Antagonistic roles of NOT1 paralogues in the timing of gene expression in *Plasmodium falciparum*

PfNOT1 paralogues and timing of gene expression

Ying Liu,¹ Ragini Rai,¹ Lei Zhu,¹ Changqing Zhang,^{1,#a} Frances Rocamora,^{1,#b} Mark Featherstone,^{1,*} Zbynek Bozdech^{1,*}

¹ School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

^{#a}Current Address: Department of Biological Sciences, National University of Singapore, Singapore 117558, Singapore

^{#b}Current Address: School of Medicine, Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, USA

*Corresponding authors:

zbozdech@ntu.edu.sg (ZB)

msfeatherstone@gmail.com (MF)

Abstract

NOT1 is the scaffold of the CCR4-NOT complex, a highly conserved multi-protein complex that regulates gene expression in eukaryotes. As opposed to most eukaryotes in which NO1 is encoded by a single gene, malaria parasites, *Plasmodium falciparum*, carry two NOT1 paralogues, PfNOT1.1 and PfNOT1.2. Here we showed that the two PfNOT1 proteins function as mutually exclusive scaffolds within the PfCCR4-NOT protein complexes that are abundantly located in the parasite cytoplasm. Intriguingly, the two PfNOT1 paralogues appear to have directly opposing functions in regulation of mRNA abundance across the *P. falciparum* IDC, in which PfNOT1.1 and PfNOT1.2 induces and suppresses transcript abundance during their active transcription, respectively. Targeted disruption of either of the *PfNOT1* gene causes defective growth and lower invasion rates presumably due to the deregulation the *P. falciparum* IDC transcriptional cascade. We also demonstrate that the regulatory function of both PfNOT1.1 and PfNOT1.2 are related to another PfCCR4-NOT subunit, *PfCaf1*, which indicates their activity during post-transcriptional regulation. Indeed RNA decay studies suggest the active role of both PfNOT1 proteins in regulation of mRNA stability in a directly opposing manner.

Author summary

CCR4-NOT complex is a highly conserved multi-protein complex that regulates gene expression in eukaryotes. NOT1 serves as the scaffold of the complex and plays important roles in gene regulation both transcriptionally and post-transcriptionally. As opposed to other eukaryotes, *P. falciparum* encodes two paralogues of PfNOT1, raising the question as to the significance to

possess an additional copy of PfNOT1 in the parasite. Here we described antagonistic regulatory functions of two PfNOT1 paralogues in gene expression during the 48-hour intraerythrocytic developmental cycle. We also reported that their regulatory functions are predominantly post-transcriptional and proposed a model in which distinct PfCCR4-NOT complexes defined by mutually exclusive PfNOT1 scaffolds differentially regulate PfCAF1 function in mRNA decay. This study highlights the importance of post-transcriptional regulation in *P. falciparum* and provides novel insights into mechanisms of gene regulation in this organism. The unique presence of two PfNOT1 paralogues may also open avenues for the development of new drug targets for anti-malarial control.

Introduction

Plasmodium falciparum is an *Apicomplexan* parasite responsible for the most lethal form of malaria and is therefore a major global health concern [1]. The lifecycle of this protozoan parasite consists of a sexual stage in the *Anopheles spp.* mosquito host and an asexual stage in the human host [2]. In a human red blood cell, the parasite passes through three morphological stages – ring, trophozoite and schizont – across the 48-hour intraerythrocytic developmental cycle (IDC), eventually releasing up to 32 merozoites ready to invade red blood cells in a subsequent round of infection [3]. During one round of the IDC, the expression of more than 50 % of genes displays a continuous cascade with transcript levels peaking at the point when their protein products are needed, leading to the “just in time” model of gene

expression [4]. Hence, gene expression is highly regulated across the IDC at multiple levels, ranging from epigenetic regulation, transcription and post-transcriptional regulation.

While the basic mechanism of eukaryotic transcription and general transcription components are conserved in *P. falciparum* [5, 6], most TBP-associated factors (TAF) are absent [6] and only a third of the expected number of canonical eukaryotic transcription activating proteins (TAP) were predicted in the parasite genome [7]. The presence of 27 *Apicomplexan*-specific transcription factors, ApiAP2, may compensate for the reduction in the number of conventional transcription components [8, 9]. These 27 ApiAP2 proteins display distinct expression profiles covering the entire IDC [8] and their recognition motifs are located in the promoter regions of most genes whose expression profiles are positively correlated to expression of the corresponding ApiAP2 gene [9]. In addition to transcriptional initiation, there is mounting evidence that multiple processes of post-transcriptional regulation are also critical for regulation of mRNA abundance through the *P. falciparum* IDC [10-12]. This is supported by the abundant presence RNA-binding proteins [13, 14] and RNA degradation components [15] in the *P. falciparum* genome. These include PfALBA1 [16], PfCAF1 [17] in *P. falciparum* and PyCCR4 [18], PyALBA4 [19] in rodent-specific *P. yoelii* all of which functions were directly implicated in regulation of transcript levels. Moreover, variability of mRNA half-lives across the IDC observed by several experimental approaches also suggested a significant contribution of mRNA decay in regulation of gene expression in malaria parasites [20]. Taken together, both transcriptional regulation and post-transcriptional regulation play critical roles in modulating transcript abundance in *P. falciparum*.

CCR4-NOT is a highly conserved proteins complex involved in multiple steps of gene expression regulation across the entire eukaryotic kingdom [21], present in both the nucleus and cytoplasm [22, 23]. Core subunits include NOT1, NOT2, NOT3/5 paralogues, NOT4, CAF1, CCR4, CAF40, yeast-specific CAF130 and *Drosophila*/mammalian-specific NOT10 and NOT11 [21]. Structurally, NOT1 is the largest subunit of the CCR4-NOT complex and acts as a scaffold to recruit other subunits [24]. The N-terminal domain of NOT1 interacts with NOT11 and NOT10 in *Drosophila* and mammals [25]. The central MIF4G domain associates with CAF1, which bridges CCR4 to the whole complex [24]. The association of CAF1 and CCR4 to the complex is required for their functions as deadenylases to shorten the mRNA poly(A) tail in yeast and *Drosophila* [26-28], the key step in mRNA degradation[29]. The DUF3819 domain of NOT1 interacts with CAF40 [30]. The C-terminal NOT1 domain interacts with NOT2 and NOT3/5 paralogues [31], which associate with transcription factors [32, 33] and histone modifications [34, 35]. The NOT1 domain also interacts with NOT4 [31], an E3 ubiquitin ligase involved in protein degradation [36, 37] and required for proteasome integrity in yeast [38].

In spite of its broad involvement in multiple levels of eukaryotic gene expression, only limited information exist about the biological function of the CCR4-NOT complex in malaria parasites; particularly its significance for the progression of the parasite life cycle. Initially it was shown that targeted gene disruption of *PfCaf1* affected expression of 1,031 genes, including many invasion-related genes, which leads to the release of premature merozoites in *P. falciparum* [17]. In *P. yoelii*, PyCCR4 appears to regulate gene expression in gametocytes while disruption of *PyCaf1* or *PyCcr4* results in a reduction of male gametocyte activation [18]. Moreover, PyCCR4 was show to associate with some transcripts that are dysregulated in the

parasite lacking *PfCcr4A* potentially via its intrinsic DNA nuclease activity that is dependent on Cu^{2+} [39]. These studies demonstrate the importance of the presumptive CCR4-NOT complex in *Plasmodium* parasite at multiple stages. The NOT1 subunit has been identified as a negative transcription regulator in yeast [22], although a positive effect on transcription has also been reported [40]. The association of yeast NOT1 with several transcriptional components, including TBP [41], TAFs [42] and the RNAPII elongation complex [43, 44] reinforces a role for NOT1 in transcription. Post-transcriptionally, NOT1 interacts with multiple RNA-binding proteins, including tristetraprolin [45], eIF4E-binding protein 4E-T [46], RNA helicase Xp54 [47] and m6A reader protein YTHDF [48], involved in mRNA degradation, mRNA decapping and translation repression in multiple organisms. Moreover, NOT1 in mouse germ cells interacts with the RNA-binding protein Nanos2, which is speculated to promote the localization of CCR4-NOT complex to P-bodies, the center of RNA degradation [49]. The structural role as the scaffold of the CCR4-NOT complex and implication in both transcriptional and post-transcriptional regulation makes NOT1 an ideal focus to investigate the presumptive PfCCR4-NOT complex and gene regulation in *P. falciparum*.

Identically to all characterized *Plasmodium* species, *P. falciparum* parasites encode two paralogues of PfNOT1 located on chromosome 11 (termed PfNOT1.1) and chromosome 14 (termed PfNOT1.2), raising the question as to the composition of tentative CCR4-NOT complex and its regulatory role in gene expression. In this study, we demonstrate that while the overall structure of the *P. falciparum* CCR4-NOT complex is highly conserved, PfNOT1.1 and PfNOT1.2 function as mutually exclusive scaffolds. Intriguingly, PfNOT1.1 and PfNOT1.2 appear to have antagonistic functions in regulation of gene during the IDC progression, presumably via

differential interaction of the CCR4-NOT complex with other regulatory factors. Our results suggest that the regulatory functions exerted by the two PfNOT1 paralogues is mediated by factors of mRNA decay.

Results

1. Four conserved CCR4-NOT subunits are localized in the cytoplasm

Most core subunits of the CCR4-NOT complex are conserved in the *P. falciparum* genome, including PfNOT2, PfNOT4, PfNOT5, PfCAF1 and PfCAF40 [50] (See Table. S1). However, unlike other eukaryotes studied to date, the malaria parasites have two PfNOT1 and four PfCCR4 paralogues and conversely do not harbor homologues of CAF130, NOT10 and NOT11. While the loss of CAF130, NOT10 and NOT11 could be attributed to the overall genome streamlining documented in *Plasmodium* species [50], the duplications of the PfNOT1- and the PfCCR4-coding genes are unique amongst eukaryotic organism. Canonically, NOT1 functions as a structural scaffold protein in the CCR4-NOT complex and as such is encoded by a single gene copy in the vast majority of eukaryotic species [21]. Nonetheless, for both PfNOT1.1 and PfNOT1.2, there is a high degree of evolutionary conservation with the presence of the MIF4G, DUF3819 and NOT1 domains (Fig. S1A and S1B). There are apparent insertions of unique amino acid stretches within the MIF4G domain and the NOT1 domain in PfNOT1.2, exclusively. Sequence insertions in highly conserved domains have been reported in *Plasmodium* proteins, suggesting their rapid evolution and diversification [51]. This suggests that PfNOT1.1 may reflect the ancestral gene copy, while PfNOT1.2 is a product of recent duplication and thus may

have diverged from its original function presumably as an alternative scaffold in CCR4-NOT complex.

Table S1. Predicted CCR4-NOT subunits in *P. falciparum*.

<i>P. falciparum</i>	Gene ID	Yeast	Human	<i>Drosophila</i>
PfNOT1.1	PF3D7_1103800	CDC39	CNOT1	Not1
PfNOT1.2	PF3D7_1417200			
PfNOT2	PF3D7_1128600	CDC36	CNOT2	Regena (Rga)
PfNOT4	PF3D7_1235300	MOT42/SIG1	CNOT4	Not4
PfNOT5	PF3D7_1006100	NOT3 NOT5	CNOT3	Not3
PfCAF1	PF3D7_0811300	POP2	CNOT7 CNOT8	Pop2
PfCCR4.1	PF3D7_0519500	CCR4	CNOT6L	Twin
PfCCR4.2	PF3D7_1363500		CNOT6	
PfCCR4.3	PF3D7_0107200			
PfCCR4.4	PF3D7_0319200			
PfCAF40	PF3D7_0507600	CAF40	CNOT9	Rcd1
		CAF130		
			CNOT10	Not10
			CNOT11	Not11

The CCR4-NOT core subunits identified in yeast, human and *Drosophila* are listed. Their counterparts in *P. falciparum* were predicted by searching the PlasmoDB database and blasting protein sequences of each subunit in the *P. falciparum* proteome.

