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Supplemental Information

A Critical Role of Perinuclear Filamentous Actin in Spatial Repositioning and Mutually Exclusive Expression of Virulence Genes in Malaria Parasites

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SUPPLEMENTAL INVENTORY

1.- Supplemental Figures S1-6

2.- Supplemental Tables S1-6

3.- Supplemental Experimental Procedures and Supplemental References

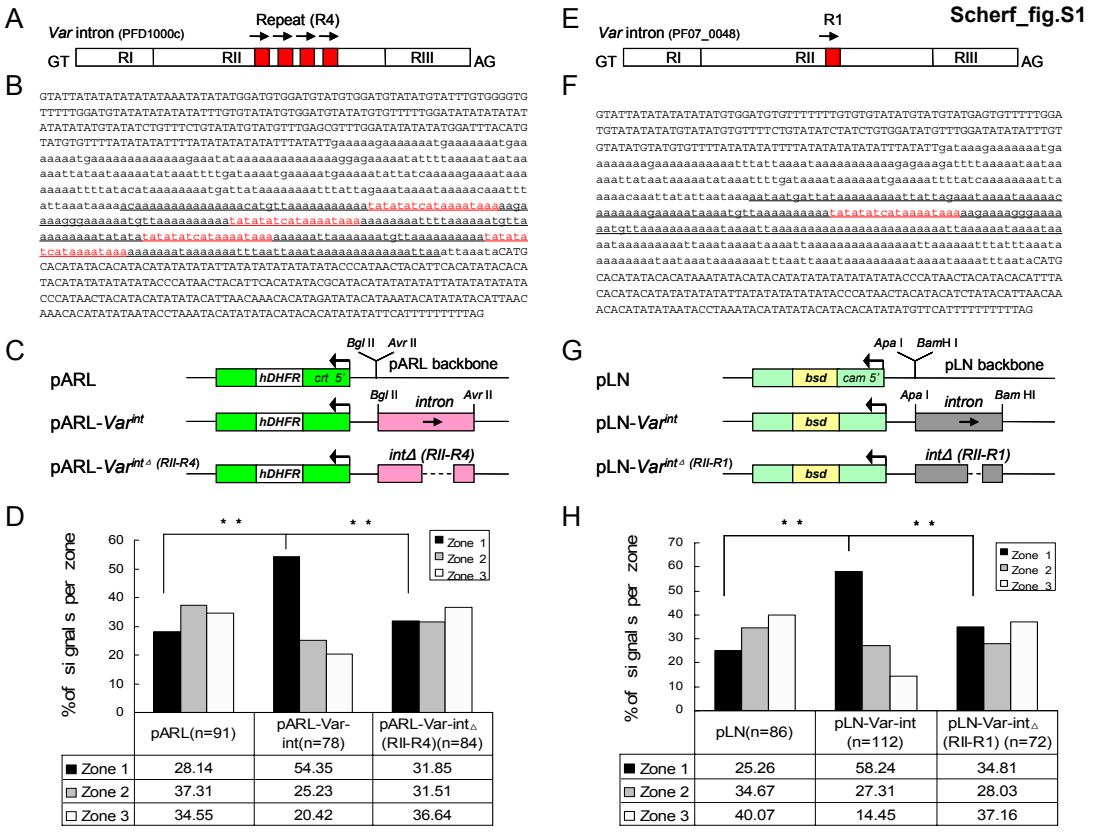

Figure S1. *Var* Introns Mediate Tethering of Episomes to the Nuclear Periphery. Related to Figure 1

(A) and (E) Conserved structure of two *var* gene introns with different number of central repeats. Left: PFD1000c *var* intron contains four 18 bp repeats (*iNPE18*) in its Region II domain (RII-R4). Right: PF07_0048 *var* intron has only one repeat (RII-R1).

(B) and (F) Full-length sequences of PFD1000c (left) and PF07_0048 (right) *var* introns, respectively. The region II (RII) sequences are labeled by lower letters. The deleted regions in the mutant intron are underlined and the *iNPE18* motifs are highlighted in red.

(C) and (G) pARL (left) or pLN (right) vector constructs with full-length intron of PFD1000c (left) or PF07_0048 (right) *var* gene, the mutant intron without the repeat region, and the vector backbone as control.

(D) and (H) Subnuclear distribution of FISH signals of episomes. The bar graph represents the position of FISH signals with respect to three concentric zones (see Figure 1A, right panel). DAPI (blue): Nuclear DNA. n = number of nuclei analyzed from two independent ring-stage samples. "**": $p<0.001$ (χ^2 test).

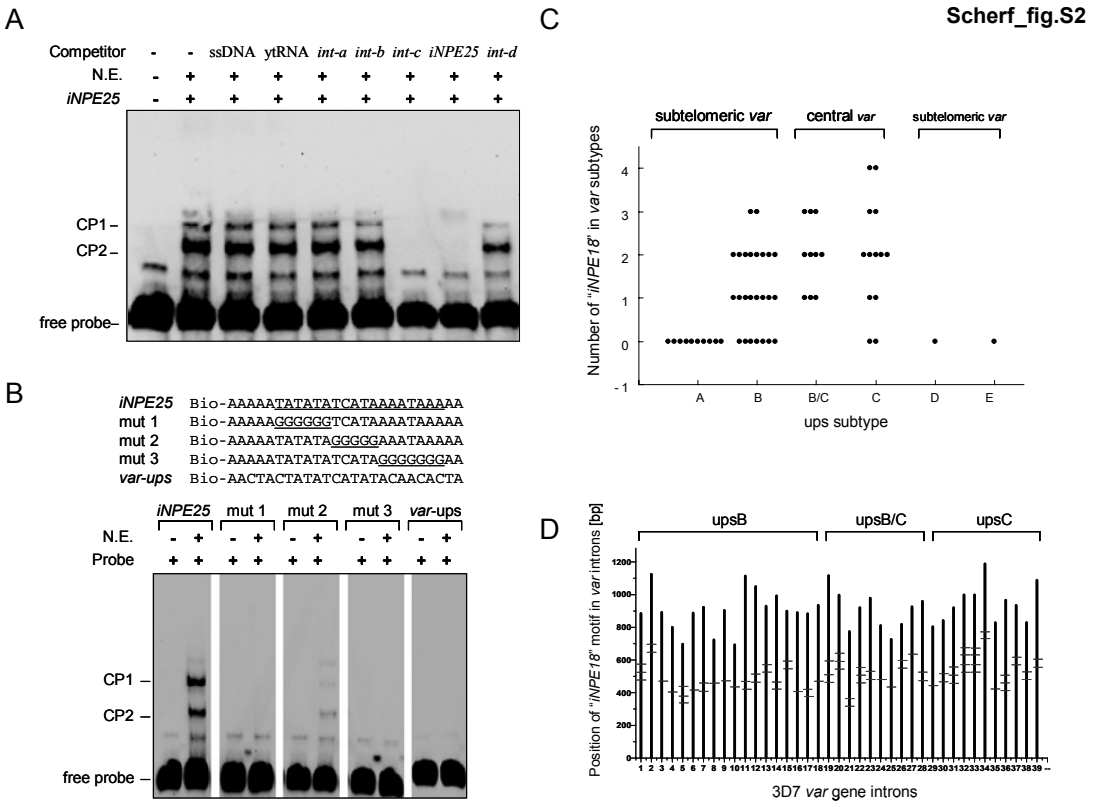

Figure S2. Features of Intron *iNPE* Sequence. Related to Figure 2

(A) Competition EMSA experiment using unlabeled competitor DNA for the *iNPE25* nuclear protein complex. Competitor DNA was added at either a 100-fold weight excess (sheared salmon sperm DNA (ssDNA) and yeast tRNA (ytRNA)) or a 100-fold molar excess (*var* intron DNA fragments). The sequences of probe and competitors are shown in Table S4. N.E. = nuclear extract.

