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CRISPR Interference of a Clonally Variant GC-Rich Noncoding RNA Family Leads to General Repression of var Genes in Plasmodium falciparum

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ABSTRACT The human malaria parasite Plasmodium falciparum uses mutually exclusive expression of the PfEMP1-encoding var gene family to evade the host immune system. Despite progress in the molecular understanding of the default silencing mechanism, the activation mechanism of the uniquely expressed var member remains elusive. A GC-rich noncoding RNA (ncRNA) gene family has coevolved with Plasmodium species that express var genes. Here, we show that this ncRNA family is transcribed in a clonally variant manner, with predominant transcription of a single member occurring when the ncRNA is located adjacent to and upstream of an active var gene. We developed a specific CRISPR interference (CRISPRi) strategy that allowed for the transcriptional repression of all GC-rich members. A lack of GC-rich ncRNA transcription led to the downregulation of the entire var gene family in ring-stage parasites. Strikingly, in mature blood-stage parasites, the GC-rich ncRNA CRISPRi affected the transcription patterns of other clonally variant gene families, including the downregulation of all Pfmc-2TM members. We provide evidence for the key role of GC-rich ncRNA transcription in var gene activation and discovered a molecular link between the transcriptional control of various clonally variant multigene families involved in parasite virulence. This work opens new avenues for elucidating the molecular processes that control immune evasion and pathogenesis in P. falciparum.

IMPORTANCE Plasmodium falciparum is the deadliest malaria parasite species, accounting for the vast majority of disease cases and deaths. The virulence of this parasite is reliant upon the mutually exclusive expression of cytoadherence proteins encoded by the 60-member var gene family. Antigenic variation of this multigene family serves as an immune evasion mechanism, ultimately leading to chronic infection and pathogenesis. Understanding the regulation mechanism of antigenic variation is key to developing new therapeutic and control strategies. Our study uncovers a novel layer in the epigenetic regulation of transcription of this family of virulence genes by means of a multigene-targeting CRISPR interference approach.

KEYWORDS ncRNA, Plasmodium falciparum, antigenic variation, mutually exclusive expression, epigenetics, var genes, virulence, malaria parasites

The protozoan parasite Plasmodium falciparum is responsible for the deadliest form of human malaria, causing hundreds of thousands of deaths every year (1), and, akin to other protozoan parasites (2), relies on the mutually exclusive expression of virulence gene families to survive within the host (2–6). Among them, the 60-member var gene family encodes PfEMP1, an important variant surface adhesion molecule (7). var genes are located in subtelomeric regions of 13 out of 14 chromosomes and in
central clusters of chromosomes 4, 6, 7, 8, and 12 (8), and their transcription is epigenetically controlled (4). Transcription of a single var gene peaks at about 12 h post invasion (hpi) (9) and is then silenced but poised during the later stages of the 48-h intraerythrocytic cycle (10, 11). All other var genes remain transcriptionally silenced throughout the cycle and are tethered in repressive heterochromatic clusters enriched in H3K9me3 and H3K36me3 at the nuclear periphery (10, 12, 13). In contrast, the active var gene is euchromatic, enriched in H3K4me3 and H3K9ac, and localizes to a distinct perinuclear expression site (10, 12, 14) (a schematic is shown in Fig. 1A). Since the first description of this expression site (14), the mechanism of activation has remained elusive. Long noncoding RNAs (lncRNA) transcribed from the var intron have been implicated in a cis activation process (15, 16); however, the necessity of the intron-derived IncRNA was questioned by a recent study showing that an intron-less var gene could be activated and silenced (17).

The initial characterization of a gene family encoding 15 GC-rich noncoding RNAs (annotated RUF6) located adjacent to central var genes suggested a role in the regulation of var genes (18, 19). Fluorescence in situ hybridization (FISH) revealed that these ncRNAs colocalized to the var gene expression site and that episomal overexpression of distinct GC-rich ncRNAs resulted in the deregulation of mutually exclusive var gene expression (18). However, their mechanism of action remains unknown. In this study, we used CRISPR interference (CRISPRi) to downregulate the entire GC-rich gene family and provide evidence for the necessity of the GC-rich ncRNA in mutually exclusive var gene activation. Our results demonstrate a clear link between the transcription of both gene families, along with other clonally variant gene families involved in malaria parasite virulence.

