



## METHOD ARTICLE

# Targeted protein degradation using deGradFP in *Trypanosoma brucei* [version 1; peer review: 2 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, degran, kinetoplastid, kinetochore

## Open Peer Review

### Approval Status

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## 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) (Keeling & Burki, 2019). Furthermore, it has been proposed that kinetoplastids may represent one of the earliest-branching eukaryotes based on a number of unique molecular features (Cavalier-Smith, 2010). 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) and CRISPR/Cas9 (Beneke *et al.*, 2017). 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

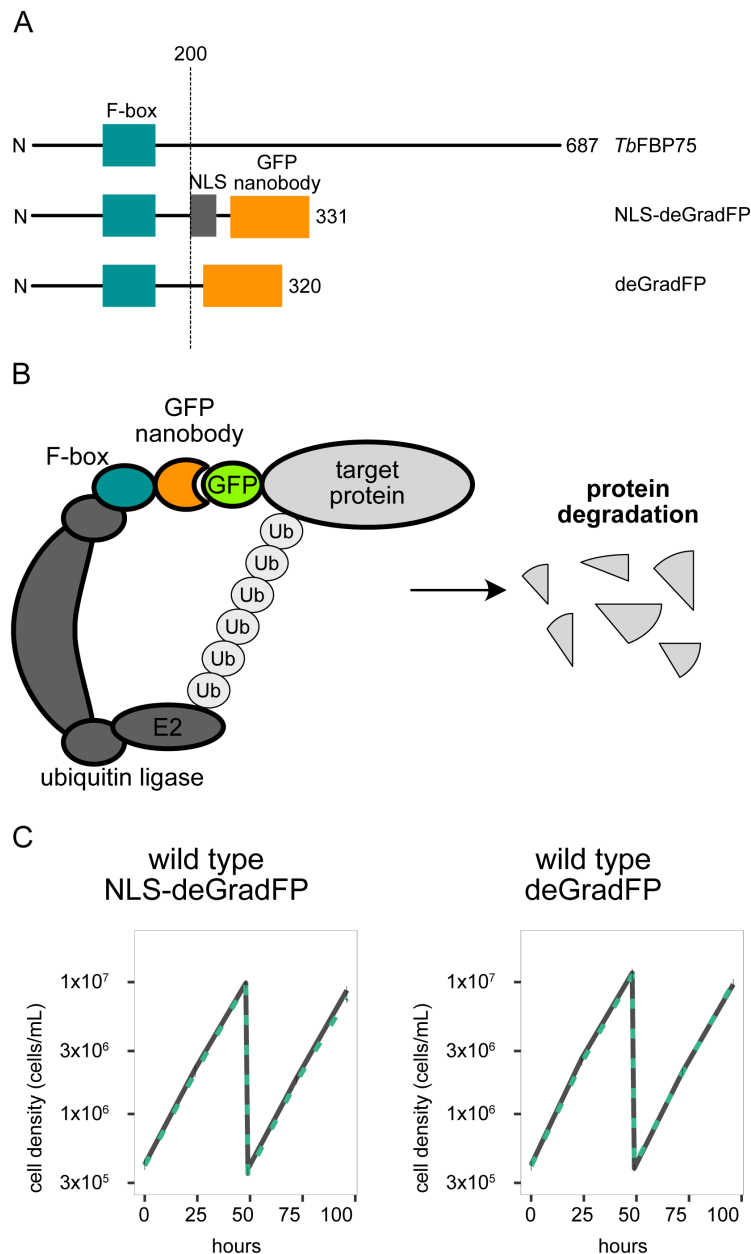
To apply deGradFP in *T. brucei*, we expressed the first 200 amino acids of Tb927.5.700 from *T. brucei* (named FBP75 herein for F-box protein 75 kDa) that contain a putative F-box fused with the anti-GFP nanobody VhhGFP4 (Saerens *et al.*, 2005) (Figure 1A, B). We made two constructs: one with a nuclear localization signal (NLS) to target nuclear proteins (pBA2675), and one without an NLS to target cytoplasmic proteins (pBA2705). In each case, the fusion protein was expressed from a derivative of pDEX777 that integrates at the 177 bp locus and allows doxycycline-inducible expression (Kelly *et al.*, 2007; Nerusheva & Akiyoshi, 2016). Induction of these deGradFP systems in wild-type procyclic cells with 1 µg/mL doxycycline did not cause any growth defect (Figure 1C).

