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Destabilization domain approach adapted for regulated protein expression in the protozoan parasite *Entamoeba histolytica*

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Abstract

A plethora of information has been gained by sequencing the genome of the human parasite *Entamoeba histolytica*, however a lack of robust genetic tools hampers experimental elucidation of gene functions. We adapted the destabilization domain (DD) approach for modulation of protein levels in *E. histolytica* using the destabilization domains of FK506 binding protein (ddFKBP) and dihydrofolate reductase (ddDHFR), respectively. In our studies, the ddFKBP appears to be more tightly regulated than ddDHFR, with minimal detectable protein in trophozoites in the absence of the stabilizing compound. The on- and off-rate kinetics for ddFKBP were rapid, with stabilization and degradation within 3 h of addition or removal of stabilizing compound, respectively. The kinetics for ddDHFR was different, with rapid stabilization (within 3 h of stabilizing compound being added) but much slower degradation (protein not destabilized until 24 h after compound removal). Furthermore, we demonstrated that for the ddFKBP, the standard stabilizing compound Shield-1 could be effectively replaced by two cheaper alternatives (rapamycin and FK506), indicating that the more cost-effective alternatives are viable options for use with *E. histolytica*. Thus, the DD approach represents a powerful method to study protein functions in *E. histolytica* and adds to the catalog of genetic tools that could be used to study this important human pathogen.

Keywords

Destabilized protein; Enteric pathogen; Amebiasis; Protein degradation; Inducible expression

1. Introduction

Entamoeba histolytica is a protozoan parasite and the causative agent of amebiasis, a major health problem affecting 50 million people and causing an estimated 100,000 deaths

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annually (WHO, 1997). Despite its global importance, insufficient data are available on the molecular basis of amebic pathogenesis. The plethora of available information from *E. histolytica* genome sequencing still requires further experimental validation of annotated gene functions (Loftus et al., 2005). While genetic tools for expression of amoebic or exogenous proteins have been developed for *Entamoeba* spp., due to variable number of nuclei, polyploidy and lack of homologous recombination in *E. histolytica* (Lopez-Revilla and Gomez, 1978; Marquez-Monter et al., 1990; Willhoeft and Tannich, 1999), knock-out technology is not currently feasible. Multiple gene knockdown approaches have been developed including regulated antisense gene expression (Sahoo et al., 2003), a double-stranded (ds)RNA-based silencing method (Kaur and Lohia, 2004), and a number of RNA interference (RNAi)-based methods (Vayssie et al., 2004; Abed and Ankri, 2008; Solis and Guillen, 2008; Linford et al., 2009; Morf et al., 2013). However, specific tools for modulation of protein abundance that could assist in elucidation of protein functions have not yet been developed.

Destabilization domain (DD)_technology enables regulation of gene product at the protein level. In the DD approach, the gene of interest is coupled to a DD, which leads to degradation of the fused protein by the proteasome (Banaszynski et al., 2006; Sellmyer et al., 2009; Egeler et al., 2011). In the presence of a stabilizing compound, the DD changes its structure, leading to a stable fusion protein (Banaszynski et al., 2006; Egeler et al., 2011). The DD is based on the mutated protein versions of FK506 binding protein (ddFKBP; Banaszynski et al., 2006) and dihydrofolate reductase (ddDHFR; Iwamoto et al., 2010; Muralidharan et al., 2011). The stabilizing compound can be Shield-1, rapamycin or FK506 for the ddFKBP system, and Trimethoprim (TMP) for the ddDHFR system. Recently, a new DD system was established based on the estrogen receptor with one of two synthetic ligands, CMP8 or 4-hydroxytamoxifen, as a stabilizing compound (Miyazaki et al., 2012).

DD approaches have been tested as fusions to diverse proteins such as kinases, cell cycle regulation proteins and small GTPases, suggesting broad applicability (Banaszynski et al., 2006). Additionally, this approach has been applied to a wide variety of different organisms including the apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii*, and the kinetoplastids *Leishmania* spp. and *Trypanosoma cruzi* (Banaszynski et al., 2006; Armstrong and Goldberg, 2007; Herm-Gotz et al., 2007; Madeira da Silva et al., 2009; Muralidharan et al., 2011; Ma et al., 2012).