To explore this further, we created independent transgenic parasites in which the endogenous allele of *PfNot1.1* and *PfNot1.2*, as well as *PfCaf1* and *PfNot4* were joined with HA epitopes by single crossover recombination (**Fig. S2A**). The correct insertion of the HA epitopes was verified by genotyping PCR for all four transgenic lines, including PfNOT1.1::HA, PfNOT1.2::HA, PfCAF1::HA and PfNOT4::HA (**Fig. S2B-E**). Western blotting analysis indicated that the bulk of PfNOT1.1 and PfNOT1.2 is present predominantly in the cytoplasm, while PfCAF1 and PfNOT4

could be found in both the cytoplasm and nucleus (**Fig. 1A-D**). This was supported by the subsequent immunofluorescent assay showing the four proteins present in the cytoplasm as a punctuate pattern (**Fig. 1E-H**). These cytosolic puncta did not overlap with aldolase or DAPI, which is consistent with previous reports for the *P. yoelii* orthologue, PyCCR4 [18] and the other three PfCCR4 paralogues [39]. This intracellular localization is consistent with other eukaryotic species suggesting the function of PfCCR4-NOT complex in posttranscriptional regulation via interactions with mRNA in the parasite cytoplasm.

2. PfNOT1.1 and PfNOT1.2 function as mutually exclusive scaffolds of the *P. falciparum* PfCCR4-NOT complexes

To further investigate the composition of the PfCCR4-NOT complex, we carried out co-immunoprecipitations coupled with mass spectrometry on the four HA-tagged proteins. Prior to the mass spectrometry, we confirmed the efficiency of immunoprecipitation of HA-tagged proteins using western blotting, showing their strong abundance in the eluent fractions (**Fig. 1I-K**). Mass spectrometry analyses of the eluent fractions detected 30 proteins for PfNOT1.1, 20 proteins for PfNOT1.2, 244 proteins for PfCAF1 and 45 proteins for PfNOT4 in two biological replicates (see full protein lists in **Table S2**). The co-immunoprecipitation studies confirmed physical interaction of PfCAF40 with PfNOT1.1, PfNOT1.2, PfCAF1 and PfNOT4; PfCAF1 with PfCCR4; and either of the PfNOT1.1 or PfNOT1.2 with PfCAF40 and PfCAF1, respectively. On the other hand PfNOT4 failed to co-immunoprecipitate PfNOT1.1 and PfNOT1.2 (**Table 1, top panel**), which is consistent with the transient character of this subunit with the CCR4-NOT complex previously demonstrated in *Drosophila* [52], human [53] and *P. yoelii* [18]. However, our data showed that PfNOT4 does associate with the PfCCR4-NOT complex given its

interaction with CAF40 and possibly CAF1. Overall, this analysis supports the presence of the four studied proteins in the PfCCR4-NOT complex, demonstrated by their interactions with each other and other canonical subunits.

Table 1. Potential interacting proteins of PfNOT1.1, PfNOT1.2, PfCAF1 and PfNOT4.

Gene ID	Description	Co-IP target			
		PfNOT1.1	PfNOT1.2	PfCAF1	PfNOT4
CCR4-NOT subunits					
PF3D7_0507600	PfCAF40	*	O	O	O
PF3D7_0811300	PfCAF1	O	O	O	
PF3D7_1103800	PfNOT1.1	O		O	
PF3D7_1417200	PfNOT1.2		O	O	
PF3D7_0519500	PfCCR4			O	
PF3D7_1235300	PfNOT4			*	O
Potential interacting proteins of the CCR4-NOT complex					
Histone and Transcription					
PF3D7_0610400	histone H3		O	O	O
PF3D7_1239200	ApiAP2 transcription factor	O			O
Ribosome and Translation machinery					
PF3D7_1424400	60S ribosomal protein L7-3		O		O
PF3D7_1447000	40S ribosomal protein S5		O		O
PF3D7_0815600	EIF3G		O		O
RNA-binding proteins					
PF3D7_1224300	PABP		O	O	O
PF3D7_1107300	PAIP1		O	O	O
PF3D7_0814200	ALBA1		O		O
PF3D7_1015900	enolase		O	O	O
PF3D7_1110400	asparagine-rich antigen		O		O
PF3D7_0703500	erythrocyte membrane-associated antigen			O	O
Other functions					
PF3D7_0524000	karyopherin beta			O	O
PF3D7_0511500	RNA pseudouridylate synthase			O	O
Unknown functions					
PF3D7_1456700	conserved Plasmodium protein	O		O	
PF3D7_1142100	conserved Plasmodium protein	O		O	
PF3D7_0813300	conserved Plasmodium protein		O		O
PF3D7_0604500	conserved Plasmodium protein			O	O

Co-immunoprecipitation and mass spectrometry analysis were performed on each HA-tagged transgenic line and the parental strain. Proteins detected in both replicates (O) or in one

replicate (*) in the transgenic lines and co-immunoprecipitated by more than one of the four studied HA-tagged proteins are listed.

Interestingly the two PfNOT1 paralogues did not co-immunoprecipitate each other. This is consistent with the model that both PfNOT1.1 and PfNOT1.2 function as scaffolds in the PfCCR4-NOT complexes in the mutually exclusive manner; given that the structure of the eukaryotic CCR4-NOT complexes contains a single NOT1 subunit. This is also consistent with the exclusivity of the other (than CCR4-NOT subunit) proteins that were co-immunoprecipitated by each of the PfNOT1 paralogues (**Table 1, bottom panel**). Particularly, PfNOT1.1 co-immunoprecipitated proteins involved in transcription (chromodomain helicase DNA-binding protein 1 homolog PF3D7_1023900, acetyltransferase PF3D7_1323300 and ApiAP2 transcription factor PF3D7_1239200), Maurer's clefts components (protein phosphatase PP1 PF3D7_1414400, exported protein PF3D7_0501000 and pyruvate kinase PF3D7_0626800) and RNA-binding (polyadenylate-binding protein PF3D7_1360900). PfNOT1.2 co-immunoprecipitated proteins involved in RNA-binding (polyadenylate-binding protein PABP PF3D7_1224300, PAIP1 PF3D7_1107300 and ALBA1 PF3D7_0814200), translation (4 ribosomal proteins and EIF3G PF3D7_0815600) and histone composition (histone H2B and histone H3) (see full protein lists in **Table S2**). Overall, the associations of proteins involved in transcription, translation and RNA-binding suggests the role of the PfCCR4-NOT complexes in regulation of *P. falciparum* gene expression that is orthologous to other eukaryotes [29]. However, the differential protein associations between the PfNOT1.1 and PfNOT1.2-containing complexes

support the notion that the two PfNOT1 paralogues modify the overall activity of the PfCCR4-NOT complex presumably giving it distinct sets of functions in gene regulatory processes.

3. Targeted disruption of PfNot1.1 or PfNot1.2 impairs parasite proliferation and invasion.

To explore this, we carried out targeted gene disruptions for both *PfNot1.1* and *PfNot1.2* in independent parasite lines. First, we disrupted the *PfNot1.1* coding region by inserting a human dihydrofolate reductase (*hDHFR*) cassette via double crossover recombination (Δ PfNot1.1) (**Fig. 2A**). The cloned parasite line was obtained by limiting dilution and the construct insertions was verified by PCR-based genotyping (**Fig. S3A**). Finally, Northern blot analysis showed a complete absence of PfNOT1.1 transcript (**Fig. 2B**). A similar strategy of gene disruption was, however, unsuccessful for *PfNot1.2* after three independent transfection experiments (**Fig. S3B**), suggesting that PfNOT1.2 might be essential for parasite viability. Thereafter, we created a conditional protein knockdown parasite line to express PfNOT1.2 fused with an *E.coli* DHFR destabilizing domain (ecDDD) at the C-terminus (dd Δ PfNot1.2) (**Fig. 2C**). In this system, the transgenic protein is stabilized by the inclusion of the trimethoprim (TMP) ligand in the culture medium. Indeed, the ecDDD insertion was achieved by single crossover recombination and confirmed by genotyping PCR (**Fig. S3C**). Using this strategy, we achieved a significant reduction of PfNOT1.2 expression at the schizont stage after removing TMP for three consecutive IDCs (**Fig. 2D and Fig. S3D**).

Disruption of *PfNot1.1* and conditional knockdown of *PfNot1.2* both resulted in significantly lower proliferation rates across three consecutive IDCs (**Fig. 2E, 2F**). In particular, Δ PfNot1.1 and dd Δ PfNot1.2 exhibited considerable decreases in invasion rate with more moderate

defects in Δ PfNot1.2 (**Fig. 2G, 2H**), possibly due to incomplete knockdown. This proliferation defect is not associated with the numbers of merozoites produced per schizont by each transgenic line that was comparable to their parental line (**Fig. S3E and S3F**). Moreover, maximum likelihood-based statistical analysis of the IDC transcriptomes showed no significant differences in the progression of Δ PfNot1.1 and Δ PfNot1.2 (**Fig. S3G and S3H**). These results suggest that reduced proliferation caused by deactivation of PfNOT1.1 or PfNOT1.2 results from impaired egress and/or invasion process, likely as a reflection of a decreased fitness of the mutant merozoites.

4. PfNOT1.1 and PfNOT1.2 have opposing effects on transcript abundance

Given the importance of CCR4-NOT in regulation of gene expression, we explored the role of two PfNOT1 paralogues in maintenance of the global transcriptional pattern across the *P. falciparum* IDC. In particular, we wished to assess the impact of *PfNot1.1* disruption and *PfNot1.2* knockdown on the global mRNA abundance pattern by analyzing the transcriptomes of the parental and transgenic lines. Transcriptional profiles of each parasite line were assessed by microarray at six time points across one IDC in biological triplicates as previously described [4]. Compared to their parental lines, we observed significant changes in mRNA abundance ($p < 0.05$) with > 1.5 fold-change for 715 genes and 202 genes in Δ PfNot1.1 and Δ PfNot1.2, respectively (See gene lists in **Table. S3**). A total of 782 genes were significantly affected by either *PfNot1.1* disruption or *PfNot1.2* knockdown across the IDC particularly at the trophozoite and schizont stages (**Fig. 3A**). Interestingly, in Δ PfNot1.1, the majority of mRNA transcripts exhibited decreased levels while in Δ PfNot1.2 the trend was towards upregulation. The

opposing pattern of these transcriptional changes in Δ PfNot1.1 and dd Δ PfNot1.2 was also supported by negative Spearman coefficients between the overall transcriptional profiles at each time point (**Fig. 3B**). Indeed, there is a statistically significant overlap ($p < 0.001$) between these two genes sets with 135 common genes (**Fig. 3C**), 114 of which display opposing changes at the corresponding time points. The changes in mRNA abundance determined by microarray were also validated by quantitative reverse transcriptase-PCR (qRT-PCR) for a random selection of genes at several time points (**Fig. 3D**). Closer inspection of the mean-centered mRNA expression profiles revealed that the apparent transcriptional suppression in Δ PfNot1 (**Fig. 3A, left heat map**) corresponds to delayed profiles of the differentially expressed genes during their active transcription (**Fig. 3A, right panel, green curves**). Conversely, the transcriptional inductions in dd Δ PfNot1.2 (**Fig. 3A, right heat map**) correspond to accelerated patterns of the IDC mRNA abundance profiles (**Fig. 3A, right panel, red curves**). Taken together, these observations suggest that PfNOT1.1 and PfNOT1.2 play roles in the timing of the mRNA levels during the “just-in-time”-like transcriptional cascade in the *P. falciparum* IDC during their active transcription (rise of the individual transcript levels prior to their peak abundance). Given that the deletion of PfNOT1.1 caused general down regulations of the transcript levels, it is feasible to suggest that the endogenous functions of this paralogues of the CCR4-NOT scaffold subunit contributes to “inductions” of transcript levels; possibly via mRNA stabilization (see below). *Vice versa*, PfNOT1.2 likely contributes to transcriptional “suppressions”.