We first targeted KKT3, a kinetochore protein that constitutively localizes at kinetochores (Akiyoshi & Gull, 2014). KKT3 does not show any obvious fluctuation in its abundance during the cell cycle, implying that it is a stable protein. In fact, the half-life of KKT3 has been estimated to be much longer than transiently-localized kinetochore proteins (Tinti *et al.*, 2019). 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 deGradFP in this cell line caused more severe growth defects than RNAi (Figure 2A and B) (Marcianò *et al.*, 2021). Microscopy analysis confirmed significant depletion of KKT3 at six hours (Figure 2B). The fact that induction of deGradFP in wild-type cells did not cause any growth defect (Figure 1C) means that the observed growth defect was due to specific degradation of YFP-tagged KKT3.

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 3), 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; 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). Furthermore, it is essential that GFP-fusion

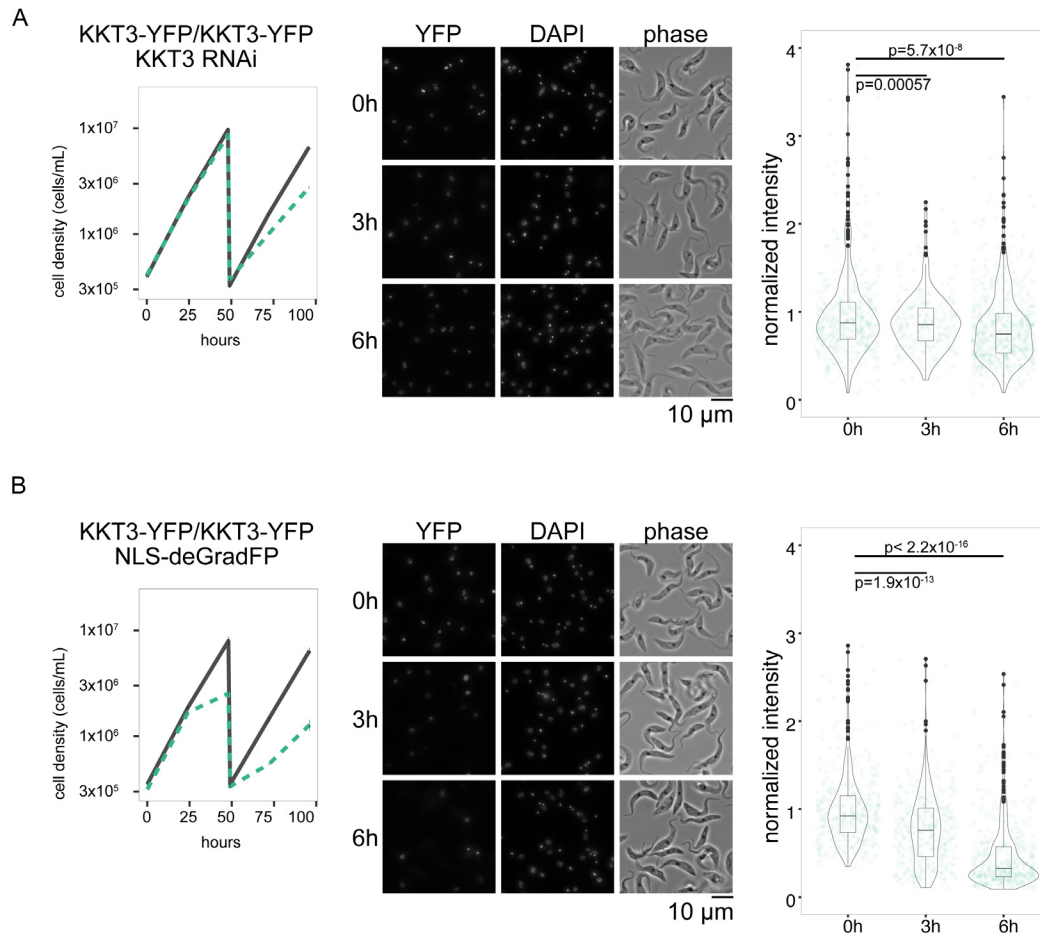


**Figure 1. deGradFP in *Trypanosoma brucei*.** (A) Schematic of *TbFBP75*, NLS-deGradFP, and deGradFP, highlighting the putative F-box domain, NLS, and GFP nanobody (vhhGFP4). (B) deGradFP forms a complex with an endogenous ubiquitin ligase complex which transfers the ubiquitin to the target protein tagged with GFP. Ubiquitin-tagged target proteins are then degraded by the 26S proteasome. (C) Growth curve for wild-type procyclic cells with NLS-deGradFP (left) or deGradFP (right). deGradFP was induced with 1  $\mu$ g/ml doxycycline and cultures were diluted at day 2. Gray lines are uninduced controls. Green dashed dashed are doxycycline-treated cells. N=3. Error bars are SEM. Cell line: BAP2395, BAP2511.

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).

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*.



