Nuclear Pores and Perinuclear Expression Sites of \textit{var} and Ribosomal DNA Genes Correspond to Physically Distinct Regions in \textit{Plasmodium falciparum}

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The human malaria parasite \textit{Plasmodium falciparum} modifies the erythrocyte it infects by exporting variant proteins to the host cell surface. The \textit{var} gene family that codes for a large, variant adhesive surface protein called \textit{P. falciparum} erythrocyte membrane protein 1 (PiEMP1) plays a particular role in this process, which is linked to pathogenesis and immune evasion. A single member of this gene family is highly transcribed while the other 59 members remain silenced. Importantly, \textit{var} gene transcription occurs at a spatially restricted, but yet undefined, perinuclear site that is distinct from repressed \textit{var} gene clusters. To advance our understanding of monoallelic expression, we investigated whether nuclear pores associate with the \textit{var} gene expression site. To this end, we studied the nuclear pore organization during the asexual blood stage using a specific antibody directed against a subunit of the nuclear pore, \textit{P. falciparum} Nup116 (PiNup116). Ring and schizont stage parasites showed highly polarized nuclear pore foci, whereas in trophozoite stage nuclear pores redistributed over the entire nuclear surface. Colocalization studies of \textit{var} transcripts and anti-PfNup116 antibodies showed clear dissociation between nuclear pores and the \textit{var} gene expression site in ring stage. Similar results were obtained for another differentially transcribed perinuclear gene family, the ribosomal DNA units. Furthermore, we show that in the poised state, the \textit{var} gene locus is not physically linked to nuclear pores. Our results indicate that \textit{P. falciparum} does form compartments of high transcriptional activity at the nuclear periphery which are, unlike the case in yeast, devoid of nuclear pores.

As shown in yeast and mammals, nuclear pores can define compartments of high transcriptional activity at the nuclear periphery (1, 2). Furthermore, relocation and tethering of specific genes to the nuclear pore complex provide transcriptional memory that enables quicker reactivation of those genes (3). In the human malaria parasite \textit{Plasmodium falciparum}, the major virulence factor, encoded by the \textit{var} gene family, is transcribed at a yet undefined site at the nuclear periphery in a monoallelic fashion. Hence, the possibility that nuclear pores play a role in monoallelic \textit{var} gene activation is an appealing hypothesis.

\textit{P. falciparum} causes the most severe form of malaria, a parasitic disease killing hundreds of thousands people a year (4, 5). Clinical symptoms of malaria arise during its 48-h replication cycle within human red blood cells. After invasion of a blood cell by a merozoite, the parasite remains in ring stage for 24 h postinvasion (hpi). In the trophozoite stage (25 to 38 hpi), it grows and replicates its genome. Segregation of the chromosomes into the newly forming daughter nuclei and cytokinesis occur in the schizont stage (38 to 48 hpi). Subsequently, 16 to 32 newly formed merozoites will be released upon rupture of the infected red blood cell and start a new infectious cycle.

This intracellular parasite expresses several proteins that are transported to the red blood cell surface and that have multiple functions, such as sequestering infected red blood cells to the vascular endothelium (6). These proteins are exposed to recognition by the host immune system. Hence, to prolong infection and ensure effective transmission, the parasite has evolved sophisticated strategies for immune evasion. Antigenic variation allows the parasite to change its gene expression profile by switching between different members of gene families coding for surface proteins (7).

\textit{var} is the best-studied variant gene family coding for the major virulence surface protein \textit{P. falciparum} erythrocyte membrane protein 1 (PiEMP1). Monoallelic expression governs the transcription of only a single gene of the 60-member gene family, whereas all other genes remain silenced (8). Expression of the \textit{var} gene peaks around 10 to 14 hpi (9), but during later developmental stages it remains in a state "poised" for reactivation in the next cycle (10). Recently, a histone methyltransferase, PfSet10, has been shown to colocalize specifically with the poised \textit{var} gene (11).

