Asparagine repeat function in a *Plasmodium falciparum* protein assessed via a regulatable fluorescent affinity tag

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One in four proteins in *Plasmodium falciparum* contains asparagine repeats. We probed the function of one such 28-residue asparagine repeat present in the *P. falciparum* proteasome lid subunit 6, Rpn6. To aid our efforts, we developed a regulatable, fluorescent affinity (RFA) tag that allows cellular localization, manipulation of cellular levels, and affinity isolation of a chosen protein in *P. falciparum*. The tag comprises a degradation domain derived from *Escherichia coli* dihydrofolate reductase together with GFP. The expression of RFA-tagged proteins is regulated by the simple folate analog trimethoprim (TMP). Parasite lines were generated in which full-length Rpn6 and an asparagine repeat-deletion mutant of Rpn6 were fused to the RFA tag. The knockdown of Rpn6 upon removal of TMP revealed that this protein is essential for ubiquitinated protein degradation and for parasite survival, but the asparagine repeat is dispensable for protein expression, stability, and function. The data point to a genomic mechanism for repeat perpetuation rather than a positive cellular role. The RFA tag should facilitate study of the role of essential genes in parasite biology.

malaria | conditional expression | polyasparagine | amino acid repeat | trinucleotide repeat

Malaria is a parasitic disease that causes high mortality and morbidity in tropical and subtropical regions of the world (1). The causative agents of malaria in humans are intracellular parasites of the genus *Plasmodium*. The most deadly of these parasites is *Plasmodium falciparum*. The proteome of *P. falciparum* is replete with amino acid repeats; about 25% of all proteins in *P. falciparum* contain asparagine repeats (2). These repeats are found in proteins with diverse functions and structures (2). Proteins containing asparagine repeats are prone to forming amyloid fibrils (3); however, despite their abundance, there is no evidence that *P. falciparum* proteins form these tangles in vivo. Many hypotheses have been proposed to explain the function of these repeats and how they could have arisen (4, 5), but there is a paucity of experimental evidence to support any of the suggestions. It has been proposed that asparagine repeats in the *P. falciparum* proteome are evolving under positive selection (6). Alternatively, it has been suggested that the propagation and evolution of these repeats is occurring primarily through neutral mechanisms (4). We set out to determine what role, if any, these repeats play in protein function and other aspects of cellular biology within the parasite.

Ideally, one would want to study a protein that has a single, large asparagine repeat, a fairly small size, and a predicted function that provides some clues as to its biological role. One protein that fulfills these criteria is the proteasome lid regulatory subunit 6, Rpn6 (PlasmoDB ID: PF14_0025). The Rpn6 protein has a single, large 28-residue asparagine repeat that is present in a *Plasmodium*-specific insertion. Rpn6 is a nonenzymatic component of the proteasome. It is thought to be important for the assembly of the proteasome in yeast (7). In *Drosophila melanogaster* (8) and *Trypanosoma brucei* (9) this protein was found to be essential for the survival of the organism. It has a clear predicted function, although the homology with Rpn6 proteins from other organisms is low (Fig. S1). Its function in *P. falciparum* is unknown.

The tools available to study the function of genes, especially essential genes, in *P. falciparum* are limited. Because of the haploid nature of the parasite, it often is not feasible or informative to use knockout technology to study essential genes, even though this technology is well established (10). Conditional expression systems such as the tetracycline-repressor system (11) have not been generally useful. FK506-binding protein degradation domain (ddFKBP)-based conditional expression was reported recently to work in the parasite (12–14). However, the small molecule that is used to stabilize the ddFKBP, Shield-1, is expensive and is somewhat toxic to the parasite at the concentrations required for protein stabilization (13).

A degradation domain based on the *Escherichia coli* dihydrofolate reductase (DHFR) enzyme was reported recently (15). The DHFR degradation domain (DDD) is stabilized by inexpensive folate analogs such as trimethoprim (TMP) (15). We combined the DDD with GFP and HA sequences to make a regulatable fluorescent affinity (RFA) tag (Fig. 1*A*) that allows us to probe aspects of protein biology such as its localization and interacting partners.

In this study, we report the use of the DDD in *P. falciparum*, the development of the RFA tag for studying Rpn6 function, and the role of the Rpn6 asparagine repeat.

Results

Validation of the DDD in *P. falciparum*. We assessed episomal expression of YFP tagged with the *E. coli* DDD in *P. falciparum*. C-terminal and N-terminal fusions were driven by the constitutive heat shock protein 86 (*hsp86*) promoter (YFP-DDD and DDD-YFP, respectively). Parasites were grown in the presence of varying TMP concentrations for 24 h to test the ability of the ligand to stabilize the DDD in the parasite. Whole-cell protein extracts from these parasites then were analyzed by Western blots probed with anti-GFP antibody (Fig. 1*B*). Parasites transfected with DDD-YFP showed a nearly complete absence of YFP when grown with no TMP, whereas YFP-DDD parasites showed a minimal amount of protein with no TMP (Fig. 1*B*). In both cases, there was a dose-dependent increase in protein stabilization.

RFA Tagging of Rpn6 and Rpn6 Asparagine Repeat Deletion Mutant Several tags have been used to study protein function in *P. falciparum*. Individually these tags allow the study of one aspect of protein function. We wanted to construct an all-inclusive system