Crucially, the PfNOT1-driven transcriptional alterations did not affect the progression of the entire IDC cascade (see **Fig. S3G and S3H**) but was confounded to a distinct subsets of the IDC-driven transcriptionally regulated genes. In order to evaluate the biological relevance of the

antagonistic regulatory relationship between PfNOT1.1 and PfNOT1.2 we inspected the functional relevance of each differentially expressed gene cluster (**Fig. 3A right panel**). At the early trophozoite stage, the dysregulated genes are mainly involved in basic cellular and biochemical pathways such as components of apicoplast and mitochondrion, DNA repair, DNA replication, mitosis, transcription, ATP synthesis and metabolic pathways (pyrimidine metabolism, purine metabolism and redox metabolism). Notably, many transcripts encoding egress- and invasion-related genes, merozoite components and merozoite motility proteins were dysregulated at the late trophozoite and the schizont stages, which may lead to the defective invasion rate and subsequent proliferation as observed in Δ PfNot1.1 and Δ PfNot1.2. Transcriptional changes were also observed in abundant transcripts encoding exported proteins and Maurer's cleft components, which are associated with erythrocyte remodeling and parasite virulence [54, 55]. Taken together, all the above mentioned affected cellular and biochemical functionalities may represent key processes in the parasite development and thus their dysregulation may lead to suboptimal formation of the invasive merozoites that have a lower potential for successful invasion.

5. PfNOT1.1 and PfNOT1.2 regulate gene expression post-transcriptionally

Considering the canonical function of CCR4-NOT complex, the antagonistic activities of PfNOT1.1 and PfNOT1.2 in gene expression may happen at the transcriptional and/or post-transcriptional regulatory steps of mRNA levels. To study this, we explored the effect of the *PfNot1.1* deletion on both transcription and mRNA decay. To study transcription, we assessed the recruitment of the elongating form of RNAPII at six time points across the IDC by chromatin-immunoprecipitation using Ser2/5-P antibody raised previously [10]. Transcriptional status of

RNAPII is known to be associated with sequential changes of phosphorylation at the C-terminal domain (CTD) of RPB1, with phosphorylation of Ser5 alone during transcriptional initiation and phosphorylation of both Ser2 and Ser5 (Ser2/5-P) when the polymerase is engaged in elongation [56]. Here, we observed a total of 150 genes that displayed significantly differential enrichment of RPB1 (> 1.5 fold-change, $p < 0.05$) between Δ PfNot1.1 and its parental strain across the IDC (**Table S4**). In contrast to differential gene expression, the differential RPB1 occupancy in Δ PfNot1.1 was most apparent at ring and trophozoite stages (**Fig. S4A**). Indeed there was a minimal overlap between the 150 genes with differential RPB1 enrichment and the 715 genes with differential mRNA abundance with only 17 genes in both gene sets is statistically insignificant (Binomial $p > 0.05$) (**Fig. 4A**). Moreover, no Spearman correlation could be observed between RPB1 occupancy and differential mRNA profiles at each time point (**Fig. S4B**). Thus, our data indicates that while deletion of *PfNot1.1* does affect the recruitment of the elongating form of RNAPII, changes in transcriptional elongation are not directly responsible for the altered mRNA abundance of the 715 genes in Δ PfNot1.1.

In eukaryotic organisms, NOT1-CAF1 interaction is required for the function of CAF1 for mRNA decay and deadenylation [26-28]. To test whether this association is conserved in *P. falciparum*, we compared significant transcriptional changes of 782 genes observed in Δ PfNot1.1 or dd Δ PfNot1.2 to those in a transgenic line with disrupted *PfCaf1* (Δ PfCaf1) reported previously [17]. Strikingly, differential transcript abundance in Δ PfNot1.1 is positively correlated to that of Δ PfCaf1 (**Fig. 4B and Fig. S4C**), demonstrating that functional correlation of PfNOT1 and PfCAF1 is conserved in *P. falciparum*. Conversely, we observed a negative correlation between differential transcript abundance in dd Δ PfNot1.2 and Δ PfCaf1, suggesting that the opposite

effects of *PfNot1.1* disruption and *PfNot1.2* knockdown on transcript abundance might be due to their opposing roles in modulating PfCAF1 function.

The removal of the mRNA poly(A) tail by CAF1 deadenylase and CCR4 deadenylase is a key step in the initiation of mRNA decay [29]. Therefore, we next assessed the possibility of that PfNOT1 paralogues mediate regulation of mRNA abundance via mRNA decay. Here we utilized the concept of transcription blocking with actD as established previously [57]. Briefly, synchronized cultures of Δ PfNot1.1 and the parental strain were incubated with 20 μ g/ml actD or DMSO vehicle for 4 hours at the trophozoite stage (24 hour post-invasion, hpi) in biological duplicates. Subsequently, we carried out comparative analysis of transcriptomes of the resulting parasites, including initial sample at 24 hpi, actD-treated sample and actD-untreated sample (**Fig. 4C**). Inhibition of transcription by actD was validated by lower transcript abundance in the actD-treated parasites compared to that in the actD-untreated parasites for both Δ PfNot1.1 and the parental strain (**Fig. 4D**). We then calculated differential transcript abundance (transcriptional changes between the actD-untreated sample and the initial sample), newly-synthesized transcript abundance (transcriptional change between the actD-untreated sample and the actD-treated sample) and decayed transcript abundance (transcriptional change between the initial sample and the actD-treated sample) within the 4-hour incubation for each parasite line. When comparing the amount of decayed transcript between Δ PfNot1.1 and the parental strain, a lower level of mRNA decay was observed for the majority of transcripts in Δ PfNot1.1 (**Fig. 4E**), suggesting that the loss of *PfNot1.1* does indeed affect mRNA decay. This observation is consistent with the functional correlation of NOT1.1 and CAF1 reported in eukaryotes that NOT1.1-CAF1 interaction is required for the function of PfCAF1 in mRNA decay [26-28].

Furthermore, we observed that the changes in differential transcript abundance between Δ PfNot1.1 and the parental strain are highly correlated with the changes in decayed transcript abundance (**Fig. 4E**, Spearman coefficient = -0.851, $p < 0.001$), but not correlated with the changes in newly-synthesized transcript abundance (**Fig. 4F**, Spearman coefficient = 0.195, $p < 0.05$), suggesting that PfNOT1.1 regulates gene expression predominantly by influencing mRNA decay but not transcription at the trophozoite stage.

Discussion

The existence of one copy of NOT1 as the scaffold of the CCR4-NOT complex has been reported in yeast [58], *Drosophila* [59], human cells [25] and *Trypanosoma brucei*, a parasitic kinetoplastid belonging to the *Euglenozoa* phylum [60]. Two copies of PfNOT1-encoding genes in *P. falciparum* is contrasting the majority of eukaryotes but is consistent with other *Plasmodium* species, such as *P. yoelii* [18], making it a unique feature in the *Plasmodium* genus. The presence of two copies of NOT1 is the likely result of gene duplication with the insertions of unique amino acid stretches in PfNOT1.2 MIF4G and NOT1 domains evolving with time. NOT1 is an essential gene in yeast [61], human HEK293T cells [62] and Trypanosome [60]. The requirement for two NOT1 paralogues in *Plasmodium* has therefore been unclear. In this study, we successfully produced a full loss-of-function transgenic line for *PfNot1.1* but not *PfNot1.2*. Consistent with this, an attempt to inactivate multiple genes in *P. berghei* was unsuccessful in knocking out *PbNot1.2* [63]. On the other hand, *PfNot1.1* and *PfNot1.2* have been suggested to be dispensable genes in *P. falciparum* given the recovery of *PiggyBac* transposon-insertions into

the coding regions of both PfNOT1 paralogues [64]. However, the essential or dispensable character of both genes is presumptive in these two studies given that the failure to inactivate *PbNot1.2* may be a simple result of a failure of the targeting vector, and *PiggyBac* insertions in the *PfNot1.2* coding region may not have resulted in a complete loss of function. Our data provides direct evidence that PfNOT1.1 is dispensable in parasite viability and that PfNOT1.2 might be refractory to disruption during the IDC (although a possibility of a failure of the targeting vector cannot be excluded). These findings raise questions as to why PfNOT1.1 as a more canonical eukaryotic NOT1 is dispensable whereas eukaryotic NOT1 is essential in multiple organisms. Moreover, PfNOT1.2 might possess functions independent from PfNOT1.1 that make PfNOT1.2 refractory to be disrupted. This possibility is supported by the distinct proteins co-immunoprecipitated by PfNOT1.1 and PfNOT1.2 in this study.

The composition of the CCR4-NOT complex is highly conserved across eukaryotes, including NOT1, NOT2, NOT4, NOT5, CAF1, CCR4 and CAF40 [29]. Our Co-IP data suggests that PfCCR4-NOT complexes comprise of either PfNOT1.1 or PfNOT1.2 scaffold in the mutually exclusive manner, PfCAF1, PfCAF1-associating PfCCR4, PfCAF40 and possibly PfNOT4, which associates with PfCAF40 and PfCAF1. In the *P. yoelii* study, PyCCR4 associated with PyNOT1.1, PyNOT1.2, PyCAF1, PyCAF40 and PyNOT2 in schizont parasites. PyCCR4 also co-immunoprecipitated PyNOT5 and PyNOT4 with much lower SAINT stringency [18]. Taken together, the composition of the CCR4-NOT complexes is conserved in *Plasmodium* parasites with NOT4 and NOT5 being less stable components. The weak association of NOT4 with the rest of the complex is expected as NOT4 is a less tightly associated subunit in mammals and *Drosophila* [52, 53]. Contrary to our finding in *P. falciparum*, the association of PyNOT2 with the PyCCR4-NOT complex may result

from an additional fixation step the *P. yoelii* study that allows the detection of weakly associated NOT2. Moreover, the weak or missing association of NOT2 and NOT5 with the complex is surprising given the highly conserved interacting domains predicted in both PfNOT1 paralogues, PfNOT2 and PfNOT5. On the other hand, the composition of the CCR4-NOT complex is distinct in another parasite *T. brucei*, in which TbCAF1 co-immunoprecipitated TbNOT1, TbNOT2 and TbNOT5 and no homologues of CCR4 and NOT4 were found in its genome [60]. Notably, the *P. yoelii* study and the *T. brucei* study both reported a punctate distribution of PyCCR4 and TbCAF1 predominantly in the cytoplasm, consistent with our findings on the cytoplasmic localization of four PfCCR4-NOT subunits, suggesting that the post-transcriptional regulatory functions of the CCR4-NOT complex(es) are highly conserved across protozoans though composition varies slightly.