**Figure 2. deGradFP depletes a nuclear protein KKT3 more efficiently than RNAi. (A)** KKT3 knockdown by RNAi. Cell line: BAP2512, **(B)** KKT3-YFP depletion by deGradFP with NLS. Cell line: BAP2513. (Left) Growth curve. RNAi or deGradFP was induced with 1  $\mu$ g/ml doxycycline and cultures were diluted at day 2. Gray lines indicate uninduced controls. Green dashed lines are doxycycline-treated cells. N=4 (RNAi) and 3 (deGradFP). Error bars are SEM. (Centre) Examples of cells at 0 h, 3 h, and 6 h after induction. YFP and DAPI images are maximum intensity projection. (Right) Plot of total YFP signal inside the nucleus (>239 cells in each condition). Data was normalized with the mean value at 0 h. P-values were calculated by Welch two sample t-test.

## Methods

### Plasmids

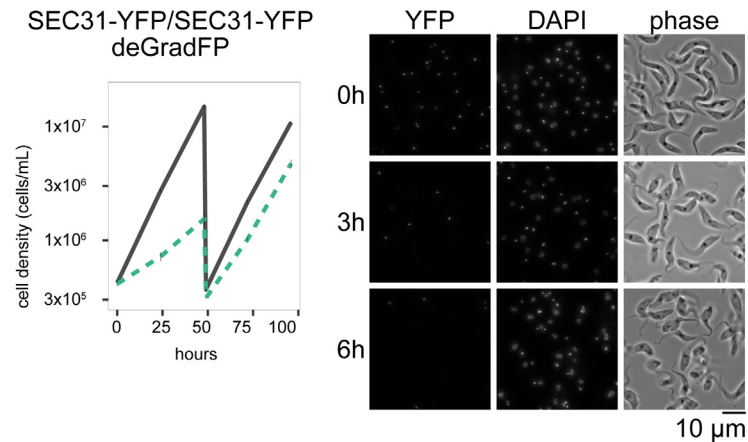
All plasmids used in this study are listed in [Table 1](#). To make pBA2675 (Inducible expression of FBP75<sup>1-200</sup>-NLS-VhhGFP4: NLS-deGradFP for nuclear proteins), synthetic DNA BAG181 (GeneArt, Thermo Fisher) was digested with HindIII/BamHI and subcloned into pBA310 cut with the same enzymes. The NLS sequence was derived from the La protein ([Marchetti et al., 2000](#)). To make pBA2705 (Inducible expression of FBP75<sup>1-200</sup>-VhhGFP4: deGradFP for cytoplasmic proteins), NLS was removed from pBA2675 by PCR with BA3647/BA3648. 12.5  $\mu$ L of 2x PrimeSTAR MAX (Takara), 1  $\mu$ L of 3 ng/ $\mu$ L pBA2675 plasmid, 1  $\mu$ L each of 10  $\mu$ M forward and reverse primers, and 11  $\mu$ L 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  $\mu$ L of DpnI (NEB) at 37°C for 1 hr and was transformed into NEB 5-alpha competent *E. coli* (NEB). To make pBA1061 (hairpin RNAi targeting 2562–3072 bp of the KKT3 coding sequence), synthetic DNA BAG55 (GeneArt, Thermo Fisher)

was 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  $\mu$ g/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  $\mu$ L of 2x PrimeSTAR MAX (Takara), 1  $\mu$ L of 30 ng/ $\mu$ L template pPOT plasmid, 1  $\mu$ L each of 10  $\mu$ M



**Figure 3. Depletion of a cytoplasmic protein SEC31 by deGradFP.** (Left) Growth curve. deGradFP was induced with 1 µg/ml doxycycline and cultures were diluted at day 2. Gray line indicates an uninduced control. Green dashed line is doxycycline-treated cells. N=4. Error bars are SEM. (Right) Examples of cells at 0 h, 3 h, and 6 h after induction. YFP and DAPI images are maximum intensity projection. Cell line: BAP2518.

