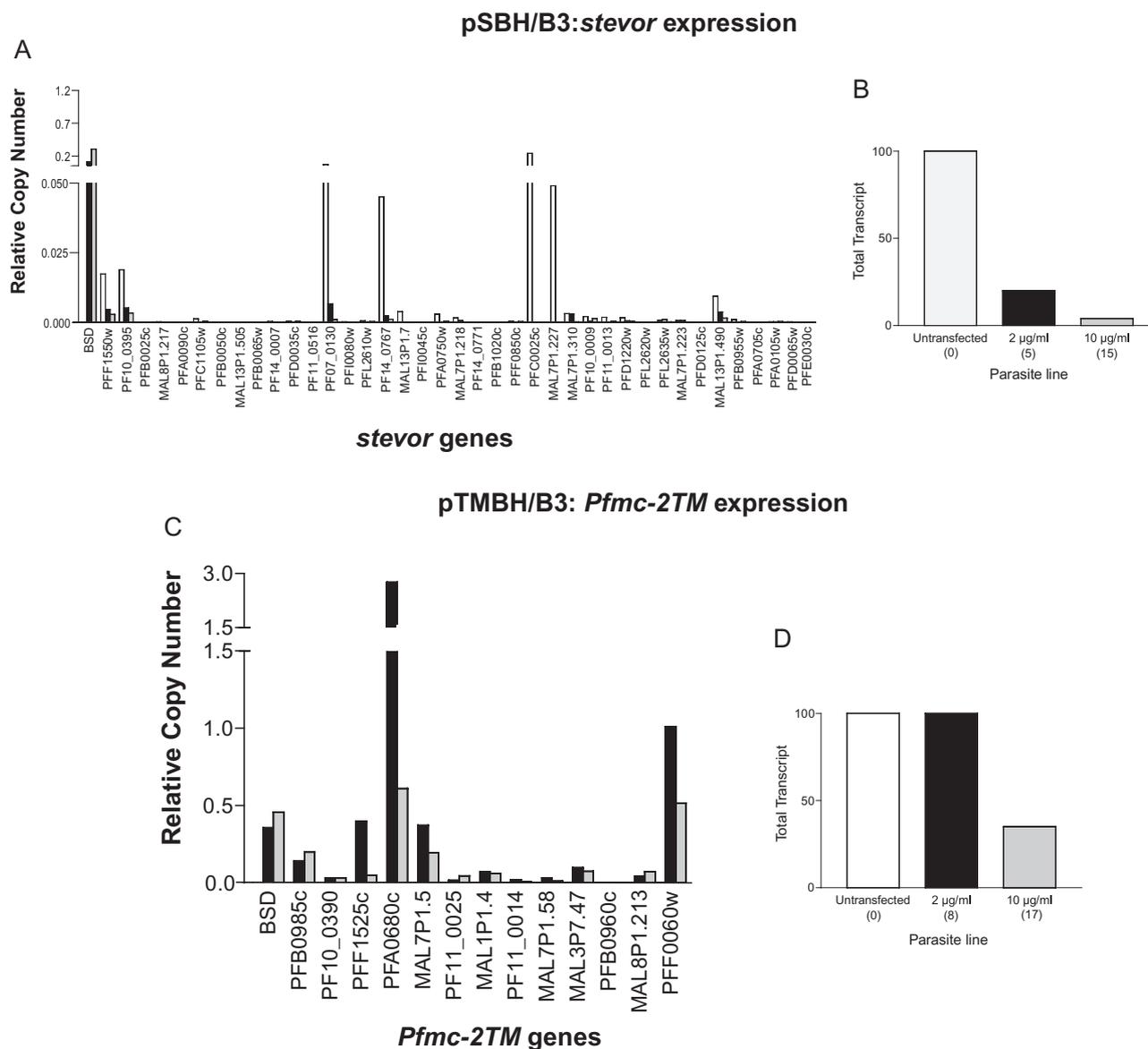
B. Genomic *bsd* copy number in each transformed parasite line under low (black bars) and high (grey bars) drug doses. In each line, the number of *bsd* copies, and therefore promoter copies, increases under higher drug pressure. Copy numbers were determined using q-PCR and comparison with the single-copy genes P60-seryl-tRNA synthetase (PF07\_0073), P61-fructose biphosphate aldolase (PF14\_0425) and P100-actin (PFL2215).

To determine if the reduced endogenous gene expression was simply a side effect of the increased blasticidin dose, parallel experiments were performed using the plasmid pHIRH in which *bsd* is driven by a *var* intron promoter (Epp *et al.*, 2008b). No reduction in *var* expression was observed (data not shown), demonstrating that the effect was not a non-specific result of large plasmid copy numbers or exposure to high blasticidin concentrations. Thus, the promoter titration method can be used as a general tool for the specific downregulation of multicopy gene families in *P. falciparum*.

#### *var*, *rifin*, *stevor* and *Pfmc-2TM* gene families share a common titratable factor

Previous work using episomal *var* promoters to downregulate expression of the *var* gene family indicated that other non-*var*, housekeeping genes were not affected (Dzikowski and Deitsch, 2008). However, it is possible that certain subsets of genes share specific transcription factors, and thus the transcriptional downregulation observed with high copy numbers of episomes carrying *var* promoters might not be exclusive to the *var* gene family. In order to assess the effect of *var* promoter titration on expression of all other genes in the *P. falciparum*

genome, microarray analysis was performed on untransfected parasites and those carrying pVBH in the presence of  $10 \mu\text{g ml}^{-1}$  blasticidin. RNA was taken from a parasite population 14–20 h after RBC invasion at a time when both *var* and *rifin* genes are highly expressed. The overall abundance of the majority of mRNA transcripts was relatively unchanged when comparing the two parasite populations (Pearson correlation value 0.85–0.96), confirming that the transfection and blasticidin selection did not result in large scale alterations in the parasite transcriptome, and that the parasite populations were at roughly the same point in the asexual cycle when the RNA was harvested. As expected, members of the *var* gene family were substantially downregulated (Pearson correlation 0.54), as previously observed by qRT-PCR. Notably, expression of *rifin*, *stevor* and *Pfmc-2TM* family members was also significantly reduced between  $10 \mu\text{g ml}^{-1}$  and no blasticidin control, suggesting they may share a titratable factor with the *var* gene family (Fig. 3). These gene families share several attributes, including location within specific subtelomeric and internal chromosomal clusters as well as transcriptional activation and silencing in a clonally variant fashion (Dzikowski *et al.*, 2006a). We therefore investigated whether the *var*, *rifin*, *stevor* and *Pfmc-2TM* multicopy gene families share a limited, titratable nuclear



**Fig. 2.** Downregulation of endogenous *stevor* and *Pfmc-2TM* expression by increased copy numbers of the plasmids pSBH and pTMBH respectively.