The degradation of mRNA plays important roles in the modulation of gene expression and quality control of mRNA biogenesis. The rate-limiting step of mRNA degradation is deadenylation, the shortening of the poly(A) tail by mRNA deadenylases, including the Pan2/Pan3 complex, the CCR4-NOT complex [23] and the vertebrate-specific DAN/PARN enzyme [65]. CCR4 and CAF1 are the major eukaryotic deadenylases while Pan2/Pan3 and PARN may play secondary roles [66]. Although both CCR4 and CAF1 possess nuclease domain and deadenylase activity [67], studies in yeast suggest that CCR4 is the predominant catalytic subunit of the deadenylase and CAF1 mainly functions to attach CCR4 to the complex [68, 69]. In contrast, CAF1 is suggested to be more important in deadenylation than CCR4 in *C. elegans* [70], *Drosophila* [71] and human HTGM5 fibrosarcoma cells [60]. Similarly, in Trypanosome a protozoan lacking CCR4 homologue, TbCAF1 was shown to function directly in deadenylation in

the total RNA and degradation of four mRNAs [60]. In *P. yoelii*, disruption of *PyCcr4* does not affect the length of the poly(A) tail of a PyCCR4-affected/bound transcript in gametocytes, suggesting that CCR4 is not the predominant deadenylase in *Plasmodium* parasites at least in the gametocytes [18]. In the same study, truncated PyCAF1 leads to a comparable but more pronounced effect on parasite transmission compared to the *PyCcr4* disruption, hinting that PyCAF1 plays a more important role in mRNA metabolism as reported in eukaryotes except for yeast. However, the mechanism underlying the function of CAF1 in gene regulation has not been described for either *P. yoelii* [18] or *P. falciparum* [17]. In this study, we showed the functional correlation of PfNOT1.1 and PfCAF1 as well as the effect of disrupted *PfNot1.1* in mRNA decay, providing the first direct evidence on the role of the PfCCR4-NOT complexes in mRNA degradation in *Plasmodium* parasites. In addition, CAF1 plays a structural role linking CCR4 to the rest of the complex and this association is needed for the deadenylation activities of CCR4 [26, 27]. This association is conserved in *Plasmodium* parasites given the similar phenotypes of parasites lacking *PyCaf1* or *PyCcr4* in *P. yoelii* [18]. Our data cannot distinguish whether the effect of *PfNot1.1* disruption on mRNA decay is the direct result of impairment of PfCAF1 activity, or non-exclusively, the indirect effect of altered PfCCR4 attachment to PfCCR4-NOT complexes.

Although NOT1 itself does not contain any nuclease domains, it is associated with deadenylation and mRNA degradation by affecting the function of CAF1 deadenylase through the NOT1-CAF1 interaction [29]. Our data on the physical interaction and the functional correlation of PfNOT1.1 and PfCAF1 indicate that this association in eukaryotes is very likely conserved in the parasite. In the absence of PfNOT1.1, free PfCAF1 deadenylation function is

impaired, leading to defective mRNA decay as we observed in Δ PfNot1.1. The decreased mRNA decay affects transcript abundance and results in abnormal timing of gene expression, reflected by the shifted expression pattern we observed in Δ PfNot1.1. On the other hand, the negative functional correlation of PfNOT1.2 and PfCAF1 suggests an inhibitory role of PfNOT1.2 on PfCAF1 function. A reduced protein level of PfNOT1.2 could release PfCAF1 from the PfNOT1.2 complex, allowing it to attach to the PfNOT1.1 complex and function in mRNA degradation, which can be tested by assessing mRNA decay in $\Delta\Delta$ PfNot1.2 in future studies. Overall, we propose that PfNOT1.1 and PfNOT1.2 compete to be the scaffold for PfCAF1 binding (**Fig. 4G**). PfNOT1.1 facilitates PfCAF1 function as reported in other eukaryotes, whereas PfNOT1.2 sequesters PfCAF1 and renders it inactive or unavailable. Parasites could favor the formation of one PfNOT1-CAF interaction or the other depending on host response, environmental, metabolic or stage-specific cues, thereby allowing the parasite to modulate gene expression according to circumstance.

In conclusion, we have shown that PfNOT1.1 and PfNOT1.2 are mutually exclusive scaffolds of cytoplasmic CCR4-NOT complexes in *P. falciparum*. PfNOT1.1 and PfNOT1.2 play critical roles in regulating transcript abundance across the IDC. The assessment of the recruitment of the elongating form of RNAPII and mRNA decay in Δ PfNot1.1 underscores the predominant role of PfNOT1.1 in mRNA decay. We propose that PfNOT1.1 and PfNOT1.2 compete to be the scaffold for PfCAF1 binding with PfNOT1.1 facilitating PfCAF1 function and PfNOT1.2 sequestering PfCAF1. Our study significantly extends our knowledge of the CCR4-NOT complex in *Plasmodium* parasites. This study supports the importance of post-transcriptional regulation in *P. falciparum* and provides novel insights into mechanisms of gene regulation in this organism.

The unique presence of two PfNOT1 paralogues may open avenues for the development of new drug targets for anti-malarial control.

Methods

Malaria Culture

Asexual blood stage of *P. falciparum* T996 strain and transgenic lines were cultured in RPMI-HEPES medium (Gibco), supplemented with 0.25% Albumax (Gibco), 0.2% sodium bicarbonate (Sigma), 100 μ M hypoxanthine (Sigma) and 10 μ g/ml Gentamycin (Gibco) [72] with 2% hematocrit. Rings were synchronized with 5% sorbitol at 4 hpi and 20 hpi for two continuous IDC to obtain highly synchronized culture [73].

For proliferation rate, parasitemia at the trophozoite stage, starting from 0.1% synchronized culture was measured every IDC using Giemsa-stained thin blood smear. Invasion rate, calculated as the ratio of daughter merozoite parasitemia versus the starting synchronized schizont parasitemia, measures how many merozoites produced from one schizont can successfully invade RBCs. The number of merozoite was counted in 50 late schizonts under microscopy for each parasite line in biological duplicates.

Creation of transgenic parasite lines

To create transgenic lines with HA-tag in C-terminal endogenous gene, the plasmid was integrated into genome by single crossover recombination. The homology region of PfNOT1.1 and PfNOT4 as a portion of 3' genomic locus up to but not including the stop codon, was PCR-

amplified from T996 genomic DNA and inserted into pTCherryHA plasmid [74] between restriction sites XmaI and BsrGI. The homology region of PfNOT1.2 was inserted into HSP101-HADB plasmid [75] between restriction sites XhoI and EagI to construct pLPfNOT1.2 plasmid. The primers 3HA_F and 3HA_R were annealed by heating at 95°C for 5 min and cooling down at 25°C for one hour. The annealed primer pair was then inserted into pLPfNOT1.2 plasmid between restriction sites XmaI and BglII. The homology region of PfCAF1 was inserted into pTCherryHA plasmid between restriction sites XmaI and NheI.

To disrupt the endogenous *PfNot1.1* by double crossover recombination, two homology regions were inserted into pCC1 plasmid [76] to flank a *hDHFR* cassette between restriction sites SacII and SpeI as well as EcoRI and NcoI to create pCC1-PfNOT1.1KO plasmid. Similarly, pCC1-PfNOT1.2KO plasmid was created to disrupt the endogenous *PfNot1.2*.

To tag the C-terminal endogenous *PfNot1.2* with three HA epitopes and an ecDHFR destabilizing domain, a portion of the 3' genomic locus of *PfNot1.2* up to but not including the stop codon was amplified and inserted into HSP101-HADB plasmid between restriction sites, XhoI and AvrII, to construct pLPfNOT1.2-HA-ecDDD plasmid. The parasite was co-transfected with a plasmid expressing hDHFR in order to confer resistance to toxicity of the stabilizing ligand, TMP. The parental line was also transfected with the plasmid expressing hDHFR.

P. falciparum T996 strain at ring stage was transfected with plasmids by electroporation, established elsewhere [77]. The selection drugs used were 2.5 nM WR99210 (Chemopharma) for the *hDHFR* selective marker, 2.5 µg/ml Blasticidin S (Invitrogen) for the *BSD* selective marker and TMP (Sigma) ligand for the ecDHFR destabilizing domain. Once observing the parasites, the drug cycling was performed to increase the population of parasites with successful integration.

The single clone of parasites was obtained by limiting dilution [78], in which the culture was diluted to a concentration of 0.3 parasite/well in the 96-well plate. The integration of plasmids into the endogenous loci was verified by PCR on genomic DNA extracted using the DNeasy Blood & Tissue Kit (Qiagen).

Immunoprecipitation and mass spectrometry

Saponin-lysed schizont pellet (400 μ l) of the parental line and HA-tagged transgenic lines was lysed with 5 pellet volumes of Lysis Buffer (20 mM HEPES, pH7.8; 10 mM KCl; 1 mM EDTA, pH8.0; 0.65% Nonidet P-40; 1 mM DTT; 1 mM PMSF; 1x protease inhibitor) to obtain cytoplasmic lysate and nucleus pellet, which was then lysed with 2.5 pellet volumes of Extraction Buffer (20 mM HEPES, pH7.8; 400 mM KCl; 1 mM EGTA, pH8.0; 1 mM EDTA, pH8.0; 1 mM DTT; 1 mM PMSF; 1x protease inhibitor) and diluted with 2.5 pellet volumes of Dilution Buffer (20 mM HEPES, pH7.8; 1 mM EDTA, pH8.0; 0.2% Triton X-100; 30% glycerol; 2 mM DTT; 1 mM PMSF; 1x protease inhibitor) to obtain nuclear lysate. Cytoplasmic lysate and nuclear lysate were combined, a portion of which was stored as input lysate and the rest was subjected to immunoprecipitation by HA magnetic bead (Pierce) for 16 hours. Proteins bound on the beads were eluted in 1x Laemmli Buffer supplemented with 0.1 M DTT by boiling at 100°C for 10 min. Prior to mass spectrometry, the efficiency of immunoprecipitation was confirmed by western blotting. The bound proteins in biological duplicates were analyzed by LC-MS/MS mass and the peptides were matched to the PlasmoDB database [79] using Mascot search software with MS tolerance of 10 ppm and MS/MS tolerance of 0.8 Da [80].

Western blotting

To obtain total protein lysate, saponin-lysed schizont pellet was lysed with 4 pellet volumes of Total Lysis Buffer (4% SDS; 0.5% Triton X-100; 50% 1x PBS) by boiling at 100°C for 10 min. HA-tagged proteins were detected using 1:1,000 anti-HA antibody (Roche) and 1:5,000 anti-rat antibody (Abcam). Actin as a loading control for immunoprecipitation blot was detected using anti- β -actin antibody (Sigma) and 1:2,000 anti-mouse antibody (Cell Signaling). Aldolase as a cytoplasmic marker was detected by 1:2,000 anti-aldehyde antibody (Abcam) and 1:2,000 anti-rabbit antibody (GE Health). Histone H3 as a nuclear marker was detected using 1:1,000 anti-histone H3 antibody (Abcam) and 1:2,000 anti-rabbit antibody. The ladder used for proteins larger than 240 kDa was 12 μ l HiMark Pre-Stained Protein Standard (Thermo Scientific). The ladder used for proteins smaller than 240 kDa was 2.5 μ l BLUltra Prestained Protein Ladder (GeneDireX). The secondary antibodies were conjugated with HRP, signals of which was exposed to an X-ray film (Canon) or captured in ImageQuant LAS4000 Chemiluminescent and Fluorescence Imaging System (Fujitsu Life Sciences) and the quantified using ImageJ [81].