(B) EMSA of nuclear extracts with biotin-labeled mutagenised *iNPE25* probes. Wide type and mutant *iNPE25* probes (wt, mut1, mut2 and mut3) were used in EMSA assay to identify the critical sites involved in the DNA-protein interaction. The *iNPE18* motif is underlined in *iNPE25* sequence, and short blocks of base pairs were replaced by poly (G) (underlined). As control a 25 bp DNA element derived from the upstream region of an *upsC*-type *var* gene (PFD1000c) was used.

(C) Distribution of *iNPE18* motif in each subtypes of 3D7 *var* gene family.

(D) The localization of *iNPE18* in individual *var* introns is shown as a short horizontal line. All 3D7 *var* genes with 1-4 *iNPE18* copies in their introns are shown (total 39).

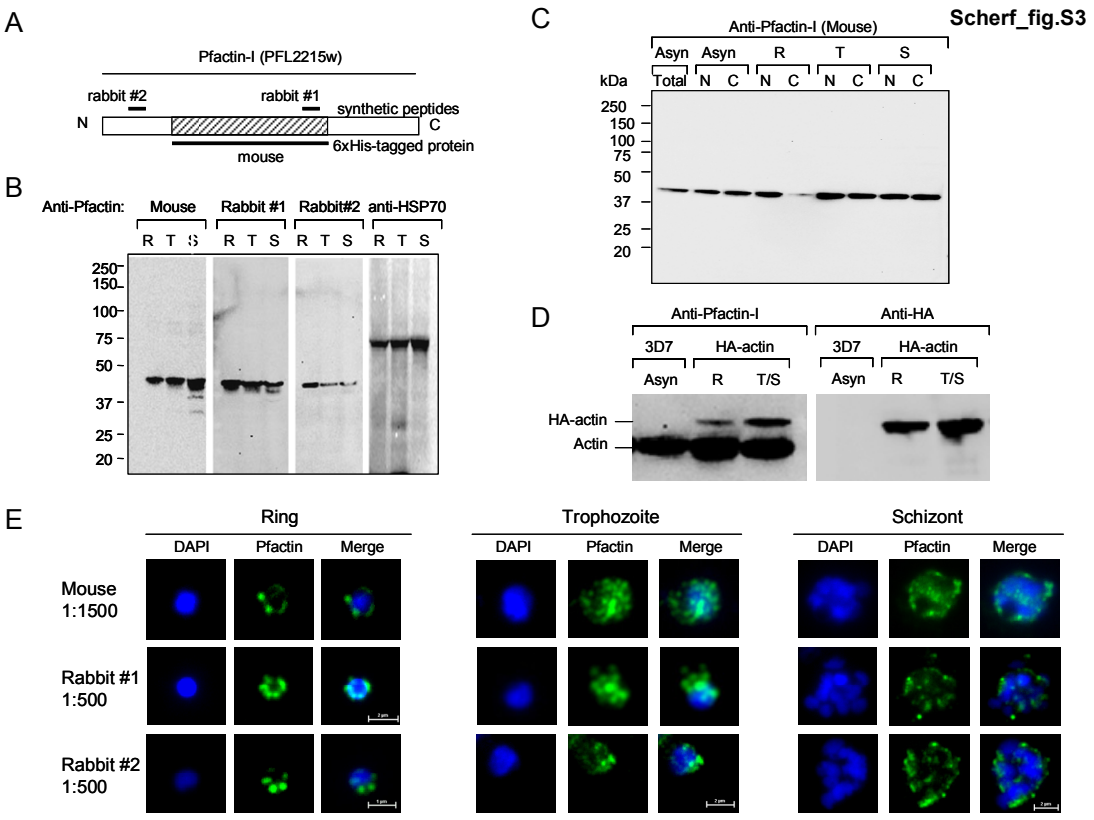

Figure S3. Characterization of Pfactin-I by Western-blot and IFA. Related to Figure 3

(A) Schematic representation of Pfactin-I. The regions selected for preparations of two rabbit antibodies and one mouse antibody are shown respectively.

(B) Western blot analysis of total parasite extracts from different asexual blood stages with mouse and rabbit anti-Pfactin-I antibodies. Each lane was loaded with total extract from ~$5x10^6$ synchronized parasites and anti-HSP70 antibody was used as a loading control.

(C) Western blot assay of total protein extract, nuclear and cytoplasmic fractions of asynchronous or synchronous parasites at different intraerythrocytic stages with mouse antibody against Pfactin-I. The amount of nuclear and cytoplasmic fractions of a given stage corresponded to an equal parasite numbers. N: nuclear extract; C: cytoplasmic extract.

(D) Western blot assay of HA-actin transfectant with rabbit (#2) anti-Pfactin-I (left) and anti-HA antibodies (right), respectively. Total extracts of ring or trophozoite/schizont-stage HA-actin transfected parasites were used with 3D7 as control.

(E) IFA of different asexual blood stage developmental forms with antibodies against Pfactin-I (mouse, rabbit #1 and rabbit #2). Nuclei were stained with Dapi (blue), and Pfactin-I is shown in green.