A. Change in *stevor* RNA levels following increase in copy number of pSBH. Expression levels are shown of endogenous *stevor* genes in pSBH/B3 under low (black bars) and high (grey bars) blasticidin doses. Untransfected B3 parasites (carrying no episome) are represented by white bars. All RNA samples were taken at 24 h post invasion.

B. Representation of data depicted in A. Total *stevor* transcript levels of untransfected B3 parasites (white bar) and pSBH transfected B3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses.

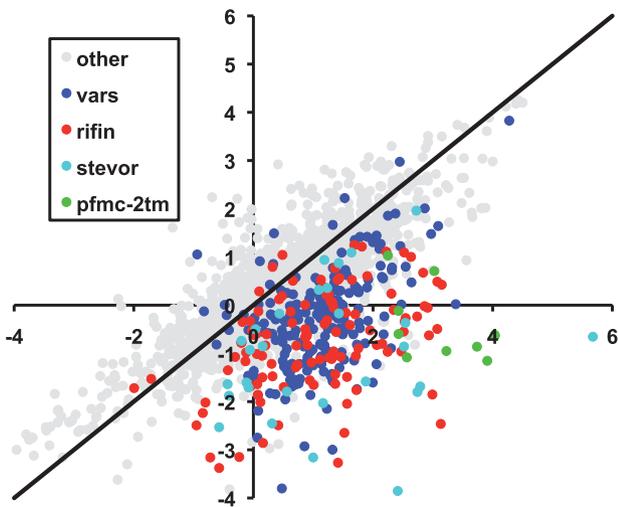
C. Change in *Pfmc-2TM* RNA levels following increase in copy number of pTMBH. Expression levels are shown of endogenous *Pfmc-2TM* genes in pTMBH/B3 parasites under low (black bars) and high (grey bars) blasticidin doses. All RNA samples were taken at 28 h post invasion.

D. Representation of data depicted in C. Total *Pfmc-2TM* transcript levels of untransfected B3 parasites (white bar) and pTMBH transfected B3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses. In both parasite lines, the episomal promoters repress expression of their own endogenous gene family. Relative copy numbers are measured with respect to the control housekeeping gene P61-fructose biphosphate aldolase (PF14\_0425) using the formula  $2^{-\Delta\Delta CT}$  with NF54 gDNA as the calibrator.

factor and thus can be co-repressed through promoter titration.

B3 parasites transfected with pVBH (pVBH/B3) were assayed by qRT-PCR for changes in endogenous *stevor*

and *Pfmc-2TM* expression. In these experiments, untransfected parasites were also included for comparison. RNA was harvested from synchronized cultures at 24 or 28 hpi for investigation into *stevor* or *Pfmc-2TM*



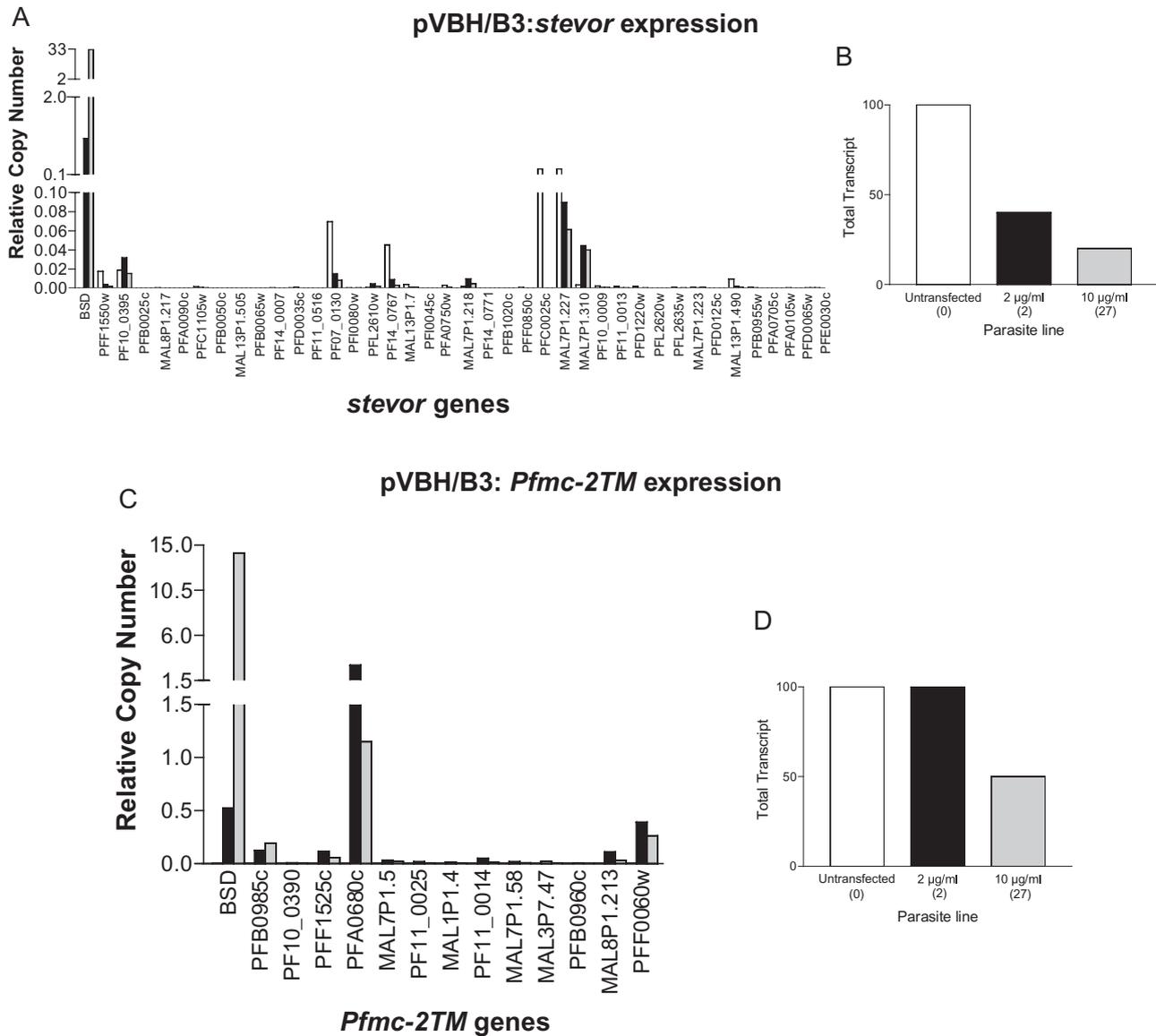
**Fig. 3.** Downregulation of expanded subtelomeric gene families when selecting for increased copy number of the pVBH plasmid using high blasticidin concentrations. The ratios of transcript signals obtained by DNA microarray under no versus  $10 \mu\text{g ml}^{-1}$  blasticidin are plotted. In grey are the intensity values for all genes excluding members of the *var*, *rifin*, *stevor* and *Pfmc-2TM* gene families. Overall, transcript levels intensities remain unchanged under drug selection and are evenly distributed around a line with a slope = 1 (black). However, the majority of the *var*, *rifin*, *stevor* and *pfmc-2TM* show a clear decrease in intensity upon selection with  $10 \mu\text{g ml}^{-1}$  blasticidin. Both axes represent  $\log_2(\text{Cy}3/\text{Cy}5)$  intensity values.