Immunofluorescence assays

The parental line and HA-tagged transgenic lines were prepared for immunofluorescence assays as previously described [82]. Asynchronous culture in blood smears were fixed in 4% paraformaldehyde and 0.0075% glutaraldehyde for 30 min, permeabilized in 0.1% Triton X-100 for 10 min, blocked in 3% bovine serum albumin for 1 hour, incubated with 1:100 anti-HA antibody and 1:1,000 anti-aldehyde antibody overnight at 4°C, and eventually incubated with 1:1,000 DAPI (Thermo scientific), 1:1,000 Alexa Fluor 488 goat anti-rat (Thermo scientific) and 1:1,000 Alexa Fluor 555 goat anti-rabbit (Thermo scientific) in dark for 1 hour. Fluorescence

images were taken using a Olympus IX83 Inverted Microscope equipped with a Plan Apo 100x oil objective lens, processed by Metamorph software and merged by ImageJ software.

Transcriptional profiling by microarray

Total RNA isolated by TRIzol/chloroform method, first strand cDNA synthesis and the subsequent SMART amplification of double-stranded cDNA were established elsewhere [83]. For each microarray hybridization, 4 µg sample cDNA was coupled with Cy5-dye and 4 µg reference cDNA, a pooling of 3D7 parasite harvested at 8 timepoints across IDC, was coupled with Cy3-dye. The microarray chip, containing 11,005 probes covering 5,061 genes in *P. falciparum* genome, was scanned by Tecan PowerScanner and oligos with bad signals were flagged for deletion. Background subtraction, normalization and filtering of raw signal intensities were accomplished by the “limma” package in R [84]. Expression of each gene was averaged from the log₂-transformed expression of oligos for the specific gene. The parasite age for each sample was subsequently estimated with respect to a reference IDC transcriptome (T996 strain) based on a Spearman Rank Correlation Coefficients as previously established [85] and modified [86].

Genes showing significantly differential transcript abundance between the transgenic lines and the parental strain were filtered with a fold change cutoff greater than 1.5, $p < 0.05$ (pairwise Student’s t-test). Hierarchical clustering was carried out using Cluster 3.0 [87] and visualized using Java Treeview [88]. Raw microarray data was submitted to the NCBI GEO public database. Gene set enrichment analysis [89] was carried out on genes with differential transcript abundance to find out enriched functional pathways. Correlation of differential transcript

abundance in different parasite lines was assessed based on Spearman Rank Correlation Coefficient.

Quantitative real time PCR

Extracted total RNA was reverse transcribed into cDNA using SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Transcript levels of selected targets and the internal control, serine-tRNA ligase, in 20 ng cDNA were measured by real-time PCR in triplicates using SYBR Select Master Mix (Thermo Scientific). The $\log_2$ -transformed fold-change of transcript abundance ($-\Delta\Delta C_T$) was calculated using the equation:

$$\Delta\Delta C_T = (C_{T \text{ target}} - C_{T \text{ control}})_{\text{transgenic line}} - (C_{T \text{ target}} - C_{T \text{ control}})_{\text{parental line}}$$

Chromatin-immunoprecipitation

At 6 timepoints across one IDC, 1.5 ml iRBCs with 5% parasitemia were crosslinked with 0.1% formaldehyde and chromatin immunoprecipitation was performed as previously described [83]. Briefly, parasite was lysed with Buffer A (1 mM EDTA, pH8.0; 0.25% NP40; 1x protease inhibitor; 2 mM PMSF) and the resulting nuclei pellet was lysed with SDS Lysis Buffer (1% SDS; 50 mM Tris, pH8.1; 10 mM EDTA; 1x protease inhibitor; 2 mM PMSF), sonicated into 200-1,000 bp DNA fragments and diluted with ChIP Dilution Buffer (0.01% SDS; 1.1% Triton X-100; 16.7 mM Tris, pH8.1; 1.2 mM EDTA; 167 mM NaCl; 1x protease inhibitor; 2 mM PMSF). A portion of chromatin DNA was stored for input DNA and the rest was incubated with protein A agarose /salmon DNA beads for 2 hours aiming to reduce unspecific binding and then incubated with anti-Ser2/5-P antibody for 16 hours. The antibody:protein was immunoprecipitated by pre-blocked protein A agarose/salmon DNA beads and eluted. The beads were washed twice with Low Salt Wash

Buffer (0.1% SDS; 2 mM EDTA; 20 mM Tris-Cl, Ph8.1; 150 mM NaCl), thrice times with High Salt Wash Buffer (0.1% SDS; 2 mM EDTA; 20 mM Tris-Cl, Ph8.1; 500 mM NaCl), once with LiCl Wash Buffer (0.25 M LiCl; 1% NP-40; 1% deoxycholic acid; 1 mM EDTA; 10 mM Tris-Cl, pH8.1) and twice with TE Buffer (10 mM Tris-Cl, pH8.1; 1 mM EDTA). The antibody:protein was eluted in Elution Buffer (1% SDS; 0.1 M sodium bicarbonate). ChIP DNA and input DNA were reverse-crosslinked, amplified and hybridized on microarray chip in biological triplicates. A fold-change cutoff more than 1.5, $p < 0.05$ (pairwise Student's t-test) was employed to filter genes with significantly differential RPB1 recruitment.

mRNA decay assay

To stop transcription, the synchronized Δ PfNot1.1 and the parental strain at 24 hpi was incubated in 20 μ g/ml actD (Thermo Scientific) or DMSO for 4 hours, as established previously [57]. For each parasite line, the initial sample at 24 hpi, the actD-treated sample at 28 hpi and the actD-untreated sample at 28 hpi were harvested in biological replicates. Transcriptional profile of each sample was assessed by microarray as described above. Transcript abundance is represented as the cy5-channel reading of 11,005 microarray oligo, which was normalized to the microarray scanning intensity. For each parasite line within the 4-hour incubation, the amount of differential transcript abundance, decayed transcript and newly-synthesized transcript were calculated. Changes in these parameters were compared between Δ PfNot1.1 and the parental strain.

Primer list

591 The primers are in the 5'- to 3'- orientation. The 21-bp extension for ligation-independent
592 cloning, described elsewhere [90] is indicated in the small case.

593 pTPfNOT1.1-3HA_HR_F: aacaaatagatctccccgggTTCATAACTCTTCACAATCACATC

594 pTPfNOT1.1-3HA_HR_R: gtacgggtaaagctgtacagTTCTTTAAATGTTAAATTTTTTTTTTCTGG

595 pTPfNOT4-3HA_HR_F: aacaaatagatctccccgggAGATGATGAAGATGAAGAAGATGAC

596 pTPfNOT4-3HA_HR_R: gtacgggtaaagctgtacagTGCACTTAAATTTAGCTTCTCATTC

597 pLPfNOT1.2-3HA_HR_F: gacactatagaactcgagTTCATGGGTGAACCTATATCATC

598 pLPfNOT1.2-3HA_HR_R: ttatataactcgacgcggccgagatctcagtcgccgggAACGCGCCCTAGGCATGCTTG

599 3HA_F:

600 CCGGGTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGC

601 TTGAA

602 3HA_R:

603 GATCTTCAAGCGTAATCTGGAACATCGTATGGGTAAGCGTAATCTGGAACATCGTATGGGTAAGCGTAATCTGGAACATCGTA

604 TGGGTAC

605 pTPfCAF1-3HA_HR_F: atctccccgggAATGAAGGAATTATTATGCATG

606 pTPfCAF1-3HA_HR_R: gccatgctagcGTTATCATAAAAATATTATGGTCCTTAG

607 pCC1-PfNOT1.1KO_HR1_F: tccaatggcccctttccgcggGATCGAAAAATAATGATGTG

608 pCC1-PfNOT1.1KO_HR1_R: aagacagatcttcgtactagtTTTGGTACTTGTGTTGATA

609 pCC1-PfNOT1.1KO_HR2_F: tatttattaaatctagaattcCTCATCCATAATTATAGGAGAAG

610 pCC1-PfNOT1.1KO_HR2_R: gccagcctaggagtccatggAGTTCCTCTAATTTCTGACTATC

611 pCC1-PfNOT1.2KO_HR1_F: tccaatggcccctttccgcggGAGGATGGATTACCATGA

612 pCC1-PfNOT1.2KO_HR1_R: aagacagatcttcgtactagtGAATGTGTTCAATTTCACTT

613 pCC1-PfNOT1.2KO_HR2_F: tatttattaaatctagaattcGATATGGTAGATACAAATGGAG

614 pCC1-PfNOT1.2KO_HR2_R: gccagcctaggagttccatggCTTATGTGATCTTGATTCTTC

615 pLPfNOT1.2-3HA-ecDDD_HR_F: gacactatagaactcgagTTCATGGGTTGAACTTATATCATC

616 pLPfNOT1.2-3HA-ecDDD_HR_R: aacatcgtatgggtacctaggAACGCGCCCTAGGCATGCTTG

617 PfNOT1.1-HA_1: ATATATAGGAATGTCATTACCATC

618 PfNOT1.1-HA_2: GTGTATATATAAACACAAGATGTG

619 PfNOT1.1-HA_3: TAATATAGAAAAAGTTACATGCACG

620 PfNOT1.1-HA_4: CCTAAGTCATTAAGGTACTAACTTAG

621 PfNOT1.2-HA_1: CTTTATTATCACCTTTAAGAATTCCTG

622 PfNOT1.2-HA_2: GTTAAATATTTAATTAGGAAAGGATAACATG

623 PfNOT1.2-HA_3: GTATATTGGGGTGATGATAAAATGAAAG

624 PfNOT1.2-HA_4: AGTGCCACCTGACGTCTAAGAAAC

625 PfCAF1-HA_1: GAGGACCAACAAGAAGTAGATC

626 PfCAF1-HA_2: CATATACATTATTATGTGTGAGTTC

627 PfCAF1-HA_3: TAATATAGAAAAAGTTACATGCACG

628 PfCAF1-HA_4: CCTAAGTCATTAAGGTACTAACTTAG

629 PfNOT4-HA_1: ATGATAATGAAGAAGATCAAGAG

630 PfNOT4-HA_2: ATATGTCACCTTTGTTTTGTTTC

631 PfNOT4-HA_3: TAATATAGAAAAAGTTACATGCACG

632 PfNOT4-HA_4: CCTAAGTCATTAAGGTACTAACTTAG

633 PfNOT1.1KO_5: ATGTACAAATGGATGAAGAATCTG

634 PfNOT1.1KO_6: ATATTCTTGACATGTTCTTTATGC

635 PfNOT1.1KO_7: TTACCAAATAATCTGAACGCAAAC

636 PfNOT1.1KO_8: AGATTCTATAGTATTTAAGGATGGTTG

637 PfNOT1.1KO_9: TTTTTCTTTTATCATGCACATTGG

638 PfNOT1.1KO_10: GAAGTATATGAGAAGAATGATTAAGC

639 PfNOT1.1KO_11: TATATCCAATGGCCCCCTTC

640 PfNOT1.1KO_12: GCGGTGTGAAATACCGCAC

641 PfNOT1.2KO_5: GAAGAAAAGAAAAAGCAAGGAG

642 PfNOT1.2KO_6: TTTTATTCGTCACACTATCATTTG

643 PfNOT1.2KO_7: ATATGAACAGTATCAACAATATGAAC

644 PfNOT1.2KO_8: TGATTCATATGTTTTTCATCTTCTAC

645 PfNOT1.2KO_9: CAAAATGCTTAAGACAGATCTTC

646 PfNOT1.2KO_10: CTTTTACAATATGAACATAAAGTACAAC

647 PfNOT1.2KO_11: TATATCCAATGGCCCCCTTC

648 PfNOT1.2KO_12: GCGGTGTGAAATACCGCAC

649 PfNOT1.2-HA-ecDDD_13: CTTTTATTATCACCTTTAAGAATTCCTG

650 PfNOT1.2-HA-ecDDD_14: GTTAAATATTTAATTAGGAAAGGATAACATG

651 PfNOT1.2-HA-ecDDD_15: TTTCCATGCCGATAACGTGATCTAC

652 PfNOT1.2-HA-ecDDD_16: AGTGCCACCTGACGTCTAAGAAAC

653 The reverse primer contains the promoter sequence of T7 RNA polymerase for in-vitro
654 transcription, shown in small cases.