**Table 1. Plasmids used in this study.**

Name	Description
pPOTv7 (eYFP, Hyg)	PCR-based eYFP-tagging vector, hygromycin marker (Dean <i>et al.</i> , 2015)
pPOTv7 (eYFP, G418)	PCR-based eYFP-tagging vector, G418 marker (Dean <i>et al.</i> , 2015)
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 <sup>1–200</sup> -NLS-VhhGFP4, integrate at 177 bp, phleomycin marker (deGradFP for nuclear proteins)
pBA2705	Inducible expression of FBP75 <sup>1–200</sup> -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 <i>et al.</i> , 2012)
PCF 1339	TREU927 procyclic cells that expresses TetR, T7 RNAP, and Cas9 (Alves <i>et al.</i> , 2020)
BAP2395	FBP75 <sup>1–200</sup> -NLS-VhhGFP4
BAP2511	FBP75 <sup>1–200</sup> -VhhGFP4
BAP2464	KKT3-YFP/KKT3-YFP
BAP2512	KKT3-YFP/KKT3-YFP, KKT3 hairpin RNAi
BAP2513	KKT3-YFP/KKT3-YFP, FBP75 <sup>1–200</sup> -NLS-VhhGFP4
BAP2466	SEC31-YFP/SEC31-YFP
BAP2518	SEC31-YFP/SEC31-YFP, FBP75 <sup>1–200</sup> -VhhGFP4