transcript levels respectively. Both families showed significant repression, with greater repression when parasites were grown under high doses of blasticidin (Fig. 4A and C). When compared with expression levels in untransfected B3 parasites, *stevor* transcript levels showed an 80% reduction (Fig. 4B). Similar to the TMBH/B3 line (Fig. 2D), *Pfmc-2TM* transcript levels showed no repression under low levels of blasticidin, but displayed an approximately 50% reduction in expression levels when the high dose parasites were compared with the untransfected parasites (Fig. 4D). Taken together with the microarray data indicating a *var* promoter-induced downregulation of the *rifin* family, it appears that these four families share a titratable nuclear factor necessary for their expression.

In the reciprocal experiment, C3 parasites carrying the *rifin*, *stevor* or *Pfmc-2TM* promoter constructs (pRBH/C3, pSBH/C3 and pTMBH/C3 respectively) were assayed at 18 h after invasion by qRT-PCR for changes in endogenous *var* gene expression. Increased copy numbers of pRBH and pSBH caused a 75% and 61% reduction in *var* gene expression respectively (Fig. 5B and D). Interestingly, titration with pTMBH had no effect on total *var* gene expression (Fig. 5E), although there is some indication that *var* gene switching may have occurred. The idea that switching could have been induced by titration with pTMBH seems unlikely, but cannot be ruled out in this experiment. The extent of repression of the *var*

family by these promoters correlates with their timing of peak expression within the 48 h replicative cycle of the parasite (Fig. 5G). Temporally, *var* genes are expressed first within the cycle (16–18 hpi), followed by *rifin* (18–23 hpi), *stevor* (22–32 hpi) and finally *Pfmc-2TM* genes (26–30 hpi) (Kaviratne *et al.*, 2002; Kyes *et al.*, 2003; Lavazec *et al.*, 2007). Of these families, *rifin* expression overlaps the greatest with *var* family expression and we observed the greatest effect on *var* expression in response to high copy numbers of *rifin* promoters. *stevor* family expression overlaps that of *var* less, and we observed a correspondingly smaller degree of downregulation of *var* expression by the episomal *stevor* promoters. Finally, the *Pfmc-2TM* family promoters, with peak expression 10–12 h following *var*, show no effect. In order to confirm that the co-regulatory effects were specific to these gene families and not simply temporal in nature, qRT-PCR primer sets were made to *sbp1* (PFE0065w) and the gene encoding antigen 332 (PF11\_0507). Both of these genes are expressed during late rings, coincident with *var* genes. If the co-regulation was exclusively temporal in nature, one would expect a *var* promoter to downregulate these similarly timed genes. When pVBH/C3 under low and high doses of blasticidin were assayed, no change in expression for either of these genes was observed (data not shown). Taken together, as the temporal expression patterns of the *rifin*, *stevor* and *Pfmc-2TM* gene families moved away from that of the *var* family, the episomal promoter-induced downregulation of *var* genes became less pronounced. Thus, there appears to be a temporal aspect to this transcriptional co-regulation of these specific gene families.

The function of the proteins encoded by the *rifin*, *stevor* and *Pfmc-2TM* multicopy gene families are not known. Proteins from all three families have similar domain structures comprised of signal peptide, PEXEL/HT erythrocyte trafficking motifs, and two predicted transmembrane domains. Moreover, the genes are found in similar genomic locations, suggesting that they might share common mechanisms of transcriptional regulation. We therefore investigated what effect downregulating members of the *stevor* gene family would have on *Pfmc-2TM* expression, and vice versa. The parasite lines pTMBH/B3 and pSBH/B3 were selected on  $10 \mu\text{g}$  blasticidin and assayed by qRT-PCR for downregulation of endogenous *stevor* and *Pfmc-2TM* genes respectively. Though pTMBH/B3 showed no change in *stevor* expression (data not shown), pSBH/B3 exhibited a threefold to sixfold upregulation of *Pfmc-2TM* transcripts (Fig. 6). The upregulation of *Pfmc-2TM* expression in response to *stevor* promoter titration was consistent and reproducible in several independent experiments and suggests two possible interpretations.



**Fig. 4.** Episomal *var* promoters downregulate endogenous *stevor* and *Pfmc-2TM* expression.

A. Change in *stevor* RNA levels following increase in copy number of pVBH. Expression levels are shown of endogenous *stevor* genes in pVBH/B3 parasites under low (black bars) and high (grey bars) blasticidin doses. Untransfected B3 parasites (carrying no episome) are represented by white bars. All RNA samples were taken at 24 h post invasion.

