A Trypanosome Trifecta: an independently tunable Triple Inducer System for genetic studies in *Trypanosoma brucei.*

Matt J. Romprey[1], Raveen Armstrong[1], Peter McGill[1] and Michele M. Klingbeil*[1,2].

[1]Department of Microbiology, University of Massachusetts, Amherst, MA United States of America

[2]Institute for Applied Life Sciences, University of Massachusetts, Amherst, MA United States of America

*Corresponding author

E-mail: mklingb@umass.edu (MMK)

ABSTRACT

Trypanosoma brucei is a model unicellular parasite for cellular and molecular genetic studies, but tools for more multiplexed experiments are limited. Tetracycline (Tet)-inducible gene regulation has been a long-standing and effective approach for overexpression, RNAi and genome wide screens. The single inducer Tet-On system has been foundational for studying essential genes that are required for biological processes and identifying potential drug targets. To achieve greater flexibility in experimental design, we capitalized upon previously described dual inducer systems that combined

vanillic acid (Van) or cumate (Cym) with Tet as inducers. Here we report the development of a triple inducer system combining Cym, Van and Tet repressor elements to selectively regulate the expression of three genetic elements within a single cell line called PHITER. As proof of principle to demonstrate independent control, we adapted the previously characterized Van/Tet dual inducer RNAi complementation cell line, IBComp^{VaT} for additional Cym inducible expression of an eGFP reporter. We provide evidence that each of the inducer elements operate independently with no evidence of leaky expression. In this system, Cym induction is specific and tunable. Additionally, simultaneous triple induction for *POLIB* RNAi complementation with wildtype POLIB and eGFP expression phenocopied the near complete rescue of *POLIB* RNAi previously reported. This system enables the study of complex and pleiotropic phenotypes through more sophisticated experimental design.

INTRODUCTION

The African trypanosome, *Trypanosoma brucei*, is an early diverging unicellular parasite that causes the Neglected Tropical Disease human African trypanosomiasis and a related wasting disease in cattle called nagana (1). In addition to medical and economic importance, *T. brucei* has served as a tractable model organism with many advanced genetics tools and insights into fundamental biological processes based on their distinctive biology. Examples include extensive post-transcriptional gene regulation,

41 mitochondrial RNA editing which overturned years of molecular central dogma and
42 unusual metabolic adaptations (2, 3). Another example of extreme biology is their
43 mitochondrial DNA network called kinetoplast DNA (kDNA). Trypanosomes have one of
44 the most topologically complex mitochondrial genomes in nature composed of
45 maxicircles and minicircles that are catenated into a single network.

46 The kDNA structure and replication mechanism are divergent from all other eukaryotes
47 and is essential for parasite survival and life cycle completion (4). A hallmark of kDNA
48 replication is the topoisomerase II-mediated minicircle release and attachment
49 mechanism, while maxicircles replicate still catenated within the network (5, 6, 7).
50 Remarkably, minicircle progeny are attached to the network while still containing at least
51 one gap. This “mark” on replicated progeny is a possible mechanism to ensure each
52 circle is replicated just once per cell cycle. However, persistent gaps (ssDNA regions)
53 negatively impact genome stability and can lead to replication stress, suggesting that the
54 DNA damage tolerance response during kDNA replication is essential and therefore
55 notably different in trypanosomes (8, 9).

56 The complexity of kDNA replication is also reflected in the many additional factors not
57 present in mammalian nucleoids including 6 DNA polymerases (Pol), 6 helicases, 3
58 topoisomerases, 2 primases, 2 ligases and 2 origin binding proteins among others.
59 Greater than 50 proteins have roles in the replication and segregation of the DNA network
60 as well as maintaining the kDNA structural integrity and have been summarized
61 elsewhere (10, 11). Interestingly, many of the kDNA replication factors have non-
62 redundant roles discovered using RNAi for reverse genetic analysis of gene function. For

63 example, *T. brucei* uses three family A DNA Pol paralogs (POLIB, POLIC, POLID) that
64 are independently essential suggesting specialized roles in maintaining the kDNA
65 network (12, 13, 14, 15). The 29-13 tetracycline (Tet)-On single inducer system has
66 been crucial for single gene and genome-wide RNAi studies over the past 2 decades
67 (16).