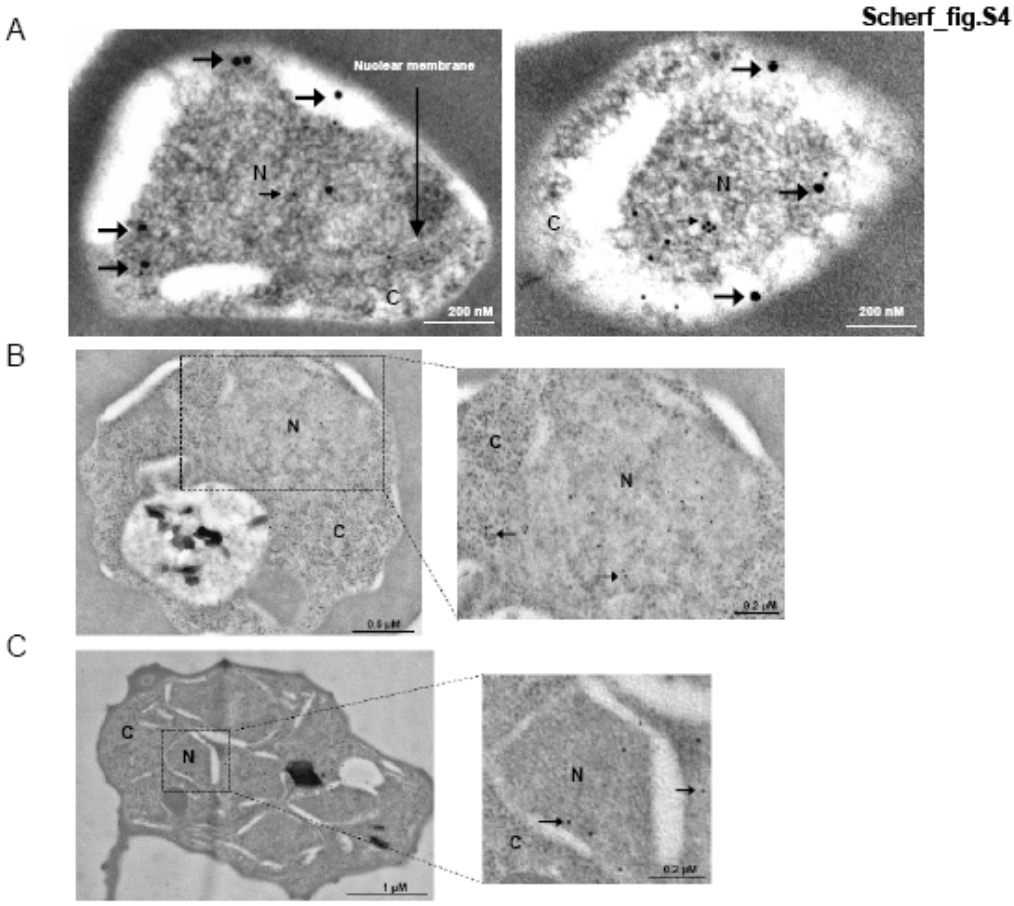

Figure S4. Immunoelectron Microscopy of Ultrathin Sections of Parasites at Different Intraerythrocytic Developmental Stages. Related to Figure 4

(A) Ring stage: labeling with mouse anti-Pfactin-I (20 nm gold grain, bold arrows), and rabbit anti-histone H3 (10 nm gold grain, slim arrows), respectively. Nuclear membrane is shown in the left panel.

(B) Trophozoite and (C) Schizont labeling with mouse anti Pfactin-I (10 nm gold grain) shown by arrow. N: nucleus. C: Cytoplasm.

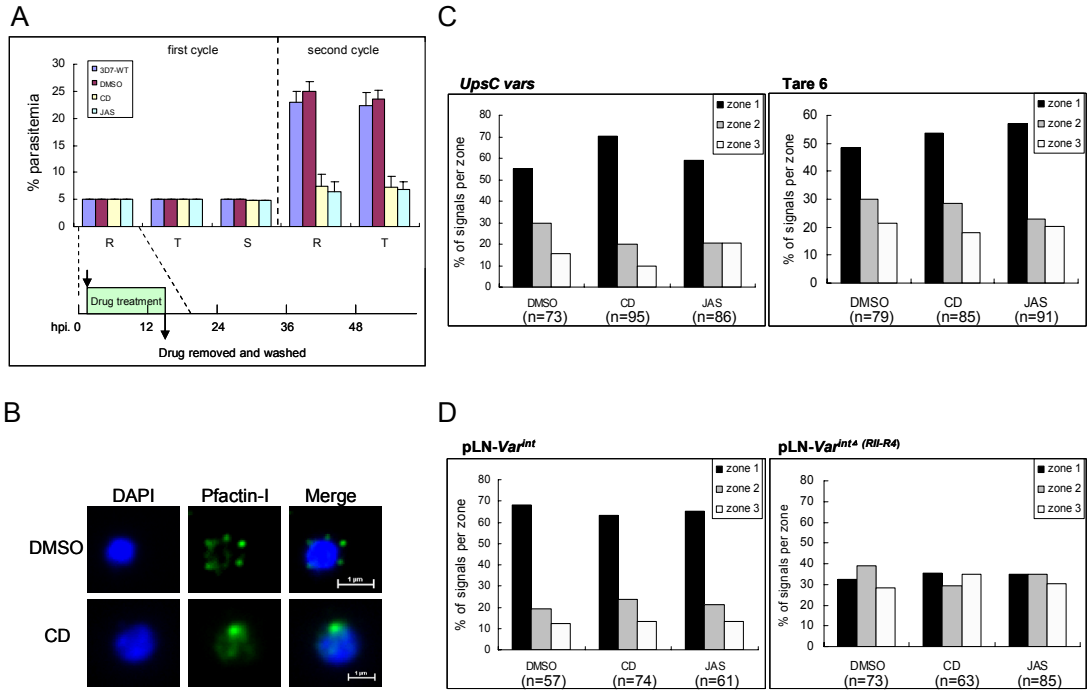

Figure S5. Treatments of Parasites with Actin Inhibitors. Related to Figure 5.

(A) Parasitemia counting of parasite cultures treated with DMSO, CD and JAS, respectively. The non-treated 3D7 clone was used as control. The window of drug treatments (10 hr) are shown during the ring stage (see Figure 5A, assay 1). hpi: hours post invasion. The data are represented as mean +/- SEM.

(B) IFA assay of ring-stage parasites treated with DMSO or CD for 10 hr. Rabbit (#2) antibody against Pfactin-I was used here (green). DAPI: nuclear DNA.

(C) Subnuclear localization of central *var* genes and episomes carrying a *var* intron in parasites treated with actin inhibitors. Parasites were grown for 10 hr in the presence of CD, JAS or DMSO (control parasites) as described in Figure 5A (assay 1). FISH analysis of central *var* genes and episomes was performed in rings as the procedure described in Figure 1C. n: nuclei counted.

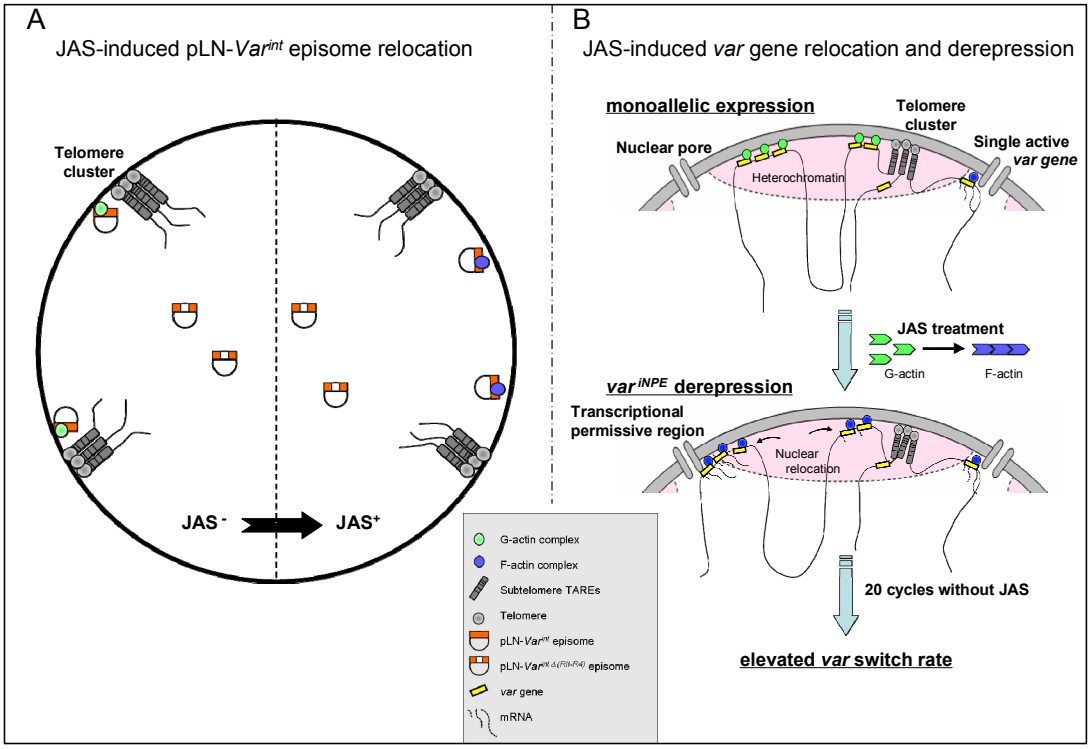

Figure S6. Model Showing JAS Induced Nuclear Spatial Reorganization of Episomes and Transcriptional Changes in *Var* Genes. Related to Figure 6

(A) Episomes carrying a *var* intron (*iNPE*-type) locate preferentially to the nuclear periphery. In JAS-treated parasites, these episomes tend to dissociate from telomere clusters. Episomes carrying an intron that have deleted the *iNPE* region show a random nuclear distribution.