RESULTS

GC-rich ncRNA shows predominant transcription of a single member when adjacent to an active var gene. Recently, RNA FISH was used to demonstrate the physical association of GC-rich ncRNAs with the expression site of central and subtelomeric var genes (18). Given the restricted genomic location of the GC-rich genes adjacent to some, but not all, central var genes (Fig. 1A), we investigated whether the transcription of GC-rich and var multigene families is coordinated. To this end, we generated an array of P. falciparum 3D7 wild-type (WT) clones and performed paired-end RNA sequencing (RNA-seq) analysis at 12 hpi to determine the transcriptional profile of the highly homologous GC-rich ncRNA and var gene families. In three clones, a single member of the 15 GC-rich genes was predominantly transcribed (Fig. 1B; see also Fig. S1A in the supplemental material). In the same clones, we observed mutually exclusive transcription of the central var gene adjacent to the upstream region of the active GC-rich gene (Fig. 1B and Fig. S1A). Notably, when the active central var gene lacks an adjacent GC-rich gene, ncRNA transcripts from several loci were detected, but at levels much lower than those in the former situation (Fig. 1C). Since subtelomeric var genes are prone to switch faster in culture, we were able to isolate only a clone with a dominant subtelomeric var gene that also expressed a second central var gene. In this clone, we observed low-level transcription from several GC-rich ncRNA genes, in addition to the dominant GC-rich ncRNA adjacent to the transcribed central var (Fig. S1B). Additionally, we performed receptor panning with chondroitin sulfate A (CSA) to enrich for parasites expressing a single subtelomeric member called var2csa. In this parasite panned line, we observed predominant transcription of var2csa and low levels of transcripts from several GC-rich genes (Fig. S1B). Taken together, these data suggest that the GC-rich gene family is expressed in a clonally variant manner related to the chromosomal location of the active var gene. GC-rich genes are predominantly transcribed from a single locus when adjacent to and upstream of an active central var gene.

CRISPRi of the entire GC-rich gene family leads to transcriptional downregulation of the var gene family. To determine the role of GC-rich ncRNA in var gene expression regulation, we aimed to downregulate the transcription of all GC-rich ncRNA
FIG 1  GC-rich ncRNA and var clonal variation. (A) Schematic model showing repressed var gene perinuclear clustering and active var gene relocation to the active expression site colocalizing with GC-rich ncRNA. ncDNA, noncoding DNA. (B) Transcriptional profile of var genes and GC-rich ncRNA at 12 hpi in WT clones assessed by RNA sequencing. Chromosome schematics (modified from reference 44) highlight the active var genes and GC-rich genes in different clones. Clones 3D7 B6 (blue) and 3D7 D6 (red) have predominant transcription of the GC-rich ncRNA adjacent to their active var locus. (C) Transcriptional profile of var genes and GC-rich ncRNA at 12 hpi in clones 3D7 C9 (green) and 3D7 A9 (yellow), which do not have a GC-rich gene upstream from their respective active central var loci and which have several GC-rich ncRNA transcripts at lower levels. The values are the means of the RPKM from two independent experiments at 12 hpi. The results for further clones are shown in Fig. S1 in the supplemental material.
genes. An attempted simultaneous knockout of all 15 highly homologous members was unsuccessful, likely due to the widespread distribution of the GC-rich genes or the diversity of their up- and downstream regions required for such an approach. Thus, we developed a CRISPR interference (CRISPRi) (20) strategy for multigene families to block transcription via binding of dead Cas9 (dCas9), a mutated Cas9 protein lacking endonuclease activity. We designed a single guide RNA (sgRNA) targeting a homologous region common to all GC-rich gene members (Fig. 2A). We transfected the 3D7 G7 parasite strain (a WT clone expressing the central var gene PF3D7_0412700) with the pUF-dCas9-GFP-3HA plasmid and either pL8-gRNA-GC-tc or a control plasmid with a scrambled guide RNA (pL8-gRNA-control). Chromatin immunoprecipitation (ChIP) of dCas9 followed by quantitative PCR (qPCR) with universal GC-rich gene primers showed...
a strong enrichment of dCas9 at GC-rich ncRNA loci in two independent CRISPRi clones but not in two scrambled control clones (Fig. S2A). ChIP followed by massively parallel DNA sequencing (ChIP-seq) analysis of dCas9 showed enrichment at all 15 GC-rich gene loci in both CRISPRi clones but not in the scrambled control (at 12 hpi in Fig. 2B and 24 hpi in Fig. S2B).