Epigenetic regulation, such as histone posttranslational lysine modification, is an essential component in monoallelic expression (12). A second essential factor is the spatial regrouping of \textit{var} genes at the nuclear periphery (13, 14). Apparently, this organization is important to establish a default silencing pathway via facultative heterochromatin.

Silent and active \textit{var} genes localize to the nuclear periphery, and \textit{var} gene activation requires the relocation to a perinuclear site that remains undefined to date (13). This has raised the hypothesis that a predefined \textit{var} gene expression site, similar to the expression site body for variant surface glycoprotein (VSG) genes in \textit{Trypano-}
falciparum (15), might exist in *P. falciparum*. Besides var genes, rRNA is among the most highly expressed RNA species in plasmodia. rRNA genes, such as the 18S RNA, cluster in the nucleolus, a specific perinuclear subcompartiment (16). Data from model organisms suggested that proximity to the nuclear pore could enable more efficient transcription of highly expressed genes (17).

Ultrastructural analysis using focused-ion-beam scanning electron microscopy (EM) (“slice and view”) has enabled the first visualization of nuclear pore organization in three dimensions (3D) during the *P. falciparum* asexual blood stages (18). This technique, however, does not allow labeling of molecular markers to link morphological features to functional studies.

In this study, we investigate nuclear pore distribution throughout the parasite’s blood stage development by immunofluorescence using a specific antibody raised against a nuclear pore subunit, PiNup116. By combining immunofluorescence and RNA fluorescence in situ hybridization (FISH), we tested the hypothesis whether nuclear pores are associated with the var gene expression site and if var gene poising requires its sequestration to a nuclear pore. We show that perinuclear gene transcription sites and var gene poising do not depend on nuclear pore association in *P. falciparum*.

**MATERIALS AND METHODS**

**Parasite culture and synchronization.** *P. falciparum* blood stage parasites were cultivated as described previously (19). Parasite culture was synchronized by sorbitol lysis during ring stage, subsequent plasmagel enrichment in schizont stage, and then by another sorbitol treatment at 6 h postinvasion. Synchronized parasites were harvested at 4% hematocrit and if necessary by sorbitol lysis during ring stage, subsequent plasmagel enrichment in schizont stage, and then by another sorbitol treatment at 6 h postinvasion. Synchronized parasites were harvested at 4% hematocrit and 5% parasitemia. Parasite development was monitored by Giemsa staining.

**Antibody.** In order to produce specific antibodies against a plasmoidal nuclear pore protein (PiNup116; PF3D7_1473700) rabbits were immunized with the synthetic peptide PQTSSGTYPQNTS conjugated to keyhole limpet hemocyanin (KLH) to generate a polyclonal anti-PiNup116 antibody according to standard protocols of GenScript (catalog number SC1031). Serum from immunized rabbits was affinity purified on a column with resin coupled to the antigenic peptide. After multiple washes, the eluate was collected, neutralized, and dialyzed with phosphate-buffered saline (PBS).

**Western blotting.** After lysis of infected red blood cells with 0.15% saponin, nuclear and cytoplasmatic fractions of the isolated parasites were separated by mechanical douncing as described previously (20). After being washed in PBS, nuclei were directly extracted in Laemmli buffer. Protein extracts were separated on a 4 to 12% SDS-PAGE gel (Criterion, Bio-Rad) and analyzed by Western blotting using the rabbit anti-PiNup116 antibody at 1:500, Horseradish peroxidase (HRP)-conjugated anti-P. falciparum aldolase (ab39805; Abcam) at 1:1,000 and rabbit anti-histone 3 (ab1791; Abcam) at 1:2,000 were used to assess purity of cytoplasmatic and nuclear fractions as described previously (21). Secondary antibody detection was done with anti-rabbit-HRP (GE health care) at 1:5,000. A SuperSignal West Pico ECL Western blotting kit (Thermo Scientific) was used to develop blots.