68 Among the many improvements to trypanosome molecular biology constructs,
69 independent dual inducible systems were developed using Tet and vanillic acid (Van) or
70 Tet and cumate (Cym) (17, 18). We recently reported the use of a dual inducer system
71 for independently regulating RNAi and overexpression through the addition of Van and
72 Tet, respectively. Simultaneous induction in this system allowed for robust RNAi
73 complementation to study the kDNA replication protein POLIB (19). RNAi
74 complementation studies and their associated control experiments can now be
75 performed within a single cell line.

76 This highly tractable system could be expanded for further experimental flexibility by
77 adding a third inducer, thus allowing for the independent regulation of 3 genetic
78 elements in one cell line. In this triple inducer system, RNAi complementation could still
79 operate using the Van and Tet inducer elements to concurrently control RNAi and
80 overexpression, while the Cym inducer elements would allow for tunable regulation of
81 an additional gene. A triple inducer system would allow expression of an additional gene
82 during RNAi complementation, such as one that impacts kDNA genome stability. This
83 can allow dissection of protein domain function during normal replication fork
84 progression and possibly evaluate overlapping roles of kDNA replication and repair.

85 Here we describe the establishment of a triple inducer parental cell line that expresses
86 all three repressor elements and demonstrate independent, and tunable Cym inducible
87 expression using an eGFP reporter. To evaluate the full potential of a triple inducible
88 system, we built upon the previously characterized Van/Tet dual inducer cell line for
89 POLIB RNAi complementation (19) as proof of principle to demonstrate that all three
90 elements can function independently and simultaneously. This tool overcomes key
91 limitations and opens new avenues for trypanosomatid functional genomics.

92

93 **MATERIALS AND METHODS**

94 For primer sequences refer to Table S1.

95 For cell lines and associated modifications used in this study refer to Table S2.

96

97 **DNA constructs**

98 **Single marker cumate inducible system**

99 The hygromycin resistance gene was PCR amplified from pKOHyg (20) using primers
100 UM147 and UM149 for subsequent Gibson assembly (NEB) with EcoRI and NcoI
101 digested pSmOxNUS (18) to create pSmOxNUSHyg. The cumate repressor (CymR)
102 gene and the hygromycin resistance gene (Hph) were then PCR amplified from
103 pSmOxNUSHyg using primers UM159 and UM160 for Gibson assembly with StuI and
104 BsiWI digested pDEX-CuO (18) to create pCuRO-eGFP.

105

106 **Cumate repressor plasmid**

107 The CymR and Hph genes were PCR amplified from pSmOxNUSHyg using primers
108 UM192 and UM193 for subsequent Gibson assembly with NheI and SpeI digested
109 pJ1173 (17) to create pCymRHyg thus removing the T7 RNA polymerase, tetracycline
110 repressor (TetR) and a puromycin resistance gene from pJ1173.

111

112 **Cumate inducible expression vector**

113 The neomycin resistance gene was PCR amplified from pC-PTP-NEO (Shimanski
114 2005) using primers UM184 and UM185 for subsequent Gibson assembly (NEB) with
115 BsiWI and StuI digested pDEX-CuO (18) to create pCuO-eGFP.

116

117 **Trypanosome cell culture and transfection**

118 The *Trypanosoma brucei brucei* procyclic cell line SMUMA (**S**ingle **M**arker **UMA**ss)
119 contains the integrated plasmid pJ1173 (17) carrying T7 RNA polymerase (T7RNAP),
120 tetracycline repressor (TetR) and vanillic acid repressor (VanR). SMUMA cells were
121 cultured at 27 ^{o}C in SDM-79 medium supplemented with 15% heat-inactivated fetal
122 bovine serum and 1how to µg/ml puromycin (Puro) (19). The IBComp^{VaT} cell line (19)
123 was cultured in SDM-79 medium supplemented with blasticidin (15 µg/mL), phleomycin
124 (2.5 µg/mL) and puromycin (1 µg/mL).