(B) Model showing *var* genes either tethered directly via their intron *iNPE* sequence (internal *upsC* and *upsB* type *var* genes) or via telomere-mediated tethering (*upsA*-type) to the nuclear periphery. A single *var* gene is expressed at a distinct perinuclear region that has not yet been identified (here shown at a nuclear pore). JAS-treatment induces the reorganization of *var* genes carrying introns with *iNPE* sequences. We hypothesize that *var* relocation is linked to the observed transcriptional activation and elevated *var* switch rate.

Table S1. List of Primers Used in this Study. Related to Figure 1

Items	Name	Primers (5'→3')[a]
pLN constructs	*upsC-int (PF1000c)*	GCGCG*gggccc*GTATTATATATATATATAAATATATATGG
		GCGCG*ggattc*CTAAAAAAAAATGAATATATATGTG
	RII-R4 (PF1000c)	GCGCG*gggccc*AAAAAAAAAAAAAAACATG
		GCGCG*ggattc*GTGCATGTATTTAATTTA
	int$^{\Delta}$*(RII-R4) (PF1000c)*	TTAATTTAAT TTTTATTTAATAAATTTG
		TTAAATAAAA ATTAAATTAAATACATGCAC
	Exon I (PF1000c)	GCGCG*gggccc*GATAAATTAACAAAAGATAAATCTG
		GCGCG*ggatcc*TGGGTCCAAGACTAGGAGTGAC
	Exon II (PF1000c)	GCGCG*gggccc*GATTATGATATACCGACAAAACT
		GCGCGggatccTGTACATTGGTGGGGTTGGTG
pARL constructs	*upsC-int or int*$^{\Delta}$*(RII-R4) (PF1000c)*	GCGCG*agatct*ATATGGATGTGGATGTATGTGG
		GCGCG*cctagg*CTAAAAAAAAATGAATATATATGTG
pLN-Ap-HLA	ACT_{5UTR}	GCGCG*gggccc*TATATTTGTATGTATAGCCA
		GCGCG*cctagg*TTTTACAGATATATAAATAGTAC
	HLA	P5:GCGCG*cctagg*ATGTACCCTTACGATGTGCCTGATTACGCGT ATCCCTATGACGTACCAGACTATGCATAC
		Pb:GCGCGGTCGACTGCTGCTGCTGCAGCATAATCCGGAACG TCATAAGGGTATGCATAGTCTGGTACG
		Pc:GCGCGgtcgacGCAGCAGCAGCAGGAGAAGAAGATGTTCA AGC
		Pd: GCGCGcttaagGAAACATTTTCTGTGGACAATAC
qRT-PCR primers for twelve reference genes	SSP2 (*PF13_0201*)	FORWARD: TAACTTGTATGCTGATTCTGC
		REVERSE: GATTTCTCTTTTTCTTGACC
	LSA-1 (*PF10_0356*)	FORWARD: CAACAGAGGGAATAGTAGAG
		REVERSE: ATTTTGTATGTCAGCAGATT
	Pf11-1 (*PF10_0374*)	FORWARD: ATTTACGCTTTAGAGGATT
		REVERSE: CCTTCTTATGTTTCGGTTT
	S47 (*PF13_0248*)	FORWARD: ATTGTATGCTTTCATCTCA
		REVERSE: CGTTCTTCTTCATTTGGTA
	TRAP (*PFA0200w*)	FORWARD: ACATTGTGATGCGTGGTCT
		REVERSE: TCAGCTTCTTCAAAGGTTC
	Pf48-45 (*PF13_0247*)	FORWARD: AGAAAATGATACAAACCCTA
		REVERSE: TACCATAGCACTTCTCCCT
	Pf13_0170	FORWARD: GGCACTTCAAGGGTACCT
		REVERSE: TAATATAGCCTCACAAGC
	PFL0090c	FORWARD: AAGAGATGCATGTTGGTC
		REVERSE: GTACCCCAATCACCTACA
	FBA (*PF14_0425*)	FORWARD: TGTACCACCAGCCTTACCAG
		REVERSE: TTCCTTGCCATGTGTTCAAT
	ACT (*PFL2215w*)	FORWARD: AGCAGCAGGAATCCACACA
		REVERSE: TGATGGTGCAAGGGTTGTAA
	Pf40S (*PFC0295c*)	FORWARD: ATCGAATCTCAAGAAGCAA
		REVERSE: AATAAACCAGCCCAATGTC
	Lact (*PF13_0141*)	FORWARD: AATCGTTTTAGTTGGCTCAG
		REVERSE: GCCAAATCGTCATAAGTGTT
primers for FISH probes of reference gene loci	SSP2 (*PF13_0201*)	FORWARD: TATACGTCGTCATAATTGGGTG
		REVERSE: CGTGCTTATTTTCGGGTTTCT
	S47 (*PF13_0248*)	FORWARD: GTGAACTATTAAGCTCAACAC
		REVERSE: TCTGGTTTTGTTGGTAAGGCT
	PFA0195w (near TRAP)	FORWARD: ATGAGAAAAACAAAAGGAAG
		REVERSE: TGTTAATACTTGTTTTATTCC
	Pf13_0170	FORWARD: GATGACACGAACCCAGTTACC
		REVERSE: GTCGTAGGTACATCTCAACTC
	FBA (*PF14_0425*)	FORWARD: TACCAGCAGATGTTGCCGAAG
		REVERSE: GAAGCACCTGCATTTTCACCAC
	PFC0285c (near Pf40s)	FORWARD: ATGAATTCTGTTATGCCTGATG
		REVERSE: CTTCTATCTTATCACAATATCC
	PF13_0143 (near Lact)	FORWARD: CATCATCCATTATGGAGATCAG
		REVERSE: TGTTGAATAAATCATTTAGAC

qRT-PCR primers for ChIP	upsC-var-int (PFD1000c)	FORWARD: TCATAAAATAAAAAGAAAAGGG REVERSE: TATGTATGTGTATATGTGCATG
	Var2CSA-ups (PFL0030c)	FORWARD: TGCTTCATAAATAAAACATGCAA REVERSE: TTCTTGTTTTTCTAAAATACCTCTCTT
Primers for EMSA probes[b] (PF07_0048)	int-a	FORWARD : Bio-GTATTATATATATATATGTGG REVERSE : ATTATTTTTAAAATCTTTCTC
	int-b	FORWARD : Bio-AATAAAAAAAAAAAAGAGAAAG REVERSE : TATTTTTTTTTTAACATTTTA
	int-c	FORWARD : Bio-CAAAAAAAGAAAAATAAAATG REVERSE : TTTTTTTTTATTTAAATAAAT
	int-d	FORWARD : Bio-TTTAAATAAAAAAAAATAATAA REVERSE : CTAAAAAAAAAATGAACATATATG

[a] The sequence of restriction enzyme site was shown in lower case.

[b] full sequences are shown in Table S3.