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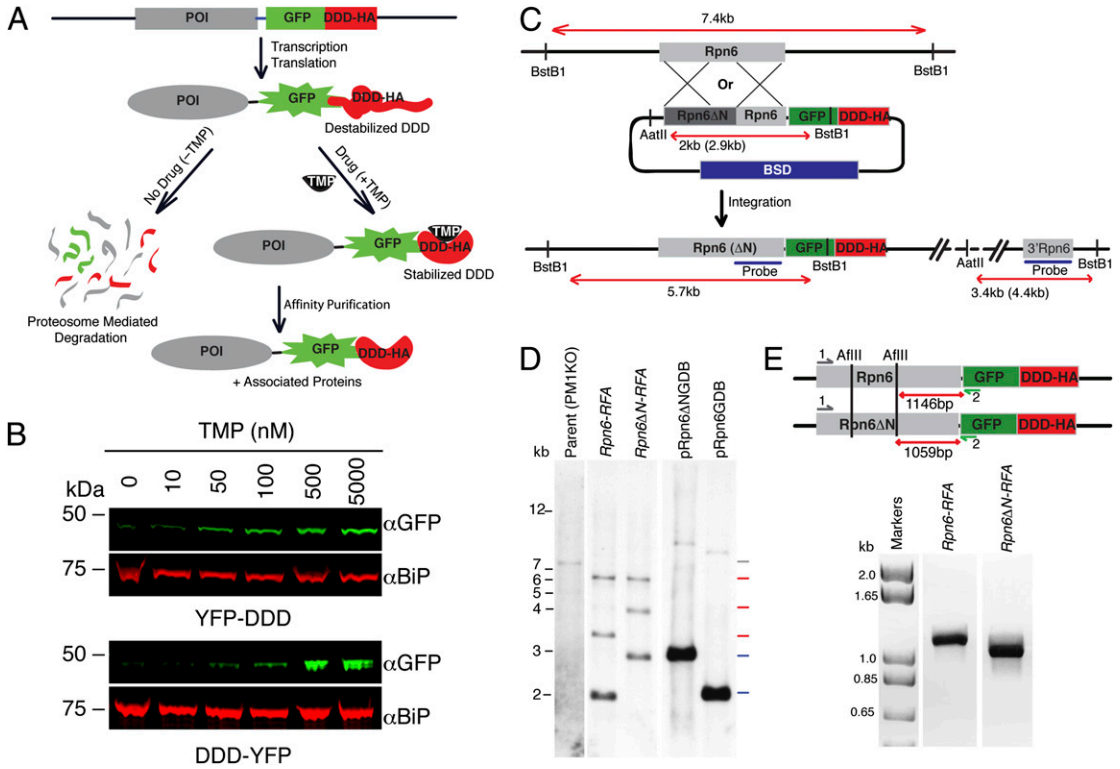

Fig. 1. Utilizing the DDD and the RFA tag in *P. falciparum*. (*A*) Outline of the RFA tag scheme. The protein of interest (POI) is RFA-tagged (GFP+DDD+HA), allowing protein knockdown, live fluorescence microscopy, and affinity purification of associated proteins. (*B*) Stabilization of DDD by TMP. Synchronized ring-stage parasites transfected with YFP-DDD (*Upper*) or DDD-YFP (*Lower*) were incubated for 24 h with different TMP concentrations (as indicated). Western blots of whole-cell protein extracts were probed with anti-GFP antibody. BiP was the loading control. (*C*) Scheme showing the strategy used to incorporate the RFA tag at the 3′ end of the endogenous locus by single-crossover homologous integration. Plasmid (pRpn6GDB or pRpn6ΔNGDB) was transfected into the parent strain, PM1KO. The plasmids contained a BSD cassette for positive drug selection. BstBI and AatII restriction sites and the probe used to detect integration along with the expected sizes are indicated (in brackets for Rpn6ΔN). (*D*) Southern blots of genomic DNA and plasmid DNA digested with BstBI and AatII. Bands expected from a single-crossover recombination event were seen in *Rpn6-RFA* and *Rpn6ΔN-RFA* integrant clones (red lines). The plasmid bands also were seen in *Rpn6-RFA* and *Rpn6ΔN-RFA* integrant clones (blue lines), suggesting that a plasmid concatamer integrated into the gene, a common occurrence in *P. falciparum*. A single band was seen for the parental strain (PM1KO, gray line) that was absent in the integrant clones. This lane was exposed for a longer time than the other lanes to visualize the band. The expected bands also were seen for the plasmid controls pRpn6GDB and pRpn6ΔNGDB (blue lines). (*E*) PCR products from *Rpn6-RFA* and *Rpn6ΔN-RFA* genomic DNA, generated by the primers 1 and 2. (*Upper*) Map showing AflII restriction sites and the expected size of restriction fragments. (*Lower*) Restriction fragments generated after AflII digest of PCR products. The 87-bp difference between *Rpn6-RFA* and *Rpn6ΔN-RFA* indicates deletion of the asparagine repeat.

that allowed protein regulation, live fluorescence microscopy, and affinity purification. For this purpose we generated a plasmid (termed "pGDB") in which GFP (in place of YFP in the episomal constructs above) was cloned in frame with the C-terminal DDD and an HA tag at its 3′ end to make the RFA tag (Fig. 1*A* and Fig. S2). The plasmid also contained a blasticidin (BSD) resistance cassette that allows us to select for plasmid integration and protein stabilization using two orthogonal drugs (Fig. S2).

We used a single-crossover homologous recombination strategy to fuse the RFA tag to the endogenous Rpn6 gene, PF14_0025 (Fig. 1*C*). To generate *Rpn6-RFA* parasites, a 1.1-kb sequence at the 3′ end of the Rpn6 coding sequence was cloned in frame with the RFA tag in pGDB vector, creating the plasmid pRpn6GDB (*Materials and Methods*). To delete the asparagine repeat (amino acids 425–453), the complete coding sequence of Rpn6 was cloned into the pGDB vector in frame with the RFA tag, and the deletion mutant was generated via site-directed mutagenesis, creating the plasmid pRpn6ΔNGDB.

TMP, the drug that is used to stabilize the DDD, is toxic to parasites (Fig. S3). We therefore transfected pRpn6GDB and pRpn6ΔNGDB into parasites containing a human DHFR (hDHFR) marker integrated via double crossover into a nonessential gene, Plasmepsin I (PM1KO) (16). The presence of the hDHFR marker renders the parasites resistant to TMP (Fig. S3). After two cycles on and off BSD, successful integration of the RFA tag was obtained for both Rpn6 and Rpn6ΔN (Fig. 1*D*), creating *Rpn6-RFA* and *Rpn6ΔN-RFA* parasite lines. During drug cycling, parasites always were grown in the presence of 5 μM TMP to stabilize Rpn6 protein in parasites in which the RFA tag had integrated into the gene. The deletion of the asparagine repeat at the Rpn6 gene locus in *Rpn6ΔN-RFA* was confirmed by sequencing as well as by AflII digest of the PCR product obtained using a primer at the 5′ end of the Rpn6 gene and a reverse primer corresponding to the 5′ end of GFP (Fig. 1*E*).

In *Rpn6-RFA* and *Rpn6ΔN-RFA*, the presence of the RFA-tagged proteins was detected via live fluorescence microscopy (Fig. 2*A*) and via Western blot of parasite extracts probed with anti-GFP (Fig. 2*B*) and anti-HA (Fig. S4). Rpn6 and Rpn6ΔN were expressed in all intraerythrocytic life cycle stages and were located in the cytoplasm (Fig. 2).