125 To create the Cym triple inducer reporter cell line, CuRO-eGFP, NotI linearized pCuRO-
126 eGFP was transfected into SMUMA cells via nucleofection to integrate into the 177 bp
127 locus and selected with 50 µg/mL hygromycin (Hyg). To create the triple inducer
128 PHITER parental cell line that expresses three repressor elements (CymR, TetR, VanR),
129 SMUMA (19) was transfected with HindIII digested pCymRHyg via nucleofection to
130 integrate into the α/β tubulin array and selected with 50 µg/mL Hyg. Verification of all
131 three repressors elements in PHITER clones are provided in Fig S1. Four clonal cell
132 lines were chosen based on doubling times and subsequently transfected via
133 nucleofection with NotI linearized pCuO-eGFP for integration into the 177 bp repeat
134 region and selected with 50 µg/mL G418 to screen for Cym inducible protein
135 expression. These populations are called $eGFP^{PHITER}$ and were not further dilution
136 cloned. All other transfected cell lines were additionally supplemented with the
137 appropriate selectable drug prior to limiting dilution to obtain clonal cell lines.

138 The POLIB dual inducer cell line $IBComp^{VaT}$ clone P5D1 (19) was transfected with
139 HindIII digested pCymRHyg and selected with 50 µg/mL Hyg. Clone P1D8 was chosen
140 for this study based on doubling time and named $IBComp^{PHIT}$ (Puro, Hyg, Inducible
141 Triad). $IBComp^{PHIT}$ was subsequently transfected with NotI linearized pCuO-eGFP and
142 selected with 50 µg/mL G418 to create the IBComp-$eGFP^{PHIT}$ reporter cell line.
143 Repressor expression was verified by northern blot and clone P1F12 was chosen for
144 this study.

145

146 **Inducible expression**

147 Expression of eGFP was induced with 25 µg/mL of Cym for 48 hr for both triple inducer
148 reporter cell lines CuRO-eGFP and eGFP^{PHITER}. In the proof-of-concept cell line,
149 IBComp-eGFP^{PHIT}, RNAi was induced by addition of Van (250 µM dissolved in DMSO)
150 and expression of an ectopic recoded version of wild type POLIB PTP-tagged variant
151 (POLIBWT) was induced by addition of Tet (4 µg/mL). Cultures were supplemented
152 daily with Van and/or Tet to maintain RNAi and protein expression (19). For eGFP
153 expression, cells were induced with Cym (3 µg/mL dissolved in DMSO) on transfer
154 days. To assess if Cym induction was reversible, IBComp-eGFP^{PHIT} cells were grown for
155 2 days in the presence of 3 µg/mL Cym only or in the presence of all three inducers.
156 Cells were then pelleted, and then washed once in 1X phosphate buffered saline (PBS)
157 to remove the inducer. Cells were resuspended and grown in media lacking Cym for an
158 additional 3 days. Cells were then harvested at indicated time points for SDS-PAGE and
159 western blot analyses.

160

161 **SDS PAGE & western blotting**

162 Cells were pelleted, washed with 1X PBS, supplemented with 1X protease inhibitor
163 cocktail (Roche), resuspended and stored at -80 ˚C. Protein samples were fractionated
164 in a sodium-dodecyl-sulfate polyacrylamide gel (150 V) and transferred overnight (90
165 mA) onto a PVDF membrane. Membranes were then blocked in a 5% w/v non-fat dry
166 milk solution for 2 hrs.

167 PTP-tagged POLIB was detected with peroxidase anti-peroxidase soluble complex
168 (PAP, 1:2000, Sigma). eGFP was detected using anti-GFP rabbit polyclonal antibody

169 (1:1000, 1 hr, Abcam) followed by HRP conjugated goat anti-rabbit polyclonal antibody
170 (1:5000, 1 hr, Pierce). TetR was detected using anti-TetR mouse monoclonal antibody
171 (1:1000, 1 hr, Takara). Loading control was detected with mouse monoclonal anti-Ef1α1
172 (1:15000, 1 hr, Santa Cruz Biotechnologies) and HRP conjugated goat anti-mouse
173 polyclonal antibody (1:2500, 1 hr, Sigma). Membranes were washed thrice with 1X Tris
174 Buffered Saline + Tween 20 (TBST) after each antibody incubation. Chemiluminescent
175 substrate (SuperSignal West Pico Plus, ThermoFisher) was used to detect protein.
176 Band intensities were quantified using ImageJ software (http://imagej.nih.gov/ij/).

