METHOD ARTICLE

REVISED Targeted protein degradation using deGradFP in *Trypanosoma brucei* [version 2; peer review: 4 approved]

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Abstract

Targeted protein degradation is an invaluable tool in studying the function of proteins. Such a tool was not available in *Trypanosoma brucei*, an evolutionarily divergent eukaryote that causes human African trypanosomiasis. Here, we have adapted deGradFP (degrade green fluorescent protein [GFP]), a protein degradation system based on the SCF E3 ubiquitin ligase complex and anti-GFP nanobody, in *T. brucei*. As a proof of principle, we targeted a kinetoplastid kinetochore protein (KKT3) that constitutively localizes at kinetochores in the nucleus. Induction of deGradFP in a cell line that had both alleles of KKT3 tagged with yellow fluorescent protein (YFP) caused a more severe growth defect than RNAi in procyclic (insect form) cells. deGradFP also worked on a cytoplasmic protein (COPII subunit, SEC31). Given the ease in making GFP fusion cell lines in *T. brucei*, deGradFP can serve as a powerful tool to rapidly deplete proteins of interest, especially those with low turnover rates.

Keywords

Trypanosoma brucei, targeted protein degradation, deGradFP, degron, kinetoplastid, kinetochore

Open Peer Review

Approval Status ✓ ✓ ✓ ✓

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version 2 (revision) 19 Oct 2022				
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REVISED **Amendments from Version 1**

In the revised manuscript, we added new results showing that CFB1C's F-box does not cause growth defects in KKT3-YFP cells, that expression of deGradFP that lacks an NLS does not cause growth defects in KKT3-YFP cells, and that NLS-deGradFP did not work on YFP-KKT18. We also made changes to respond to referee comments.

Any further responses from the reviewers can be found at the end of the article

Introduction

Kinetoplastids are a group of unicellular flagellated eukaryotes found in diverse environmental conditions (d'Avila-Levy *et al.*, 2015). They belong to the phylum Euglenozoa (Discoba/Excavata) and are evolutionarily distant from commonly studied model eukaryotes such as yeasts, worms, flies, and humans (Opisthokonta) (Cavalier-Smith, 2010; Keeling & Burki, 2019). Understanding their biology could therefore provide insights into the extent of conservation/divergence among eukaryotes and lead to a deeper understanding of biological systems and evolution of eukaryotes. Importantly, three neglected tropical diseases are caused by parasitic kinetoplastids: African trypanosomiasis, Chagas disease, and leishmaniasis (Horn, 2022; Rao *et al.*, 2019). Human African trypanosomiasis (sleeping sickness) is caused by *Trypanosoma brucei*, which also causes the cattle disease, nagana, that leads to weight loss and anemia in livestock and imposes a huge burden on economic development in affected regions. Understanding the biology of kinetoplastids could facilitate the design of new drugs against kinetoplastid parasites.

Inducible depletion of a target protein is an essential tool in biology (Prozzillo *et al.*, 2020). In *Trypanosoma brucei*, this can be achieved by RNAi (Alsford *et al.*, 2011; Ngô *et al.*, 1998) and Tet-off system (Merritt & Stuart, 2013) at the RNA level, as well as by conditional knockout at the gene level using Cre-LoxP (Kim *et al.*, 2013). Although powerful in many cases, these approaches are not efficient in reducing the level of proteins that have slow turnover rates. Targeted degradation tools could circumvent this problem and have been used in other organisms (Damerow *et al.*, 2015; Madeira da Silva *et al.*, 2009; Nabet *et al.*, 2018; Nishimura *et al.*, 2009; Uhlmann *et al.*, 2000; Wheeler *et al.*, 2015). However, such tools were not available in *Trypanosoma brucei*, to our knowledge.

In this study, we have adapted the deGradFP (degrade green fluorescent protein) system which was originally established in *Drosophila melanogaster* (Caussinus *et al.*, 2011). It relies on the expression of VhhGFP4 fused with a truncated F-box protein. VhhGFP4 is an anti-GFP nanobody that recognizes GFP and some derivatives such as yellow fluorescent proteins (YFP) and Venus (Saerens *et al.*, 2005), while an F-box protein is a substrate-recognition subunit of the SKP1–CUL1–F-box (SCF) E3 ubiquitin ligase complex that catalyzes the ubiquitylation of target proteins (Petroski & Deshaies, 2005). In deGradFP, a substrate-recognition domain of an F-box protein is replaced by VhhGFP4 so that GFP-fusion proteins are ubiquitylated by the SCF complex, leading to their degradation via the 26S proteasome pathway (Caussinus & Affolter, 2016). deGradFP or modified versions have been used in mammalian cells, *Caenorhabditis elegans*, zebrafish, and plants (Shin *et al.*, 2015; Sorge *et al.*, 2021; Wang *et al.*, 2017; Yamaguchi *et al.*, 2019). Here, we show that deGradFP successfully depletes a kinetochore protein and a COPII subunit in the procyclic form of *T. brucei* cells.

Results

To establish a targeted protein degradation method in *T. brucei*, we chose a protein that has a slow turnover rate. KKT3 is a kinetochore protein that constitutively localizes at kinetochores and does not show any obvious fluctuation in its abundance during the cell cycle, implying that it is a stable protein (Akiyoshi & Gull, 2014). In fact, the half-life of KKT3 has been estimated to be much longer than transiently-localized kinetochore proteins (Tinti *et al.*, 2019). To apply deGradFP in *T. brucei*, we made a construct that expresses an F-box domain fused with a nuclear localization signal (NLS) and the anti-GFP nanobody VhhGFP4 (Saerens *et al.*, 2005) (Figure 1A, B). The fusion protein was expressed from a derivative of pDEX777 that integrates at the 177 bp repeats on minichromosomes and allows doxycycline-inducible expression (Kelly *et al.*, 2007; Nerusheva & Akiyoshi, 2016). Putative F-boxes of Tb927.5.700 and Tb927.1.4580 (called cyclin-like F-box protein 1C, CFB1C (Benz & Clayton, 2007)) were tested. When expressed in cells that have KKT3-YFP as the sole copy of KKT3 (KKT3-YFP/kkt3Δ) (Figure 1C), the construct containing Tb927.5.700 caused growth defects, implying that expression of Tb927.5.700$^{1\text{-}200}$-NLS-VhhGFP4 led to degradation of KKT3-YFP. In contrast, expression of CFB1C$^{1\text{-}200}$-NLS-VhhGFP4 did not cause strong growth defects. This means that expression of VhhGFP4 (at least when fused to the F-box of CFB1C) was not sufficient to disrupt the function of KKT3-YFP. We therefore used the F-box from Tb927.5.700 (named FBP75 herein for F-box protein 75 kDa) for subsequent experiments. Besides the construct with an NLS to target nuclear proteins (pBA2675: NLS-deGradFP), we also made another one without an NLS to target cytoplasmic proteins (pBA2705: deGradFP). Induction of these deGradFP systems in wild-type procyclic cells with 1 μg/mL doxycycline did not cause any growth defect (Figure 1D), showing that expression of deGradFP itself does not cause growth defects.