655 PfNOT1.1_probe 1_F: ACCAGTAGCTGTCGATAGAGC

656 PfNOT1.1_probe 1_R: actgactgtaatacagactcactatagggACGTAATGGTTCTTGCATGTAGC

657 PfNOT1.1_probe 2_F: CATCAGAAACAAATATCATCGATCC

658 PfNOT1.1_probe 2_F: actgactgtaatacagactcactatagggGTGTATATATAAACACAAGATGTG

659 PfNOT1.1_probe control_F: TGGAAGAATGTGCCGAACCTTG

660 PfNOT1.1_probe control_R: actgactgtaatacagactcactatagggGCACATAATGGTTGTTCTGAGGT

661 **Data availability**

662 Microarray data is available in GEO database: transcriptional profiling of Δ PfNot1.1 and
663 $\Delta\Delta$ PfNot1.2 (GSE142275); recruitment of RNAPII in Δ PfNot1.1 (GSE142276); mRNA decay
664 analysis in Δ PfNot1.1 (GSE142527). All data has been deposited in two datasets in Mendeley
665 Data Repository (DOI: 10.17632/gnytrrsjcn.1).

666

667 **Acknowledgment**

668 Y. L. is a recipient of Nanyang Graduate Scholarship from Nanyang Technological University,
669 Singapore. This work was supported by an AcRF Tier 1 grant from the Singapore Ministry of
670 Education (#RG41/09) and grants from the Singapore National Medical Research Council
671 (#NMRC/CBRG/0029/2013; CBRG12nov104) to MF; and AcRF Tier 2 grant from the Singapore
672 Ministry of Education (#MOE2017-T2-2-030 (S)) and Singapore National Medical Research
673 Council (#NMRC/OFIRG/0040/2017) to ZB. We would like to thank Brendan Crabb (pTCherryHA),

Alan Cowman (pCC1) and Daniel Goldberg (HSP101-HADB) labs for sharing plasmid vectors. We also thank Siu Kwan Sze lab for performing mass spectrometry.

Author Contribution

Conceptualization: Y.L., Z.B. and M.F.; Formal Analysis: Y.L.; Funding Acquisition: Z.B. and M.F.; Investigation: Y.L.; Methodology: Y.L. and R.R.; Project Administration: C.Q.Z. and F.R.; Software: L.Z.; Supervision: Z.B. and M.F.; Writing – Original Draft Preparation: Y.L.; Writing – Review & Editing: Y.L., Z.B. and M.F..

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of Interests

The authors declare no competing interests.

References

1. WHO, *World malaria report*. World health organization, Geneva, Switzerland, 2017.
2. Pasvol, G., *Protective hemoglobinopathies and Plasmodium falciparum transmission*. Nature Genetics, 2010. **42**: p. 284.
3. Cowman, A.F. and B.S. Crabb, *Invasion of Red Blood Cells by Malaria Parasites*. Cell, 2006. **124**(4): p. 755-766.
4. Bozdech, Z., et al., *The Transcriptome of the Intraerythrocytic Developmental Cycle of Plasmodium falciparum*. PLOS Biology, 2003. **1**(1): p. e5.
5. Lanzer, M., et al., *Plasmodium: Control of Gene Expression in Malaria Parasites*. Experimental Parasitology, 1993. **77**(1): p. 121-128.

- 698 6. Callebaut, I., et al., *Prediction of the general transcription factors associated with RNA*
699 *polymerase II in Plasmodium falciparum: conserved features and differences relative to other*
700 *eukaryotes*. BMC genomics, 2005. **6**: p. 100-100.
- 701 7. Coulson, R.M.R., N. Hall, and C.A. Ouzounis, *Comparative Genomics of Transcriptional Control in*
702 *the Human Malaria Parasite Plasmodium falciparum*. Genome Research, 2004. **14**(8): p. 1548-
703 1554.
- 704 8. Campbell, T.L., et al., *Identification and Genome-Wide Prediction of DNA Binding Specificities for*
705 *the ApiAP2 Family of Regulators from the Malaria Parasite*. PLOS Pathogens, 2010. **6**(10): p.
706 e1001165.
- 707 9. De Silva, E.K., et al., *Specific DNA-binding by Apicomplexan AP2 transcription factors*.
708 Proceedings of the National Academy of Sciences, 2008. **105**(24): p. 8393-8398.
- 709 10. Rai, R., et al., *Genome-wide analysis in Plasmodium falciparum reveals early and late phases of*
710 *RNA polymerase II occupancy during the infectious cycle*. BMC genomics, 2014. **15**(1): p. 959-959.
- 711 11. Gopalakrishnan, A.M., et al., *Plasmodium falciparum: Preinitiation complex occupancy of active*
712 *and inactive promoters during erythrocytic stage*. Experimental Parasitology, 2009. **121**(1): p. 46-
713 54.
- 714 12. Sims, J.S., et al., *Patterns of gene-specific and total transcriptional activity during the*
715 *Plasmodium falciparum intraerythrocytic developmental cycle*. Eukaryotic cell, 2009. **8**(3): p.
716 327-338.
- 717 13. Reddy, B.P.N., et al., *A bioinformatic survey of RNA-binding proteins in Plasmodium*. BMC
718 genomics, 2015. **16**: p. 890-890.
- 719 14. Ay, F., et al., *Three-dimensional modeling of the P. falciparum genome during the erythrocytic*
720 *cycle reveals a strong connection between genome architecture and gene expression*. Genome
721 Research, 2014.
- 722 15. Hughes, K.R., et al., *From cradle to grave: RNA biology in malaria parasites*. Wiley
723 Interdisciplinary Reviews: RNA, 2010. **1**(2): p. 287-303.
- 724 16. Vembar, S.S., et al., *The PfAlba1 RNA-binding protein is an important regulator of translational*
725 *timing in Plasmodium falciparum blood stages*. Genome Biology, 2015. **16**: p. 212.
- 726 17. Balu, B., et al., *CCR4-associated factor 1 coordinates the expression of Plasmodium falciparum*
727 *egress and invasion proteins*. Eukaryotic cell, 2011. **10**(9): p. 1257-1263.
- 728 18. Hart, K.J., et al., *Plasmodium male gametocyte development and transmission are critically*
729 *regulated by the two putative deadenylases of the CAF1/CCR4/NOT complex*. PLoS pathogens,
730 2019. **15**(1): p. e1007164-e1007164.
- 731 19. Muñoz, E.E., et al., *ALBA4 modulates its stage-specific interactions and specific mRNA fates*
732 *during Plasmodium yoelii growth and transmission*. Molecular microbiology, 2017. **106**(2): p.
733 266-284.
- 734 20. Painter, H.J., et al., *Genome-wide real-time in vivo transcriptional dynamics during Plasmodium*
735 *falciparum blood-stage development*. Nature Communications, 2018. **9**(1): p. 2656.
- 736 21. Collart, M.A. and O.O. Panasenko, *The Ccr4-Not complex*. Gene, 2012. **492**(1): p. 42-53.
- 737 22. Collart, M.A. and K. Struhl, *CDC39, an essential nuclear protein that negatively regulates*
738 *transcription and differentially affects the constitutive and inducible HIS3 promoters*. The EMBO
739 journal, 1993. **12**(1): p. 177-186.
- 740 23. Tucker, M., et al., *The Transcription Factor Associated Ccr4 and Caf1 Proteins Are Components of*
741 *the Major Cytoplasmic mRNA Deadenylase in Saccharomyces cerevisiae*. Cell, 2001. **104**(3): p.
742 377-386.
- 743 24. Xu, K., et al., *Insights into the structure and architecture of the CCR4-NOT complex*. Frontiers in
744 genetics, 2014. **5**: p. 137-137.

- 745 25. Mauxion, F., B. Prève, and B. Séraphin, *C2ORF29/CNOT11 and CNOT10 form a new module of*
746 *the CCR4-NOT complex*. RNA biology, 2013. **10**(2): p. 267-276.
- 747 26. Bawankar, P., et al., *NOT10 and C2orf29/NOT11 form a conserved module of the CCR4-NOT*
748 *complex that docks onto the NOT1 N-terminal domain*. RNA biology, 2013. **10**(2): p. 228-244.
- 749 27. Basquin, J., et al., *Architecture of the Nuclease Module of the Yeast Ccr4-Not Complex: the Not1-*
750 *Caf1-Ccr4 Interaction*. Molecular Cell, 2012. **48**(2): p. 207-218.
- 751 28. Petit, A.-P., et al., *The structural basis for the interaction between the CAF1 nuclease and the*
752 *NOT1 scaffold of the human CCR4-NOT deadenylase complex*. Nucleic acids research, 2012.
753 **40**(21): p. 11058-11072.
- 754 29. Collart, M.A., *The Ccr4-Not complex is a key regulator of eukaryotic gene expression*. Wiley
755 interdisciplinary reviews. RNA, 2016. **7**(4): p. 438-454.
- 756 30. Sgromo, A., et al., *A CAF40-binding motif facilitates recruitment of the CCR4-NOT complex to*
757 *mRNAs targeted by Drosophila Roquin*. Nature Communications, 2017. **8**: p. 14307.
- 758 31. Bhaskar, V., et al., *Structure and RNA-binding properties of the Not1–Not2–Not5 module of the*
759 *yeast Ccr4–Not complex*. Nature Structural & Molecular Biology, 2013. **20**: p. 1281.
- 760 32. Villanyi, Z., et al., *The Not5 subunit of the ccr4-not complex connects transcription and*
761 *translation*. PLoS genetics, 2014. **10**(10): p. e1004569-e1004569.
- 762 33. Venturini, G., et al., *CNOT3 is a modifier of PRPF31 mutations in retinitis pigmentosa with*
763 *incomplete penetrance*. PLoS genetics, 2012. **8**(11): p. e1003040-e1003040.
- 764 34. Peng, W., et al., *Regulators of cellular levels of histone acetylation in Saccharomyces cerevisiae*.
765 Genetics, 2008. **179**(1): p. 277-289.
- 766 35. Mulder, K.W., et al., *Regulation of histone H3K4 tri-methylation and PAF complex recruitment by*
767 *the Ccr4-Not complex*. Nucleic acids research, 2007. **35**(7): p. 2428-2439.
- 768 36. Albert, T.K., et al., *Identification of a ubiquitin-protein ligase subunit within the CCR4-NOT*
769 *transcription repressor complex*. The EMBO journal, 2002. **21**(3): p. 355-364.
- 770 37. Panasenko, O., et al., *The Yeast Ccr4-Not Complex Controls Ubiquitination of the Nascent-*
771 *associated Polypeptide (NAC-EGD) Complex*. Journal of Biological Chemistry, 2006. **281**(42): p.
772 31389-31398.
- 773 38. Panasenko, O.O. and M.A. Collart, *Not4 E3 ligase contributes to proteasome assembly and*
774 *functional integrity in part through Ecm29*. Molecular and cellular biology, 2011. **31**(8): p. 1610-
775 1623.
- 776 39. Jiang, N., et al., *Identification and characterization of DNA endonucleases in Plasmodium*
777 *falciparum 3D7 clone*. Malaria Journal, 2018. **17**(1): p. 232.
- 778 40. Liu, H.Y., et al., *The NOT proteins are part of the CCR4 transcriptional complex and affect gene*
779 *expression both positively and negatively*. The EMBO journal, 1998. **17**(4): p. 1096-1106.
- 780 41. Lee, T.I., et al., *Interplay of Positive and Negative Regulators in Transcription Initiation by RNA*
781 *Polymerase II Holoenzyme*. Molecular and Cellular Biology, 1998. **18**(8): p. 4455-4462.
- 782 42. Deluen, C., et al., *The Ccr4-not complex and yTAF1 (yTaf(II)130p/yTaf(II)145p) show physical and*
783 *functional interactions*. Molecular and cellular biology, 2002. **22**(19): p. 6735-6749.
- 784 43. Kruk, J.A., et al., *The multifunctional Ccr4-Not complex directly promotes transcription*
785 *elongation*. Genes & development, 2011. **25**(6): p. 581-593.
- 786 44. Babbarwal, V., J. Fu, and J.C. Reese, *The Rpb4/7 module of RNA polymerase II is required for*
787 *carbon catabolite repressor protein 4-negative on TATA (Ccr4-not) complex to promote*
788 *elongation*. The Journal of biological chemistry, 2014. **289**(48): p. 33125-33130.
- 789 45. Sandler, H., et al., *Not1 mediates recruitment of the deadenylase Caf1 to mRNAs targeted for*
790 *degradation by tristetraprolin*. Nucleic acids research, 2011. **39**(10): p. 4373-4386.