Stabilization of Rpn6-RFA and Rpn6Δ*N*-RFA with TMP. To assess the efficacy of the DDD within the RFA tag, synchronized early-trophozoite-stage *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites were incubated with varying amounts of TMP for 24 h. We monitored protein levels by live fluorescence microscopy (Fig. 3*A*) and by Western blots (Fig. 3*B*). There was a dose-dependent response to TMP (Fig. 3 *A* and *B*). Synchronized early-trophozoite-stage *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites were washed and incubated in medium without TMP to test the time course of protein degrada-

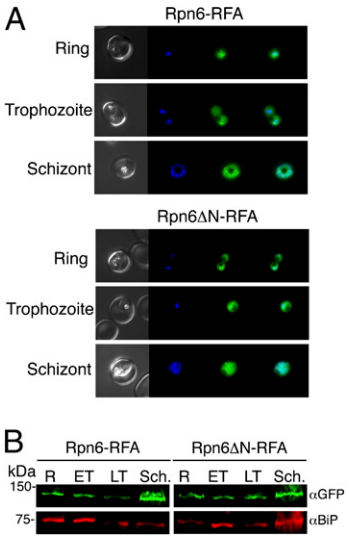

Fig. 2. Intraerythrocytic expression of Rpn6 and Rpn6ΔN. (*A*) Stages of the erythrocytic life cycle of *Rpn6-RFA* (*Upper*) and *Rpn6ΔN-RFA* (*Lower*) parasites observed by live fluorescence microscopy. Images left to right are phase, DAPI, GFP, and fluorescence merge. (*B*) Western blot of lysates from different erythrocytic life cycle stages of *Rpn6-RFA* (*Left*) and *Rpn6ΔN-RFA* (*Right*) parasites, probed with anti-GFP antibody. BiP is the loading control. ET, early trophozoites; LT, late trophozoites; R, rings; Sch., schizonts.

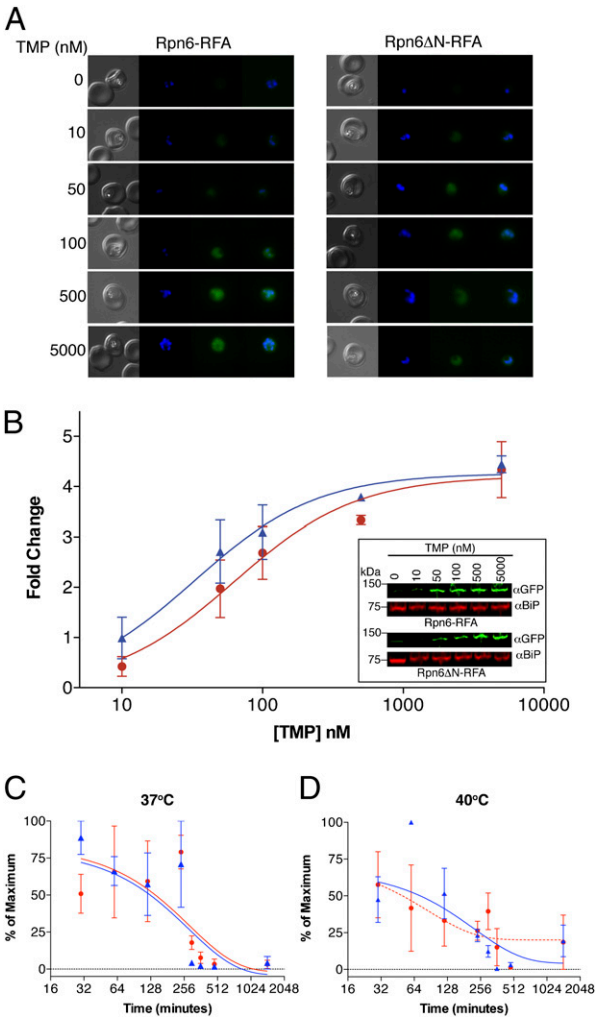

Fig. 3. Regulation of RFA-tagged proteins. (*A*) Live fluorescence images of *Rpn6-RFA* (*Left*) and *Rpn6ΔN-RFA* (*Right*) parasites incubated for 24 h in different TMP doses (as indicated). Images left to right are phase, DAPI, GFP, and fluorescence merge. (*B*) Stabilization of Rpn6-RFA (red filled circle) and Rpn6Δ*N*-RFA (dark blue filled triangle) by TMP. Data are shown as fold change over the no-TMP sample. GFP signal from Western blots was normalized against BiP. (*Inset*) One of four independent TMP dose–response Western blots. BiP served as the loading control. (*C* and *D*) Change in the amount of protein over time when *Rpn6-RFA* (red filled circle) and *Rpn6ΔN-RFA* (dark blue filled triangle) parasites were incubated without TMP and quantified in three independent experiments. (Sample Western blots are shown in Fig. S5.) BiP served as the loading control. The experiment was done at two temperatures, 37 °C (*C*) and 40 °C (*D*). Error bars represent SE from three independent experiments.

tion (Fig. 3 *C* and *D*). The half-life of tagged Rpn6 was about 6 h. Thus, the RFA tag can promote rapid degradation of fused proteins upon removal of the stabilizing ligand TMP.

Asparagine repeats are known to affect protein stability and induce amyloid fibril formation (3). We compared Rpn6-RFA and Rpn6Δ*N*-RFA protein levels at different TMP concentrations (Fig. 3 *A* and *B*) and at different times after removal of TMP (Fig. 3 *C* and *D*). Protein stability also can be affected by stress, especially heat shock, and *P. falciparum* is known to be very sensitive to heat shock stress (17). Therefore Rpn6-RFA and Rpn6Δ*N*-RFA protein half-lives also were measured at 40 °C (Fig. 3*D*). Stability at an intermediate TMP concentration, 100 nM, was tested also (Fig. S5). In all cases, levels of tagged protein with or without the asparagine repeat were similar.

Rpn6 Is an Essential Protein. Rpn6 is known to be essential for growth in some organisms (8, 9), but the importance of this protein in *P. falciparum* is not known. We grew synchronized ring-stage *Rpn6-RFA* and *Rpn6ΔN-RFA* in the presence or absence of 5 μM TMP and monitored parasite growth by flow cytometry over several days (Fig. 4*A*). In the absence of TMP, parasites did not grow, and no live parasites were seen when Giemsa-stained blood smears were observed by microscopy. Parasite growth also was measured after 48 h in different TMP doses; growth was dependent on the presence of TMP with an EC_{50} of about 15 nM (Fig. 4*B*). Rpn6 is essential for the intraerythrocytic growth of *P. falciparum*.

To test if the presence of the asparagine repeat in Rpn6 has an effect on the ability of parasites to deal with a global protein unfolding stress, *Rpn6-RFA* and *Rpn6ΔN-RFA* were incubated at 37 °C or 40 °C for 6 h, then were maintained at 37 °C, and their growth was monitored over several days (Fig. 4 *C*–*F*). There was no difference in the growth characteristics of the two parasite lines either with or without heat shock. There was a threshold of TMP concentration, about 150 nM, that was absolutely required to deal with the heat shock stress, and this threshold was the same for both *Rpn6-RFA* and *Rpn6ΔN-RFA*. The parasite requires Rpn6 for growth within the red blood cell.