Here, we explore the DD approach as a method for genetic manipulation of regulated protein expression in *E. histolytica*. We show that regulated protein levels are achievable and we characterize the kinetics and efficiency of DD-induced protein degradation. Importantly, we determine that in *E. histolytica* trophozoites the ddFKBP approach is more tightly regulated than ddDHFR, with almost no stable protein detectable in trophozoites with the ddFKBP in the absence of a stabilizing compound. We also confirmed that stabilizing compound Shield-1 can be effectively replaced by the cheaper alternatives rapamycin and FK506. Furthermore, we determined that the two DD approaches showed different off-rate kinetics in *E. histolytica*. Overall we expect that this approach will expand the arsenal of tools for genetic manipulation in *E. histolytica*.

2. Materials and methods

2.1. Plasmid generation

Constructs were generated to express fusion proteins of yellow fluorescent protein (YFP) with N- and C-terminal DDs based on mutated ddFKBP and ddDHFR and a haemagglutinin (HA) tag (Fig. 1). The YFP, DD and HA inserts were directly amplified and cloned from mammalian over-expression plasmids provided by the Wandless group (Rakhit et al., 2011) into the *NheI* site of the *Entamoeba* over-expression plasmid pKT-3M (Saito-Nakano et al., 2004) and named pE8 to pE11. In order to generate a ddDHFR-YFP-HA expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE8 using the forward primer OLE12 and the reverse primer OLE13, and cloned into pKT-3M using the *NheI* site (Saito-Nakano et al., 2004), resulting in plasmid pE19 (Fig. 1). To generate an HA-YFP-ddDHFR expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE9 using the forward primer OLE14 and reverse primer OLE15, and cloned into pKT-3M using the *NheI* site (Saito-Nakano et al., 2004), resulting in plasmid pE20 (Fig. 1). To generate a ddFKBP-YFP-HA expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE10 using the forward primer OLE16 and the reverse primer OLE17, and cloned into pKT-3M using the *NheI* site (Saito-Nakano et al., 2004), resulting in plasmid pE21 (Fig. 1). To generate an HA-YFP-ddFKBP expression plasmid for *E. histolytica*, the fusion protein insert was amplified from pE11 using the forward primer OLE18 and reverse primer OLE19, and cloned into pKT-3M using the *NheI* site (Saito-Nakano et al., 2004), resulting in plasmid pE22 (Fig. 1). Correct orientation of all inserts was confirmed by sequencing. Sequences of all primers are listed in Table 1.

2.2. Parasite culture and transfection

For generation of transgenic parasites, *E. histolytica* strain HM-1:IMSS trophozoites were transfected using a previously published protocol (Baxt et al., 2010). Briefly, trophozoites were seeded in 35 mm Petri dishes and transfected with 10-20 µg of plasmid DNA using SuperFect (Qiagen, USA) reagent. The transfected parasites were allowed to grow for 24 h, and drug selection started at 1 µg/ml of G418 drug selection and then increased in a stepwise manner to 6 µg/ml of G418 until a stable cell line was achieved. All experiments were done with trophozoites grown at 12-48 µg/ml of G418.

2.3. Western blot analysis

Western blot analysis was performed as described previously (Pearson et al., 2013). Briefly, parasites were harvested, washed once and lysed in lysis buffer containing different protease inhibitors. Lysates (40-80 µg of total protein) were subjected to 12% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked, incubated in primary anti-HA antibody (Cell Signaling, USA) (1:1,000 dilution) overnight at 4°C and secondary HRP-conjugated anti-mouse antibody (Jackson Immuno Research, USA) (1:10,000 dilution) for at least 1 h at room temperature before detection with ECL+ (GE Healthcare, USA) on the Kodak Image Station 4000R (Kodak, USA) or with film (GE Healthcare). After mild stripping, the same membrane was used to detect actin using a pan-specific mouse anti-actin antibody (MP Biomedicals, USA) (1:1,000 dilution) for at least 1 h at room temperature and the secondary HRP-conjugated anti-mouse

antibody (Jackson Immuno Research) (1:10,000 dilution) for at least 1 h at room temperature.