46. Nishimura, T., et al., *The eIF4E-Binding Protein 4E-T Is a Component of the mRNA Decay Machinery that Bridges the 5' and 3' Termini of Target mRNAs*. Cell Reports, 2015. **11**(9): p. 1425-1436.
47. Waghray, S., et al., *Xenopus CAF1 requires NOT1-mediated interaction with 4E-T to repress translation in vivo*. RNA (New York, N.Y.), 2015. **21**(7): p. 1335-1345.
48. Du, H., et al., *YTHDF2 destabilizes m6A-containing RNA through direct recruitment of the CCR4–NOT deadenylase complex*. Nature Communications, 2016. **7**: p. 12626.
49. Suzuki, A., et al., *NANOS2 interacts with the CCR4–NOT deadenylation complex and leads to suppression of specific RNAs*. Proceedings of the National Academy of Sciences, 2010. **107**(8): p. 3594-3599.
50. Gardner, M.J., et al., *Genome sequence of the human malaria parasite Plasmodium falciparum*. Nature, 2002. **419**(6906): p. 498-511.
51. Aravind, L., et al., *Plasmodium Biology: Genomic Gleanings*. Cell, 2003. **115**(7): p. 771-785.
52. Temme, C., et al., *Subunits of the Drosophila CCR4–NOT complex and their roles in mRNA deadenylation*. RNA (New York, N.Y.), 2010. **16**(7): p. 1356-1370.
53. Lau, N.-C., et al., *Human Ccr4–Not complexes contain variable deadenylase subunits*. Biochemical Journal, 2009. **422**(3): p. 443-453.
54. Maier, A.G., et al., *Exported proteins required for virulence and rigidity of Plasmodium falciparum-infected human erythrocytes*. Cell, 2008. **134**(1): p. 48-61.
55. Mundwiler-Pachlatko, E. and H.-P. Beck, *Maurer's clefts, the enigma of Plasmodium falciparum*. Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(50): p. 19987-19994.
56. Cho, E.-J., *RNA polymerase II carboxy-terminal domain with multiple connections*. Experimental & Molecular Medicine, 2007. **39**: p. 247.
57. Shock, J.L., K.F. Fischer, and J.L. DeRisi, *Whole-genome analysis of mRNA decay in Plasmodium falciparum reveals a global lengthening of mRNA half-life during the intra-erythrocytic development cycle*. Genome Biology, 2007. **8**(7): p. R134.
58. Bai, Y., et al., *The CCR4 and CAF1 Proteins of the CCR4–NOT Complex Are Physically and Functionally Separated from NOT2, NOT4, and NOT5*. Molecular and Cellular Biology, 1999. **19**(10): p. 6642-6651.
59. Temme, C., M. Simonelig, and E. Wahle, *Deadenylation of mRNA by the CCR4–NOT complex in Drosophila: molecular and developmental aspects*. Frontiers in genetics, 2014. **5**: p. 143-143.
60. Schwede, A., et al., *A role for Caf1 in mRNA deadenylation and decay in trypanosomes and human cells*. Nucleic acids research, 2008. **36**(10): p. 3374-3388.
61. Maillet, L., et al., *The essential function of not1 lies within the Ccr4-not complex*¹¹Edited by J. Karn. Journal of Molecular Biology, 2000. **303**(2): p. 131-143.
62. Ito, K., et al., *The role of the CNOT1 subunit of the CCR4–NOT complex in mRNA deadenylation and cell viability*. Protein & cell, 2011. **2**(9): p. 755-763.
63. Bushell, E., et al., *Functional Profiling of a Plasmodium Genome Reveals an Abundance of Essential Genes*. Cell, 2017. **170**(2): p. 260-272.e8.
64. Zhang, M., et al., *Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis*. Science (New York, N.Y.), 2018. **360**(6388): p. eaap7847.
65. Körner, C.G., et al., *The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of Xenopus oocytes*. The EMBO Journal, 1998. **17**(18): p. 5427-5437.
66. Parker, R. and H. Song, *The enzymes and control of eukaryotic mRNA turnover*. Nature Structural & Molecular Biology, 2004. **11**(2): p. 121-127.
67. Tucker, M., et al., *Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae*. The EMBO Journal, 2002. **21**(6): p. 1427-1436.

68. Goldstrohm, A.C., et al., *PUF Protein-mediated Deadenylation Is Catalyzed by Ccr4p*. Journal of Biological Chemistry, 2007. **282**(1): p. 109-114.
69. Viswanathan, P., et al., *Mouse CAF1 Can Function As a Processive Deadenylase/3' - 5' - Exonuclease in Vitro but in Yeast the Deadenylation Function of CAF1 Is Not Required for mRNA Poly(A) Removal*. Journal of Biological Chemistry, 2004. **279**(23): p. 23988-23995.
70. Molin, L. and A. Puisieux, *C. elegans* homologue of the Caf1 gene, which encodes a subunit of the CCR4-NOT complex, is essential for embryonic and larval development and for meiotic progression. Gene, 2005. **358**: p. 73-81.
71. Temme, C., et al., *A complex containing the CCR4 and CAF1 proteins is involved in mRNA deadenylation in Drosophila*. The EMBO journal, 2004. **23**(14): p. 2862-2871.
72. Cranmer, S.L., et al., *An alternative to serum for cultivation of Plasmodium falciparum in vitro*. Transactions of the Royal Society of Tropical Medicine and Hygiene, 1997. **91**(3): p. 363-365.
73. Lambros, C. and J.P. Vanderberg, *Synchronization of Plasmodium falciparum Erythrocytic Stages in Culture*. The Journal of Parasitology, 1979. **65**(3): p. 418-420.
74. Meissner, M., et al., *Tetracycline analogue-regulated transgene expression in Plasmodium falciparum blood stages using Toxoplasma gondii transactivators*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(8): p. 2980-2985.
75. Beck, J.R., et al., *PTEX component HSP101 mediates export of diverse malaria effectors into host erythrocytes*. Nature, 2014. **511**(7511): p. 592-5.
76. Maier, A.G., et al., *Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination*. Mol Biochem Parasitol, 2006. **150**(1): p. 118-21.
77. Wu, Y., et al., *Transfection of Plasmodium falciparum within human red blood cells*. Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(4): p. 973-977.
78. Trager, W., et al., *Clones of the malaria parasite Plasmodium falciparum obtained by microscopic selection: their characterization with regard to knobs, chloroquine sensitivity, and formation of gametocytes*. Proceedings of the National Academy of Sciences of the United States of America, 1981. **78**(10): p. 6527-6530.
79. Aurrecochea, C., et al., *PlasmoDB: a functional genomic database for malaria parasites*. Nucleic acids research, 2009. **37**(Database issue): p. D539-D543.
80. Perkins, D.N., et al., *Probability-based protein identification by searching sequence databases using mass spectrometry data*. ELECTROPHORESIS, 1999. **20**(18): p. 3551-3567.
81. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis*. Nature methods, 2012. **9**(7): p. 671-675.
82. Chêne, A., et al., *PfAlbas constitute a new eukaryotic DNA/RNA-binding protein family in malaria parasites*. Nucleic Acids Research, 2012. **40**(7): p. 3066-3077.
83. Bozdech, Z., S. Mok, and A. Patkar, *DNA Microarray-Based Genome-Wide Analyses of Plasmodium Parasites*. Vol. 923. 2013. 189-211.
84. Smyth, G.K., *Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments*. Vol. 3. 2004. Article 3.
85. Lemieux, J.E., et al., *Statistical estimation of cell-cycle progression and lineage commitment in Plasmodium falciparum reveals a homogeneous pattern of transcription in ex vivo culture*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(18): p. 7559-7564.
86. Mok, S., et al., *Artemisinin resistance in Plasmodium falciparum is associated with an altered temporal pattern of transcription*. BMC genomics, 2011. **12**: p. 391-391.
87. de Hoon, M.J.L., et al., *Open source clustering software*. Bioinformatics, 2004. **20**(9): p. 1453-1454.

88. Saldanha, A.J., *Java Treeview—extensible visualization of microarray data*. Bioinformatics, 2004. **20**(17): p. 3246-3248.
89. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(43): p. 15545-15550.
90. Jeong, J.Y., et al., *One-step sequence- and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies*. Appl Environ Microbiol, 2012. **78**(15): p. 5440-3.

Figure captions and legends

Figure 1. Four conserved CCR4-NOT subunits are localized in the cytoplasm. Expression of HA-tagged PfNOT1.1 (**A**), PfNOT1.2 (**B**), PfCAF1 (**C**) and PfNOT4 (**D**) was tested by western blotting using anti-HA antibody of total lysates, cytoplasmic lysates (Cyto) scored by aldolase and nuclear lysates (Nuc) scored by histone H3. The location of the full-length protein is indicated by arrows. Four proteins were detected predominantly in the cytoplasmic lysates. (**E-H**) Four studied proteins were visualized as cytosolic puncta by the immunofluorescent assay using anti-HA antibody at the ring, trophozoite and schizont stages. Nuclei were stained with DAPI and aldolase immunoreactivity was used as a cytoplasmic maker. Scale bars are 1 micron. Immunofluorescent assay on the parental strain served as a negative control (Fig. S2F in supplemental figures). (**I-K**) Immunoprecipitation of HA-tagged PfNOT1.1, PfNOT1.2, PfCAF1 and PfNOT4 was verified by western blotting. HA-tagged proteins were detected in the input and eluent but not the unbound supernatant of each HA-tagged transgenic line by anti-HA antibody. Immunoprecipitation of the parental strain lysate (par) served as a negative control.

Comparable signals of actin verified similar loading of each parasite sample. The sizes of the ladder are given at the left.

