

# An FKBP destabilization domain modulates protein levels in *Plasmodium falciparum*

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**To enhance the repertoire of molecular tools for studying malaria parasite biology, we adapted a ligand-regulatable FKBP protein destabilization domain (ddFKBP) for use in *P. falciparum*. We destabilized the reporter yellow fluorescent protein (YFP) and the *P. falciparum* protease falcipain-2 in a ligand-reversible manner by tagging with ddFKBP. The swollen food vacuole phenotype of falcipain-2 knockout parasites could be rescued in a Shld1 ligand-dependent fashion by falcipain-2–ddFKBP expression.**

New tools are needed to study the biology of malaria parasites. Knockout technology is well established in *P. falciparum* but the study of essential genes is difficult in this haploid organism<sup>1</sup>. The tetracycline-regulatable expression system has been adapted for use in *P. falciparum*<sup>2</sup> but has not yet found wide applicability. RNAi manipulation of this parasite has been reported but does not appear to be generally functional, and typical RNAi machinery is absent from the organism's genome<sup>3</sup>. Gene expression potentially can be downregulated at multiple points, from transcription to post-translational control.

Recently a destabilization domain technique has been developed in which an engineered version of human FKBP12 (F36V, L106P) (ddFKBP) is fused to the N or C terminus of a protein, promoting degradation of the fusion protein<sup>4</sup>. In the presence of a ddFKBP ligand, degradation is mitigated, allowing regulation of protein levels in mammalian cells.

We sought to adapt this system for use in *P. falciparum*. The original study<sup>4</sup> used an analog of rapamycin named Shld1 because both of the common FKBP12 ligands FK506 and rapamycin have pleiotropic effects on mammalian cells. In *P. falciparum*, FK506 and rapamycin are toxic at levels lower than the concentrations used to stabilize the FKBP12 mutant<sup>5</sup>. To ensure that Shld1 is not toxic to *P. falciparum*, we grew the parasites in medium containing Shld1 for 3 d and then measured parasitemia using flow cytometry<sup>6</sup>. At the concentrations used for ddFKBP stabilization, there was no inhibition of parasite growth (data not shown).

To allow different genes and promoters to be used easily in the system, we used the multisite Gateway system (Invitrogen) to create plasmids for transfection into the parasites<sup>7</sup> (Supplementary Fig. 1 and Supplementary Methods online). To test the system, we used the *P. falciparum* *hsp86* promoter to drive expression of ddFKBP fused to the N or C terminus of YFP (ddFKBP-YFP and YFP-ddFKBP, respectively). We transfected 3D7 cells with plasmids encoding these fusions and cultured the cells under drug selection for 2–3 weeks, to establish episomal expression lines.

We grew cells containing a gene encoding ddFKBP-YFP (from two independent transfections) in the absence of Shld1 or in the presence of 1  $\mu\text{M}$  Shld1 for 24 h. We isolated whole-cell protein extracts from these parasites, and analyzed the extracts by western blot. Extracts from cells transfected with a gene encoding ddFKBP-YFP showed a near-complete loss of YFP protein when Shld1 was absent, but we observed high protein levels in the presence of Shld1 (Fig. 1a). Extracts from cells transfected with a gene encoding YFP-ddFKBP (two independent transfections) had amounts of YFP comparable to those of the N-terminal fusions when Shld1 was present. In the absence of Shld1, there was a several-fold reduction in the amount of YFP, but some YFP was still detectable in most experiments. Similar results are seen for mammalian cells<sup>4</sup> and *Toxoplasma gondii* (see an accompanying paper in this issue of *Nature Methods*<sup>8</sup>).

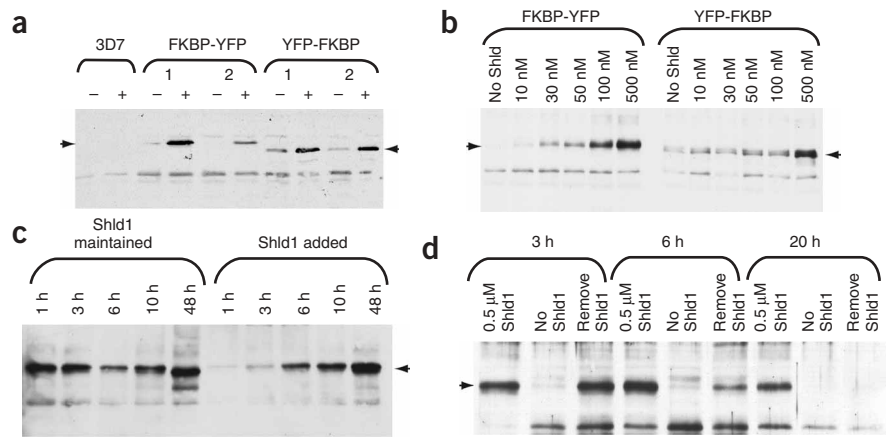
We treated ddFKBP-YFP-expressing parasites with varying amounts of Shld1 for 24 h and measured ddFKBP-YFP levels by western blot (Fig. 1b). At very low concentrations of Shld1, there was little difference in protein levels compared to those in the absence of ligand. At higher Shld1 concentrations, protein levels rose to the robust levels seen at 0.5  $\mu\text{M}$ . Higher concentrations, up to 3  $\mu\text{M}$ , gave comparable YFP signal to that seen at 0.5  $\mu\text{M}$  (data not shown). This suggests that in *P. falciparum*, lower concentrations of Shld1 are sufficient for full expression of the protein than what is seen in mammalian cells. We observed comparable results for YFP-ddFKBP, with a plateau at 0.1  $\mu\text{M}$ , where YFP levels were comparable to those observed in the absence of Shld1. This likely occurs because, as noted above, YFP-ddFKBP does not lead to complete degradation of the protein.