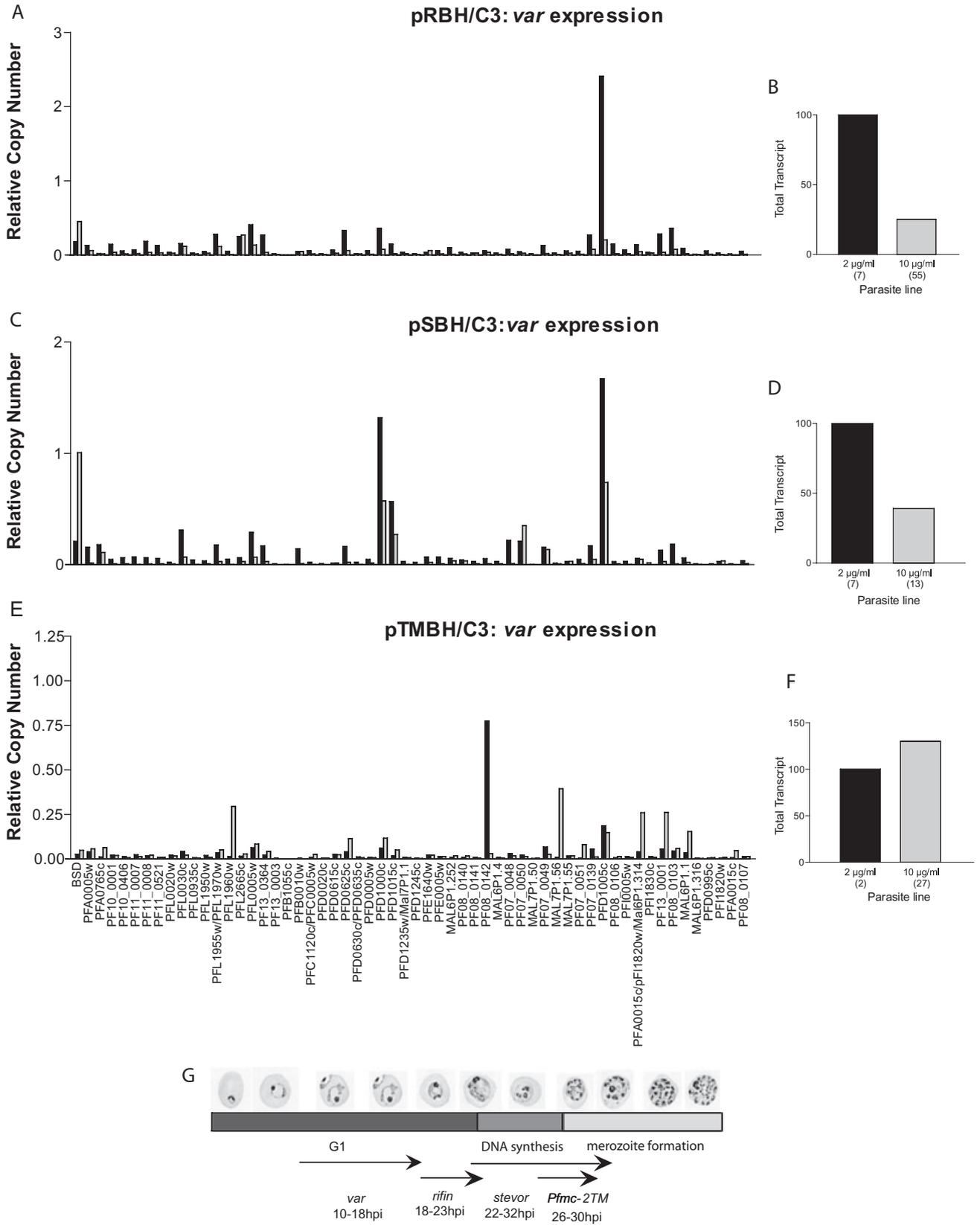
B. Representation of data depicted in A. Total *stevor* transcript levels of untransfected B3 parasites (white bar) and pVBH transfected B3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses.

C. Change in *Pfmc-2TM* RNA levels following increase in copy number of pVBH. Expression levels are shown of endogenous *Pfmc-2TM* genes in pVBH/B3 parasites under low (black bars) and high (grey bars) blasticidin doses. All RNA samples were taken at 28 h post invasion.

D. Representation of data depicted in C. Total *Pfmc-2TM* transcript levels of untransfected B3 parasites (white bar) and pVBH transfected B3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses. Relative copy numbers are measured with respect to the control housekeeping gene P61-fructose biphosphate aldolase (PF14\_0425) using the formula  $2^{-\Delta\Delta CT}$  with NF54 gDNA as the calibrator.

First, the episomal *stevor* promoter could be titrating a factor that represses *Pfmc-2TM* expression. Alternatively, the proteins encoded by the two families may be functionally redundant and therefore the *Pfmc-2TM* family could be upregulated in pSBH/B3 in order to compensate for the downregulation of *stevors* (shown

above, Fig. 2A). The possibility that expression of members of these families might be required for parasite viability is supported by our inability to drive down endogenous *Pfmc-2TM* expression to less than 65% (Fig. 2C), suggesting an important role for the corresponding protein.



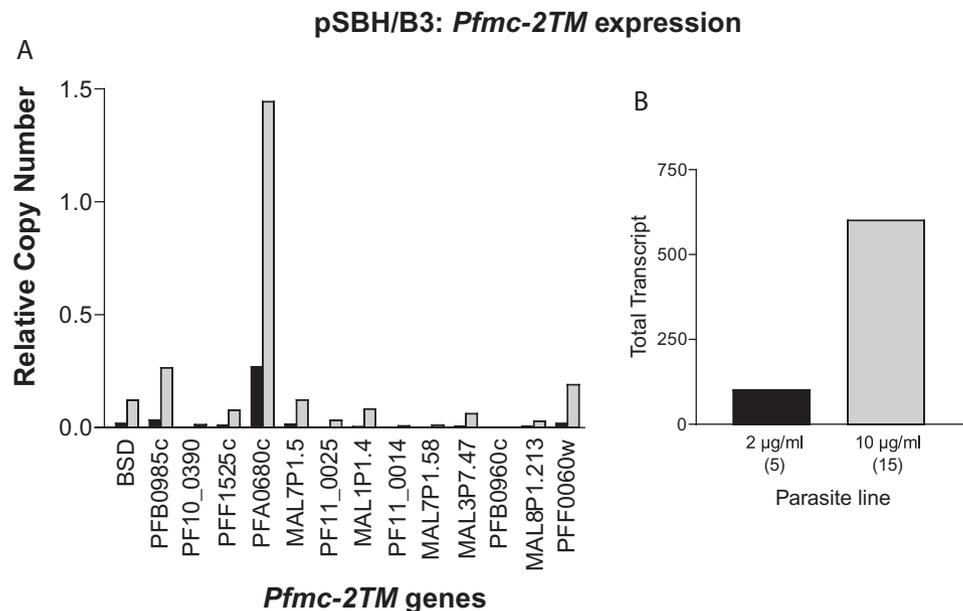
**Fig. 5.** *var*, *rifin*, *stevor* and *Pfmc-2TMs* share a common factor required for efficient expression.