Asparagine Repeat of Rpn6 Is Dispensable for Proteasome Function. Rpn6 is part of the proteasome lid. To test if its essential nature results from a role in proteasome function, we incubated synchronized early-trophozoite-stage *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites with varying TMP concentrations. After 24 h, whole-cell protein lysates were analyzed for ubiquitinated proteins via Western blot (Fig. 5). Ubiquitinated proteins started to accumulate as the dose of TMP decreased and the levels of Rpn6 decreased (Fig. 5). Rpn6, but not the asparagine repeat, was essential for proper disposal of ubiquitinated proteins.

To test further if Rpn6 is a proteasome subunit in *P. falciparum*, Rpn6-associated proteins were isolated by pull down using an anti-GFP antibody and were identified by MS (Table 1). Profiles from *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites were nearly identical. We identified nearly 30 proteasome subunits in pull downs with whole-cell protein extracts from both *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites. This result strongly suggests that Rpn6 is

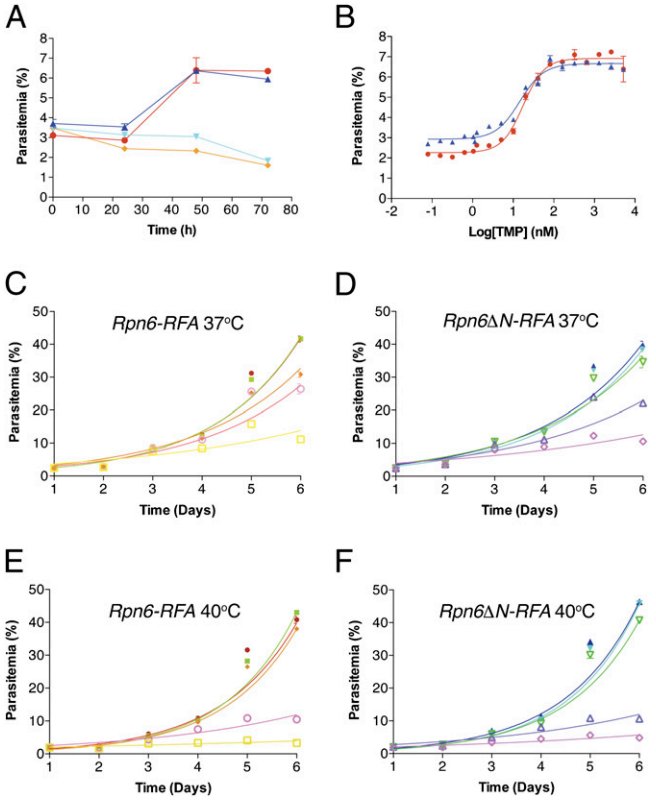

Fig. 4. Rpn6 is an essential gene. (*A*) Synchronous ring-stage parasites were grown with (*Rpn6-RFA,* red filled circle; *Rpn6ΔN-RFA*. dark blue filled triangle) or without (*Rpn6-RFA*, orange filled diamond; *Rpn6ΔN-RFA*, light blue inverted filled triangle) 5 μM TMP, and their growth was monitored over 3 d by flow cytometry. (*B*) Asynchronous *Rpn6-RFA* (red filled circle) and *Rpn6ΔN-RFA* (dark blue filled triangle) parasites were incubated with different TMP concentrations, and their growth was measured after 48 h by flow cytometry. (*C*) Asynchronous *Rpn6-RFA* parasites were incubated with different TMP concentrations [5 μM (red filled circle); 0.312 μM (green filled square); 0.156 μM (orange filled diamond); 0.078 μM (open purple circle); or 0.039 μM (open yellow square)] at 37 °C, and their growth was monitored over 6 d by flow cytometry. (*D*) Asynchronous *Rpn6ΔN-RFA* parasites were incubated with different TMP concentrations [5 μM (dark blue filled triangle); 0.312 μM (light blue inverted filled triangle); 0.156 μM (inverted open green triangle); 0.078 μM (open purple triangle); and 0.039 μM (open purple diamond)] at 37 °C, and their growth was monitored over 6 d by flow cytometry. (*E*) Asynchronous *Rpn6-RFA* parasites were incubated with different TMP concentrations (symbols as in *C*) and were heat shocked for 6 h at 40 °C. After 6 h the cultures were transferred to 37 °C, and their growth was monitored over 6 d by flow cytometry. (*F*) Asynchronous *Rpn6ΔN-RFA* parasites were incubated with different TMP concentrations (symbols as in *D*) and were heat shocked for 6 h at 40 °C. After 6 h the cultures were transferred to 37 °C, and their growth was monitored over 6 d by flow cytometry. Error bars represent SE from one of three independent experiments done in triplicate.

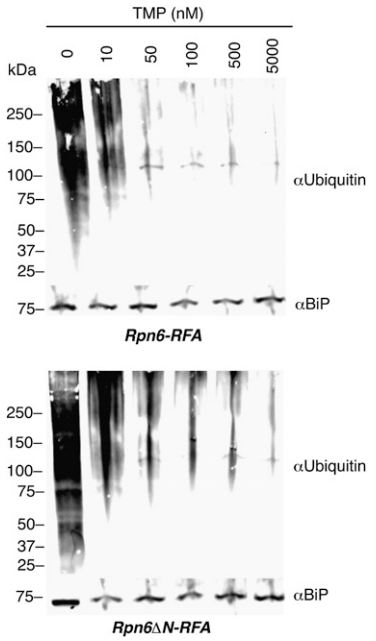

Fig. 5. Ubiquitinated protein degradation depends on Rpn6. Synchronous early-trophozoite-stage *Rpn6-RFA* (*Upper*) and *Rpn6ΔN-RFA* (*Lower*) parasites were incubated for 24 h in different TMP concentrations (as indicated). Western blots of whole-cell protein extracts were probed with anti-ubiquitin antibody to assess the accumulation of ubiquitinated proteins in parasites. BiP was the loading control.

indeed a component of the proteasome and that the asparagine repeat does not affect its associations.

Several proteasome inhibitors have been well characterized, and some, such as MG132, have been shown to be toxic to *P. falciparum* (18). The toxicity of MG132 is assumed to result from its inhibition of the proteasome, which is required for parasite growth. We tested MG132 for its effect on parasite growth after 24 h in different TMP doses (Fig. S6). Different TMP doses correspond to different Rpn6 levels, and Rpn6 levels have a direct effect on proteasome function. We expected parasites in higher TMP doses to be more resistant to MG132, and vice versa. However, the IC_{50} values for MG132 were unaffected by the TMP dose in both *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites (Fig. S6*C*). This result suggests that MG132 kills parasites not by its inhibition of proteasome function but via some other mechanism.