We next used a cell line in which both alleles of KKT3 were C-terminally tagged with YFP using a PCR-based method in one transfection step (Dean *et al.*, 2015). We found that induction of NLS-deGradFP in this cell line caused more severe growth defects than RNAi (Figure 2A and B) (Marcianò *et al.*, 2021). We did not observe severe growth defects with deGradFP without NLS in KKT3-YFP cells, showing the importance of NLS to target nuclear proteins in our system (Figure 2C). Microscopy analysis confirmed that NLS-deGradFP caused more significant depletion of KKT3 at six hours than RNAi (Figure 2D and E). The fact that

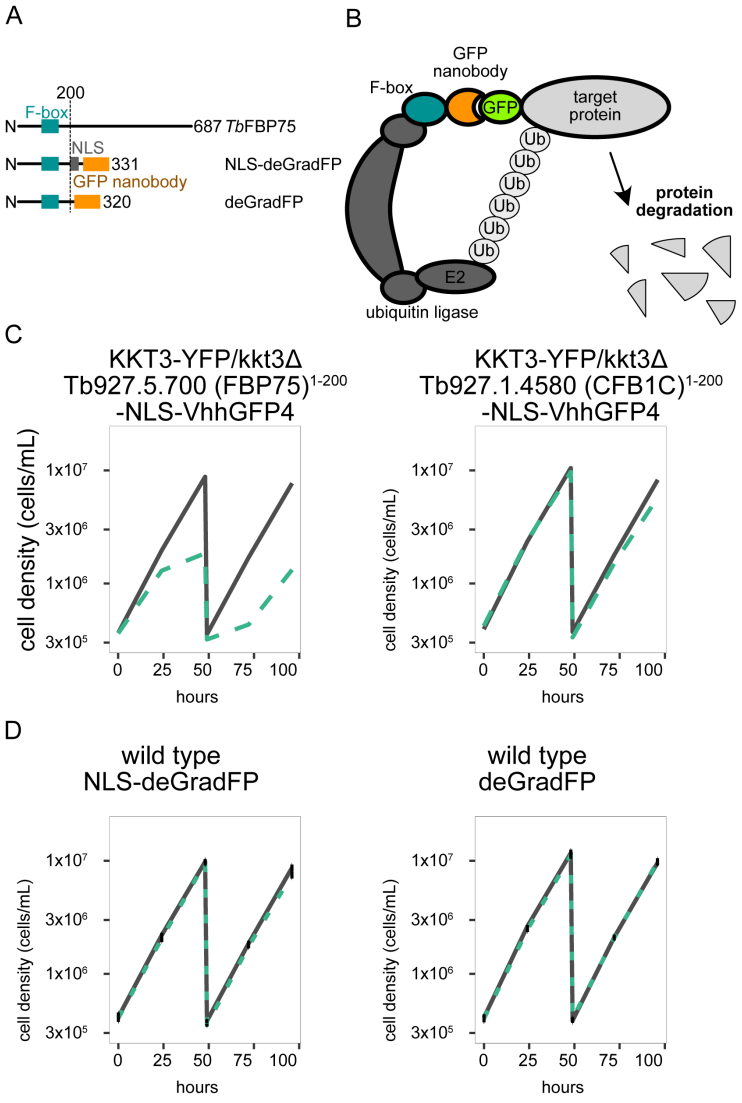

Figure 1. deGradFP in *Trypanosoma brucei*. (**A**) Schematic of *Tb*FBP75 (Tb927.5.700), NLS-deGradFP, and deGradFP, highlighting the putative F-box domain, NLS, and GFP nanobody (vhhGFP4). (**B**) deGradFP forms a complex with an endogenous ubiquitin ligase complex which transfers ubiquitins to the target protein tagged with GFP. Ubiquitylated target proteins are then degraded by the 26S proteasome. (**C**) Growth curve for KKT3-YFP/kkt3Δ with induction of Tb927.5.700 $(FBP75)^{1\text{-}200}$-NLS-VhhGFP4 (left) and Tb927.1.4580 $(CFB1C)^{1\text{-}200}$-NLS-VhhGFP4 (right). N=2. Representative growth curves are shown. Cell line: BAP2398, BAP2399 (**D**) Growth curve for wild-type procyclic cells with NLS-deGradFP (left) or deGradFP (right). N=3. Error bars are SEM. Cell line: BAP2395, BAP2511. For all growth curves, deGradFP was induced with 1 μg/ml doxycycline and cultures were diluted at day 2. Gray lines are uninduced controls. Green dashed lines are doxycycline-treated cells. N=3. Error bars are SEM. Cell line: BAP2395, BAP2511.

induction of NLS-deGradFP in wild-type cells did not cause any growth defect (Figure 1D) means that the observed growth defect was due to specific degradation of YFP-tagged KKT3. In the deGradFP system, YFP-tagged target proteins ubiquitylated by the SCF ubiquitin ligase complex are degraded by the proteasome system (Caussinus & Affolter, 2016). Consistent with this possibility, addition of the proteasome inhibitor MG132 prevented degradation of KKT3-YFP (Figure 2F), suggesting that our deGradFP system in *T. brucei* relies on the proteasome-dependent protein degradation pathway as expected. We note that NLS-deGradFP did not work well for another kinetochore protein KKT18 (Figure 2G). In contrast, RNAi-mediated depletion of KKT18 caused growth defects and reduction of protein.

We next targeted a cytoplasmic protein SEC31 using a deGradFP construct that lacks an NLS. SEC31 is a subunit of COPII and localizes at the endoplasmic reticulum (ER) exit site (Hu *et al.*, 2016). Both alleles of SEC31 were C-terminally tagged in a CRISPR cell line (Beneke *et al.*, 2017). Induction of deGradFP caused a strong growth defect (Figure 3A and B), which is apparently more severe than RNAi-mediated depletion of SEC31 reported in a previous study (Hu *et al.*, 2016). These results therefore show that deGradFP can efficiently deplete both nuclear and cytoplasmic proteins in *T. brucei*.

Discussion

In *T. brucei*, it is easy to tag genes at the endogenous locus using plasmid- or PCR-based methods (Beneke *et al.*, 2017;