A. Change in *var* RNA levels following increase in copy number of pRBH. Expression levels are shown of endogenous *var* genes in pRBH/C3 parasites under low (black bars) and high (grey bars) blasticidin doses.  
 B. Representation of data depicted in A. Total *var* transcript levels of pRBH transfected C3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses.  
 C. Change in *var* RNA levels following increase in copy number of pSBH. Expression levels are shown of endogenous *var* genes in pSBH/C3 parasites under low (black bars) and high (grey bars) blasticidin doses.  
 D. Representation of data depicted in C. Total *var* transcript levels of pSBH transfected C3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses.  
 E. Change in *var* RNA levels following increase in copy number of pTMBH. Expression levels are shown of endogenous *var* genes in pTMBH/C3 parasites under low (black bars) and high (grey bars) blasticidin doses.  
 F. Representation of data depicted in E. Total *var* transcript levels of pTMBH transfected C3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses.  
 All RNA was harvested at 18 h post invasion (hpi). Relative copy numbers are measured with respect to the control housekeeping genes P60-seryl-tRNA synthetase (PF07\_0073) using the formula  $2^{-\Delta\Delta CT}$  with NF54 gDNA as the calibrator.  
 G. Temporal expression pattern of *var*, *rifin*, *stevor* and *Pfmc-2TMs* during the 48 h *P. falciparum* life cycle. Peak expression of *var* genes is at 16–18 hpi, followed by *rifin* at 18–23 hpi, *stevors* at 22–32 hpi, and finally *Pfmc-2TMs* at 26–30 hpi. Episomal *rifin*, *stevor* and *Pfmc-2TM* promoters downregulate endogenous *var* expression. The level of co-regulation, however, correlates with the temporal order of gene expression of these families during the 48 h replicative cycle.

#### Active *rifin* and *var* promoters colocalize at a subnuclear expression site

It has been suggested that localization to a subnuclear expression site might play a role in control of *var* gene expression (Duraisingh *et al.*, 2005; Ralph *et al.*, 2005; Voss *et al.*, 2006; Lopez-Rubio *et al.*, 2009). Recently, it has been demonstrated that active *var* promoters colocalize greater than 90% of the time at a specific spot in the parasite nucleus (Dzikowski *et al.*, 2007). As *var*, *rifin*, *stevor* and *Pfmc-2TM* genes appear to share a titratable nuclear factor, we were interested to determine if active

promoters from these four families colocalize to the same subnuclear position. For *var* and *rifin* genes, the time periods during the asexual cycle in which they are expressed overlap, therefore we performed DNA-FISH on the parasite line pRBH/C3, which like its parent line C3, predominantly expresses the *var* gene PFD1005c (Frank *et al.*, 2007). Localization of biotin-labelled probes specific to the active *var* gene PFD1005c or inactive *var* gene PFL0030c was compared with a fluorescein-labelled blasticidin probe which specifically hybridized with the episomal plasmid pRBH. The active episomal *rifin* promoter colocalized with the active endogenous *var* gene,



**Fig. 6.** pSBH upregulates *Pfmc-2TM* expression.

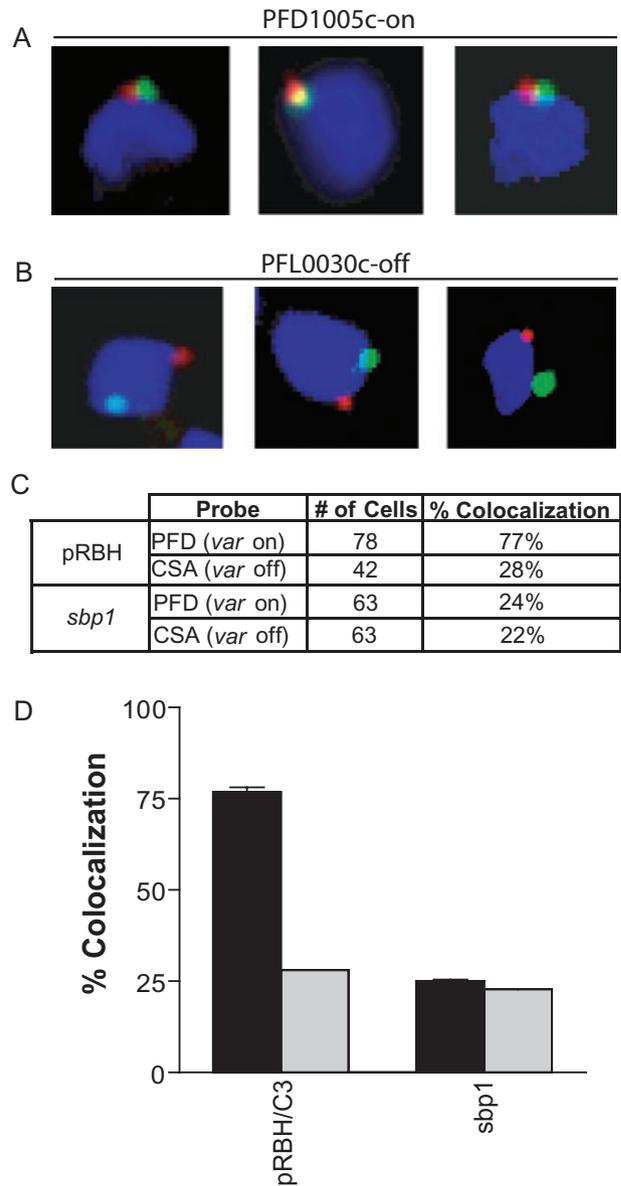
A. Expression levels of endogenous *Pfmc-2TM* genes in pSBH/B3 parasites under low (black bars) and high (grey bars) blasticidin doses.  
 B. Representation of data depicted in A. Total *Pfmc-2TM* transcript levels of pSBH transfected B3 parasites under low (black bar) and high blasticidin doses (grey bar). The approximate copy number of the transfected episome for each drug concentration is shown in parentheses.  
 RNA was harvested at 28 hpi and relative copy numbers were measured with respect to the control housekeeping gene P100-actin (PFL2215) using the formula  $2^{-\Delta\Delta CT}$  with NF54 gDNA as the calibrator.