Discussion

The study of essential genes in *P. falciparum* has met with limited success. The only conditional expression technology that is widely applicable in the parasite is the ddFKBP-based system (12–14), whose ligand is prohibitively expensive and toxic to the parasite (13). In this study, we tested the feasibility of using the DDD in *P. falciparum*. The DDD is stabilized by a commercially available and inexpensive ligand, TMP. We found that this domain can modulate protein levels, whether episomally expressed or integrated into the genome.

Because of the difficulties in tagging genes in *P. falciparum*, we wanted to use the DDD in combination with other tags to provide a one-stop approach for functional characterization of genes. In this work, we used GFP and an HA sequence along with the C-terminal DDD to generate the RFA tag (Fig. 1*A*). We used the RFA tag to tackle an important biological question in the malaria parasite. About one fourth of the entire proteome of *P. falciparum* contains asparagine repeats (2). Although most of the work on these repeats has relied on bioinformatic and statistical analysis (2, 4–6), almost nothing is known about the effect of these repeats on the cellular function of proteins. In the present work we carried out detailed characterization of the asparagine repeat in *P. falciparum* Rpn6 protein. We chose to study the Rpn6 protein because it has a single, large, well-defined asparagine repeat (28 asparagines) and a predicted function. However, despite a predicted function, the homology of *P. falciparum* Rpn6 to that from other organisms is low, and the parasite Rpn6 contains a large insertion that could be involved in a parasite-specific function (Fig. S1).

Our results demonstrated that we could generate parasite lines in which Rpn6 was fused to the RFA tag (Fig. 1 *C*–*E*). The presence of GFP in the RFA tag allowed us to follow Rpn6 fused to it by live fluorescence microscopy (Fig. 2*A*), and HA allowed us to probe for the protein in Western blots (Fig. S4). Rpn6 is a highly expressed protein (Fig. 2*B* and Fig. S4) and is located within the

Table 1. Proteins associated with Rpn6-RFA and Rpn6Δ*N*-RFA

PlasmoDB I.D.	Protein function	Unique peptides with Rpn6-RFA	Unique peptides with Rpn6Δ*N*-RFA
PF14_0632	26S proteasome subunit	29	62
PFB0260w	Proteasome 26S regulatory subunit	28	42
PFL2345c	Tat-binding protein homolog (proteasome subunit)	28	29
MAL13P1.190	Proteasome regulatory component	22	23
PFD0665c	26S proteasome AAA-ATPase subunit RPT3	23	22
PF11_0314	26S protease subunit regulatory subunit 6a	21	26
PF13_0063	26S proteasome regulatory subunit 7	17	17
PF14_0025	Proteasome subunit, Rpn6	25	32
PF11_0303	26S proteasome regulatory complex subunit	19	23
PF10_0081	26S proteasome regulatory subunit 4	17	22
PF10_0298	26S proteasome subunit	19	21
PF08_0109	Proteasome subunit α type 5	12	18
PF13_0033	26S proteasome regulatory subunit	18	19
MAL8P1.128	Proteasome subunit α	13	11
MAL13P1.343	Proteasome regulatory subunit	15	14
PF10_0174	26s proteasome subunit p55	12	21
PF07_0112	Proteasome subunit α type 5	10	10
PF08_0054	heat shock 70-kDa protein	13	11
PF14_0716	Proteasome subunit α type 1	8	7
PFI0630w	26S proteasome regulatory subunit	8	10
MAL13P1.270	Proteasome subunit	8	7
PFC0520w	26S proteasome regulatory subunit S14	7	10
PF11_0177	Deubiquinating/deneddylating enzyme	7	11
PF13_0304	Elongation factor-1 α	9	6
PFC0745c	Proteasome component C8	6	6
PFL2215w	Actin I	5	3
PFI1545c	Proteasome precursor	6	5
PFF0420c	Proteasome subunit α type 2	7	9
PF14_0676	20S proteasome β 4 subunit	6	8
MAL8P1.142	Proteasome β-subunit	6	3
PFE0915c	Proteasome subunit β type 1	6	10
PF13_0282	Proteasome subunit	4	5
PF13_0346	60S ribosomal protein L40/UBI	3	6
PF14_0138	Conserved protein	5	6
MAL13P1.351	Conserved *Plasmodium* protein		13
PFL0340w	Conserved *Plasmodium* protein		5
PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase	6	

All proteins for which more than four unique peptides were identified in *Rpn6-RFA* or *Rpn6ΔN-RFA* pulldowns are shown.

cytoplasm of the parasite (Fig. 2*A*). The degradation of Rpn6-RFA and Rpn6Δ*N*-RFA was rapid upon TMP removal, disappearing within about 8 h, even under heat shock (Fig. 3 and Fig. S5).

The presence of the asparagine repeat did not affect expression levels, localization, or half-life of *P. falciparum* Rpn6. The ability to delete the asparagine repeat in the endogenous locus suggested either that Rpn6 is not essential or that the repeat is dispensable during intraerythrocytic growth. The latter possibility is supported by the fact that the removal of TMP results in parasite death (Fig. 4*A*), because TMP modulates protein levels via RFA-tag stabilization, and removal of TMP results in degradation of RFA-tagged Rpn6 protein within the cell (Fig. 3 *A* and *B*). Indeed, *Rpn6-RFA* and *Rpn6ΔN-RFA* parasite lines are completely dependent on TMP for their survival (Fig. 4 *B–D*) and for surviving a heat shock stress (Fig. 4 *E* and *F*).

Because Rpn6 is a subunit of the proteasome lid, we investigated its role in proteasome function. We found that it is required for disposal of ubiquitinated proteins by the proteasome (Fig. 5), suggesting that it is required for proper proteasome function. Indeed, we find that *P. falciparum* Rpn6 immunoprecipitation selectively brings down the entire proteasome (Table 1). This result suggests that the essentiality of the Rpn6 gene is tied to its requirement for proteasome function. The asparagine repeat in Rpn6 was not required for its interaction with other subunits of the proteasome, its function in the proteasome, or the survival of the parasite.

One protein, PF14_0598, was found only in the pulldown of the asparagine repeat-containing Rpn6. It is an abundant glycolytic protein that frequently comes down in pulldowns with unrelated proteins (19). The other two proteins come down only with the asparagine-deletion mutant. It is unlikely that the mutant has a gain of function (i.e., the asparagine repeat protects against association). Therefore, we do not believe that these proteins are significant differential interactors, although the current evidence does not rule out this possibility.