2.4. Stabilization and kinetic experiments

To determine whether the stabilizing compounds were inhibitory to parasite growth, trophozoites were grown in the presence of the stabilizing compounds (1 μ M Shield-1 or TMP or 10 μ M FK506 or rapamycin) in TYI medium for up to 20 days with routine sub-culturing. Parasite cultures in diluent alone were maintained as controls. Parasite growth was monitored by obtaining cell counts at each sub-culture occurrence.

For the proof of principle experiments, trophozoites were grown to 80 to 100% confluence and protein stabilization induced for 24 h with stabilizing compound (1 μ M Shield-1 or TMP or 10 μ M FK506 or rapamycin). Alternatively they were incubated with stabilizing compound for 24 h and removed from drug, washed and cultivated in media without stabilizing compound for 24 h. After collection, parasites were prepared for western blot analysis.

For determination of on-rate kinetics, parasites were induced in fresh media containing 12 μ g/ml or 48 μ g/ml of G418 and stabilizing compound (1 μ M of Shield-1 or TMP; or 10 μ M FK506 or rapamycin). Parasites were collected 0, 3, 6, 9, 12 and 24 h after exposure to stabilizing compound and prepared for western blot analysis. For measurement of off-rate kinetics, parasites were induced in fresh media containing 12 μ g/ml or 48 μ g/ml of G418 and stabilizing compound. After incubation for 24 h with stabilizing compounds, parasites were washed once and incubated in cell media with the corresponding G418 concentration but without stabilizing compound. Parasites were collected 0, 3, 6, 9, 12 and 24 h later and processed for western blot analysis.

3. Results

3.1. Regulated expression of YFP upon addition of stabilizing compounds

The overall approach for use of the DD is outlined in Fig. 2. To demonstrate regulated expression of YFP, parasites were grown to 80 to 100% confluence and induced for 24 h with stabilizing compound (1 μ M Shield-1 or TMP; or 10 μ M FK506 or rapamycin). Alternatively, they were induced for 24 h with stabilizing compound, and then removed from drug, washed and cultivated in media without stabilizing compound for 24 h. The data demonstrate that YFP-HA could be stabilized using both approaches, and that stabilization is independent of whether the DD is located on the N- or C-terminal of the protein of interest (Fig. 3). We did, however, note some persistence of YFP in the absence of stabilizing compound using the ddDHFR DD approach, compared with the tight regulation with ddFKBP (Fig. 3). This indicates that there is constant generation of fusion protein, but in the absence of a stabilizing compound, protein degradation occurs faster than protein synthesis. The ddFKBP system, however, appears less leaky than the ddDHFR system and may reflect a more efficient degradation pathway induced by the ddFKBP compared with the ddDHFR. In systems in which the DD approach is functional, both systems work as seen in mammalian cells (Banaszynski et al., 2006; Sellmyer et al., 2009), *T. gondii* (Herm-Gotz et

al., 2007), *P. falciparum* (Armstrong and Goldberg, 2007), *Leishmania* spp. (Madeira da Silva et al., 2009) and *T. cruzi* (Ma et al., 2012). However, it not certain that DD can be adapted to every organism as establishment of DD was difficult for the model organism *Saccharomyces cerevisiae* (Rakhit et al., 2011).

The compound Shield-1 is a chemical derivative of FK506 and rapamycin, and thus we tested the usability of FK506 and rapamycin as cheaper alternatives for the ddFKBP system. We found that YFP-HA can also be effectively stabilized with 10 μ M rapamycin or 10 μ M FK506 using the ddFKBP, albeit with slightly less stringent regulation (Fig. 3). That rapamycin and FK506 stabilized fusion protein in *E. histolytica* is similar to results obtained in mammalian cells and *Leishmania major* (Banaszynski et al., 2006; Madeira da Silva et al., 2009). Thus, we expect that the cheaper alternatives rapamycin and FK506 may increase the attractiveness of the ddFKBP system for use in *E. histolytica*. However, given the slightly less stringent regulation, it will be important for the level of regulation and functional correlation to be determined for each protein of interest.