PFD1005c, 77% of the time compared with 28% with the inactive *var* promoter PFL0030c (Fig. 7) indicating that these two active promoters generally occupy the same subnuclear position. Similar to the data reported by Dzikowski *et al.* the two spots were usually found immediately adjacent to one another, rather than completely overlapping, suggesting a fairly large transcriptionally active region (Dzikowski *et al.*, 2007). Attempts to do similar experiments with pSBH/C3 and pTMBH/C3 were complicated by the fact that the periods of active transcription of *stevors* and *Pfmc-2TMs* do not substantially overlap with that of *var* genes and because in both of these cultures significant *var* gene switching had occurred (Fig. 5), making the *var* gene expression pattern heterogenous. Therefore, no conclusions can be drawn regarding these promoters.

From this data, it appears that the active episomal *rifin* promoter does indeed localize to the same position in the nucleus as an active *var* promoter. The reliance of the two families on the same titratable factor, along with their similar temporal expression patterns supports this finding. Previously, we observed that an unrelated gene (PFF1125c) also colocalized with active *var* promoters ~75% of the time, suggesting that there might be a limited number of active transcription sites within the nucleus, and thus transcriptionally active genes might tend to colocalize frequently regardless of promoter type. To investigate this phenomenon further, we performed FISH with probes to *sbp1* (PFE0065W), a ring-stage specific gene, and the active or inactive *var* genes PFD1005c and PFL0030c. However, unlike PFF1125c, colocalization between the active *sbp1* gene and the active *var* gene was only ~25%, suggesting that the subnuclear spot is not occupied equally by all active promoters. What dictates the subnuclear position of different promoters at different times therefore remains difficult to predict and deserves additional investigation.

## Discussion

The ability to investigate gene function through knockouts has proven to be a powerful tool for understanding the biology of malaria parasites. However, in instances where knockouts are lethal or where numerous genes have redundant functions, simple gene knockouts are not informative. In the case of multicopy gene families, with the exception *var* genes, it has not been possible to simultaneously knockout the expression of all members of a family. For example, targeting up to 150 genes for disruption, as would be necessary in the case of the *rifin* family, is not possible with current technology. The lack of RNAi in *Plasmodium* makes the use of this type of RNA-based approaches also infeasible. Here we have shown that the promoter titration method can be used for the specific



**Fig. 7.** Active *var* and *rifin* promoters colocalize at a transcriptionally active subnuclear spot. For DNA-FISH, the nuclei are stained with DAPI (blue), and the pRBH episomes are detected with a *bsd* probe (green). Endogenous *var* genes [PFD1005c-on, or PFL0030c (CSA)-off] were detected with gene-specific probes (red). Ring stage parasites transfected with pRBH were probed with the *bsd* probe plus either the PFD1005c-on or PFL0030c-off probe. A. Examples of cells scored as colocalizing. B. Examples of cells scored as non-colocalizing. C. Quantification of colocalization of the active episome (green) or the control gene *sbp1* with either the active *var* gene (PFD) or inactive *var* gene (CSA) (red) in each parasite line. Percentages and cell numbers are an average of three separate, blinded counts. D. Graphical representation of data in C. Black bars represent colocalization with active *var* gene (PFD). Grey bars represent colocalization with inactive *var* gene (CSA).

downregulation of large multicopy gene families. In addition, promoter titration allows stepwise downregulation of gene expression, thereby potentially enabling the detection of intermediate phenotypes that might be obscured by lethality in a complete knockout. Specific downregulation of large gene families may give insight into gene phenotypes which we have as of yet been unable to observe. This technique, though very useful, does have caveats that need to be considered. For example, although promoter titration with a *var* promoter caused no detectable change in expression of ~90% of *P. falciparum* genes, downregulation of other multicopy families was observed. Thus, it is necessary to ensure the specificity of the episomal promoter chosen, keeping in mind that gene families which share promoter sequences could also share limited regulatory factors.

In the case of the *vars*, *rifins*, *stevors* and *Pfmc-2TMs*, the possibility that they share a limiting nuclear factor necessary for expression might be due to the similarities in how the genes are regulated and in the roles the encoded proteins play in RBC modification. All are multicopy gene families primarily located in subtelomeric regions which undergo expression switching (Dzikowski *et al.*, 2006a). Additionally, like PfEMP1 encoded by *var* genes, both RIFINs and STEVORs have been localized to the infected red blood cell surface and this, along with their hypervariability and abundance in the genome, has implicated them in antigenic variation (Kyes *et al.*, 1999; Niang *et al.*, 2009). Furthermore, the RIFINs, STEVORs and PfMC-2TMs share a common protein structure, potentially suggesting a common function (Lavazec *et al.*, 2006). It might not be surprising therefore that the four families share a common titratable nuclear factor necessary for their transcription. Although it has been shown that the pattern of *rifin*, *stevor* and *Pfmc-2TM* expression is not linked to that of *var* genes (Sharp *et al.*, 2006; Lavazec *et al.*, 2007), it is possible that similar mechanisms play a hand in regulating their expression. Chromatin modification, subnuclear localization, and interactions with DNA regulatory elements have all been shown to impact *var* gene expression and silencing, and some or all of these mechanisms may require the titratable factor detected here to regulate transcription of these other multicopy gene families.

It has been hypothesized that the members of the two transmembrane superfamily might play a role in antigenic variation and cytoadherence, rosetting, and/or solute pore structure (Helmbly *et al.*, 1993; Kyes *et al.*, 1999). The specific downregulation of these families by promoter titration had the potential to elucidate a phenotype; unfortunately, the downregulation of the *rifin*, *stevor* and *Pfmc-2TM* families showed no obvious phenotype in cultured parasites other than an initial decrease in growth rate that is likely due to the blastic-

din pressure. PfEMP1, encoded by *var* genes, is widely recognized as the major *P. falciparum* virulence factor and its expression is thought to be necessary for parasite persistence *in vivo*. Nonetheless, both Voss *et al.* (2006) and Dzikowski *et al.* (2006b) showed that parasites that do not express PfEMP1 also display no detectable phenotype *in vitro*. The similar lack of an *in vitro* phenotype may suggest a role for RIFINs, STEVORs and PfMC-2TMs in direct interactions with the host. It is intriguing that parasites in which *stevor* expression was downregulated repeatedly exhibited a notable upregulation of *Pfmc-2TM* transcripts, and that we were unable to completely abolish *Pfmc-2TM* expression by promoter titration. It is possible that the proteins encoded by the two gene families are functionally redundant and necessary for parasite viability, and thus *Pfmc-2TM* family upregulation compensates for the downregulation of the *stevors* in the same line. Inter-family compensation could also explain the lack of phenotype in the titrated parasites. In this light it would be interesting to observe the phenotype of parasites with complete knockdowns of all three families (*rifins*, *stevors* and *Pfmc-2TMs*), if indeed the triple knockdown proves to be viable.