**Freeze-fracture and cryo-scanning electron microscopy.** Infected red blood cells were fixed in 2.5% glutaraldehyde in PBS overnight at 4°C and washed three times with PBS. One drop of blood cell pellet was pipetted into a cryo-freezer hat type B (G3467; Agar Scientific), a 100-nm EM grid (G2410PD; Agar Scientific) was placed on top, and the sample was closed up by a second drop and cryo-freezer hat. Sandwiched samples were high-pressure frozen in an HPM 010 (Bal-Tec) freezing machine. After samples were frozen, they were transferred into an Alto 2500 cryo-transfer system (Gatan) and fractured manually. Sublimation was carried out at −105°C for 2 min. Then samples were coated with 9.5 nm of chromium in a 681 High Resolution Ion Beam Coater (Gatan).

Fractured cells were imaged with a JSM-6700F electron microscope (Jeol) using a yttrium aluminum garnet (YAG) detector at 5 kV and −120°C.

**Immunofluorescence and RNA FISH.** Infected red blood cells were lysed with 0.015% saponin in RPMI medium, and the released parasites were fixed in suspension with 4% paraformaldehyde in PBS overnight at 4°C. Parasites were then deposited on number 1.5 cover glasses, permeabilized for 15 min with 0.1% Triton X-100, and subjected to immunofluorescence staining and RNA FISH as described previously (22). FISH probes were PCR amplified from genomic DNA using the primers 5′-CA GTAGTCTAGTCTGTG-3′ and 5′-TAATGATCTTCCGGAGG-3′ for 18S rRNA and 5′-CACACCACTGACGTCTGCAA-3′ and 5′-CTA GTGGATAGGATGGGTG-3′ for PF3D7_0412700 mRNA and labeled by nick translation using a Fluorescein-High Prime kit (Roche). For sequential immunofluorescence assay and RNA FISH, parasites were first hybridized with FISH probes at 37°C overnight. After they were washed, they were postfixed in 4% paraformaldehyde in PBS for 15 min and then subjected to immunofluorescence staining. Antibodies were diluted 1:200 in 0.5% pork skin gelatin in PBS for anti-PiNup116 and at 1:300 for mouse anti-hemagglutinin (JHA ab18181; Abcam) and detected with Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) diluted 1:2,000. Images were captured using a Nikon Eclipse 80i microscope with a CoolSnap HQ2 camera (Photometrics). NIS Elements, version 3.0, software (Nikon) was used for acquisition, and Fiji software (http://fiji.sc) was used for analysis.

**Quantitative analysis of images.** Nuclear pore polarization was quantified by scoring whether all detectable PiNup116 fluorescence dots localized within one quadrant of the nucleus (see Fig. 2C). Colocalization was scored in cell nuclei positive for both fluorescent signals when they overlapped while using similar contrast adjustments throughout all images. Scoring was performed by direct optical observation using Fiji software (http://fiji.sc). For each analysis, images from three independent replicates were combined. For the movie in the supplemental material, a z-stack was acquired and deconvolved using Huygens software (SVI).