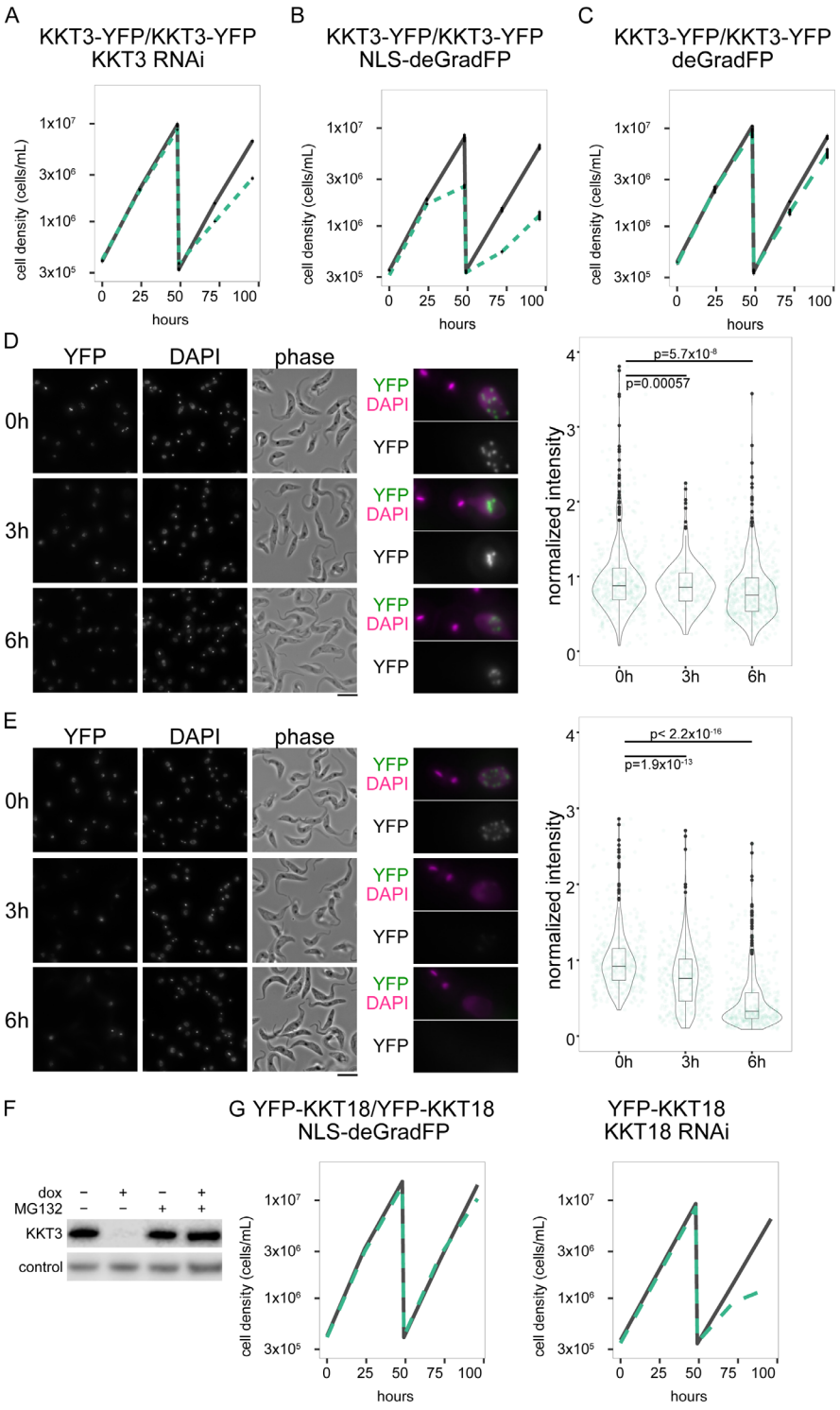

Figure 2. deGradFP depletes a nuclear protein KKT3 more efficiently than RNAi. (**A**, **B**, **C**) Growth curves for KKT3 knockdown by RNAi (**A**: N=4, cell line: BAP2512), KKT3-YFP depletion by deGradFP with NLS (**B**: N=3, cell line: BAP2513) and KKT3-YFP with deGradFP without NLS (**C**: N=3, cell line: BAP2514). Error bars are SEM. (**D**, **E**) Images of KKT3 knockdown by RNAi (**D**: cell line: BAP2512), and KKT3-YFP depletion by deGradFP with NLS (**E**: cell line: BAP2513). (Left) Examples of cells at 0 h, 3 h, and 6 h after induction. YFP and DAPI images are maximum intensity projection. Bars, 10 μm. (Centre) Examples of cells from left images. (Right) Plot of total YFP signal inside the nucleus (>239 cells in each condition). Data were normalized with the mean value at 0 h. P-values were calculated by Welch two sample t-test. (**F**) KKT3-YFP cells with NLS-deGradFP were incubated with either 1 μg/mL doxycycline or 10 μM MG132, or both for 6 hours. Control is untreated cells. KKT3-YFP was detected by immunoblot using anti-GFP antibody. PFR2 was used as a loading control. Cell line: BAP2513. (**G**) Growth curve for YFP-KKT18 with NLS-deGradFP (left) and KKT18 RNAi (right). Representative growth curves are shown. N=2 (NLS-deGradFP) and 3 (RNAi). Cell lines: BAP2593, BAP2122. For all growth curves, RNAi or deGradFP was induced with 1 μg/ml doxycycline and cultures were diluted at day 2. Gray lines are uninduced controls. Green dashed lines are doxycycline-treated cells.

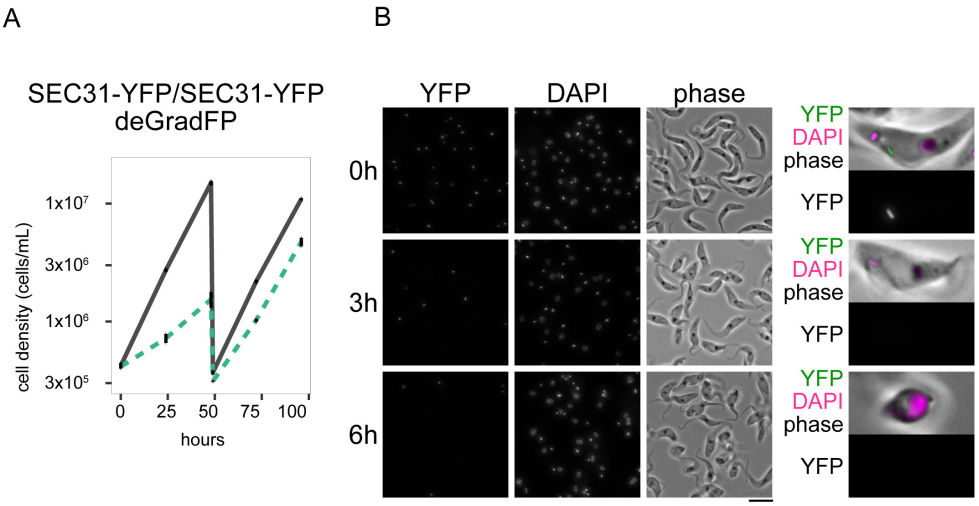

Figure 3. Depletion of a cytoplasmic protein SEC31 by deGradFP. (**A**) Growth curve for SEC31-YFP with deGradFP. N=4. Error bars are SEM. (**B**) Examples of cells at 0 h, 3 h, and 6 h after induction. YFP and DAPI images are maximum intensity projection. Bar, 10 µm. Cell line: BAP2518. For all growth curves, RNAi or deGradFP was induced with 1 µg/ml doxycycline and cultures were diluted at day 2. Gray lines are uninduced controls. Green dashed lines are doxycycline-treated cells.

Dean *et al.*, 2015; Kelly *et al.*, 2007; Kovářová *et al.*, 2022). Taking advantage of the inducible expression system (Poon *et al.*, 2012; Wirtz & Clayton, 1995), we have shown that deGradFP can induce targeted protein degradation in *T. brucei*. The depletion kinetics is faster than the RNAi-mediated depletion method, at least for KKT3. Our results therefore show that deGradFP can be a powerful tool in characterizing depletion phenotypes in *T. brucei*. It is, however, important to note that deGradFP has some limitations. For example, it has been suggested that deGradFP does not work if GFP is not accessible (Caussinus *et al.*, 2011; Caussinus & Affolter, 2016). It is also essential that target proteins have lysines that can be ubiquitylated by deGradFP. Furthermore, it is critical that GFP-fusion proteins retain enough functionality to support cell growth because the deGradFP system utilizes the VhhGFP4 nanobody that recognizes GFP or its derivatives. If necessary, this system could be modified to use nanobodies against other epitope tags or even the protein of interest itself to induce degradation of the target (Aguilar *et al.*, 2019).