Table S2. Distribution of *iNPE18* Motif in Genome of Various Species. Related to Figure 2

Species	100% identity	1 mismatch	Location
Plasmodium falciparum [a]	85	+48	Intronic (limited to *var* and pseudo *var* genes)
Plasmodium reichenowi [a, d]	21	+30	Intronic (*var*-like genes)
P.vivax [b]	1	+11	intergenic
Plasmodium knowlesi [a]	0	+3	-
Plasmodium chabaudi [a, d]	1	+10	intergenic
Plasmodium berghei [a]	0	+17	-
P.yoelii [b]	1	+19	intergenic
Human [c]	12	+40	intergenic
Mouse [c]	9	+27	intergenic
Rat [c]	6	+18	intergenic
Drosophila [c]	0	+2	-
Saccharomyces cerevisiae [c]	0	+2	-
Schizosaccharomyces pombe [c]	0	0	-

The *iNPE* motif was searched in various species by genome BLAST server with the following genome databases:
a. Sanger Database (http://www.sanger.ac.uk/Projects/P_falciparum/);
b. PlasmoDB (http://plasmodb.org/plasmo/);
c. NCBI Genome Resource (http://www.ncbi.nlm.nih.gov/genome/guide/);
d. Shotgun reads or contigs.

Table S3. List of Probes Used in this Study. Related to Figure 3

Probe (5'-biotin)[a]	**Sense-strand sequence (5'→3')[b]**
int-a (PF07_0048)	GTATTATATATATATATGTGGATGTGTTTTTTTGTGTGTATATGTATGTATGAGTGTTTTTGGA TGTATATATATGTATATGTGTTTTCTGTATATCTATCTGTGGATATGTTTGGATATATATTTGT GTATATGTATGTGTTTTATATATATTTTATATATATATATTTATATTGATAAAGAAAAAAATGA AAAAAAAGAAAAAAAAAAATTTATTAAAATAAAAAAAAAAAGAGAAAGATTTTAAAAA TAAT
int-b (PF07_0048)	AATAAAAAAAAAAAGAGAAAGATTTTAAAAATAATAAAAATTATAATAAAAATATAAATT TTGATAAAATAAAAAATGAAAAATTTTATCAAAAAAAATTAAAAACAAATTATATTAATAA AAATAATGATTATAAAAAAAAAATTATTAGAAATAAAATAAAAACAAAAAAAGAAAAATA AAATGTTAAAAAAAAAATA
int-c (PF07_0048)	CAAAAAAAGAAAAATAAAATGTTAAAAAAAAAAATATATATCATAAAATAAAAAGAAAAG GGAAAAAATGTTAAAAAAAAAAATAAAATTAAAAAAAAAAAAAAAAAAAAAAATTA AAAAATAAAATAAAATAAAAAAAAATTAAATAAAATAAAATTAAAAAAAAAAAAAAATT AAAAAATTTATTTAAATAAAAAAAAA
int-d (PF07_0048)	TTTAAATAAAAAAAAATAATAAATAAAAAAATTTAATTAAATAAAAAAAAATAAAATAAA ATTTAATACATGCACATATACACATAAATATACATACATATATATATATATACCCATAACTA CATACACATTTACACATACATATATATATTATATATATATATACCCATAACTACATACATCTA TACATTAACAAACACATATATAATACCTAAATACATATATACATACACATATATGTTCATTTT TTTTTTAG
int-c1	TCAAAAAAAGAAAAATAAAATGTTAAAAAAAAAAATATATATCATAAAATAAAAAGAAAA GGGAAAAAATGTTAAAAAAAAAAATAAAATTAA
int-c2	AAAAATAAAATTAAAAAAAAAAAAAAAAAAAAAAAAATTAAAAAATAAAATAAAATAAA AAAAAATTAAAT
int-c3	TAAAAAAAAATTAAATAAAATAAAATTAAAAAAAAAAAAAAATTAAAAAATTTATTTAAA TAAAAAAAAA
int-c1.1	BCAAAAAAAGAAAAATAAAATGTTAAAAAAAAAAATATATATC
int-c1.2	AAAAAAATATATATCATAAAATAAAAAGAAAAGGGAAAAAATGTTAAAAAA
int-c1.2.1	AATGTTAAAAAAAAAATATATATC
int-c1.2.2	AAAAATATATATCATAAAATAAAAA
int-c1.2.3	ATAAAATAAAAAGAAAAGGGAAAAAATGTTAAAAAA
int-c1 probe	AAATATATATCATAAAATAAAAAGAAAAGGGAAAAAATGTTAAAAAAAAAATATATATCA TAAAATAAAAAAAAAAAATTTTA AAAAATGTTA
iNPE25	AAAAATATATATCATAAAATAAAAA
iNPE18	TATATATCATAAAATAAA
Scrambled	CTTTGATTAGATTTAAAATTGAAAGTTAAAAAAATTAATAG

a. the *int-a*, *b*, *c*, and *d* sequences were also used a competitors without biotin label in competition EMSA.

b. the sequence of *iNPE18* motif is underlined.

Table S4. Summary of LC-MS/MS Analysis of *Int-c1* Binding Protein Complexes. Related to Figure 4

Gene group	Identical	Exp.1	Exp.2
Pfactin-I	1	1	1
DNA/RNA binding proteins[a]	9	9	12
Ribosomal proteins	17	19	29
Conserved unknown Plasmodium proteins	9	22	14
Proteasome proteins	0	1	12
Acyl-CoA synthetase, etc	6	12	8
Phosphatase or kinase	5	9	12
Heat shock proteins (Chaperonin)[a]	3	6	6
Histone	2	5	2
Plasmodium exported protein	0	4	0
DNAJ protein	0	4	0
Eukaryotic translation initiation factor	2	2	2
14-3-3 protein[a]	1	1	1
others	17	38	27
sum	72	133	126

a. putative candidates recruiting multiple proteins by protein-protein interactions.

14-3-3 proteins: are conserved regulatory molecules expressed in all eukaryotic cells, and able to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors.

Replication Protein A (RPA): plays a central role physically interacting with many proteins involved in DNA replication, recombination, nucleotide excision repair, and transcriptional.

Heat shock proteins (Chaperonins)

Table S5. Subnuclear Position of Reference Gene Loci Determined by FISH Assay. Related to Figure 6

Gene	PlasmoDB Accession	Length (bp)	Chromosome location	Transcriptional status in rings	Subnuclear position (FISH)
SSP2	PF13_0201	1725	central	silent	nuclear periphery
LSA-1	PF10_0356	3489	subtelomere	silent	nuclear periphery[a]
Pf11-1	PF10_0374	28692	subtelomere	silent	nuclear periphery[a]
S47	PF13_0248	1320	central	silent	Random
TRAP	PFA0200w	492	central	silent	Random
Pf48-45	PF13_0247	1347	central	silent	Random
13_0170	PF13_0170	2757	central	active	Random
L0090c	PFL0090c	657	subtelomere	active	nuclear periphery[a]
FBA	PF14_0425	1538	central	active	Random
ACT	PFL2215w	1131	central	active	Random*
Pf40S	PFC0295c	426	central	active	Random
Lact	PF13_0141	951	central	active	Random

a. these gene loci had already been analyzed by Freitas-Junior, L.H., et al., 2000, and Lopez-Rubio, J.J., et al., 2009.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid Constructs Used in Transfections

Transfection vectors pLN-ENR-GFP (Nkrumah et al., 2006) and pARL-GFP (Crabb et al., 2004) were modified for cloning of *var* introns and control sequences. Briefly, the whole *pfenr-gfp* expression cassette was removed from pLN-ENR-GFP, and various DNA fragments described in text were inserted into the *Apa* I/*Bam*H I multi-cloning site. For pARL-GFP, the *crt* promoter was replaced by digestion with *Bgl* II and *Avr* II with various DNA sequence of interest. The primers used in constructing these transfection plasmids are listed in Table S5. The digestion map of various expression cassettes in each plasmid was analyzed before transfections. All cloned DNA fragments were sequenced before and validated after transfection experiments by PCR to exclude DNA rearrangments.