Western blot analyses with stabilizing compound added for 24 h was adequate to demonstrate stabilization of the HA-YFP protein. However, in certain situations, parasites may need stabilized protein for longer periods of time to allow for functional assays. Thus, we have tested growth and viability of *E. histolytica* trophozoites in each stabilizing compound by continuous culture. No alteration in growth kinetics, parasite viability or changes in cellular morphology were noted during continuous culture for up to 20 days (data not shown). Thus, the destabilization approach should be applicable to situations in which longer-term protein stabilization is needed, although for each phenotype of interest the specifics of parasite viability will need to be determined.

3.2. Kinetics of YFP induction stabilized by DD

In order to determine the time-frame of protein stabilization, *E. histolytica* trophozoites were induced in media containing 12 μ g/ml or 48 μ g/ml of G418 and 1 μ M stabilizing compound Shield-1 or TMP. Parasites were collected 0, 3, 6, 9, 12 and 24 h after exposure to stabilizing compound and prepared for western blot analysis. In both systems, we could detect stabilized fusion protein as early as 3 h after addition of stabilizing compound (Fig. 4A). The time-frame observed in *E. histolytica* using the ddFKBP approach was similar to mammalian cells in which proteins were stabilized between 4 to 24 h after addition of stabilizing compound (Sellmyer et al., 2009).

Stabilized protein levels were reached after 3 h and remained constant for up to 24 h in the ddDHFR systems in *E. histolytica* (Fig. 4B). In contrast, stabilized protein levels were reached after 16 - 24 h in mammalian NIH3T3 cells (Iwamoto et al., 2010). Both, N- and C-terminal DD fusion proteins behaved similarly for the ddDHFR systems in *E. histolytica*, which is in contrast to data in mammalian cells where C-terminal ddDHFR is more effective than the N-terminal ddDHFR in stabilizing the fusion protein (Iwamoto et al., 2010).

3.3. Kinetics of YFP decrease after removal of stabilizing compounds

For measurement of off-rate kinetics, parasites were induced in fresh media containing 12 µg/ml or 48 µg/ml of G418 and 1 µM stabilizing compound Shield-1 or TMP. After incubation for 24 h, parasites were washed once and incubated in media but without stabilizing compound and were collected 0, 3, 6, 9, 12 and 24 h later for western blot analysis.

Using the ddFKBP system, we could not detect significant amounts of stabilized protein after 3 h, suggesting a quick degradation of fusion protein in the absence of Shield-1 for the N- and C-terminal ddFKBP constructs (Fig. 4C). This observation was similar to results seen in mammalian cells, where protein levels returned to basal amounts after 2 to 4 h without stabilizing compound (Sellmyer et al., 2009). A more detailed analysis of the degradation kinetics of N- and C-terminal tagged fusion proteins showed a quicker return of the N-terminal fusion protein to basal levels (1 h) compared with the C-terminal fusion protein (3 h) in *E. histolytica* (Fig. 5). This was similar to results seen in mammalian cells and *P. falciparum* (Banaszynski et al., 2006; Armstrong and Goldberg, 2007), where ddFKBP at the C-terminus was less effective in destabilizing YFP than when placed at the N-terminus. Similar to Shield-1, we could not detect significant amounts of ddFKBP fusion protein 3 h after removal from drug, when we used 10 µM rapamycin or FK506 to stabilize the fusion protein (Fig. 6).

In the ddDHFR system, we could still detect stabilized protein 12 h after removal from 1 µM TMP for the both N- and C-terminal constructs, and could detect background protein levels at 24 h, indicating that the fusion protein with ddDHFR takes longer to degrade than the fusion protein with ddFKBP (Fig. 4D). In contrast to the above result, degradation of the ddDHFR constructs after removal of TMP was similar between N- and C-terminal constructs and reached background levels after 6 h in mammalian NIH3T3 cells (Iwamoto et al., 2010).