Our data suggest that the asparagine repeat in one highly expressed protein, Rpn6, does not have a discernable effect on protein function or cellular biology. It is, of course, possible that asparagine repeats may not have the same role in every protein in *P. falciparum*, but the results do support the notion that the evolution of these repeats is happening via protein-neutral mechanisms (5). Whether there is selection at the genome level is not addressed by our experiments (6). We have demonstrated the utility of the RFA tag in interrogating the role of the Rpn6 protein in *P. falciparum* biology. The data presented here establish the feasibility of using the RFA tag to study essential genes in *P. falciparum*. The RFA tag facilitates comprehensive analysis of gene product function in this important but difficult-to-study organism.

Materials and Methods

Plasmid Construction. The episomal vector was constructed from the plasmid containing Plasmepsin II in frame with GFP (pPM2GT) (20) by inserting the *Hsp86* promoter between the AatII and XhoI (New England Biolabs) sites.

YFP-DDD and DDD-YFP were introduced into this vector using XhoI and EagI (New England Biolabs) sites.

The DDD (mutations: N18T and A19V) was amplified using the primer 5′-TACAAATAAGCGGCCGATCAGTCTGATTGCGGCGTTAGCGGTAGATCACG-3′ and another primer encoding the HA-tag, 5′-TAACTCGACGCGGCCGTCA-AGCGTAATCTGGAACATCGTATGGGTATCGCCGCTCCAGAATCTCAAAGCAAT-AGCTGTGAGAG-3′. The PCR product was introduced into the PM2GT vector (20) using the EagI restriction site and the In-Fusion cloning system (Clontech) that ensured insertion in the proper orientation. The pGDB plasmid was generated by replacing the hDHFR cassette from pPM2GT (20) plasmid with a BSD cassette from pRZ-TK-BSD2 (21) using SalI and BglII (New England Biolabs) restriction sites. The integration plasmid, pRpn6GDB, was created by introducing a 1.1-kb PCR product from the 3′ end of the Rpn6 ORF into pGDB. The PCR was done using the forward primer 5′-CACTATAGA-ACTCGAGCTTCTCAAAATAAAACAAAAAGTATAAGTCAAATAAGTCCC-3′ and the reverse primer 5′- CTGCACCTGGCCTAGGTATCGTCAATTGAGCTTTTTCA-TATAATATGTCTACAGACTCC-3′. Inserting the entire 2-kb Rpn6 ORF (forward primer, 5′- CACTATAGAACTCGAGATGGATAACTTTGAAGAAATCGA-AAGAGGTTACAAAGAAATAG-3′ and the same reverse primer as before) into pGDB created the integration plasmid, pRpn6ΔNGDB. The PCR products in both cases were introduced into pGDB between the XhoI/AvrII (New England Biolabs) restriction sites using the In-Fusion PCR cloning kit (Clontech). The Rpn6 deletion mutant Rpn6ΔN was generated using the QuikChange site directed mutagenesis kit (Stratagene) with the forward primer 5′-A-CGTTAAATAGAGAAAATGAAAACATTGATCTAAAAAATGATAGTAGTAGTA-GTAGTTTTTGTAGT-3′ and the reverse primer 5′-ACTACAAAAACTACTACTA-CTACTATCATTTTTTAGATCAATGTTTTCATTTTCTCTATTTAACGT-3′.

Parasite Culturing and Transfection. Parasites were cultured and synchronized as described earlier (22). Transfections were carried out as described (23). Parasites transfected with episomally maintained plasmids (YFP-DDD and DDD-YFP) were selected and maintained as described earlier (23). Parasites transfected with the pGDB vectors for integration underwent positive selection 48 h after transfection with 2.5 μg/mL BSD (Calbiochem) and 5 μM TMP (Sigma). Integration was detected after two rounds of BSD cycling. TMP always was present in the medium after its initial introduction. Integrant clones were isolated by limiting dilution.

Southern Blot. Genomic DNA for Southern blots was isolated using the Qiagen Blood and Cell Culture kit. Southern blots were performed as described earlier (20) using 1 μg of DNA digested overnight with the restriction enzymes AatII and BstBI (New England Biolabs). Integrations were screened using a probe for the 3′ end of the Rpn6 ORF.

Microscopy. Live parasites were stained with 2 μM Hoescht 33342 (Molecular Probes) and were observed using an Axioscope Microscope (Carl Zeiss Microimaging) as described previously (20). The images were collected using a fixed exposure time in both GFP and DAPI channels for all samples. Images were analyzed and processed using ImageJ (National Institutes of Health) and Adobe Photoshop.

Western Blot, Protein Quantification, and Immunoprecipitation. Parasites were collected, and the host erythrocytes were permeabilized selectively by treatment with cold 0.04% Saponin in PBS for 10 min, followed by a wash in PBS. Western blots were performed as described previously (20). Lysates from 1×10^7 parasites were loaded per lane. The antibodies used in this study were mouse monoclonal anti-GFP (1:4,000), JL8 (Clontech); mouse monoclonal anti-HA (1:3,000), 3F10 (Roche); mouse monoclonal anti-ubiquitin (1:1,000), P4D1 (Santa Cruz); rabbit polyclonal anti-binding immunoglobulin protein (anti-BiP) (1:20,000), MRA-20 (MR4; ATCC). The signal was detected using IRDye 680CW (1:15,000) conjugated donkey anti-rabbit IgG (LI-COR Biosciences) and IRDye 800CW (1:20,000) conjugated goat anti-mouse IgG (LI-COR Biosciences) on the Odyssey infrared imager (LI-COR Biosciences). The Western blot images were processed and analyzed and GFP and BiP signals were quantified using the Odyssey infrared imaging system software. The protein dose–response and decay data were fit using standard dose–response and single exponential decay equations in GraphPad Prism, v. 5.0 (nonlinear least-squares analysis).

For immunoprecipitation, 4×10^8 parasites were treated with cold 0.04% saponin in PBS for 10 min and washed once with PBS. The purified parasites were lysed with cold 0.5% Triton X-100 in PBS with protease inhibitor mixture (Roche), and the insoluble fraction was separated by centrifuging the lysates at 4,000 × *g* for 5 min at 4 °C. The soluble fraction was incubated with 150 μL Protein A Dynabeads (Invitrogen) and 0.5 μg mouse monoclonal anti-GFP, 3E6 (Invitrogen), for 1 h at 4 °C. The beads then were washed four times in PBS containing protease inhibitor mixture (Roche). The immunoprecipitated complex was solubilized with SDS/PAGE sample buffer, and the proteins were fractionated by 10% SDS/PAGE. The proteins resolved on 10% SDS/PAGE were excised, trypsinized, and identified by MS-MS (Fingerprints Proteomics Facility, College of Life Sciences, University of Dundee, Dundee, United Kingdom) (24). A pull down using anti-GFP from PM1KO (parent) lysates acted as the control.