For pLN-HA-Pfactin-I construct, the full-length DNA sequence was amplified from 3D7 genome, and three copies of HA tag (YPYDVPDYA) were ligated to the N-terminus of Pfactin-I via a linker sequence (AAAAVDAAAA) containing a *Sal* I site as previously described (Deligianni et al., 2011). This HA-Linker-actin gene (HLA) was cloned into pLN vector via digestion of *Avr*II and *Afl* II. The *calmodulin* promoter of pLN-HLA was replaced by 1.5 kb 5'UTR of PFL2215w encoding Pfactin-I with *Apa* I and *Avr* II sites, and the resulting construct was named pLN-HA-Pfactin-I. The primers used in this construct are listed in Table S5.

Parasite Transfection

Plasmid constructs were transfected into the *P.falciparum* 3D7 line by direct electroporation as described previously (Fidock and Wellems, 1997). Briefly, cultures were synchronized to ring-stage at ~5% parasitemia by sorbitol treatment. 200 µl infected RBCs were mixed with 100 µg of

plasmid DNA and transferred into a 0.2 cm cuvette (Bio-rad) and electroporated using Gene Pulser (Bio-Rad) under the condition of 0.31 kV and 950 μF. Electroporated cells were transferred immediately into a 75 cm^2 culture flask (Falcon) with 12 ml of complete media containing 400 μl fresh RBCs, then incubated under standard cultivation conditions. The transfected cultures were maintained under 2.5 μg/ml BSD drug selection pressure until the episomal transfectants were established in three to five weeks.

Treatment of Parasite Culture with Actin Inhibitors

Cytochalasin D (Zygosporium mansonii, Sigma) and Jasplakinolide (Jaspis johnstoni, Invitrogen) were prepared as 10 mM stock solutions in DMSO, respectively. Parasite culture was synchronized by sorbitol lysis during the young ring stage followed with plasmion treatment at early schizont stage. Synchronized young ring-stage parasite culture of *P. falciparum* 3D7 (0-5 hours post-invasion) were aliquoted into different flasks at 2.0% hematocrit and ~5% parasitemia, then incubated at 37℃ with 10 μM Cytochalasin D, 10 μM Jasplakinolide, DMSO (0.1%, v/v) or without any drug, respectively for 3 hr (Assay 1) or 10 hr (Assay 2). The cultures were harvested for parasite fixation and total RNA extraction (Assay 1), or washed twice with incomplete medium and to be maintained in drug-free medium for additional twenty cycles (Assay 2). After remove of drugs (Assay 1+2) from ring stage, parasite development to schizont stage was monitored by Giemsa staining.

Quantitative Reverse Transcription PCR

Total RNA of synchronous parasite culture was extracted using Trizol reagent (Invitrogen) according to the protocol as described previously (Kyes et al., 2000). The potential genomic DNA contamination were removed by DNase I treatment with DNA-free kit (Ambion). 500 ng total

RNA was subjected to reverse transcription reaction with a mixture of oligo dT and random hexmer primers as reverse primers in a 20μl reaction according to the manufacture's recommendations (Invitrogen). All runs were performed using an Eppendorf Mastercycler detection system in a 20 μl reaction contained 0.5 μl cDNA, 1×SYBR Green I Mastermix, 0.2 μM specific primer pair for individual gene or *var* gene subtypes tested, and each reaction was run in duplicate. *Seryl-tRNA synthetase* (PF07_0073) was used as endogenous control. The qRT-PCR reaction conditions were as follows: 95 °C for 10 s, followed by 40 cycles of 94 °C for 20 s, 50 °C for 20 s and 62 °C for 20 s. The threshold cycle (Ct) is defined as the cycle number at which the fluorescence passes a pre-determined threshold. In our analyses, quantification of *var* genes with respect to *Seryl-tRNA synthetase* was calculated using the equation: $\Delta Ct = Ct_{var\ subgroup} - Ct_{Seryl\text{-}tRNA\ synthetase}$. ΔCts were then converted to relative copy numbers with the formula $2^{-\Delta Ct}$. The fold change of experimental group to reference group was calculated as $2^{-\Delta\Delta Ct}$. Dissociation curves were generated for each real-time RT-PCR to verify the specificity of amplification. For the transcription level assay of individual *var* genes and twelve endogenous control genes, we used the *var* primer pairs published else (Salanti et al., 2003) and listed in Table S5.

Antibodies

Rabbit antibodies against Pfactin-I were produced by Rabbit Polyclonal Antibody service from GenScript Corporation (USA). Briefly, two synthetic peptides (#1: N-QSSDIEKSYELPDGC-C, #2: N-RPKNPGIMVGMEEKC-C) conjugated with Keyhole Limpet Hemocyanin (KLH) were used to immunize rabbits according to the standard protocols of GenScript, and the Pfactin-I-specific polyclonal antibodies were affinity-purified with the peptides. To prepare the polyclonal mouse antibody against recombinant Pfactin-I (rPfactin-I), a DNA fragment of 564 bp was amplified from genomic DNA of *P.falciparum* 3D7 strain with the following primers:

rPfactin-I-F：5’ CGG*GGATCC*TATCCAATAGAACATGGTATTG 3’

rPfactin-I-R：5’ CGG*CTCGAG*TTATCTAAATCTTTCATTACCTACAG 3’

The PCR product was cloned into the *E.coli* expression vector pET-28a-c (+) by restriction enzyme digestion of *Bam*H1 and *Xho*I and expressed in *E.coli BL21* strain as his6-tagged protein according to the manufacturer's recommendation (Novagen). The expressed rPfactin-I was purified with Ni-NTA agarose (Qiagen) and emulsified with complete freund's adjuvant (Sigma) to immunize five mice. The total IgG was isolated with Protein A sepharose (Pierce) from the pooled mouse sera.

Production of recombinant AP2 domain in *E.coli*

To express the predicted DNA-binding AP2 domain of PF11_0091 in *E.coli* (Campbell et al., 2010), the sequence of DDKVKGVYFSKSPRGVGKWNAYFQIANNKRLFTSFSVSKYGYN EARKLSILKRTEWEKEYKHH was amplified from 3D7 genomic DNA with the following primers:

rAP2-P5: 5’GCGCG *GGATCC* GATGATAAGGTTAAGGGTGTTTAC

rAP2-P3: 5’GCGCG *GAATTC* TTA ATGATGCTTATACTCTTTTTCC

The resulting PCR product was cloned into the *E.coli* expression vector pGEX-4T-1 by restriction enzyme digestion of *Bam* H1 and *EcoR* I, and expressed in the *E.coli BL21* strain as GST-tagged protein according to the manufacturer's recommendation (Novagen). The expressed rAP2 was purified with Glutathone Sepharose 4B according to the standard protocol (GE Healthcare). The elution fractions were analyzed by 4-12% SDS-PAGE followed by Coomassie staining (Bio-Rad), then the positive elutions were pooled and dialyzed in 1xPBS (Gibco) with centrifugal filters (Millipore).