4. Discussion

The DD approach was established to study the functional role of a diverse range of proteins such as cysteine proteases, kinases, cell cycle regulatory proteins, GTPases and transcription factors in many different organisms (Banaszynski et al., 2006; Armstrong and Goldberg, 2007; Herm-Gotz et al., 2007; Madeira da Silva et al., 2009; Ma et al., 2012). The advantage of this approach is the direct regulation of gene products at the protein level with rapid stabilization and destabilization kinetics. However, it is not always possible to adapt the DD system to an organism, as it did not function in *S. cerevisiae* (Rakhit et al., 2011). Although this approach does have some drawbacks, such as only functioning with cytoplasmic proteins (Madeira da Silva et al., 2009), it is a powerful and flexible system for analysis of protein function. Thus, we aimed to establish the DD for *E. histolytica*, a system in which standard genetic tools are problematic and an increased repertoire of genetic approaches is needed.

Our data demonstrate that the DD approach worked in *E. histolytica* for regulated protein expression. The on- and off-rate experiments with YFP fusion using the ddFKBP produced

results similar to those in mammalian cells (Sellmyer et al., 2009), while the on- and off-rate experiments with YFP fusion using the ddDHFR produced results different to those in mammalian cells (Iwamoto et al., 2010). Shield-1 and its cheaper alternatives rapamycin and FK506 could stabilize fusion proteins with ddFKBP and that TMP could stabilize fusion proteins with ddDHFR, similar to results found in other organisms (Banaszynski et al., 2006; Armstrong and Goldberg, 2007; Herm-Gotz et al., 2007; Madeira da Silva et al., 2009; Ma et al., 2012).

Interestingly, the C-terminal fusion of ddFKBP was less effective in destabilizing than N-terminal in mammalian cells and *Plasmodium* (Banaszynski et al., 2006; Armstrong and Goldberg, 2007), an observation that recapitulated for the ddFKBP system in *E. histolytica*. Furthermore, the overall on- and off-rate kinetics of the ddFKBP system in *E. histolytica* were similar to those found in other organisms (Banaszynski et al., 2006; Armstrong and Goldberg, 2007; Herm-Gotz et al., 2007; Madeira da Silva et al., 2009; Sellmyer et al., 2009; Ma et al., 2012). In contrast, both the N-terminal or C-terminal DD fusion proteins behaved similarly for the ddDHFR DD system in *E. histolytica*, which differs from the observation in mammalian cells in which C-terminal ddDHFR is more effective in stabilizing the fusion protein than the N-terminal ddDHFR fusion protein (Iwamoto et al., 2010). Additionally, the on-rate kinetics is faster and the off-rate kinetics is slower in *E. histolytica* than in mammalian cells using the ddDHFR approach (Iwamoto et al., 2010).

The DD approach is a versatile method to modulate protein levels in an organism and has proven to be an important technique to study gene function. This approach has been applied to inducibly express potentially lethal proteins such as dominant negative alleles of essential genes (Herm-Gotz et al., 2007) or inhibitory proteins including the J-domain of the CDPK1 protein in *P. falciparum* (Azevedo et al., 2013). Dominant negative approaches have been successful at dissecting protein function in *E. histolytica* (Katz et al., 2002; Welter et al., 2005) and thus the DD approach should be readily adaptable for this use. The ability to control protein levels in *E. histolytica* with both the ddFKBP and the ddDHFR system, and to use either N- or C-terminal fusions of ddDHFR, will further enhance the utility of both DD approaches in *E. histolytica*. Depending on the required outcome, both systems have their merits. For tighter and more rapid regulation the ddFKBP is more favorable than ddDHFR. For slower and more constant expression of the inducible protein, the ddDHFR system may be preferred. To avoid leakiness in the DD system, it is advisable to check the stringency of destabilization by evaluation of N- and C-terminal tagged protein (Herm-Gotz et al., 2007; Sellmyer et al., 2009), or use of further mutated versions of the DD with possibly tighter regulation (Chu et al., 2008). A switch to the new estrogen receptor-based DD system may be another experimental approach (Miyazaki et al., 2012). An additional approach is to combine the tetracycline-inducible system with the DD approach as has been done in mammalian cells, further tightening the ability to control protein levels (Hamann et al., 1997; Ramakrishnan et al., 1997; Armstrong and Goldberg, 2007; Senkel et al., 2009). Thus, several additional experimental avenues are available to make further use of the DD approach. Overall, adaption of the DD technology to *E. histolytica* is an important new tool for studying gene function of this significant human pathogen.