177

178 **RNA isolation and northern analysis**

179 5 x 10^7 cell equivalents were pelleted and washed with PBS for total RNA isolation with
180 TRIreagent (Sigma-Aldrich). RNA was run on a 1.5% agarose/7% formaldehyde gel
181 overnight at 25 V then transferred to a Pall 60208 Biodyne B Membrane as previously
182 described (13). RNA was cross-linked at 1200 J/cm^2 using a UV Stratalinker 1800
183 (Stratagene). Specific ^{32}P-labelled probes were generated as described previously (14,
184 19) using PCR products for CymR, TetR, VanR, and POLIB (Table S1) using the
185 Random Primers DNA labeling system (Invitrogen). Tubulin probes were generated from
186 a 650 bp gel extracted product from pZJM (21). Hybridization and wash conditions were
187 described previously (21). Specific mRNAs were detected and quantified using a
188 Typhoon 9500 Phosphorimager (GE Healthcare) and normalized to the tubulin signal.

189 **IF microscopy**

190 Cells were harvested at 1000 *x g*, washed once and resuspended in 1X PBS then
191 adhered to poly-L-lysine coated slides (5 min). Cells were fixed with 3 %
192 paraformaldehyde (5 min), washed twice with PBS + 0.1 M Glycine, then permeabilized
193 with 0.05 % Triton X-100 (5 min). Cells were washed thrice with 1x PBS. POLIBWT was
194 detected with rabbit polyclonal anti-protein A (1:1000, 1 hr, Sigma) followed by
195 secondary antibody Alexa Fluor 594 goat anti-rabbit (1:250, 1 hr, ThermoFisher). eGFP
196 was detected using rabbit polyclonal anti-GFP antibody (1:500, 1 hr, Abcam) followed by
197 secondary antibody Alexa Fluor 488 goat anti-rabbit (1:250, 1 hr, ThermoFisher). DNA
198 was stained with 1 µg/ml 4′-6′-diamidino-2-phenylindole (DAPI) and slides were
199 mounted with Vectashield (Vector Laboratories). Images were acquired using an
200 inverted Nikon Ti2-E wide-field fluorescence microscope with a Nikon Plan Apo λ 60x
201 1.45 numerical aperture objective lens. Z stacks with a step size of 0.2 µm were
202 deconvoluted using the Landweber algorithm within NIS Elements, a closed-source
203 analysis tool developed by Nikon Instruments.

204

205 **RESULTS**

206 **Design and Establishing a Triple Inducer System**

207 In our work to expand the molecular toolbox for functional genomics in *Trypanosoma*
208 *brucei*, we transitioned from using the well establish single inducer Tet-ON system (22)
209 to a dual inducer system for robust RNAi complementation studies. Dual induction relies
210 on the procyclic cell line SMUMA that constitutively expresses Tet and Van repressors
211 as well as T7 RNA polymerase (19). We identified an opportunity to provide additional

212 flexibility by incorporating the previously reported cumate (Cym) inducible components
213 (18, 23) that could allow for the independent regulation of three genetic elements in a
214 single cell line. In this triple inducer system, RNAi complementation would still operate
215 using the Van and Tet inducer elements, while the Cym inducer elements would allow
216 for tunable regulation of additional genes.

217 Initially, we set out to design a vector that housed both the cumate repressor (CymR)
218 and a cumate (Cym) inducible eGFP reporter under a single drug selectable marker to
219 integrate into the 177 bp locus (Supp Fig1A). We transfected pCuROeGFP into the dual
220 inducer parental cell line SMUMA. Of 10 clonal cell lines, only 4 displayed varied
221 cumate inducible eGFP expression following 48 hr of 25 μg/mL Cym induction with
222 detectable eGFP expression in uninduced controls indicating the system was leaky
223 (Supp Fig1B).