**RESULTS**

**Nuclear pore distribution changes throughout the developmental blood stages.** Scanning electron microscopy images of freeze-fractured infected red blood cells show clusters of pores on the nuclear surface (Fig. 1A). Their symmetric structure and diameter are consistent with the expected organization of the nuclear pore complex. Surprisingly, only one subunit protein of nuclear pores, PiNup100 (23, 24), has been annotated in the genome of *P. falciparum* (www.plasmoDB.org). To investigate nuclear pore positioning, we evaluated a novel antibody against a putative nuclear pore protein (PF3D7_1473700). It shows homology with the *Saccharomyces cerevisiae* nucleoporin Nup116 (34% identity and 50% similarity). Rat serum raised against this protein has been previously used for nuclear pore labeling in *P. falciparum* (19, 25). In this work, a polyclonal affinity-purified rabbit antibody was raised against a 15-amino-acid synthetic peptide from the central region of PiNup116 (Fig. 1B). We validated antibody specificity by Western blotting. In protein extracts from asynchronous parasite culture, we detected a major band of 110 kDa, which migrates slightly lower than the predicted size of 142 kDA. This protein is highly enriched in the nuclear fraction (Fig. 1C). The efficient separation of the nuclear and cytoplasmatic fractions was controlled for with anti-histone 3 and anti-aldolase antibodies, respectively (Fig. 1C, lower panels). To further characterize this nuclear pore protein, we carried out immunofluorescence localization studies on highly synchronized blood stage parasites throughout their intraerythrocytic development. We compared immunofluorescence staining of nuclear pores in early ring stages (around 12 hpi) with trophozoite stages (around 32 hpi) and late, segmented schizont stages.
The anti-PfNup116 signal concentrates in foci at the nuclear periphery. Interestingly, nuclear pores formed a polarized cluster in very early rings. Most ring stages showed a single dot, whereas about 30% showed two dots, and very few cells showed three dots (Fig. 2B). As parasites progress through their cycle, nuclear pores redistribute around the nuclear envelope, and signal intensity increases. During schizont stages the number of nuclear pore foci per nucleus diminishes again, most likely to a single dot, but due to spatial proximity the signals can only sometimes be attributed to individual nuclei (see Movie S1 in the supplemental material). While early-stage cells have only one or two nuclear pore foci, the number of foci increases markedly in late-stage cells, whereas frequently individual clusters are not distinguishable any longer (Fig. 2A).

Also, the polarization of nuclear pores in the ring stage is lost in the trophozoite stage (Fig. 2C).

Our immunofluorescence data using a nuclear pore marker are in agreement with previous ultrastructural data on nuclear pore distribution throughout the intraerythrocytic development cycle (18). This localization pattern indicates that our molecular pore marker is a valuable tool for functional studies in *P. falciparum*.

Active var gene and 18S RNA expression sites do not associate with nuclear pores. Nuclear pores could form an "anchor" site required for monoallelic var gene expression. Further, proximity to nuclear pores could facilitate transcription and mRNA export of highly expressed genes. To test whether the var gene expression site colocalizes with nuclear pores, we combined RNA FISH of an active var gene with anti-PfNup116 immunostaining. rRNA, expressed in the nucleolus, is the most highly transcribed RNA species in *Plasmodium*. Hence, we also tested whether nuclear pores would position closely to the 18S RNA transcription sites.

As var gene expression peaks around 10 to 14 hpi, we analyzed tightly synchronized ring stage parasites. We used the 3D7-G7 clone, which predominantly expresses the var gene PF3D7_0412700 against which our FISH probe was targeted (19). No significant colocalization of nuclear PF3D7_0412700 mRNA signal with the nuclear pore signal could be detected (Fig. 3A). Similarly, 18S RNA expression sites rarely colocalized with the PfNup116 signal (Fig. 3B). Since at this early stage the pores are still polarized, scoring colocalization with 18S RNA (*n* = 32) and PF3D7_0412700 mRNA (*n* = 33) was greatly facilitated (Fig. 3C). The frequency of random colocalization can be estimated by geometric calculation. The colocalization images we analyzed are two-dimensional. Ring stage nuclei have a diameter of 1 μm and therefore a circumference of 3.14 μm. The diameters of the fluorescence signals we see for the nuclear pore antibody and the FISH probe range from 0.2 to 0.3 μm. If both signals localize randomly on a circle of 3.14-μm circumference (nuclear periphery), their...
predicted colocalization frequency ranges from 12 to 19%. These values are in the range of observed colocalization frequencies reported in Fig. 3C. Our results strongly suggest that nuclear pores do not recruit or host the var gene expression site. Also, the other known perinuclear expression site, containing the ribosomal DNA (rDNA) genes, does not associate to nuclear pores.

var gene poising is not associated with nuclear pores. In yeast, it has been shown that certain regulated genes are tethered to the nuclear pore complex for extended periods of time, which enables rapid reactivation of transcription upon external stimulation (3). Similarly, var genes remain in a poised state during the trophozoite and schizont stages before being reactivated at the start of the next cycle. We tested whether var gene poising occurs at the nuclear pore. A putative methyltransferase, PfSet10, which is expressed only in later stages, has recently been shown to colocalize specifically with the poised var gene (11).