The function of the F-box protein used in this study (FBP75) remains unknown. We also do not know which SKP1 or cullin proteins interact with FBP75 and whether those proteins are expressed in other life stages. It therefore remains unknown whether FBP75-based deGradFP works in bloodstream form cells. If it does not work, other F-box proteins could be utilized to deplete proteins of interest in bloodstream form cells (Benz & Clayton, 2007; Rojas *et al.*, 2017). In any case, it is our hope that deGradFP will prove to be a useful protein degradation tool to facilitate studies of *Trypanosoma brucei*.

Methods

Plasmids

All plasmids used in this study are listed in Table 1. To make pBA2675 (inducible expression of FBP75$^{1–200}$-NLS-VhhGFP4: NLS-deGradFP for nuclear proteins) and pBA2676 (inducible expression of CFB1C$^{1–200}$-NLS-VhhGFP4), synthetic DNA BAG181 (pBA2675) and BAG182 (pBA2676) (GeneArt, Thermo Fisher) were digested with HindIII/BamHI and subcloned into pBA310 cut with the same enzymes. The NLS sequence was derived from the La protein (Marchetti *et al.*, 2000). To make pBA2705 (Inducible expression of FBP75$^{1–200}$-VhhGFP4: deGradFP for cytoplasmic proteins), NLS was removed from pBA2675 by PCR with BA3647/BA3648. 12.5 µL of 2x PrimeSTAR MAX (Takara), 1 µL of 3 ng/µL pBA2675 plasmid, 1 µL each of 10 µM forward and reverse primers, and 11 µL of MilliQ water were mixed (30 cycles of 98°C 10 s, 55°C 15 s, 72°C 35 s). The PCR reaction was incubated with 1 µL of DpnI (NEB) at 37°C for 1 hr and was transformed into NEB 5-alpha competent *E. coli* (NEB). To make pBA871 (hairpin RNAi targeting 49–518 bp of KKT18 coding sequence) and pBA1061 (hairpin RNAi targeting 2562–3072 bp of the KKT3 coding sequence), synthetic DNA BAG34 (KKT18) and BAG55 (KKT3) (GeneArt, Thermo Fisher) were digested with HindIII/BamHI and subcloned into pBA310 cut with the same enzymes.

Trypanosome cells

All cell lines used in this study were derived from the TREU 927 procyclic form cells and are listed in Table 2. SmOxP927 expresses Tet repressor and T7 RNA polymerase (Poon *et al.*, 2012), while PCF 1339 expresses Tet repressor, T7 RNA polymerase, and the Cas9 nuclease (Beneke *et al.*, 2017). Cells were grown at 28°C in SDM-79 medium (Life Technologies, Thermo Fisher) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 7.5 µg/mL hemin, as well as puromycin (Sigma) and appropriate drugs (Brun & Schönenberger, 1979).

To make the homozygous KKT3-YFP cell line, two YFP-tagging cassettes were amplified from pPOTv7 (YFP, Hyg) or pPOTv7 (YFP, G418) (Dean *et al.*, 2015) by PCR using BA1821/BA1822 (Table 3). 25 µL of 2x PrimeSTAR MAX (Takara),

Table 1. Plasmids used in this study.

Name	Description
pPOTv7 (eYFP, Hyg)	PCR-based eYFP-tagging vector, hygromycin marker (Dean *et al.*, 2015)
pPOTv7 (eYFP, G418)	PCR-based eYFP-tagging vector, G418 marker (Dean *et al.*, 2015)
pBA101	YFP-KKT18 tagging at endogenous KKT18 locus, hygromycin (Akiyoshi & Gull, 2014)
pBA871	Inducible expression of KKT18 hairpin RNAi (targeting 49–518 bp of KKT18 coding sequence), integrate at 177 bp, phleomycin marker
pBA310	Inducible expression vector, integrate at 177 bp, phleomycin marker (Nerusheva & Akiyoshi, 2016)
pBA1061	Inducible expression of KKT3 hairpin RNAi (targeting 2562–3072 bp of KKT3 coding sequence), integrate at 177 bp, phleomycin marker
pBA2675	Inducible expression of FBP75$^{1-200}$-NLS-VhhGFP4, integrate at 177 bp, phleomycin marker (NLS-deGradFP for nuclear proteins)
pBA2676	Inducible expression of CFB1C$^{1-200}$-NLS-VhhGFP4, integrate at 177 bp, phleomycin marker
pBA2705	Inducible expression of FBP75$^{1-200}$-VhhGFP4, integrate at 177 bp, phleomycin marker (deGradFP for cytoplasmic proteins)

Table 2. Trypanosome cell lines used in this study.

Name	Description
SmOxP927	TREU927 procyclic cells that expresses TetR and T7 RNAP (Poon *et al.*, 2012)
PCF 1339	TREU927 procyclic cells that expresses TetR, T7 RNAP, and Cas9 (Alves *et al.*, 2020)
BAP167	YFP-KKT18 (Akiyoshi & Gull, 2014)
BAP2122	YFP-KKT18, KKT18 RNAi
BAP1123	KKT3-YFP/kkt3Δ (Nerusheva *et al.*, 2019)
BAP2398	KKT3-YFP/kkt3Δ, FBP75$^{1-200}$-NLS-VhhGFP4
BAP2399	KKT3-YFP/kkt3Δ, CFB1C$^{1-200}$-NLS-VhhGFP4
BAP2395	FBP75$^{1-200}$-NLS-VhhGFP4
BAP2511	FBP75$^{1-200}$-VhhGFP4
BAP2464	KKT3-YFP/KKT3-YFP
BAP2512	KKT3-YFP/KKT3-YFP, KKT3 RNAi
BAP2513	KKT3-YFP/KKT3-YFP, FBP75$^{1-200}$-NLS-VhhGFP4
BAP2514	KKT3-YFP/KKT3-YFP, FBP75$^{1-200}$-VhhGFP4
BAP2466	SEC31-YFP/SEC31-YFP
BAP2518	SEC31-YFP/SEC31-YFP, FBP75$^{1-200}$-VhhGFP4
BAP2567	YFP-KKT18/YFP-KKT18
BAP2593	YFP-KKT18/YFP-KKT18, FBP75$^{1-200}$-NLS-VhhGFP4

1 µL of 30 ng/µL template pPOT plasmid, 1 µL each of 10 µM forward and reverse primers, and 22 µL of MilliQ water were mixed (30 cycles of 98°C 10 s, 55°C 15 s, 72°C 1 min). 50 µL of PCR products were transfected into SmOxP927 (Poon *et al.*, 2012) by electroporation. Transfected cells were selected by addition of 50 µg/mL hygromycin (Sigma) and 30 µg/mL G418

Table 3. Sequence of primers and synthetic DNA used in this study.