Figure 2. Targeted disruption of *PfNot1.1* or *PfNot1.2* impairs parasite proliferation and invasion. (A) The endogenous *PfNot1.1* was disrupted by the *hDHFR* cassette by double crossover recombination. (B) Northern hybridization analysis detected *PfNot1.1* transcript in the parental strain but not Δ PfNot1.1 at the ring (R), trophozoite (T) and schizont (S) stages with the probe shown as red boxes in Fig. 1A. The loading control is Serine-tRNA ligase transcript. (C) HA epitopes and an ecDDD destabilizing domain were fused to PfNOT1.2 in the C-terminus by single crossover recombination. (D) Expression of tagged PfNOT1.2 was detected by western blotting in biological triplicates. Intensities were quantified by ImageJ and normalized to actin expression level and then to the expression level in 10 μ M TMP, the concentration employed to maintain the culture. Comparable protein intensities for different concentrations of TMP ligand indicates that 2 μ M TMP is sufficient to stabilize the ecDDD. Tagged PfNOT1.2 was conditionally knocked down significantly after removing TMP for three IDCs ($p < 0.05$). (E, F) Parasitemia was counted in synchronized cultures with biological triplicates across three IDCs. Parasite proliferation rate of Δ PfNot1.1 and dd Δ PfNot1.2 was significantly lower compared to that of the parental strain. (G, H) Two transgenic lines showed significantly lower invasion rate compared to that in the parental strain in biological triplicates. All data are represented as mean $\pm$ standard deviation from three biological triplicates and statistic test was performed with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (**).

Figure 3. PfNOT1.1 and PfNOT1.2 have opposing effects on transcript abundance. (A) Left panel. A total of 782 genes display significantly differential mRNA abundance (> 1.5 fold-change, $p < 0.05$) in either Δ PfNot1.1 or dd Δ PfNot1.2 at six timepoints, representing early ring (ER, 4 hpi), middle ring (MR, 12 hpi), late ring/early trophozoite (ET, 20 hpi), late trophozoite (LT, 28 hpi), early schizont (ES, 36 hpi) and late schizont (LS, 44 hpi). A hierarchical clustering of $\log_2$ -transformed ratios of mRNA abundance in the transgenic lines to that in the parental strain is shown in left panel with 6 clusters categorized based on the timepoint of drastic changes in mRNA abundance. The scale bar at the bottom shows increased abundance in red and decreased abundance in green in the transgenic lines. **Middle panel.** In each cluster, average mean-centered expression of genes at each timepoint was calculated in the parental strain, Δ PfNot1.1 and dd Δ PfNot1.2 and then plotted across the IDC. **Right panel.** The enriched GO/MPM pathways in each cluster are listed. **(B)** Spearman coefficient between Δ PfNot1.1 and dd Δ PfNot1.2 at each timepoint indicates significant negative correlation at the trophozoite and schizont stages. **(C)** Disruption of *PfNot1.1* or *PfNot1.2* altered mRNA abundance of 715 genes and 202 genes, respectively with a significant overlap of 135 genes ($p < 0.001$). **(D)** qRT-PCR analysis was performed on random genes at several timepoints in biological triplicates and data are represented as mean $\pm$ standard deviation. Changes in transcript abundance were consistent with those assessed by microarray analysis, verifying the accuracy of genome-wide microarray.

Figure 4. PfNOT1.1 and PfNOT1.2 regulate gene expression post-transcriptionally. (A) A total of 150 genes showing significantly differential RNAPII recruitment in Δ PfNot1.1, 17 of which displays significant changes in mRNA abundance (Binomial $p > 0.05$). **(B)** Spearman correlation coefficient calculated between differential mRNA abundance in three transgenic lines demonstrates a positive correlation between Δ PfNot1.1 and Δ PfCaf1 as well as a negative correlation between Δ PfNot1.2 and Δ PfCaf1. **(C)** In Δ PfNot1.1 and the parental strain, mRNA decay was assessed using actD or DMSO vehicle followed by microarray analysis. Transcript abundance for each microarray oligo was derived from microarray readings. **(D)** In the presence of actD, transcription inhibition was validated by lower transcript abundance in the actD-treated parasite compared to that in the actD-untreated parasite for both Δ PfNot1.1 and the parental strain. Changes in decayed transcript abundance **(E)** and changes in newly-synthesized transcript abundance **(F)** are plotted against changes in differential transcript abundance between Δ PfNot1.1 and the parental strain within the 4-hour incubation. Changes in transcript abundance are highly correlated to changes in decayed transcript abundance, suggesting that mRNA decay is the key factor that leads to altered transcript abundance in Δ PfNot1.1. **(G)** Proposed mechanism of PfNOT1.1- and PfNOT1.2-mediated gene regulation. PfNOT1.1 and PfNOT1.2 compete to be the scaffold for PfCAF1 to form either a PfNOT1.1-CAF1 complex or PfNOT1.2-CAF1 complex. The two forms function oppositely in regulating mRNA abundance, possibly by controlling PfCAF1 function in mRNA decay. Genes being regulated in this manner are involved in merozoite egress, merozoite invasion, parasite virulence and other cellular processes.

Supporting information captions

Figure S1. Conserved domains of the NOT1 orthologues in different organisms. (A) The length and location of four conserved domains, including DUF2363, MIF4G, DUF3819 and NOT1, are proportional to the length of protein sequence. **(B)** The conserved domains were pair-wise blasted using the NCBI BLASTp program. The lengths and the similarities of the identified regions were calculated. Due to the insertions in the MIF4G domain and the NOT1 domain of PfNOT1.2, two parts were blasted separately.

Figure S2. The CCR4-NOT subunits were successfully tagged with HA epitopes. (A) The endogenous gene was fused with three HA epitopes by single crossover recombination. **(B-E)** The integration of plasmids was verified by genotyping PCR. Parental loci (par) were detected in the parental lines but not in the transgenic lines, confirming the cloned parasites does not contain contamination of the parental lines. Integrated loci (int) and episomal loci (epi) were detected in the transgenic lines. **(F)** The parental strain was stained with anti-HA antibody, serving as a negative control of the immunofluorescent assay. Nuclei were stained with DAPI and aldolase immunoreactivity was used as a cytoplasmic maker. No signals were detected by anti-HA antibody, confirming that the detected signals in **Fig. 1E-H** are specific. Scale bars are 1 micron.

Figure S3. Genotyping PCR and additional phenotypes of Δ PfNot1.1 and dd Δ PfNot1.2. Genotyping PCR was performed to verify the integration of the plasmids into the genome in the

(A) *PfNot1.1* knockout transgenic line, (B) *PfNot1.2* knockout transgenic line and (C) *PfNot1.2* conditional knockdown transgenic line. Detected genomic loci include parental locus (par, par1 and par2 flanking two homology regions, respectively), integrated locus (int, int1 and int2) and episomal locus (epi, epi1 and epi2). (D) Representative western blotting of HA-ecDDD-tagged PfNOT1.2 displayed reduction of protein expression level after removing TMP for three cycles. The failure to detect the full-length NOT1.2-HA-ecDDD protein at 542 kDa is possibly due to the incomplete stabilization of the fusion protein by TMP. The sizes of the ladder are given at the left. (E, F) The number of merozoite in each schizont, counted in 50 iRBCs in biological duplicates, is comparable between the transgenic lines and the parental strain. Data are represented as mean $\pm$ standard deviation. Statistic test was performed at each timepoint using pairwise Student's t-test with $p > 0.05$ shown as non-significant (ns). (G, H) Estimated developmental ages of parasites harvested at 6 timepoints across the IDC were comparable between the transgenic lines and the parental strain at biological triplicates. Data are represented as mean $\pm$ standard deviation. Statistic test was performed at each timepoint using pairwise Student's t-test with $p < 0.05$ (*), which was not applicable for identical ages for triplicates (na).

Figure S4. PfNOT1.1 regulates transcript abundance by affecting post-transcriptional regulation instead of transcriptional regulation. (A) Heatmap of differential RNAPII recruitment for 150 genes (left panel) and corresponding differential mRNA abundance (right panel) across the IDC are represented by $\log_2$ -transformed differences in Δ PfNot1.1 versus the parental strain. The scale bar at the bottom shows increased abundance in red and decreased

abundance in green in the transgenic lines. **(B)** The Spearman correlation coefficient was calculated between differential RNAPII recruitment and differential mRNA abundance of 150 genes in Δ PfNot1.1 at each timepoint, showing no correlations. **(C)** Significantly differential transcript abundance of 782 genes in Δ PfNot1.1 or dd Δ PfNot1.2 is shown as the log₂-transformed ratio of expression in the transgenic lines versus the parental strain. Differential mRNA abundance of the corresponding genes in Δ PfCaf1 was adapted [17]. The scale-bar is given at the bottom.

Table S2. List of proteins co-immunoprecipitated by HA-tagged PfNOT1.1, PfNOT1.2, PfCAF1 and PfNOT4. HA-tagged proteins were co-immunoprecipitated by Pierce HA magnetic beads and bound proteins were identified by LC-MS/MS mass spectrometry in the transgenic lines and the parental strain at the schizont stage in biological replicates. The mass spectrometry data was matched to the PlasmoDB database using Mascot search software and the sequenced peptides were identified based on Mascot probability scoring [80]. The ions score was calculated for each peptide to measure how well the peptide sequence matches the database sequences. The Mascot protein score, as the summed ions score of all peptides identified in one protein reflects the confidence of this protein detected in the eluent. The proteins detected only in the transgenic lines or detected in both the parental strain and the transgenic lines with a log₂-transformed ratio of Mascot score in the transgenic lines versus that in the parental strain more than 1.5, were selected as positive hits. The proteins identified as positive hits in both biological replicates are listed in this file and ranked based on the average Mascot score.

1039

1040 **Table S3. List of genes with significantly differential mRNA abundance in Δ PfNot1.1 and**
 1041 **dd Δ PfNot1.2.** The differential mRNA abundance is represented by the $\log_2$ -transformed ratio of
 1042 mRNA abundance in the transgenic lines versus that in the parental strain. The significant
 1043 change is defined as an expression fold-change more than 1.5 (corresponding to the $\log_2$ ratio
 1044 at 0.585) and p-value (pairwise Student's t-test) less than 0.05. In Δ PfNot1.1 and dd Δ PfNot1.2,
 1045 there are 715 genes and 202 genes significantly differentially expressed, respectively. Six
 1046 clusters of genes, categorized according to the timepoint of the most drastic changes in mRNA
 1047 abundance are also indicated. A total of 782 genes with significant changes in mRNA abundance
 1048 in either of two transgenic lines and a total of 135 genes showing significantly differential
 1049 mRNA abundance in both Δ PfNot1.1 and dd Δ PfNot1.2 are listed as well.

1050

1051 **Table S4. List of genes with significantly differential recruitment of RNAPII in Δ PfNot1.1.**
 1052 Recruitment of the RPB1 with phosphorylated Ser2 and Ser5 was assessed by chromatin-
 1053 immunoprecipitation followed by microarray analysis. The differential recruitment was
 1054 calculated between Δ PfNot1.1 and the parental strain at six timepoints across the IDC. The
 1055 significant change is defined as a fold-change more than 1.5 and p-value (pairwise Student's t-
 1056 test) less than 0.05. The values are given in the form of $\log_2$ -transformed differential
 1057 recruitment. The order of the genes corresponds to the heatmap in Fig. S4A in supplemental
 1058 figures.

Figure 1

Figure 2

-0.122 -0.037* -0.478 -0.780 -0.624 -0.718

Figure 3

Binomial $p > 0.05$

<https://doi.org/10.1101/2020.02.12.945477>

CC-BY 4.0 International license

0.462	0.311	0.388	0.526	0.442	0.701
-0.141	-0.162	-0.303	-0.521	-0.564	-0.522

Transcription

Decay

Figure 4