To determine the transcriptional effect of GC-rich gene CRISPRi, we performed reverse transcription-quantitative PCR (RT-qPCR) with universal GC-rich ncRNA primers for two clones each of the CRISPRi and scrambled control lines. The housekeeping gene fructose-bisphosphate aldolase (PF3D7_1444800) was used for normalization. CRISPRi clones showed significantly reduced levels of GC-rich ncRNA compared to the control line clones at 12 and 24 hpi (see Fig. 2C and 4A, respectively).

The global transcriptional effects of GC-rich gene CRISPRi were analyzed by RNA-seq. Strikingly, two independent clones of the CRISPRi lines exhibited a global downregulation of var genes, suggesting a role for the GC-rich ncRNA in the activation of var gene transcription (Fig. 3A). Conversely, scrambled control clones showed transcription of a single dominant var gene (Fig. 3A), similar to wild-type clones (Fig. 1B), suggesting that the expression of dCas9 alone does not affect var gene transcription. Additionally, a rescue experiment was conducted by removing the drug pressure required to maintain the plasmids and using negative drug selection to ensure plasmid removal from the CRISPRi lines (Fig. S3). Rescue control clones recovered var mutually exclusive transcription (Fig. 3A). Differential gene expression analysis of CRISPRi and scrambled control clones with a false discovery rate (FDR) cutoff of 0.01 returned 125 genes, 115 (92%) of which were downregulated in the CRISPRi lines in three independent replicates (Fig. 3B and C, Fig. S4, and Table S1). Among these downregulated genes were 13 GC-rich genes, 23 var genes (including the active var gene in the scrambled control clones, PF3D7_0712000), and several rif genes (Table S1). Differentially expressed genes other than GC-rich genes were validated for the lack of presentation of off-target dCas9 binding (Table S2).

GC-rich ncRNA CRISPRi affects other multigene families encoding variant surface antigens. GC-rich ncRNA is highly transcribed at the same later time point (~24 hpi) as the rif and stever genes (21, 22). Interestingly, several virulence gene families with a transcriptional peak in the blood-stage cycle later than that for var genes showed significant transcriptional downregulation upon GC-rich gene CRISPRi, even at 12 hpi (Fig. 3B and Table S1). To investigate a potential role for GC-rich ncRNA in the transcriptional control of these gene families, we performed RNA-seq and differential expression analysis in the CRISPRi lines at 24 hpi. The total number of genes significantly differentially expressed (FDR cutoff, 0.01) between the CRISPRi clones and the scrambled control clones at 24 hpi was 77, of which the majority (77%) were downregulated (Fig. 4B, Fig. S7, and Table S1) in three independent replicates. Besides GC-rich genes, most significantly downregulated genes belonged to multigene families encoding variant surface antigens with 2 transmembrane (2TM) domains (23): rif, Pfmc-2TM, and stever (Fig. 4B). These three multigene families exhibited a transcription downregulation of most members in the CRISPRi lines compared to their expression in the control lines (Fig. 4C). In the case of the Pfmc-2TM gene family, the global...