Name	Description
BA3647	CACCTGCTCCACCGTCCTCCATGTGCGGCA
BA3648	GGAGGACGGTGGAGCAGGTGGAGCAGGTGT
BA1821	GTAATGGAGTTTGTGAGGTGCTTGATGAGGAAAAATTCCCCCTTTCGGAGGAACTCAACCAGATGCTCTACGGTGGCGTGGGTTCTGGTAGTGGTTCC
BA1822	GAAATGCGACAGCAGACGGAAACGGAAAAAAAAATAAAAAAAAAGAGAGGGCTATCTGTAATTCTTTACGTACATCACTTCCAATTTGAGAGACCTGTGC
BA523	TATGTCTGTTTATTGCCCAC
BA2352	GATCGATC GCGGCCGC TTTTCAGTTGCTATAGGCCT
BA3633	GCTCAAGGGAAATGTGGAAAGAGCTCGCCACTAAGCACTTTTCGGCAATTCAACATATTAATAACCTCAAGTTTCTGCAGGGTTCTGGTAGTGGTTCCGG
BA3634	ATATATGCAACCCGGCGACAAACAAACACCGCACAGGTGCAAAGGCACACAAACATGTTTTCCTTTGAGTGCCATGTGTGCCAATTTGAGAGACCTGTGC
BA3635	GAAATTAATACGACTCACTATAGGGGCGTGGCTCGCTCACGAGCGTTTTAGAGCTAGAAATAGC
G00 (BA2931)	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATT TCTAGCTCTAAAAC
BA3638	TTGTTTAGGATCACAACGCT
BA3639	GTACAGACACGTCCGTACAA
BA3376	CACTTTATCCTTGGGAGGGGTTTGCCAACCGCGTGCCTGTGGTCTTCAGCCGTGCATTTCTTTCCTCTTTCTTCAGCCTCGTATAATGCAGACCTGCTGC
BA3377	ACCTCTACATCAGAAAGGGGAAACACACAAGCGCGCACCTCGTCCGTTGGAAGCTGCAACAACTCTTTTACGCAGGTCATACTACCCGATCCTGATCCAG
BA3378	GAAATTAATACGACTCACTATAGGATCGTTGTAGGGCGCTGACCGTTTTAGAGCTAGAAATAGC
BA385	GATCGATC GCGGCCGC ATGATTGGCTGTTATGCAAC
BA384	GATCGATC GCGGCCGC GCTGGCCCTCTTTACATGAA
BAG34	GATCGATCGATC AAGCTT GCTTGTGTGTTTCCCCTTTCTGATGTAGAGGTTACTTTAGTCCTCTGTGAATTGGGAAAACTTCTGCAGAGATGGCTTTACGCCACGCGCTACAAGTTGG TTTCCTCATTCGAGGTATTTCTTCATTTATTTGTGCACGTAGCGGACAGAGAACCGAAAAGGATAGCCAACGCTTTCCTCGGTTCATGTAAAGAGGGCCA GCACCTGTCGTTGCTGGCAGATGTATGTGAAACCCTCTTCTCACCATCCCGGATTTCCCTTCTTGCTGAAGTGGACGTAGAGCTTTTCTTAACATTCTTA AAGTTCCTCTGTGACATTCCTGTATTGAAGGATCGGCTCGGTAATGTTCTGGTGAAGATTCTCCTGGAATTCCTCTCCGCTGCGGTGGAGCGCGATGGGG ACTACCGTCAACCCCGAGCATGTGCAAGCGTGCTCATCACCCTCACACGTGGGTCAAAGGCAAACAAAGAAAAGGCGGACCCTCATTTCTAAGTACGGTC AGGTGTCGTAGCACTGCATTGAATTCGATTGCCATTCTCCGAGTGTTTTAGCGTGACGGCCGCAGGGGTCCCATAATCTTTGTTTGCCTTTGACCCACGT GTGAGGGTGATGAGCACGCTTGCACATGCTCGGGGTTGACGGTAGTCCCCATCGCGCTCCACCGCAGCGGAGAGGAATTCCAGGAGAATCTTCACCAGAA CATTACCGAGCCGATCCTTCAATACAGGAATGTCACAGAGGAACTTTAAGAATGTTAAGAAAAGCTCTACGTCCACTTCAGCAAGAAGGGAAATCCGGGA TGGTGAGAAGAGGGTTTCACATACATCTGCCAGCAACGACAGGTGCTGGCCCTCTTTACATGAACCGAGGAAAGCGTTGGCTATCCTTTTCGGTTCTCTG TCCGCTACGTGCACAAATAAATGAAGAAATACCTCGAATGAGGAAACCAACTTGTAGCGCGTGGCGTAAAGCCATCTCTGCAGAAGTTTTCCCAATTCAC AGAGGACTAAAGTAACCTCTACATCAGAAAGGGGAAACACACAAGC GGATCC GATCGATCGATC (KKT18 hairpin)