The fact that the expression of all four of the gene families studied here can be altered through promoter titration provides added emphasis to their promoters/upstream regulatory regions and the titratable factor that controls them. These elements might be involved not only in transcriptional activation, but also in the mechanism that controls mutually exclusive expression. However, the nature or identity of the titratable factor described here and by Dzikowski and Deitsch remains to be determined. Few identifiable putative specific transcription factors have been annotated in the *P. falciparum* genome (Aravind *et al.*, 2003). One notable candidate family is that of the apicomplexan AP2 (Apetala2), or ApiAP2, proteins. The *P. falciparum* genome contains 26 members of this family which show weak homology to a family of plant AP2/ERF DNA-binding proteins (Balaji *et al.*, 2005). The proteins vary both in structure and life-cycle stage of expression (De Silva *et al.*, 2008). Several members have been shown to bind *var* promoter sequences by protein binding microarrays. One in particular, PF14\_0633, is of considerable interest as its predicted consensus binding sequence is present in the *var*, *stevor* and *Pfmc-2TM* promoters used for promoter titration in this paper (Voss *et al.*, 2003; De Silva *et al.*, 2008). Additionally, this protein is expressed during the late ring stage, coincident with *var* family expression. It remains to be determined if this ApiAP2 family is involved in *var* transcriptional regulation. Further, it should be noted that several titratable factors might be involved in the regulation of these gene families, and thus the titration phe-

nomenon could be the result of a limiting amount of any protein or protein complex.

Recently it has been shown that active *var* genes localize to a specific subnuclear expression site (Duraisingh *et al.*, 2005; Ralph *et al.*, 2005; Voss *et al.*, 2006; Dzikowski *et al.*, 2007). This type of location-dependent allelic exclusion mechanism has been established for the *vsg* genes in African Trypanosomes (Navarro and Gull, 2001). It is possible that the titratable factor necessary for the expression of the four gene families discussed here is not a molecule or transcription factor per se, but rather access to this subnuclear compartment. The large promoter-carrying episomes may simply displace the endogenous genes in this expression site, leading to their transcriptional repression. Here, an active episomal *rifin* promoter was shown to colocalize with an active endogenous *var* promoter, and episomal *rifin* promoters also displayed the greatest repressive effect on *var* gene expression, followed by lesser effects by *stevors* and *Pfmc-2TM* promoters. It is possible that all four promoter types may localize to the same subnuclear position, perhaps containing a specific transcriptional factory, at different times during the replication cycle. If this is the case, the active genes would move into and out of the transcriptional region throughout the 48 h asexual cycle at times corresponding with the expression pattern of the each gene family. As *rifin* family expression closely follows and overlaps that of the *var* family, one might expect a high level of colocalization between these two families and a greater repressive effect on *var* gene expression than *stevor* and *Pfmc-2TM* genes, which is indeed the result observed here.

## Experimental procedures

### Parasite culture

All parasites used were derivatives of the NF54 parasite line and were cultivated at 5% haematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate and 0.1 mg ml<sup>-1</sup> gentamicin. Parasites were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen. The C3, clonally expressing the *var* gene PFD1005c, and B3, predominantly expressing one *stevor* (MAL7P1.227) and one *Pfmc-2TM* (PFA0680c), lines have been described previously (Frank *et al.*, 2007; Lavazec *et al.*, 2007).

Parasite cultures were synchronized using percoll/sorbitol gradient centrifugation as previously described (Aley *et al.*, 1984; Calderwood *et al.*, 2003). Briefly, infected RBCs were layered on a step gradient of 40%/70% percoll containing 6% sorbitol. The gradients were then centrifuged at 12 000 *g* for 20 min at room temperature. Highly synchronized, late stage parasites were recovered from the 40%/70% interphase, washed twice with complete culture media and placed back in culture. To verify that this process resulted in populations of parasites that were at similar points in the cell cycle,

microarray gene expression data were compared between two separate cultures that were equivalently synchronized. This analysis showed that the overall abundance of the majority of mRNA transcripts were virtually identical when comparing the two parasite populations (Pearson correlation value 0.85–0.96), indicating that they were at equivalent points in the 48 h replicative cycle.

### Plasmid construction and parasite transfection

The plasmids pRBH, pSBH and pTMBH were constructed by replacing the *var* promoter in pVBH, as described (Dzikowski *et al.*, 2007), with a 1.6 kb portion of the promoter region from a *rifin* (PF10\_0398), *stevor* (PFF1550w) or *Pfmc-2TM* (PFF0060w). The promoter regions were amplified by PCR and the cloning sites HpaI (3') and KpnI (5') introduced using the following primer sets: PF10\_0398, 5'-ATATAGGTACCCA CTCATTAAGATTACCTTG-3', 5'-ATATAGTTAACGTGGAA CCACATTTGCC-3'; PFF1550w, 5'-ATATAGGTACCGAA CATCTCCTAACATATATG-3', 5'-ATATAGTTAACGATAAGC GGAAATTTTGTG-3'; PFF0060w, 5'-ATATAGGTACCCTCA AATATCATAGATTCTAC-3', 5'-ATATAGTTAACGTGATATAT GAAAAAGTGCTGG-3'. Parasites were transfected as described (Deitsch *et al.*, 2001b). Briefly, 0.2 cm electroporation cuvettes were loaded with 0.175 ml of erythrocytes and 100 µg of plasmid DNA in incomplete cytomix solution. Stable transfectants were initially selected on 2 µg ml<sup>-1</sup> blasticidin (Invitrogen, USA). In order to obtain parasites carrying large plasmid copy numbers, these cultures were then subjected to 10 µg ml<sup>-1</sup> blasticidin for 2 weeks or until normal growth rates were observed.