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transcription level was significantly lower in the CRISPRi lines than in the control lines, whereas the total levels of the stevor and rif gene families were not significantly lower. Altogether, these data strongly suggest that the GC-rich ncRNA is an important trans-activating element shared by at least the Pfmc-2TM and var gene families.

**DISCUSSION**

The perinuclear compartment that is key to the mutually exclusive expression of a single var gene in *P. falciparum* remains poorly understood. In a previous study, we identified the GC-rich gene family to be the first trans-acting ncRNA localizing to this expression site (18). Here, we show that this ncRNA is essential for the transcriptional activation of a single var gene, and we provide evidence that this function of the GC-rich element is shared with other clonally variant gene families.
By performing RNA-seq analysis on freshly cloned parasite lines that each transcribed a single var gene, we showed that GC-rich ncRNAs are transcribed in a clonally variant manner (Fig. 1B). We observed two profiles of GC-rich gene transcription, depending on the relative chromosomal location of the GC-rich genes and active var gene. In cases in which there was one GC-rich gene predominantly transcribed (transcribed at a level 5- to 10-fold higher than that for other members), it was always found...
adjacent to the 5’ region of an active central var gene. The ncRNA transcription profile for a clone with an active central var gene or a subtelomeric var lacking an adjacent member of the GC-rich gene family at its 5’ upstream region showed multiple ncRNA transcripts, but at levels much lower than those in the former case. It is tempting to speculate that high levels of GC-rich ncRNA transcription adjacent to a central var gene may stabilize the expression site of a central var gene over that of subtelomeric var genes. A previous study reported variable switch rates, depending on the chromosomal location of var genes, with central var genes being more stably expressed and less prone to switching than the subtelomeric ones (24). Our data suggest that varying levels of ncRNA at the var gene expression site modulate the switch rate of individual var genes. Furthermore, transcription of GC-rich genes may open the local chromatin structure and enhance the accessibility of the transcription machinery to the adjacent var gene. This hypothesis finds support from the findings of a recent study showing the increased chromatin accessibility of GC-rich genes when they are adjacent to the active var gene and/or rif gene (25).

Until recently, it was not possible to inactivate an entire multigene family dispersed over many chromosomes in P. falciparum. We adapted the CRISPRi technique for the simultaneous knockdown of the entire GC-rich multigene family by targeting a conserved region that includes part of the polymerase III (Pol III) B box, present in all 15 members. All members of this GC-rich family have unique DNA motifs (internal A and B boxes) (18, 19) found only at polymerase III-transcribed tRNA genes and short interspersed nuclear elements (SINEs) in other organisms (26), suggesting that transcription of this multigene family is mediated by Pol III. Upon downregulation of GC-rich ncRNA transcription, var gene expression was abolished, revealing an unprecedented regulatory interaction between Pol III- and Pol II-transcribed clonally variant genes.

Interestingly, the GC-rich gene family is conserved throughout all Laverania subgenera of Plasmodium, along with var genes and other clonally variant gene families involved in immune evasion, such as rif and stevor (27–29). Since GC-rich genes are transcribed at their highest levels at approximately 24 hpi, the question arises whether GC-rich ncRNA might also play a direct or indirect role in regulating clonally variant virulence gene families expressed at later stages of the asexual blood cycle, such as rif (21, 30). A previous study suggested that an activation factor may be common to multiple clonally variant families (31). Our work suggests that GC-rich ncRNA could indeed be such a factor regulating different clonally variant gene families.

Although the precise molecular mechanism of ncRNA action remains to be investigated using techniques such as chromatin isolation by RNA purification (ChIRP), we postulate that the ncRNA associates with var gene control regions and acts as an activator. Indeed, a recent study showed that Pol III-transcribed SINEs act as enhancers of Pol II gene activation in response to the depolarization of neurons (32). However, the lack of sequence homology between GC-rich genes and var loci suggests the need for additional protein factors for such a physical interaction. Alternatively, GC-rich ncRNA could interact with nascent var mRNA, stabilizing it for transcription. It is also possible that GC-rich ncRNA could participate in ncRNA-mediated HP1 eviction from heterochromatic var genes, as previously described in the fission yeast Schizosaccharomyces pombe (33). Whichever hypothesis is correct, an essential next step would be to use this ncRNA as a molecular tool to pull down interacting partners from the var expression site, elucidating the molecular mechanism of var gene activation.