Flow Cytometry. Parasite culture aliquots (5 μL) were stained with 1.5 μg/mL Acridine Orange (Molecular Probes) in PBS, and the fluorescence profiles of infected erythrocytes were analyzed on a BD FACSCanto flow cytometer (BD Biosystems). The parasitemia data were fit to standard growth curve or dose–response equations in GraphPad Prism, v. 5.0 (nonlinear least-squares analysis).

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Supporting Information

Muralidharan et al. 10.1073/pnas.1018449108

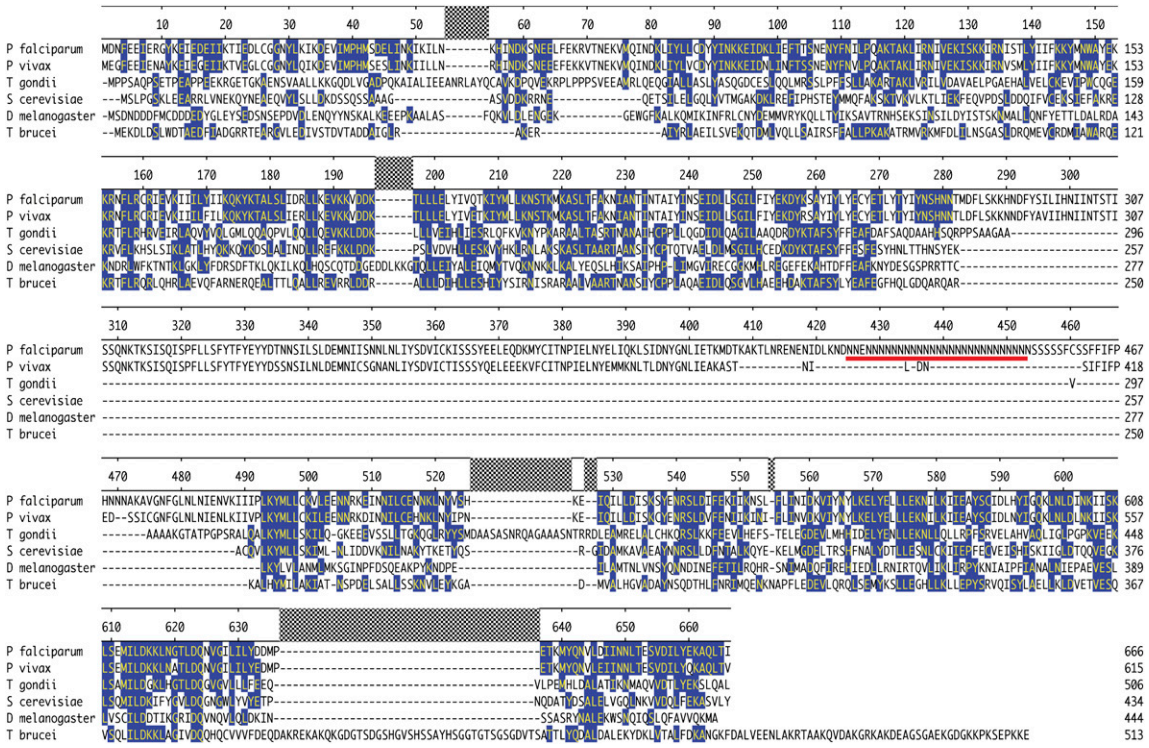

Fig. S1. Sequence alignment of *Plasmodium falciparum* proteasome regulatory subunit 6 (Rpn6) with homologs from selected organisms. The alignment was generated using ClustalW. Conserved residues are highlighted in blue. The asparagine repeat in *P. falciparum* Rpn6 that was deleted in this study is underlined in red.

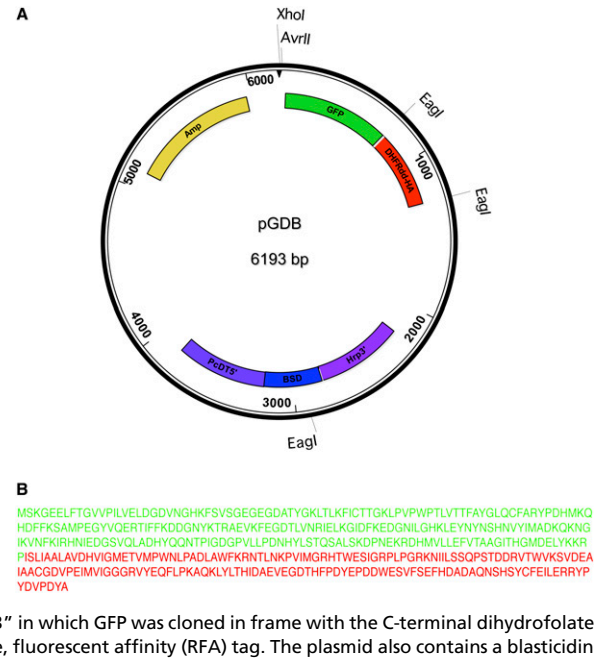

Fig. S2. Map of the plasmid termed "pGDB" in which GFP was cloned in frame with the C-terminal dihydrofolate reductase degradation domain (DDD) and an HA tag at its 3′ end to make the regulatable, fluorescent affinity (RFA) tag. The plasmid also contains a blasticidin (BSD) drug selection cassette. (*A*) The regions coding for the RFA tag and drug resistance markers are indicated. A select few restriction enzyme sites are indicated also. Any PCR product inserted using the XhoI and AvrII sites will be in frame with the RFA tag. (*B*) Protein sequence of the RFA tag. GFP is highlighted in green, and DDD-HA is highlighted in red.

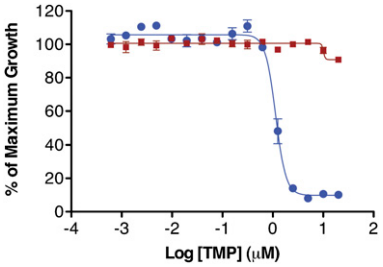

Fig. S3. Growth of parasites in the presence of trimethoprim (TMP). Asynchronous 3D7 (dark blue filled circle) and Plasmepsin I knockout (PM1KO) (red filled square) parasites were grown in different TMP concentrations, and growth was measured by flow cytometry after 72 h. TMP inhibited the growth of 3D7 parasites with an IC_{50} of 1.6 μM but did not affect the growth of PM1KO at the concentrations tested. Error bars represent SE from three experiments.