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

Name	Description
BA3647	CACCTGCTCACCGTCTCCTCATGTGGGCA
BA3648	GGAGGACGGTGGAGCAGGTGGAGCAGGTGT
BA1821	GTAATGGAGTTTGTGAGGTGCTTGATGAGGAAAAAATTCGCCCTTTTCGGAGGAACTCAACCAGATGCTCTACGGTGGCGTGGTTCCTGTTAGTGGTTCC
BA1822	GAATGCGACAGCAGCGAAACGGAAGAAAAATAAAAAAGAGAGGGCTATCTGTAATTCTTTACGTACATCACTTCCAATTTGAGAGACCTGTGC
BA523	TATGCTGTTTATTGCCAC
BA2352	GATCGATC GCGGCGCG TTTTCAGTTGCTATAGGCCT
BA3633	GCTCAAGGGAATGTGAAAGAGCTCGCCACTAAGCACTTTTCGGCAATTCACATATTAAACCTCAAGTTTCTGCAGGGTTCTGTGATGGTTCCGG
BA3634	ATATATGCAACCCGGCGACAAACACACCGCACAGGTGCAAGGCGACAAACATGTTTTCCTTTGAGTGCCATGTGTGCCAATTTGAGAGACCTGTGC
BA3635	GAATTAATACGACTACTATAGGGGGTGGCTCGCTCAGAGCGTTTITAGAGCTAGAAATAGC
G00 (BA2931)	AAAGCACCGACTCGGTGCCACTTTTTCAGTTGATAACGGACTAGCCTTATTAACTTGCTATT TCTAGCTCTAAAC
BA3638	TTGTTTAGGATCAACAGCT
BA3639	GTACAGACACGTCCTCGTACAA
BAG55	GATCGATCGATC AAGCTT ACGTACACATGCTGCTTAAACGGTGATTGGATACGCTACTACCACCTTTATCTATGGAGGAAGAGGGGAGCTCAGTCGCTGTGCACATATCATATTACG CCGGACGTACTGGGTGTACATTTTCAACCATAGTTTCTGTGCATCAGCTGTGTGAGTTGGAAACATGCTATACGTCTGTAGATCGTGTG ATATCGAGGAAGATAATGACGTGGCGTATCTTGTGTGGACAAGCATTAATGAGGAGAAGATCTACGATGCTCTCAATTGGTGCAGAACCCA CGACACATATGTTAAACAGCGGGCTCCCGTATTATGCTTTATGGCCACCAACACAGCTTCAATGCAATGGTGATCCCTTTGTATT GTTCCGTGTACTCCGTCCATATGGAACTCTATAGTGCTTATGCTCTCAACGGGCTGTGCAAGTTGTAGCGGGAAATACAGTTACGTTACGGT GGCTGATGACAAAGGGGACCTCATTTCTAAGTACGGTGACGTGTGACATGATTTGAATTCGCAATCTCCAGTGTCTTAGCGTGACGG CCGAGGGTCCATAAGTATCCAGCCACCTGATTTCCCGCTACAACACCTGCACAGCCCCGTTGGAGCATAAAGCCACTCATAGAT TTTCCATGGAAGTACAGCGCAAAATACAAAGGATCACTTGCATTGAAATGCTTTTGTGGGCAATAAAGACATAATACCGGAGA CCGCGCTGTTTAAACATGTGTGCTGGGTTTCGACCAATTGAAGGACATGTAGATCTTCTCTCATTCAGTGTGCGCAACGACAAGATA CGCGCACTCATATCTCTGATATCAACGATCAGCATGATGTTTCCAACTGACACGCTGAGTGACAGAAAACTATGGT TGAAAAATGTAAACACGATACGTCCTCCGCTGAATATGATGTACAGCGACTGAGTCGCCTCTTCTCTCATAGGATAAAAGTTGGTAGCGGTATCCA ATACCGTTTAAGCAGCATGTGACGTGGATCC GATCGATCGATC (KKI3 hairpin)
BAG181	GATCGATC AAGCTT ATGGGTGGTGAGCAGCGGTGCTGCTGTGTGACAGAGCGCGCGGCACTTCGGGTAAACGACCAACACGGATGGTAATCATCGGAGGTACG CCTGGAAATCGTTGACGACGTGGGGGCGCTTTCACCGGTTCCATGAATGGCAACACCTCCCTGCCAAAGGTTCAAACATCTGTGAGACTACGGGTG TTGGATAGCAATACTCGAAGGAGACATTTCTCGAGCGAGCTAGCTTACCTCTGAGCGCTGTGGCGTTGCAGCAGCGTCTTTCACATTCCA GTGCTACTCTTGACGAGGTGTCACTTTTGTGATCCCGAGGATCTCTGACGGTATTGGAAGTATGCCGTTTCTCTGCGCGCTGTAAAGGTCCG ATGTACCGCTGGAGGTCGTATGCCCTTCGTATGGAAGAAACAGGAGGCTCTCGCGCTGGTGCCTGAATGGCCGTCTGTGGAGGAGGTATGCCG ACAGGAAGACTTGGAGTGATTGTGTGCAACAAGCGTTTGACATGAATATTTCACATGATGATGTTGGGAAACCATTAAACATGGTGGCCGACATGGAGGACGGT ACCGTCTGGAGACACAAGCGGTACGTGAACAA GGAGCAGGTGGAGCAGGT GTCCAATGTTGGTGTGGTGGCTTTGGTGCAAGCCAGGTGGCTCTCTGCGTTTGTCTGTGCGCTTCCAGTGAACCGCTATTTCATGC GCTGTATCGCAGGCTCAGCAAGAGAGCTGAGTGGGTATGCCGTATGTCCAGCGGGTGATCTGATGCTCTATGAAGACTCCGTGAAGGCCGTTT CACCATAGCCGTGACGTGCCGTAAACCGTGTATCTGCAATGAACAGCTTGAACTCAGATACGCGCGTGTATGATGTGAACGTGGGCTTC GAGTATTTGGGCGCAAGGCAACCAGGTACCGTCTCCAGCTAA GGATCC GATCGATC (FBP75's first 600 bp, NLS, GlyAlaGly linker v2, VhhGFP.v1)

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 (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 cell line, donor DNA templates amplified from pPOTv7 (YFP, Hyg) or pPOTv7 (YFP, G418) (Dean *et al.*, 2015) with BA3633/BA3634. 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 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. 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 and SEC31-YFP) 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<sup>3</sup>). YFP total intensity inside the nucleus was measured using a redirect function in 3D Objects Counter. 3D Plots were made with ggplot in R.

### 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)
- 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)
- 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)

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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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) are freely available upon request.

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# Open Peer Review

Current Peer Review Status:  

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Version 1

Reviewer Report 29 June 2022

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**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<sup>1</sup>), based on sequences, 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.

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**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

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**Cynthia He Y** 

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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.**

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