In conclusion, we developed a novel CRISPRi system that allows for the simultaneous downregulation of the entire GC-rich multigene family. In doing so, we establish the GC-rich ncRNA as an epigenetic regulatory element that plays a role in the activation of var gene transcription and the transcription of several other clonally variant gene families. We also provide a first glimpse into the molecular process that controls the switch rates of var genes, which is currently a black box in the field of var gene transcription. The identification of a trans-activating factor of the expression site
opens novel experimental approaches to identify regulatory proteins needed for mutually exclusive var expression.

MATERIALS AND METHODS

Parasite culture and synchronization. Blood-stage *P. falciparum* parasites were cultured as previously described (12). Parasites were synchronized with a 6-h time window by sorbitol lysis during the ring stage, followed by plasma gel enrichment in the schizont stage and another sorbitol treatment 6 h afterward, corresponding to 3 ± 3 hpi. Synchronized parasites were harvested at a 3.3% hematocrit and −2 to 4% parasitemia. Parasite development was monitored by Giemsa staining.

Receptor panning. Plastic cell culture dishes were coated with 1 mg/ml CSA receptor (catalog number C9819; Sigma) in phosphate-buffered saline (PBS) overnight at 4°C. The dishes were blocked with 1% bovine serum albumin (catalog number A4503; Sigma) in PBS for 1 h at 37°C. Mature-stage infected red blood cells (iRBCs) were immobilized by plasma gel enrichment and resuspended in 8 ml of binding medium (RPMI 1640, 25 mM HEPES, pH 7.2). The culture dish was washed with binding medium, and iRBCs were added and allowed to adhere for 1 h with gentle agitation. The dish was washed until no unbound cells were observed by microscopy and bound cells were recovered with the culture medium.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). RNA was harvested from synchronized parasite cultures after saponin lysis in 0.075% saponin in PBS, followed by one wash in PBS and resuspension in the QiAzo1 reagent. Total RNA was extracted using the miRNeasy mini kit and performing on-column DNase treatment (Qiagen). Reverse transcription was achieved using a Super-Script Vilo kit (Thermo Fisher Scientific) and random hexamer primers. cDNA levels were quantified by quantitative PCR in a CFX384 real-time PCR detection system (Bio-Rad) using Power SYBR green PCR master mix (Applied Biosystems) and primers from a previous study (18). Transcript levels were quantified by normalizing the starting quantity mean to the one for a housekeeping gene (fructose-bisphosphate aldolase, PF3D7_1444800). The starting quantity means from three replicates were extrapolated from a standard curve of serial dilutions of genomic DNA.

Chromatin immunoprecipitation and next-generation sequencing. ChIP was performed as previously described (12), with some modification, using ring- or-trophozoite stage parasites (12 or 24 hpi). Sonicated chromatin (500-ng DNA content) was combined with either 0.5 μg of anti-HP1 (Genscript) or 1 μg of anti-hemagglutinin (anti-HA; catalog number Ab9110; Abcam) polyclonal rabbit antibodies. After overnight incubation, 25 μl of Dynabeads protein G (Invitrogen) was added to each sample, and an additional incubation of 2 h was conducted. Subsequent washing, cross-link reversal, and DNA extraction were carried out as described before (12). Sequencing libraries were produced with the immunoprecipitated DNA using a MicroPlex library preparation kit (v2; Diagenode) with Kapa HiFi polymerase (Kapa Biosystems) for the PCR amplification. For each ChIP sample, a control DNA corresponding to the ChIP input was processed in parallel. The multiplexed libraries were subjected to 150-bp paired-end sequencing on a NextSeq 500 sequencer (Illumina). Fastq files were obtained by demultiplexing the data using bcl2fastq software (Illumina), prior to downstream analysis. A minimum of two biological replicates were analyzed for each clone and time point.