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Name	Description
BAG55	GATCGATCGATC AAGCTT ACGTCACATGCTGCTTAACGGTGATTGGATACGCTACTACCACTTTTATCCTATGGAGGAAGAAGGAGGCGACTCAGTCGCTGTCACATATCATATTCAG CCGGGACGTACTGGTGTTACATTTTTCAACCATAGTTTTTCTGTGCACTCAGCTGTGCTGTCAGTGTTGGAACACATCGTATACGTCGTAGATCGTGTTG ATATCGAGGAAGATAATGACGTGGCGCGTATCTTGTCGTTGGCACAAGCATTGAATGAGGAGAAGAAGATCTACGATGTCCTTCAATTGGTCGAAACCCA CGACACACATATGTTAAAACAGCGGCGGTCTCCCGGTATTATGTCTGTTTATTGCCCACCACAAACAGCATTTCAATGCAATGGTGATCCCTTTGTATTT GTTCGCTGGTACCGCTTCCATATGGAAAACTCTATGAGTGGCTTTATGCTCTCCAACGGGGCTGTGCAGGTGTTTGTAGGCGGGAAATACGAGTTACGGT GGCTGGATGACAAAGGCGGACCCTCATTTCTAAGTACGGTCAGGTGTCGTAGCACTGCATTGAATTCGATTGCCATTCTCCGAGTGTTTTAGCGTGACGG CCGCAGGGGTCCCATAAGTCATCCAGCCACCGTAACTCGTATTTCCCGCCTACAAACACCTGCACAGCCCCGTTGGAGAGCATAAAGCCACTCATAGAGT TTTCCATATGGAAGCGGTACCAGCGAACAAATACAAAGGGATCACCATTGCATTGAAATGCTGTTTGTGGTGGGCAATAAACAGACATAATACCGGGAGA CCGCCGCTGTTTTAACATATGTGTGTCGTGGGTTTCGACCAATTGAAGGACATCGTAGATCTTCTTCTCCTCATTCAATGCTTGTGCCAACGACAAGATA CGCGCCACGTCATTATCTTCCTCGATATCAACACGATCTACGACGTATACGATGTGTTCCAACACTGACAGCACAGCTGAGTGCACAGAAAAACTATGGT TGAAAAATGTAACACCAGTACGTCCCGGCTGAATATGATATGTGACAGCGACTGAGTCGCCTCCTTCTTCCTCCATAGGATAAAAGTGGTAGTAGCGTATCCA ATCACCGTTAAGCAGCATGTGACGT GGATCC GATCGATCGATC (KKT3 hairpin)
BAG181	GATCGATC AAGCTT ATGGGTGGTGGAGCAGCGGTGTCGTCTGGTGACGACAGCGCCGCGGCATCTTCGGGTAACGACACCACCACAACGGATGGTAATCATGGCGGGAGGTACG CCTGGGAATGCGTTGACGACGTCGGGGGCGCTTTCAACGGTTCCATGAATGGCAACACCTCCCTGCCAAAAGGTTCAAACATCTGTGAGACTCACGGGTG TTGGAGTAAGCAAATACTCCGAAGGGACATTTGCTGCGAGCGGAGCTACGTTCACCCTGTCAGCGCTGCTTGGCGTTGCAGCAGCGTTCTTTCACTTCCA GTGTCACTCCTTGACGAGGTGTTCACTTTTTTGCATCCCGAGGATCTCTGCAGGGTATTGGAAGTATGCCGGTTTTTCTTCTCTGCCGCTGTAAGGTCCG ATCGTACCGCCTGGAGGTCCGTATGCCTTTCGCTATGGAAAAACAAGCAGGGGCTCTCGCGCGTGGTGCGTGAATGGCCGTCTGTGGAGGAGGTATGCCG ACAGGAAGACTTGGAGTCGATTTGTGTGCAACAAGCGTTTGCACATGAATATTTCAGCTGGGGAACCATTAACAACATGGTGCCGCACATGGAGGACGGT ACCGGTCGAGGACACAAGCGGTCACGTGAACAA GGAGCAGGTGGAGCAGGT GTCCAACTGGTGGAGTCTGGTGGCGCTTTGGTGCAGCCAGGTGGCTCTCTGCGTTTGTCCTGTGCCGCTTCTGGCTTCCCAGTGAACCGCTATTCCATGC GCTGGTATCGCCAGGCTCCAGGCAAAGAGCGTGAGTGGGTAGCCGGTATGTCCAGCGCGGGTGATCGTAGCTCCTATGAAGACTCCGTGAAGGGCCGTTT CACCATCAGCCGTGACGATGCCCGTAACACGGTGTATCTGCAAATGAACAGCTTGAAACCTGAAGATACGGCCGTGTATTACTGTAATGTGAACGTGGGCTTC GAGTATTGGGGCCAAGGCACCCAGGTCACCGTCTCCAGCTAA GGATCC GATCGATC (FBP75's first 600 bp, NLS, GlyAlaGly linker x2, VhhGFP4)
BAG182	GATCGATC AAGCTT ATGTTTTTGAAGGAGGGAAACAGTGAAATGCGGTTGAGCGGAGCTACGTTCTTGAAGGGCGCTGTTGATGATTTCAATGCATGTGAGTCCCCTGTTTTAT CATCCGGAAGCAGTTTGGTTCTCACAAGTCCTGCTCTGAGCACCTCAGACACGTGCCGATCTGCGATGCGTGAAACCATCCCTTCCGTTTGTGCTGGCGC AGGCCTCAGCAGTCGCTGCTCAACTGATTGCACTGTGCTGAAGTGTGTGAACCCGCTGCATGTTAAGTTCCAGGAGCCGTCAGGGTTGTCAGGGGCTGAT TCAGTGGACTCGTTTGACTCGTTGGGTGACCTTTCCGAGTTGCATGAGGAACATCAACATGTTTCTTCTGTTGCCTCTGAAAAGCCTTTGACCCTTCACG ATGTGCCGGAATCCGTCCTGTATGGTGCCATTCCATACCTTTCCCTTCCAGACGTCATTGCCTTATCACGGACATGTAAGAAGTTTCACAAACTGGTGCA GGGATACTTTGCAGTGAATGAGCATGGCGTGATGAGCATCCCCGCCTTTGACACCCGCAGCTTCATGCAGTACCGTCCAGAGAGGAAACCTCCGGTAACG ACCGGTCGAGGACACAAGCGGTCACGTGAACAA GGAGCAGGTGGAGCAGGT GTCCAACTGGTGGAGTCTGGTGGCGCTTTGGTGCAGCCAGGTGGCTCTCTGCGTTTGTCCTGTGCCGCTTCTGGCTTCCCAGTGAACCGCTATTCCATGC GCTGGTATCGCCAGGCTCCAGGCAAAGAGCGTGAGTGGGTAGCCGGTATGTCCAGCGCGGGTGATCGTAGCTCCTATGAAGACTCCGTGAAGGGCCGTTT CACCATCAGCCGTGACGATGCCCGTAACACGGTGTATCTGCAAATGAACAGCTTGAAACCTGAAGATACGGCCGTGTATTACTGTAATGTGAACGTGGGC TTCGAGTATTGGGGCCAAGGCACCCAGGTCACCGTCTCCAGCTAA GGATCC GATCGATC

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(Sigma) and cloned by dispensing dilutions into 96-well plates. Clones were screened by diagnostic PCR of genomic DNA using BA523/BA2352.

To make the homozygous SEC31-YFP and YFP-KKT18 cell lines, donor DNA templates were amplified from pPOTv7 (YFP, Hyg) or pPOTv7 (YFP, G418) (Dean *et al.*, 2015) with BA3633/BA3634 (SEC31) or BA3376/3377 (KKT18). 25 µL of 2x PrimeSTAR MAX (Takara), 1 µL of 30 ng/µL template pPOT plasmid, 1 µL each of 10 µM forward and reverse primers, and 22 µL of MilliQ water were mixed (30 cycles of 98°C 10 s, 55°C 15 s, 72°C 1 min). sgRNA template amplified with BA3635/G00 (SEC31) and BA3378/G00 (KKT18) using 12.5 µL of 2x PrimeSTAR MAX (Takara), 4 µL of 10 µM G00 primer, 4 µL of 10 µM target specific forward primer, 4.5 µL MilliQ water (35 cycles of 98°C 10 s, 60°C 30 s, 72°C 15 s). All the PCR products were mixed and purified using a QIAquick PCR purification kit (QIAGEN), eluted with 50 µL of MilliQ water, and then co-transfected into PCF 1339 (Alves *et al.*, 2020; Beneke *et al.*, 2017) by electroporation. Transfected cells were selected by addition of 50 µg/mL hygromycin (Sigma) and 30 µg/mL G418 (Sigma) and cloned by dispensing dilutions into 96-well plates. Clones were screened by diagnostic PCR of genomic DNA using BA3638/BA3639 (SEC31) and BA385/BA384 (KKT18). 5 µg of RNAi and deGradFP constructs were linearized by NotI-HF (NEB) and transfected into YFP-tagged cell lines (3 clones each for KKT3-YFP, SEC31-YFP, and YFP-KKT18) or SmOxP927 by electroporation and selected by addition of 5 µg/mL phleomycin (Sigma). For induction of deGradFP or RNAi, doxycycline (Sigma) was added to the medium to a final concentration of 1 µg/mL. Cell growth was monitored using a CASY cell counter (Roche) and plotted with ggplot in R.