We determined how quickly protein levels respond to the presence of Shld1. To do this we grew parasites expressing ddFKBP-YFP in the presence or absence of 0.5  $\mu\text{M}$  Shld1 for 2 d. We then added 0.5  $\mu\text{M}$  Shld1 to parasites that had been grown in the absence of ligand. Western blot analysis showed that 6 h after Shld1 was added, ddFKBP-YFP levels were comparable to those in parasites continuously grown in the presence of Shld1 (Fig. 1c).

To investigate how quickly protein levels respond to the absence of Shld1, we grew parasites in 0.5  $\mu\text{M}$  Shld1 for 2 d and then removed the compound. Western blot analysis of ddFKBP-YFP over

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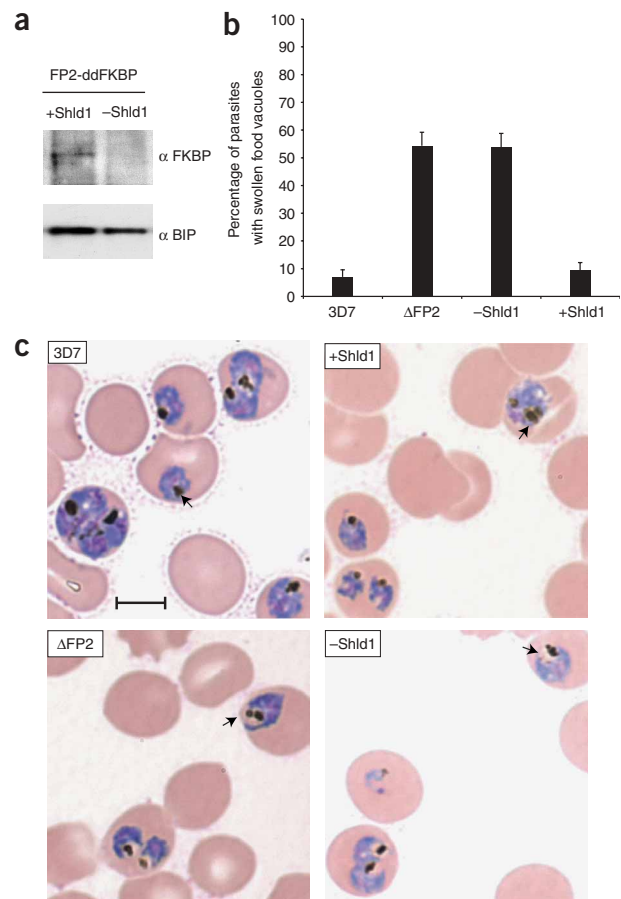
**Figure 1** | Shld1 suppression of destabilization of proteins fused to ddFKBP. **(a,b)** Western blots using anti-GFP to probe whole-cell extracts from cells expressing N- or C-terminally ddFKBP-tagged YFP. Parasites were grown for 24 h in the presence (+) or absence (-) of 1.0  $\mu$ M Shld1 (1 and 2 indicate different transfectants; **a**), or in medium containing the indicated concentrations of Shld1 **(b)**. **(c,d)** Western blots using anti-GFP to probe whole-cell extracts isolated from transfected parasites expressing ddFKBP-YFP that were grown with or without 0.5  $\mu$ M Shld1. Parasites grown without Shld1 were then shifted to medium containing 0.5  $\mu$ M Shld1 for the indicated number of hours (Shld1 added), and those grown initially with Shld1 were maintained in Shld1 **(c)**. Parasites were maintained in the presence or absence of Shld1 (Shld1, no Shld1), or Shld1 was removed from culture that had been grown in the presence of ligand (remove Shld1) for the indicated number of hours **(d)**. An arrow marks the fusion protein band. All western blots were repeated multiple times; in each case a representative blot is shown. Equal amounts of protein were loaded in each lane and an endogenous cross-reacting (lower) band was used as a loading control. BiP gave similar results when used as a loading control.



time revealed that after 6 h protein levels had dropped substantially, and by 20 h no ddFKBP-YFP was detectable (**Fig. 1d**). These results demonstrate that Shld1 acts quickly and can be used to modulate protein levels within a single round of the parasite life cycle.