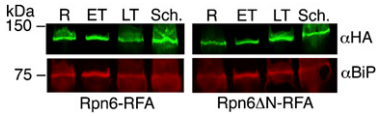

Fig. S4. Expression of Rpn6 during the erythrocytic life cycle of parasites. Western blot of lysates from different erythrocytic life cycle stages of RFA-tagged *Rpn6* (*Rpn6-RFA*) and RFA-tagged *Rpn6* asparagine repeat-deletion mutant (*Rpn6ΔN-RFA*) parasites, probed with anti-HA antibody. Binding immunoglobulin protein (BiP) is the loading control. ET, early trophozoites; LT, late trophozoites; R, rings; Sch., schizonts.

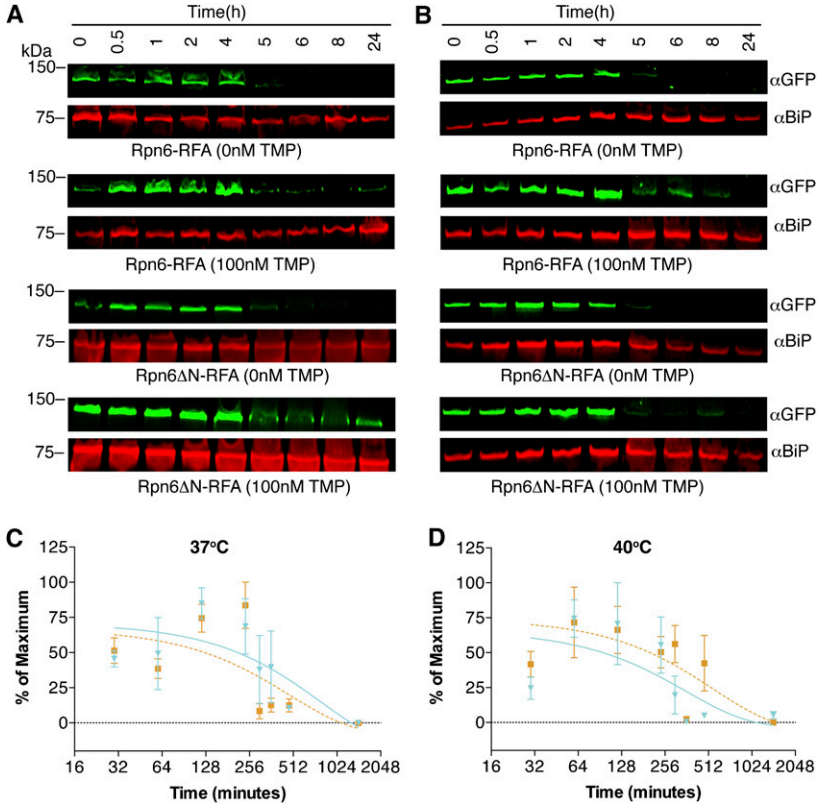

Fig. S5. Regulation of RFA-tagged proteins. Synchronized early-trophozoite-stage *Rpn6-RFA* and *Rpn6ΔN-RFA* parasites were incubated with no TMP or 100 nM TMP at 37 °C (*A*) or 40 °C (*B*). Aliquots of parasites were collected at times indicated, and Western blot of lysates probed with anti-GFP antibody is shown. BiP was the loading control. (*C* and *D*) Change in the amount of protein over time when *Rpn6-RFA* (orange filled square) and *Rpn6ΔN-RFA* (light blue inverted triangle) parasites were incubated in 100 nM TMP. The experiment was done at two temperatures, 37 °C (*C*) and 40 °C (*D*). Results shown were quantified in three independent experiments. BiP served as the loading control. Error bars represent SE from three independent experiments.

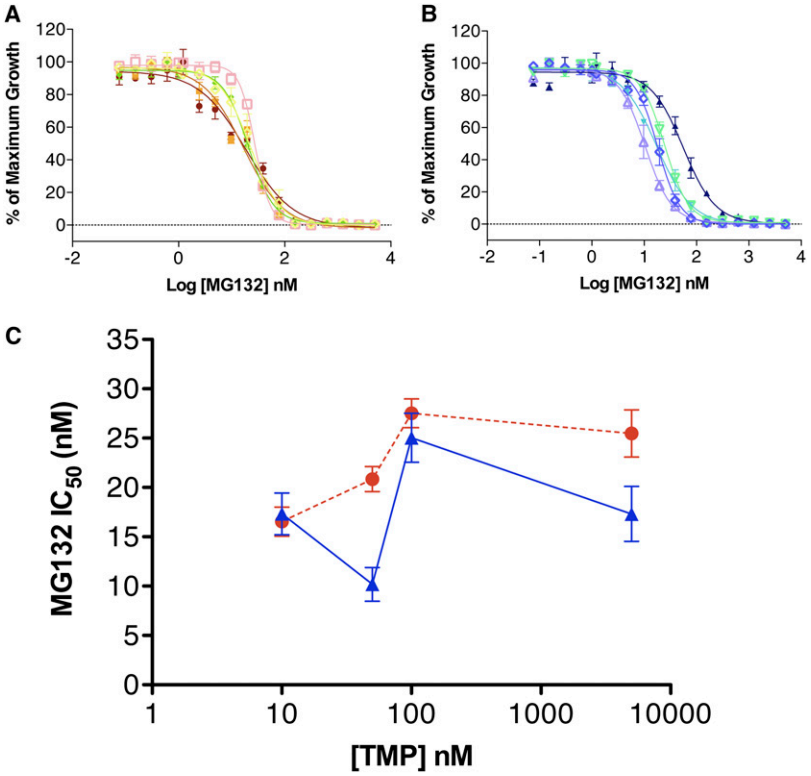

Fig. S6. Effect of the proteasome inhibitor, MG132. (*A*) Growth of asynchronous *Rpn6-RFA* parasites in different concentrations of TMP [1 nM (brown filled circle); 10 nM (tan filled square); 50 nM (green filled triangle); 100 nM (tan open square); and 5,000 nM (yellow open circle)] and MG132 was observed by flow cytometry after 24 h. (*B*) Growth of asynchronous *Rpn6ΔN-RFA* parasites in different concentrations of TMP [1 nM (dark blue filled triangle); 10 nM (light blue filled inverted triangle); 50 nM (dark blue open triangle); 100 nM (green open inverted triangle): and 5,000 nM (dark blue open diamond)], and MG132 was measured by flow cytometry after 24 h. (*C*) The change in the IC_{50} of MG132 toward *Rpn6-RFA* (red filled circle) and *Rpn6ΔN-RFA* (dark blue filled triangle) parasites with changing TMP concentrations. Error bars represent SE from three independent experiments.