Western-blot Analysis

Total parasites extract, nuclear and cytoplasmic extractions were separated respectively on 4-12% SDS-PAGE gel (Bio-rad), and analyzed by western-blot with different antibodies against Pfactin-I, Histone 3, and HSP70 respectively. Primary antibody dilutions were: anti-Pfactin-I (rabbit#1, rabbit#2), 1:500; anti-Pfactin-I (mouse), 1:1500; anti-Histone 3 (Abcam), 1:2000, anti-HSP70 (mouse, mAb 1C11, gift from Mattei D), 1:500; anti-HA (mouse, Roche, 12CA5), 1:2000. Second antibody dilutions were: anti-rabbit-HRP (Pierce) 1:5000; anti-mouse-HRP (Pierce) 1:5000. ECL western blotting kit (GE healthcare) was used to develop blots.

Preparation of EMSA probes

For EMSA probes used to identify the *var* intron binding proteins, full-length *var* intron (PF07_0048) was amplified from 3D7 genomic DNA, and subsequently cloned into TOPO TA cloning vector according to the supplier's instructions (Invitrogen). After DNA sequencing, the fragments (≥ 100 bp) were amplified from the cloned *var* intron by specific primers labeled by 5'-biotin (primer sequences were listed in Table S5). Then the PCR products were purified using Gel Extraction Kit (Qiagen), and the final elution fractions in Tris-Cl buffer (pH8.0) were used directly in EMSA assay.

For fragments (< 100 bp), 5'-biotin labeled sense-strand oligo and unlabeled antisense-strand oligo were synthesized, mixed at a ratio of 1:1.2 in Tris-Cl buffer (pH8.0), and denatured at 95℃ for 5 min, followed by annealing for 1 hr at room temperature.

EMSA and Super Shift Assay

Nuclear extract was prepared as described previously (Voss et al., 2003). EMSA reactions were performed by incubating 1 μg of crude nuclear proteins or recombinant protein (rAP2) with 20

fmol of biotin-labeled probes in EMSA buffer (20mM Hepes, pH7.8, 60mM KCl, 0.5mM EDTA, 2mM DTT, 2mM MgCl2, 0.1% Triton X-100, 10% glycerol) containing 500 ng of poly (dI-dC)(dI-dC) as nonspecific competitor DNA in a 20 μl reaction volume for 20 min at room temperature. Binding reactions were then analyzed on a 4 or 5% polyacrylamide gel in 0.5% TBE (Ambion) according to the length of probe. For the competition EMSA or super shift assay, the labeled probes were added 20 min after incubation of protein extract and competitor DNA or polyclonal antibodies. For the nuclear extract depleted in actin, 50 μg crude nuclear was incubated with 1 μg or 5 μg rabbit anti-actin antibodies respectively at 4℃ with rotation overnight, followed by adsorption with 10 μl protein A (Millipore). After a short spin, the supernatant was used in EMSA.

Affinity Purification of the DNA-binding Proteins

The streptavidin-biotin affinity purification system was used to isolate the DNA-binding proteins. 200 pmol biotinylated DNA probe (*int-c1* or Scrambled) was diluted in 1x incomplete Binding Buffer (20 mM Hepes, pH 7.8, 60mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 0.65% Nonidet P-40), and incubated with 300 μl beads (Dynabeads Myone Streptavidin T1, Invitrogen) per purification for 1 hr at 4℃. DNA-immobilized beads were harvested by magnetic instrument, and washed once with the same binding buffer.

To remove the non-specific bead-binding proteins, 6 ml crude nuclear extract corresponding to $4x10^{10}$ asynchronous parasites in 1x complete Binding Buffer (20 mM Hepes, pH 7.8, 60mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 25 μM $ZnCl_2$, 1 mM DTT, 0.1% Triton X-100, 1 mM PMSF, 1x protease inhibitor cocktail (Sigma), 50 ng/μl poly(dI-dC)(dI-dC) (Sigma), 5% glycerol) was pre-incubated with 300 μl beads with rotation for 30 min at room temperature. The beads were collected and washed with 500 μl Elution Buffer (20 mM Hepes, pH 7.8, 1mM EDTA, 10%

glycerol, 1M KCl) twice. Then the beads were re-incubated with the previous 6 ml nuclear extract. After two pre-clearances with beads, 3 ml nuclear extract per purification was used for int-c1/beads or scramble/beads, respectively. After incubation with rotation for 30 min at room temperature, the beads were harvested, and washed with 10 ml Washing Buffer (20 mM Hepes, pH 7.8, 250 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 1 mM PMSF, 1x protease inhibitor cocktail, 5% glycerol) in 15 ml tube (Corning) for at least 5 time. The tube was changed after each washing step. Then the DNA-binding proteins were eluted with 0.5 ml Elution Buffer complemented with protease inhibitors. The elution step was repeated one with another 0.5 ml Elution Buffer. The 1 ml combined eluted fraction was dialyzed with cold 1xPBS (Gibco) in Amicon Ultra (Millipore), and concentrated to a final volume of ~100 µl. The final samples were aliquoted for EMSA (5µl), SDS-PAGE followed by silver-staining or Western-blot (20µl), and LC-MS/MS analysis (75µl).

Orbitrap Mass Spectrometry

Protein reduction/alkylation and in-solution trypsin digestion – Protein sample were dissolved in 30 µl of 8 M urea were sequentially reduced and alkylated by incubation in 5 mM TCEP (30 min at 4 °C) then 10 mM iodoacetamide (30 min in the dark). Proteins were digested with 0.5 µg of Lys-C at room temperature for 5 hr at 37°C. 90 mM NH_4HCO_3 was added and digestion was continued at room temperature in the presence 0.5 µg of trypsin (Promega, WI) (overnight, 37°C). Digestion was terminated by adding formic acid and the tryptic peptides were desalted on ZipTip C18 (Millipore). LC-MS/MS analysis was performed using an UltiMate 3000 HPLC system (Dionex) in line with an LTQ-Orbitrap velos fitted with a dynamic nano-electrospray ion source (Thermo Fischer Scientific). In the peptide separation step by liquid chromatography, solvent A (2% (v/v) acetonitrile, 0.1% (v/v) formic acid) and solvent B (80% (v/v) acetonitrile, 0.1% (v/v) formic acid)

are used.

Aliquots of tryptic peptides were loaded onto a 0.3x5 mm C18 trap column (Dionex) at 30 µl/min in 100% solvent A for 5 min and subsequently back flushed onto a pre-equilibrated analytical column (PepMap C18, 75 µmx150 mm, Dionex) using a flow rate of 0.3 µl/min. Peptides were separated at 40 °C using 2 linear gradient steps (0- 60% solvent B for 90 min, 60 - 95 % solvent B for 5 min), holding at 95% solvent B for a further 6 min before returning to 0% solvent B. The LTQ-Orbitrap velos was controlled using Xcalibur 2.1 software (Thermo Fischer Scientific) and operated in data-dependent acquisition mode whereby the survey scan was acquired in the Orbitrap with a resolving power set to 60,000 (at 400 m/z). MS/MS spectra were concurrently acquired in the LTQ mass analyzer on the 20 most intense ions from the FT survey scan. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (duration 90 s, exclusion list size 500) were used. Fragmentation conditions in the LTQ were: 35% normalized collision energy, activation q of 0.25, 10 ms activation time and minimum ion selection intensity of 500 counts.