224 Our second attempt to create a triple inducer cell line relied upon separating the
225 elements onto 2 vectors similar to previous work from the He lab (18). The CymR gene
226 is housed within pCymRHyg to integrate into the α/β tubulin array and the expression
227 vector, pCuO-eGFP, contains 3 cumate operators upstream of an eGFP reporter gene
228 to integrate into the 177 bp repeat region (Fig.1A). SMUMA was transfected with
229 pCymRHyg to create a basal, triple repressor parental cell line called PHITER
230 (Puromycin Hygromycin Inducible Triad of Exogenous Repressors), and 10 clonal cell
231 lines were generated. Several PHITER clones were subsequently transfected with
232 pCuO-eGFP to characterize cumate inducible expression using eGFP as a reporter.
233 Following induction with 25 μg/mL Cym for 48 hr, four $eGFP^{PHITER}$ populations showed

234 varying levels of eGFP protein expression while no expression was detected in the
235 uninduced cells (Fig. 1B). Longer exposures (90 sec) showed no expression of eGFP in
236 the uninduced controls (Supp Fig1C). These results demonstrate that separating the
237 Cym elements onto 2 vectors eliminated the problem of leakiness in the uninduced
238 controls.
239 To demonstrate independent control of all three inducers, we adapted the previously
240 characterized RNAi complementation cell line, IBComp^{VaT} (19), to work in parallel with
241 the Cym inducible elements. IBComp^{VaT} was transfected with pCymRHyg and pCuO-
242 eGFP to create IBComp-eGFP^{PHIT} thus allowing Cym inducible eGFP expression in
243 addition to Van inducible POLIB RNAi and Tet inducible POLIBWT overexpression
244 within a single cell line (Fig. 1C). Both the pCymRHyg and pJ1173 integration events
245 occur within the α/β tubulin repeat region. Therefore, we performed northern blot
246 analyses to confirm expression of each repressor mRNA in the 4 clones using SMUMA
247 cells as a control (Fig. 1D). IBComp-eGFP^{PHIT} clone P1F12 was selected as the cell line
248 to analyze proof-of concept for triple induction based on repressor expression, and
249 similar POLIB RNAi complementation results as previously published (19).
250
251
252 **POLIB Triple Inducer System: Optimization of Cumate Inducible eGFP**
253 To determine if the triple inducer cell line IBComp-eGFP^{PHIT} was able to express eGFP
254 across a range of Cym concentrations (0.5 – 10 µg/mL) were added to the cells for 48
255 hr, cell pellets were collected and then analyzed by western blot analyses. No eGFP
256 expression was observed in uninduced cells even upon a longer exposure of 5 min (Fig.
257 S2A). Expression of eGFP was detected from 1 µg/mL Cym and consistently increased

258 as the Cym concentration increased to 10 µg/mL (Fig. S2A). Cells induced with 5 µg/mL
259 of Cym or greater showed degradation of eGFP (Fig. S2A). We further tested 3 and 5
260 µg/mL Cym for sustained expression of eGFP over an 8 day induction. While some
261 variation in the amount of eGFP was detected using 5 µg/mL, there was consistent
262 expression of eGFP using 3 µg/mL Cym throughout the 8 day induction (Fig S2B). We
263 also tested whether Cym concentrations impacted fitness of the cells. No significant
264 fitness impact was detected by inducing the cells with a range of Cym concentrations (3,
265 5, 10 µg/mL) or by expression of eGFP over an 8 day induction (Fig. S2C). Based on
266 these data, 3 µg/mL Cym was used for subsequent inductions.

267

268 **Independent Gene Expression in IBComp-eGFP^{PHIT} Cell Line**

269 One advantage of the triple inducer system is that all the single inducer controls can be
270 performed in a single cell line. To determine whether the Van, Tet and Cym inducible
271 systems could operate independently of one another, each inducer was added
272 separately to the IBComp-eGFP^{PHIT} cell line. Cells were cultured for 8 days with the
273 addition of either 3 µg/mL Cym, 4 µg/mL Tet, or 250 µM Van. After 48 hours, ectopic
274 POLIBWT and eGFP expression were assessed by western blotting and endogenous
275 *POLIB* mRNA was quantified via northern blotting. Cells induced with Tet expressed
276 POLIBWT (10.1 fold greater than SE) with no detectable eGFP, while cells induced with
277 Cym expressed eGFP with no detectable expression of POLIBWT (Fig 2A).
278 Induction with Van did not lead to expression of either POLIBWT or eGFP while
279 endogenous *POLIB* mRNA was depleted by 98% within 48 hr (Fig. 2B). Throughout the
280 8 day induction only the addition of Van led to a significant loss of fitness (LOF) with cell
281 doublings reaching only 9.8 compared to 21.5 for uninduced cells, a hallmark