Fluorescence microscopy

Cells were harvested by centrifugation at 800 g for 5 min, washed in PBS, settled onto glass slides for 5 min, and fixed with 4% paraformaldehyde for 5 min. Following three washes in PBS (5 min each), cells were permeabilized with 0.1% NP-40 in PBS for 5 min, washed three times in PBS (5 min each), and embedded in mounting media (1% 1,4-Diazabicyclo [2.2.2]octane (DABCO), 90% glycerol, and 50 mM sodium phosphate pH 8.0) containing 100 ng/mL DAPI. Images were captured at room temperature on a Zeiss Axioimager. Z2 microscope (Zeiss) installed with ZEN using a Hamamatsu ORCA-Flash4.0 camera with 63x objective lenses (1.40 NA). 22 optical slices spaced 0.24 µm apart were collected. Images were processed in ImageJ/Fiji (Schneider *et al.*, 2012). Maximum intensity projection images were generated by Fiji software (Schneider *et al.*, 2012). Total YFP intensity in the nucleus was measured using 3D Objects Counter with default settings in Fiji as follows. DAPI images were first used to segment the nucleus by removing regions that have top 0.2% intensity (that correspond to kDNA signals) and then by selecting objects that have the size of nuclei (5.3–40 μm^3). YFP total intensity inside the nucleus was measured using a redirect function in 3D Objects Counter. 3D Plots were made with ggplot in R.

Immunoblots

Cells were harvested by centrifugation (800 g, 5 min) and washed with 1 ml PBS. Then cells were resuspended in 2× LDS sample buffer (Thermo Fisher) with 0.1 M DTT. Denaturation of proteins was performed for 5 min at 95°C. SDS-PAGE and immunoblots were performed by standard methods using the following antibodies: anti-GFP (OriGene, rabbit TP401, 1:2000) for YFP-tagged proteins and L8C4 (anti-PFR2, 1:1500) (Kohl *et al.*, 1999) for a loading control. Bands were visualized by horseradish-peroxidase-conjugated sheep anti-mouse-IgG antibodies (GE Healthcare) on an ODYSSEY Fc Imaging System (LI-COR).

Data availability

Underlying data

Figshare: Extended Data for "Targeted protein degradation using deGradFP in *Trypanosoma brucei*", https://doi.org/10.6084/m9.figshare.19960244 (Ishii & Akiyoshi, 2022)

This project contains the following underlying data:

- Fig1_1.csv (raw data for growth curve of NLS-deGradFP in wild-type cells, BAP2395)

- Fig1_2.csv (raw data for growth curve of deGradFP in wild-type cells, BAP2511)

- Fig1_3.csv (raw data for growth curve of NLS-deGradFP in KKT3-YFP/kkt3Δ, BAP2398)

- Fig1_4.csv (raw data for growth curve of Tb927.1.4580$^{1-200}$-NLS-VhhGFP4 in KKT3-YFP/kkt3Δ, BAP2399)

- Fig2_1.csv (raw data for growth curve of KKT3-YFP/KKT3-YFP with KKT3 RNAi, BAP2512)

- Fig2_2.csv (raw data for YFP intensity of KKT3-YFP/KKT3-YFP with KKT3 RNAi, BAP2512)

- Fig2_3.czi (raw microscopy image, 0h control, KKT3-YFP/KKT3-YFP with KKT3 RNAi, BAP2512)

- Fig2_4.czi (raw microscopy image, 3h, KKT3-YFP/KKT3-YFP with KKT3 RNAi, BAP2512)

- Fig2_5.czi (raw microscopy image, 6h, KKT3-YFP/KKT3-YFP with KKT3 RNAi, BAP2512)

- Fig2_6.csv (raw data for growth curve of KKT3-YFP/KKT3-YFP with NLS-deGradFP, BAP2513)

- Fig2_7.csv (raw data for YFP intensity of KKT3-YFP/KKT3-YFP with NLS-deGradFP, BAP2513)

- Fig2_8.czi (raw microscopy image, 0h control, KKT3-YFP/KKT3-YFP with NLS-deGradFP, BAP2513)

- Fig2_9.czi (raw microscopy image, 3h, KKT3-YFP/KKT3-YFP with NLS-deGradFP, BAP2513)

- Fig2_10.czi (raw microscopy image, 6h, KKT3-YFP/KKT3-YFP with NLS-deGradFP, BAP2513)

- Fig2_11.csv (raw data for growth curve of KKT3-YFP/KKT3-YFP with deGradFP, BAP2514)

- Fig2_12.csv (raw data for growth curve of YFP-KKT18 with KKT18 RNAi, BAP2122)

- Fig2_13.csv (raw data for growth curve of YFP-KKT18 with NLS-deGradFP, BAP2593)

- Fig2_14.tif (raw immunoblot data for KKT3-YFP, GFP antibody)

- Fig2_15.tif (raw immunoblot data for PFR2 loading control, L8C4 antibody)

- Fig3_1.csv (raw data for growth curve of SEC31-YFP/SEC31-YFP with deGradFP, BAP2518)

- Fig3_2.csv (raw microscopy image, 0h control, SEC31-YFP/SEC31-YFP with deGradFP, BAP2518)

- Fig3_3.csv (raw microscopy image, 3h, SEC31-YFP/SEC31-YFP with deGradFP, BAP2518)

- Fig3_4.csv (raw microscopy image, 6h, SEC31-YFP/SEC31-YFP with deGradFP, BAP2518)

- Table3.csv (Sequence for primers, synthetic DNA, and plasmids)

Acknowledgments

We thank Markus Affolter for advice. We also thank Jack Sunter, Sam Dean, and Tom Beneke for sharing reagents. pBA2675 (NLS-deGradFP for nuclear proteins) and pBA2705 (deGradFP for cytoplasmic proteins) and their plasmid maps have been deposited into Addgene (ID 189997 and 189998).

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Version 1

Reviewer Report 11 July 2022

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✔ **David Horn**
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Approaches for conditional expression of specific mRNAs and/or proteins have greatly facilitated experimental analyses of protein function in cells and organisms, particularly where protein depletion results in loss-of-fitness. One such approach involves targeted protein degradation, which was not previously reported for the African trypanosome, Trypanosoma brucei, an otherwise experimentally tractable parasite that causes often lethal diseases in humans and animals.

The current study presents compelling data reporting adaptation of the deGradFP system for insect-form T. brucei; proof-of-principle is demonstrated by targeting a nuclear protein and a cytoplasmic protein. As the authors point out, the deGradFP approach relies upon the GFP-tagged protein retaining function and the VhhGFP4 nanobody being able to access the tag (meaning that the tag and the nanobody must be present in the same cellular compartment). This approach, as also noted, could be particularly powerful and effective for linking primary loss-of-function phenotypes to proteins with normally slow turnover, since protein degradation using deGradFP is rapid (detectable after 3 h), while knockdown by the widely used RNA interference approach can be relatively slow.

I suggest some minor/simple edits to improve clarity:

1. Fig. 1C, 2A, 2B, 3: The error bars are unclear. Suggest increasing the line weight.

2. Fig. 1 legend: Replace “dashed dashed” with “dashed lines”.

3. First paragraph of results: Replace “177 bp locus” with “177 bp repeats on minichromosomes”.

Some further suggestions that the authors may want to consider:

1. It’s likely that the two example proteins are degraded in a proteasome-dependent manner,

but this could be tested using a proteasome inhibitor such as bortezomib.

2. First paragraph of results: The authors could add some detail explaining how Tb927.5.700 was selected/prioritised as the source of the F-box to be fused to the nanobody.