### Genomic DNA extraction, RNA extraction and cDNA synthesis

Genomic DNA was extracted as described (Dzikowski and Deitsch, 2008). Briefly, iRBCs were pelleted and lysed with Saponin. Parasites were pelleted, washed with PBS, and taken up in 200 µl TSE buffer (100 mM NaCl, 50 mM EDTA, 20 mM Tris, pH 8), 40 µl of 10% SDS and 20 µl 6 M NaClO<sub>4</sub>. This suspension was rocked for 12 h and genomic DNA was extracted with phenol/chloroform. The resulting DNA was taken up in 100 µl dH<sub>2</sub>O.

RNA extraction and cDNA synthesis was performed as described (Dzikowski and Deitsch, 2006). Briefly, RNA was extracted from synchronized parasite cultures at 18, 24 or 28 hpi, corresponding with the peak RNA expression levels for determination of *var*, *stevor* or *Pfmc-2TM* transcript levels respectively. RNA was extracted with the TRIZOL LS Reagent® as described (Kyes *et al.*, 2000) and purified on PureLink column (Invitrogen) according to manufacturer's protocol. Isolated RNA was then treated with Deoxyribonuclease I® (Invitrogen) to degrade contaminating gDNA. cDNA synthesis was performed from 800 ng total RNA with Superscript II RNase H reverse transcriptase® (Invitrogen) with random primers® (Invitrogen) as described by the manufacturer.

### DNA microarray hybridizations

For DNA microarray hybridizations, cDNA with amino-allyl incorporated dUTPs was generated from RNA as described

(Bozdech *et al.*, 2003). As an arbitrary reference, we used pooled RNA from hourly time points of a synchronized 48 h culture of 3D7 strain parasites used. Coupling to Cy3 (reference pool) and Cy5 (experimental sample) (GE Healthcare) and array hybridizations were performed as described using a recently designed new-generation *P. falciparum*-specific long oligonucleotide DNA microarray (Hu *et al.*, 2007). The arrays were scanned using an Axon 4200A scanner and images analysed using Axon GenePix software (Axon Instruments, Union City, CA, USA). Data were normalized by the global signal intensity across both channels. The resulting data were further analysed using the Princeton University Microarray database (PUMAdb, <http://puma.princeton.edu>) for cluster analysis.

### Real-time qRT-PCR

For qRT-PCR reactions to detect transcription from all *var*, *stevor* and *Pfmc-2TM* genes present in the 3D7 genome, we used the primer sets published by Salanti *et al.* (2003), Lavazec *et al.* (2007) and Sharp *et al.* (2006) respectively (Salanti *et al.*, 2003; Sharp *et al.*, 2006; Lavazec *et al.*, 2007). PCR primers used to amplify *sbp1* (PFE0065W) were: 5'-ACGAACCAACACAATTACAG-3', 5'-AGCTGCTTCTCCA CTAAC-3', while those used to amplify *antigen 332* (PF11\_0507) were: 5'-CGAAGAAAATGTCTCATTCA-3', 5'-TCCCGAATTTTTATCTTCAT-3'. Transcript copy numbers were determined using the formula  $2^{-\Delta\Delta CT}$  as described in the Applied Biosystems User Bulletin 2 using NF54 gDNA as the calibrator. Specifically, relative copy number was calculated as 2 exponential negative [(Ct target gene in cDNA – Ct reference gene in cDNA) – (Ct target gene in gDNA – Ct target gene in gDNA)]. Copy numbers of episomal promoters were calculated using qRT-PCR of gDNA and comparing the  $\Delta CT$  of *bsd* with that of the single copy housekeeping gene P60-seryl-tRNA synthetase (PF07\_0073), P61-fructose bisphosphate aldolase (PF14\_0425) or P100-actin (PFL2215) as described (Frank *et al.*, 2006). The control gene used for the graphs shown in the figures was chosen arbitrarily and is specified in the figure legends. All qRT-PCR assays were performed at least in duplicate for each template with no apparent differences, and each experiment was completed at least twice in its entirety, again with no significant differences.

### Fluorescent in situ hybridization

DNA probes were labelled with biotin and fluorescein using Roche High-Prime Kits. Biotin was detected using streptavidin AlexaFluor 594 (Molecular Probes). Synchronous ring-stage parasites were fixed in 4% paraformaldehyde solution as described in *Methods in Malaria Research* (Mancio-Silva, Freitas-Junior, Scherf). Slides were washed twice with 2× SSPE and DNA-FISH was carried out on ring stage parasites as described by Freitas-Junior *et al.* (2000) with modifications. Briefly, probes were hybridized overnight (94°C, 2 min, 37°C, 16 h). Slides were washed in 2× SSPE, 37°C, 1 h; 0.2× SSPE 50°C, 1 h; 0.2× SSPE 37°C, 30 min; maleic acid buffer, room temperature, 10 min; 1% blocking reagent in maleic acid buffer (Roche), room temperature, 1 h; 1% blocking reagent in maleic acid buffer (Roche) plus AlexaFluor 594 (Molecular Probes), room temperature, 30 min;

three times maleic acid buffer, room temperature, 20 min. After washing, the slides were mounted in anti-fade medium and visualized using an Olympus M081 fluorescent microscope. Composite images were produced using Photoshop 6.0 and the images blindly counted by three individuals.

The PFD1005c probe was generated using the following primers: 5'-CGGATGATGGTGATACTG-3', 5'-CTCCACAT GATGGTAGAAGAC-3'. The resulting 680 bp PCR fragment from the 3' region of exon 1 was gel-extracted and biotin labelled using a Roche High-Prime kit. The PFL0030c (*var2CSA*) probe was generated using nested primers to the uORF region of this gene. Primary reaction primers: 5'-GGTACCTGAACGCTTAAAGAAACAAGG-3', 5'-CTGCA GCATTTTGTCCAACCATTAC-3'. Secondary reaction primers: GCAATGTATAACAAAAATATAAG-3', GACACCAA ATATTCCTATGTGC-3'. The resulting 1 kb PCR product was gel-extracted and biotin labelled using a Roche High-Prime kit. The *bsd* probe was made by labeling whole pCR<sup>®</sup>2.1-TOPO (Invitrogen) plasmid carrying a *bsd* cassette with fluoroscein using a Roche High-Prime kit. The *sbp1* (PFE0065W) probe was generated using the following primers: 5'-ATGTGTAGCGCAGCTCGAGC-3', 5'-TTAGGTTTCTCTAGC AACTG-3'. The resulting 1 kb PCR product was gel-extracted and fluoroscein labelled using a Roche High-Prime kit.

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