Database Searching - Peptide information was extracted from Xcalibur raw files using Thermo Proteome Discoverer v. 1.0 (Thermo Fischer Scientific) (parent ions in the mass range of 350-5000 m/z, S/N of 2). MS/MS data were analyzed using an in-house MASCOT server (Matrix Science, version 2.2) to search a decoy protein database (56332 entries) created by combination of human proteins from SwissProt (downloaded 2009 09 01) and of *Plasmodium falciparum* proteins (PlasmoDB vers.6.0, downloaded 2009 09 01) assuming trypsin digestion. Parent ion tolerance of 10 ppm and a fragment ion mass tolerance of 0.6 Da were used. S-carbamidomethylation of cysteine residues was specified as a fixed modification while cyclisation of N-terminal glutamine to pyroglutamic acid, oxidation of methionine were specified as variable modifications.

Criteria for protein identification - Scaffold (version Scaffold_3_00_06, Proteome Software Inc.,

Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0 % probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

ChIP

Ring-stage (10 hr post invasion ±5 hr) synchronized 3D7 strain transfected with pLN-HA-Pfactin-I construct were used in ChIP assay. Parasites culture ($1x10^9$ rings) was harvested and cross-linked immediately with 1% paraformaldehyde on rotation for 10 min at room temperature, and quenched with 0.125M glycine for 5 min at 4℃. The fixed culture was washed with cold 1xPBS twice, and the parasites were released from iRBC with 0.15% saponin lysis for 20 min on ice. After three washes with cold 1xPBS, nuclei were isolated by incubation with 2 ml cold Lysis Buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA pH8.0, 0.1mM EGTA pH8.0, 1mM DTT, 0.25% NP40, 1x protease inhibitors cocktail) for 30 min on ice, and followed by dounce homogenization for 200 strokes. After centrifuge for 10 min at 10000 g, 4 ℃, the nuclei was resuspended in 150µl SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH8.0) for sonication. Chromatin was sheared into 200-500 bp fragments with Bioruptor of highest power for 4x4 min at 30 sec intervals. The resulting chromatin was diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH8.0, 150 mM NaCl, 1x protease inhibitors cocktail). 250 µl chromatin was incubated with 1µg antibody (anti-HA or IgG control) for each IP overnight at 4℃,

and recovered by 50 µl protein A per sample. After extensive washes, immunoprecipitated chromatin was eluted with ChIP Elution Buffer (1% SDS, 0.1 M $NaHCO_3$), and reverse cross-linked at 65℃ for 6 hr in presence of 200 mM NaCl and proteinase K, then DNA was purified by Phenol/chloroform/isoamylic alcohol extraction for qRT-PCR assay with primers listed in Table S5 or published else (Salanti et al., 2003; Petter et al., 2011). Real-time qPCR was performed on Realplex 4 Epgradient S thermal cycler (Eppendorf) using SYBR Green (Applied Biosystems). PCR was performed in duplicates and serial dilutions of purified input DNA were measured together with the immunoprecipitated DNA samples. This allowed us to calculate the amount of target sequence in immunoprecipitated chromatin relative to the amount of target sequence in input (% input). The site specific enrichment of HA antibody were obtained with mouse IgG control. The sequences of primers used are shown in Table S3. The presented data are the mean of two independent immunoprecipitations from different parasite preparations.

Immuoelectron Microscopy (EM)

Ring-stage *P.falciparum*-infected erythrocytes with ~ 20% parasitemia were fixed in 1% glutaraldehyde/RPMI-HEPES buffer for 1 hr at 4 °C. After washing, polymerization in Agar type IX and dehydratation with ethanol, the sample was transferred in LR-White (London Resin Compagny Ltd) and polymerized for 12 hour at 4°C. Ultrathin sections were colleted and mounted on Cu/Pd grids. Free aldehyde groups were blocked by incubating the samples for 5 min in 50 mM NH_4Cl. Sections were blocked in 1x Phosphate-Buffered Saline (PBS) containing 5% (wt/vol) nonfat milk and 0.01% (wt/vol) Tween-80 followed by a washing with 1xPBS containing 0.8% BSA (fraction V, Sigma) and 0.01% Tween-80. For double-labeling of Pfactin-I and Histone 3, grid was firstly labeled with anti-Histone 3 (abcam, ab1791) (1/100) and 10 nm protein A-colloidal gold according to the protocol described above. Then the grid was washed and fixed with 1%

glutaraldehyde/H_2O for 5 min. After washing, the second labeling with mouse anti-Pfactin-I (1/50) and 20 nm protein A-colloidal gold was performed according to the same protocol. Washed sections were stained for 15 min with aqueous 4% uranyl acetate followed by a staining of 2 min with 1% lead citrate. Sections were analyzed with a JEOL JEM-1200EX electron microscope at 120 kV.

Immunofluorescence and FISH

Infected red blood cells were lysed with 0.15% saponin and the released parasites were fixed in suspension with 4% paraformaldehyde for 10 min on ice. Parasites were then deposited on microscope slides and subjected to IFA or DNA FISH in the conditions as previously described (Mancio-Silva et al., 2008). For immuno-DNA FISH, parasites were first subjected to immunofluorescence, post-fixed in 4% paraformaldeyde for 15 min, deposited on microscope slides, and hybridized with FISH probes at 72 °C for 10 minutes and then at 37 °C overnight. TARE6 probes were obtained as described before (Ralph et al., 2005). All other FISH probes were PCR amplified from genomic DNA using the primers as previously described (Lopez-Rubio., 2009) or listed on Table S3. For staining of F-actin with phalloidin, the saponin-treated ring-stage parasites were treated with 0.1% triton X-100 for 5 min at room temperature, and incubated with 0.5μM Atto 488 phalloidin (Sigma) for 30 min at room temperature. Antibody dilutions for rabbit anti Pfactin-I were 1:500, for mouse anti Pfactin-I was 1:1500, for mouse anti-HA (Roche) was 1:200, and Alexa-Fluor-488-conjugated anti-rabbit or anti-mouse IgG (Invitrogen), 1:500.

Images were captured by using a Nikon Eclipse 80i microscope with a CoolSnap HQ2 camera (Photometrics). NIS Elements 3.0 software (Nikon) was used for acquisition and ImageJ (http://rsbweb.nih.gov/ij/) for composition.

Quantitative Analysis of Subnuclear Position in FISH Asaay

The subnuclear distribution of FISH signals was described in detail previously (Lopez-Rubio et al. 2009). Briefly, the area of the nuclear section was calculated and divided into three equal surfaces as illustrated in Figure 1, panel A, right. The FISH signals were then scored as localizing to zone 1, 2 and 3. For an average nucleus, the zone1 corresponds to a diameter of 2.06 – 1.67 μm and was defined as the peripheral zone; zone 2 corresponds to a diameter of 1.67 – 1.19 μm and zone 3 the internal zone, corresponds to a diameter of 1.19 μm, which was defined by quantitative fluorescence analysis with antibodies against *P.falciparum* nuclear pore protein (PF14_0706) and DAPI staining of nuclear region (see Figure S3 and legend published by Lopez-Rubio et al., 2009). Measurements were performed using the NIS-Elements 3.0 imaging software (Nikon). The scoring was performed by direct optical observation and registered using the NIS-Elements 3.0 software. For each FISH probe images from two independent cultures were combined and analyzed.

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