To assess the effect of the degradation domain on a native *P. falciparum* protein, we fused ddFKBP to the C-terminal end of the food vacuole cysteine protease falcipain-2 (falcipain-2-ddFKBP). Fusion-protein expression was driven by the *hsp86* promoter, and we transfected the plasmid into a falcipain-2 knockout clone<sup>6</sup>. Knockout of the falcipain-2 gene gives rise to a swollen food vacuole phenotype, owing to impairment of the parasite hemoglobin degradation pathway<sup>9</sup>. This allowed us to assess the capacity of the ddFKBP fusion for conditional phenotypic rescue. We grew transfected parasites for 2 weeks under drug (WR99210) pressure to select for plasmid-carrying parasites and treated the selected parasites with or without 0.5  $\mu$ M Shld1 for 48 h. Western blot analysis with an antibody to wild-type FKBP revealed the presence of falcipain-2 when Shld1 was added to the medium and its absence in medium without ligand (**Fig. 2a**). In medium containing Shld1, we observed correction of the swollen food vacuole phenotype (**Fig. 2b,c**). These results demonstrate that native proteins can be modulated by ddFKBP and that this should be a useful tool in the study of many *P. falciparum* proteins.

Although fusion of ddFKBP to either terminus of a target protein allowed Shld1-tunable modulation of protein level, N-terminal fusion allowed more complete downregulation than C-terminal fusion, which still resulted in substantial suppression. This flexibility can be useful because placing ddFKBP at one or the other end could affect targeting and/or function. The results of the



**Figure 2** | Conditional phenotypic rescue of the swollen food vacuole phenotype in falcipain-2 knockout parasites. **(a)** Western blot using anti-FKBP to probe whole cell extract isolated from parasites transfected with plasmids expressing falcipain-2-ddFKBP (FP2-ddFKBP). Parasites were grown in the presence (+) or absence (-) of 0.5  $\mu$ M Shld1 for 48 h before extraction. BiP levels were assessed as a loading control. **(b)** Parasites possessing swollen food vacuoles were quantified by an independent investigator blinded to the parasite identities on blood smears. Error bars, s.e.m.; trophozoites counted,  $n = 100, 105, 104, 107$  for 3D7,  $\Delta$ FP2 (falcipain-2 knockout line), -Shld1, +Shld1, respectively. The difference between - and + Shld1 is significant at  $P < .001$  by the Student's *t*-test. **(c)** Light micrographs of Giemsa-stained parasites. 3D7, parental parasites;  $\Delta$ FP2, knockout parasites; +Shld1 and -Shld1:  $\Delta$ FP2 knockout parasites expressing falcipain-2-ddFKBP in the presence or absence of 0.5  $\mu$ M Shld1. Arrows mark examples of food vacuoles in each parasite line. Scale bar, 5  $\mu$ m.

experiments with falcipain-2 demonstrate that native *P. falciparum* proteins are subject to modulation by the degradation domain system. Falcipain-2 is synthesized as an integral membrane protein precursor via the secretory pathway, so there is potential for this system to work on various proteins, as observed in mammalian cells<sup>4</sup>. Applying this technique to *P. falciparum* proteins should allow for the use of this method in dominant-negative studies and even conditional knockout approaches. It may even be possible to integrate the ddFKBP sequence at the 3' end of an endogenous coding region<sup>10</sup> to generate a conditional knockdown *in situ*.

One of the limitations of the system is leakiness because the amount of degradation may not be enough to produce a phenotype for some proteins. A combination with the tetracycline-regulatable expression system<sup>2</sup> may tighten regulation in such cases. Another limitation is that some proteins do not function or mistarget when made as fusion proteins. Also, some proteins may not be degraded well despite ddFKBP fusion. Last, our episomal expression was under control of the *hsp86* promoter, which gives peak expression in trophozoite and schizont-stage parasites. Whether an early-expressed protein would be well modulated remains to be assessed. Despite these limitations, this work, and that in the accompanying report on *Toxoplasma gondii*<sup>8</sup>, suggests that ddFKBP-based modulation of protein levels may find widespread applicability in apicomplexan parasite research.

Note: Supplementary information is available on the Nature Methods website.

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#### AUTHOR CONTRIBUTIONS

C.M.A. designed and executed experiments and wrote the manuscript. D.E.G. designed experiments and wrote the manuscript.

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