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Highlights

- Regulated protein expression using a destabilization domain adapted to *Entamoeba histolytica*
- Important new technology for genetic studies in *E. histolytica* is reported
- Cheaper alternatives to Shield-1 as a stabilizing compound are functional in ameba

Fig. 1.

Establishment of a destabilization domain (DD) approach in *Entamoeba histolytica*. (A) Generation of plasmids for establishment of DD in *E. histolytica* with yellow fluorescent protein (YFP) fused to a DD and tagged with N or C-terminal haemagglutinin (HA) tags. Plasmids for mammalian expression were obtained from Banaszynski et al. (2006) and Iwamoto et al. (2010), and modified to generate plasmids pE19-pE22 which were used for testing the DD approach in *E. histolytica*. Cysteine synthase regulatory regions were used to drive protein expression. ddDHFR, destabilization domain based on mutated dihydrofolatereductase; ddFKBP, destabilization domain based on mutated FK506 binding protein.

Fig. 2. Schematic diagram of the destabilization domain (DD) approach. A protein of interest is fused to a DD. The fusion leads to an unstable protein, which is degraded. In the presence of a stabilizing compound (Shield-1, FK506, or rapamycin for FKBP a destabilization domain (ddFKBP); and Trimethoprim (TMP) for the dihydrofolate reductase (DHFR) destabilization domain (ddDHFR)), the protein is stabilized and can be detected.

Fig. 3.

Regulated expression of yellow fluorescent protein-haemagglutinin (YFP-HA) fusion controlled by destabilization domains (DD) in *Entamoeba histolytica*. Proof of principle experiments for YFP-HA expression of fusion protein, using N- or C-terminal DD, by four stabilizing compounds (Shield-1, rapamycin, FK506 and Trimethoprim (TMP)). A total of 50 µg of protein loaded for N-terminal and 80 µg of protein loaded for C-terminal DD based on mutated FK506 binding protein (ddFKBP) using 1 µM Shield-1. A total of 50 µg of protein loaded for N-terminal and C-terminal ddFKBP using 10 µM rapamycin or FK506. A total of 35 µg of protein loaded for N-terminal and 30 µg of protein loaded for C-terminal DD based on mutated dihydrofolate reductase (ddDHFR) using 1 µM TMP. Trophozoites were induced for 24 h and removed from drug for 24 h (indicated as addition of stabilizing compound and recovery, respectively). Cell lines for N-terminal ddFKBP, C-terminal ddFKBP and N-terminal ddDHFR were at 12 µg/µl of G418 drug selection. Cell line for C-terminal ddDHFR was at 48 µg/µl of G418 drug selection. UT, untransfected wild-type *E. histolytica* HM-1:IMSS trophozoites.

Fig. 4.

On and off-rate kinetics using yellow fluorescent protein-haemagglutinin (YFP-HA) *Entamoeba histolytica* parasites stabilized using different destabilization domain (DD) approaches. (A) On-rate kinetics experiments using N- and C-terminal DD based on mutated FK506 binding protein (ddFKBP) stabilized by Shield-1. Parasites were maintained at 12 $\mu\text{g}/\mu\text{l}$ of G418 drug selection and induced with 1 μM Shield-1. A total of 40 μg of protein was loaded for N-terminal ddFKBP parasites and 30 μg of protein loaded for C-terminal ddFKBP parasites. (B) On-rate kinetics experiments with N- and C-terminal DD based on mutated dihydrofolate reductase (ddDHFR) stabilized by Trimethoprim (TMP). Parasites for N-terminal ddDHFR were maintained at 12 $\mu\text{g}/\mu\text{l}$ of G418 drug selection. Parasites for C-terminal ddDHFR were at 48 $\mu\text{g}/\mu\text{l}$ of G418 drug selection. Proteins were stabilized with 1 μM TMP. A total of 50 μg of protein was loaded for N-terminal and C-terminal ddDHFR parasites. (C) Off-rate kinetics experiments for N- and C-terminal tagged fusion protein with ddFKBP. Both parasite lines were at 12 $\mu\text{g}/\mu\text{l}$ of G418 drug selection. A total of 35 μg of protein was loaded for N-terminal ddFKBP parasites and 40 μg of protein loaded for C-terminal ddFKBP parasites. Fusion protein was stabilized with 1 μM Shield-1. (D) Off-rate kinetics experiments for N- and C-terminal tagged fusion protein with ddDHFR. Both parasite lines were maintained at 12 $\mu\text{g}/\mu\text{l}$ of G418 drug selection. Protein was stabilized