We used a parasite line expressing endogenous PfSet10 tagged with hemagglutinin (HA) to carry out double immunostaining with anti-HA and anti-PfNup116 antibodies on late-stage parasites (11). For quantification, we selected cells positive for both signals but with a limited number of nuclear pore foci to clearly distinguish between colocalizing and noncolocalizing signals (Fig. 4A). In the parasites we scored (n = 45), PfSet10-HA signal rarely colocalized with the PfNup116 signal (Fig. 4B). We conclude that var gene poising is not associated with nuclear pores.

DISCUSSION

This is, to our knowledge, the first study that investigates the role of nuclear pores in monoallelic gene expression. P. falciparum is a unique organism that uses perinuclear expression sites for gene families that are mutually exclusively expressed. So far, no specific molecular marker has been identified that characterizes these sites. Given the recent evidence for roles of nuclear pores in regulating gene expression, we explored the idea of whether pores associate with the var gene expression site.

Our study supports and expands the ultrastructural data on the unusual distribution pattern of nuclear pores in P. falciparum by immunofluorescence staining (18). Strong polarization of pores, found in early-stage parasites, has been very rarely described in other cell types and organisms. In late development of mouse spermatids, nuclear pores are clustered at the caudal pole of the nucleus, opposite to the acrosomal cap (26). In budding yeast, deletion of Nup133, a nucleoporin, causes extensive clustering of nuclear pores (27). A recent paper shows that motor proteins and cytoskeletal elements are important for uniform distribution of nuclear pores in fungal nuclei, suggesting that an active process prevents their clustering (28). Weiner et al. argue that ring and schizont stage nuclei are in close contact with the plasma membrane, and therefore nuclear pores polarize to the side of the nuclear envelope with free access to the cytoplasm (18). Even though...
data on nuclear pore biology of *P. falciparum* is still very scarce, a recent study of the *P. falciparum* nuclear proteome yielded interesting candidate proteins, whose further characterization can improve understanding of nuclear pore organization (23).

Ribosomal DNA genes and *var* genes are among the most highly transcribed RNA species in *P. falciparum* (16). As neither of them is expressed in the proximity of the nuclear pore, this shows that the spatial proximity is not limiting for efficient RNA export into the cytoplasm, as has been suggested by models and *in vivo* diffusion measurements in other organisms (29). As 18S RNA is expressed in the nucleolus, we can conclude that nucleolar positioning is independent of nuclear pore localization.

What defines the *var* gene expression site at the nuclear periphery is a long-standing question in the field. Since nuclear pores have been previously associated with transcriptional activation, as proposed first in the gene-gating hypothesis (30), they are attractive candidates for recruitment sites of the activated *var* gene. Our observations, however, show that tethering to the nuclear pore does not occur for active *var* and rDNA genes. The only factor known to spatially organize *var* genes remains actin, which contributes to the tethering of central *var* genes to the nuclear periphery (25). A number of other chromatin components have been identified that are enriched at the nuclear periphery; however, no functional data are yet available that demonstrate a role of these proteins in the spatial organization of silent or active *var* genes through, e.g., chromosome end clustering (31, 32). Recently, it has been shown that spatial segregation is also important for activation of other clonally variant genes. As relocating the silent PFRH4 gene to a transcriptionally active site increases its probability of being switched on, the authors hypothesized that translocation precedes activation (33).

Gene poising is another important aspect of antigenic variation. Keeping a single gene marked for reactivation reduces switching to another member of the family and therefore prevents premature depletion of the “immunogenic repertoire.” Potentially, the poised gene could be maintained at a defined site, marked by PiSet10 (11), throughout the late parasite stages. This would provide positional memory for reactivation of the same *var* gene. If, however, positional memory exists in the context of *var* gene poising, it is not associated with the nuclear pore.

In conclusion, our work points to a distinctive perinuclear compartment involved in monallelic exclusion. The identification of molecular markers associated to this expression site is of key importance to gain new insight into the virulence process of malaria parasites.

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