3. Discussion: deGradFP may also effectively target 'nuclear' proteins, either before they reach the nucleus or by acting directly in the nucleus. Indeed, it remains unclear at this point whether the centrally located La NLS effectively directs NLS-deGradFP to the nucleus. Also, some proteins that function in both compartments may be effectively targeted by deGradFP.

4. Consider depositing key plasmids with a plasmid repository such as Addgene.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasite genetics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 July 2022

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This work reports the implementation of a degron approach for protein depletion in *T. brucei*. While *T. brucei* benefits from RNA interference, degron approaches that target the protein pool directly are likely to be valuable tools for rapid, selective, and more complete protein depletion. Ishii and Akiyoshi have identified a domain of a trypanosomatid-specific SCF E3 ubiquitin ligase complex (FBP75) that can target proteins for degradation, which is an important advance. They have fused this degron domain to a single-chain antibody that recognizes GFP, which when expressed using a tetracycline-inducible system targets FP-tagged proteins in cells, likely leading to their ubiquitination and subsequent degradation via the proteasome. Expression of the FBP75-Vhh fusion on its own does not appear to be toxic, which is essential for this method. The authors have shown degron-tag specific depletion of two proteins- the kinetochore protein KKT3, which is nuclear, and Sec31, which is a component of the COPII complex in the cytosol. They argue that the depletion of these proteins is more rapid than RNAi and provides more acute growth defects.

Overall, this work provides the exciting prospect of a functional degron system for use in trypanosomatids, which could be extremely useful as a complement to RNAi in *T. brucei* and allow the conditional depletion of essential proteins in *Leishmania* and *T. cruzi*, which is a pressing issue. I have one significant concern that could be addressed by citing specific previous results:

1. As the authors mention, the FBP75 domain has just been identified as a potential degron and we do not currently know which Skp1 or cullins the domain interacts with - this specific pathway in T. brucei is complex. Can the authors show that the Vhh antibody on its own is not sufficient to disrupt the function of or degrade the YFP-tagged proteins? It appears that the original Cassinus paper has this data (Figure 2B). Considering that a new degron candidate is being employed here, the evidence that the Vhh on its own does not cause degradation in other systems should be specifically mentioned.

Smaller comments not necessary to address in this manuscript but would be important for testing the mechanism and generality of the approach:

1. Does the expression of the Vhh-FBP75 fusion lead to the appearance of higher MW bands corresponding to ubiquitinated versions of the YFP-tagged proteins?

2. Does blocking proteasomal activity via MG132 limit the loss of YFP signal? Are the proteins mislocalized, even if they are still present in the cell?

3. It seems possible that degron-mediated depletion of proteins could provide different phenotypes compared to RNAi. Since the method could actively target proteins that are already part of stable protein complexes, such as the KKTs, ubiquitination of these proteins could lead to the direct disruption of complexes. That might provide different phenotypes compared to RNAi. So, speed of depletion is just one aspect of the potential benefits of the degron. Have the authors observed any differences in how the phenotypes manifest?

4. The authors do not provide a side-by-side comparison of the Sec31 RNAi and Sec31 degron. The paper they cite for the Sec31 RNAi shows a slower growth defect than the degron, but it should be noted that at longer time points the RNAi is showing a very lethal phenotype while the degron is showing what appears to be a rebound in the population. This could be due to a loss of expression of the Vhh-degron, which can occur with tetracycline-inducible

systems. This might be a persistent issue with this implementation of the degron approach.

5. Figure 1 legend: “Green dashed dashed” - Remove one of the “dashed.”

6. Figures 1 - 3: Error bars aren’t clear. I would recommend making them more visible by using a thicker line size.

7. Figures 2 and 3: The authors could zoom in to a smaller area for the fluorescence images so that we can see the YFP image in more detail.

8. To our knowledge, the CRISPR/Cas9 approach used/described in Beneke *et al.*, 2017 is not a conditional KO. It is described as such in the introduction.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: parasitology, chemical biology, cytoskeleton

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 29 June 2022

https://doi.org/10.21956/wellcomeopenres.19908.r51226

Christine Clayton
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I will not summarize the article since the Abstract is perfectly adequate.

This method should be extremely useful for studying the functions of proteins that are difficult to deplete by RNAi, or which show slow depletion so that it is difficult to distinguish between primary and secondary effects. The authors carefully highlight the possible drawbacks, especially not knowing whether it works in bloodstream forms, and target protein accessibility. It is likely that proteins that are in membrane-bound compartments (glycosome, mitochondrion, ER) will be less susceptible. In future, it would be interesting to know whether it was really necessary to express the nuclear-targeted version of the nanobody fusion in order to get degradation of the kinetochore protein, and what happens for proteins that shuttle between nucleus and cytoplasm. It's also possible that some "accessible" proteins will be less amenable than others, as happens with other degron systems - only testing will reveal this.

It would have been useful to supply annotated versions of the plasmid sequences and some maps. I'm sure researchers could work this out but it would save time to supply the details in advance. On the other hand it's really nice that the sequences are already supplied, and the colour-coding on the sequence Table is already very helpful.

I have a small quibble. The statement "kinetoplastids may represent one of the earliest-branching eukaryotes based on a number of unique molecular features" with the reference to Cavalier-Smith's paper in 2010 should probably be removed. Most of the arguments in that paper have since been invalidated. Cavalier-Smith's conclusion is based mainly on the absence of Tom40 and ORC complexes. Both Tom40 and ORC complexes are known to be present, although highly diverged from those of other model organisms. In contrast, for example, some Kinetoplastid protein sequences (e.g. Tom22) resemble plants more than yeast. Other remaining arguments for things that are missing or unusual are also out of date. Possibly more remarkable is the completely new kinetochore composition in trypanosomes, discovered by the current author. However, it depends on what you judge to be important - what about (for example) the novel proteins that replace histones in Dinoflagellates? Burki *et al.* (2019[1]), based on seqeunces, put all of the Excavata and various other things as branching separately, but on the same "level" as separation of Amorphea from Plants and SAR, with no way of seeing what came first. Trying to pick out a few organisms as being earliest-branching based on particular features is simply too subjective.

References

1. Burki F, Roger AJ, Brown MW, Simpson AGB: The New Tree of Eukaryotes.*Trends Ecol Evol*. **35** (1): 43-55 PubMed Abstract | Publisher Full Text

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use

by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Trypanosome gene expression

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 27 June 2022

https://doi.org/10.21956/wellcomeopenres.19908.r51223

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A deGradFP system that allows inducible, targeted protein degradation in *T. brucei* is described. As a proof-of-concept, the system is successfully used to target the degradation of one nuclear protein and one cytoplasmic protein. This is the first targeted protein degradation method reported in *T. brucei*, expanding the current molecular toolkits available in this important model organism. Rapid protein degradation makes it very useful for protein functional studies.

As the method relies on the physical recruitment of the ubiquitin ligase complex to the targeted protein through YFP binding and KKT3 is present in a protein complex, it may be worth addressing whether other components in the KKT complex could also be degraded through this process. Such information may help to understand any potential “off-target” effects of this method.

Another minor suggestion is to make the error bars larger in all the growth curves. They are barely visible in the current figures.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: T. brucei cell biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.