with 1 μ M TMP. A total of 40 μ g of protein was loaded for N-terminal and C-terminal ddDHFR parasites. UT, untransfected wild-type *E. histolytica* cell line; UI, uninduced transgenic *E. histolytica* cell line; Ind, induced *E. histolytica* cell line. Expected size of fusion proteins ddFKBP-YFP-HA or HA-YFP-ddFKBP was 40.5 kDa, of ddDHFR-YFP-HA or HA-YFP-ddDHFR was 46.5 kDa and for actin 42 kDa.

Fig. 5.

Off-rate kinetics experiments using yellow fluorescent protein-haemagglutinin (YFP-HA) *Entamoeba histolytica* parasites destabilized by a destabilization domain (DD) based on mutated FK506 binding protein (ddFKBP) approach with early time points. Parasite lines with N- and C-terminal tagged fusion protein were maintained at 12 µg/µl of G418 drug selection. A total of 40 µg of protein was loaded. Fusion protein was stabilized with 1 µM Shield-1. UT, untransfected wild-type *E. histolytica* cell line; UI, uninduced transgenic *E. histolytica* cell line; Ind, induced *E. histolytica* cell line. Expected size of fusion proteins ddFKBP-YFP-HA or HA-YFP-ddFKBP was 40.5 kDa and for actin was 42 kDa.

Fig. 6.

Off-rate kinetics experiments using yellow fluorescent protein-haemagglutinin (YFP-HA) *Entamoeba histolytica* parasites destabilized by a destabilization domain (DD) based on mutated FK506 binding protein (ddFKBP) and the stabilizing compounds rapamycin and FK506. Parasite lines with N-terminal tagged ddFKBP fusion protein were maintained at 12 $\mu\text{g}/\mu\text{l}$ of G418 drug selection. A total of 40 μg of protein loaded for N-terminal ddFKBP parasites. Fusion protein was stabilized with 10 μM rapamycin or FK506. UT, untransfected wild-type *E. histolytica* cell line; UI, uninduced transgenic *E. histolytica* cell line; Ind, induced *E. histolytica* cell line. Expected size of ddFKBP-YFP-HA protein is 40.5 kDa and actin is 42 kDa.

Table 1

Oligonucleotides for cloning of yellow fluorescent protein (YFP) fused to destabilization domain (DD) (YFP-DD) to generate plasmids pE19-pE22 in this study.

Primer name	Sequence	Resulting construct
OLE12	ATGGCTAGCGCCACCATGATCAGTCTGA TT	ddDHFR-YFP-HA
OLE13	CGGGCTAGCTCATGCGTAGTCTGGTACG	ddDHFR-YFP-HA
OLE14	GCCGCTAGCGCCACCATGTATCCGTACG	HA-YFP-ddDHFR
OLE15	GTAGCTAGCGGTCATCGCCGCTCCAG	HA-YFP-ddDHFR
OLE16	GCCGCTAGCGCCACCATGGGAGTGCAG	ddFKBP-YFP-HA
OLE17	GCCGCTAGCTTAGTCGAGTGCGTAGTCTG G	ddFKBP-YFP-HA
OLE18	GCCGCTAGCGCCACCATGTATCCGTAC	HA-YFP-ddFKBP
OLE19	GCCGCTAGCTCATTCCAGTTCTAGAAGCT C	HA-YFP-ddFKBP

The *NheI* restriction site is underlined.

ddDHFR, destabilization domain based on mutated dihydrofolate reductase; ddFKBP, destabilization domain based on mutated FK506 binding protein.