RNA library preparation and sequencing. Total RNA was subjected to rRNA depletion to ensure ncRNA and mRNA capture using a RiboCop rRNA depletion kit (Lexogen), prior to strand-specific RNA-seq library preparation using a TruSeq stranded RNA LT kit (Illumina) with Kapa HiFi polymerase (Kapa Biosystems) for the PCR amplification. The multiplexed libraries were subjected to 150-bp paired-end sequencing on a NextSeq 500 sequencer (Illumina). Fastq files were obtained by demultiplexing the data using bcl2fastq software (Illumina), prior to downstream analysis. A minimum of three biological replicates were analyzed for each clone for the knockdown experiment, and a minimum of two biological replicates were analyzed for each WT clone (see Table S3 in the supplemental material).

Plasmid construction, transfection, and plasmid removal. A sequence coding for a control guide RNA (gRNA; targeting luciferase) flanked by an autocleavable ribozyme (custom gene synthesis; Genscript) was ligated with the backbone vector pL6-eGFP (34) using the Dral and Swal restriction sites and T4 DNA ligation to obtain the pL8-gRNA-control plasmid. A sequence coding for a gRNA targeting all GC-rich ncRNA loci flanked by an autocleavable ribozyme (gBlock; Integrated DNA Technologies) was cloned into the pL8-gRNA-control plasmid using the Hpal and PmlI restriction sites, followed by Gibson assembly (In-Fusion HD cloning kit; Clontech) to generate the pL8-gRNA-GC-tc plasmid. The constructs were transformed into *Escherichia coli* XL10-Gold ultracompetent cells (Agilent Technologies) and isolated with a Nucleospin Xtra Maxiprep kit (MacHEREY-NAGEL). pUF-dCas9-GFP-HA was constructed by primer mutagenesis of pUF1-Cas9 and addition of a green fluorescent protein (GFP) tag, three HA tags, and an inducible ribowitch (35). pUF-dCas9-GFP-HA was transfected together with either pL8-gRNA-control or pL8-gRNA-GC-tc into ring-stage parasites as previously described (36) and maintained under WR99210 and DSM1 drug selection pressure. Parasite clones were obtained by limiting dilution. Rescue lines were obtained by drug removal from the CRISPRi lines and negative-selection treatment over 21 days using 5-fluorocytosine (Ancotil) to ensure pL8-gRNA-GC-tc removal. Negative selection was further maintained during parasite cloning.

ChIP-seq and RNA-seq data analysis. Fastq files were subjected to quality control using FastQC software (37). Sequencing reads were mapped to the *P. falciparum* genome (8) (PlasmoDB, v9), using the Burrows-Wheeler alignment tool (BWA-MEM) with the default settings (38). PCR duplicates were removed with the UnifiedGenomes package (39) to identify the 4 GC-rich ncRNAs fall in low-mappability regions. ChIP-seq data were normalized over the input, and likelihood ratio calculation and peak calling were performed using MACS2 software (39) with the default parameters and a false discovery rate (FDR) cutoff of 0.05. RNA-seq data gene counts were calculated using the bedtools suite of utilities (40). Differential gene expression

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analysis was performed with the R package edgeR (41) with an FDR threshold of 0.01. Normalization of the gene counts according to the number of reads per kilobase per million mapped reads (RPKM) for gene length and sequencing depth was performed using the Rlimma package (42). Data were visualized using the Integrative Genomics Viewer (43). Data availability. Sequencing data from this study are available in the GenBank repository under accession number PRJNA498234 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA498234).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.6 MB.
FIG S3, PDF file, 0.03 MB.
FIG S4, PDF file, 1.3 MB.
FIG S5, PDF file, 0.04 MB.
FIG S6, PDF file, 0.2 MB.
FIG S7, PDF file, 1.2 MB.

TABLE S1, XLSX file, 0.1 MB.
TABLE S2, XLSX file, 0.02 MB.
TABLE S3, XLSX file, 1.4 MB.

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