282 associated *POLIB* RNAi (12, 14, 19). Cells induced with Tet and Cym reached 20.8 and
283 21 doublings, respectively (Fig. 2C). These data confirm that the three inducers operate
284 independently and did not lead to promiscuous expression.
285 To determine if POLIB RNAi complementation was still functioning in the triple induction
286 system, IBComp-eGFP^{PHIT} cells were simultaneously induced with Van and Tet for 8
287 days. Consistent expression of POLIBWT was confirmed with a 7 fold increase
288 compared to the single expressor allelically tagged endogenous control (SE) (Fig. 2D).
289 Similar to RNAi alone, induction with Van and Tet resulted in a 99% knockdown of
290 endogenous *POLIB* mRNA after 48 hr of induction (Fig. 2B). Lastly, there was a near
291 complete rescue of the RNAi LOF during POLIB complementation with cells reaching
292 18.9 doublings by day 8 phenocopying results for the dual inducer system (19) (Fig.
293 2D).
294
295 **Selective and Concurrent Gene Expression in IBComp-eGFP^{PHIT} Cell Line**

296 To assess whether Cym induction interfered with Van and/or Tet inductions we next
297 evaluated whether these inducers could operate simultaneously in IBComp-eGFP^{PHIT}
298 cells. The following combinations of inducers were used across 8 days of induction;
299 Van/Cym (VC), Tet/Cym (TC), and Van/Tet/Cym (VTC) and assessed for POLIB mRNA
300 knockdown, POLIBWT expression, and eGFP expression after 48 hr. TC induction did
301 not have a significant impact on fitness (21.2 doublings) compared to uninduced cells
302 (21.6 doublings) and led to the highest amount of POLIBWT and eGFP expression with
303 no impact on endogenous *POLIB* mRNA (Fig. 3A, B, C). VC induction resulted in a
304 phenocopy of RNAi alone (Van) with the characteristic LOF (9.5 doublings), a 94%
305 reduction in *POLIB* mRNA and expression of eGFP (Fig. 3A, B, C). The full triple

306 induction (VTC) also led to a 97% reduction in *POLIB* mRNA, a similar near complete
307 rescue of the RNAi LOF and eGFP expression (Fig. 3A, B, C).
308 Compared to Tet alone, TC induction led to a 12.3 fold increase in POLIBWT, whereas
309 VT induction led to a slight decrease in POLIBWT (7 fold) that did not significantly
310 impact *POLIB* RNAi complementation (Fig. 2C, D, Fig. 3C). The triple induction with
311 VTC resulted in a 6.5 fold above endogenous POLIB levels that still allowed for
312 complementation. For VC, eGFP decreased 3.2 fold compared to Cym alone. With a
313 comparable decrease of 3.6 fold for the triple VTC inductions (Fig 3C). Although the
314 addition of Van seemed to impact the total amount of eGFP or POLIBWT being
315 produced, the simultaneous induction of three inducers still led to a near complete
316 rescue of the *POLIB* RNAi LOF with 18.8 doublings with consistent and sustained
317 expression of eGFP and POLIBWT (Fig 3A, D).
318 Lastly, fluorescence microscopy revealed that eGFP and POLIBWT expression were
319 homogenous in the cell population with POLIBWT localizing throughout the
320 mitochondrion and concentrating near the kDNA while eGFP was cytoplasmic with
321 some fluctuation in the total amount of protein being produced (Fig. 3E, F). These data
322 indicate that Cym induction did not significantly interfere with *POLIB* RNAi or
323 overexpression of POLIBWT and highlight that Van induction might lead to decreased
324 expression levels for genes under the control of Cym and Tet induction.
325
326 **Temporal Expression of eGFP during RNAi Complementation**

327 Another advantage of the triple induction system would be to control when a gene of
328 interest is expressed while performing RNAi complementation. Therefore, we tested
329 whether eGFP expression could be controlled temporally during Cym induction and

330 *POLIB* RNAi complementation. Following 48 hr of Cym induction, removal of Cym
331 resulted in a decrease in eGFP expression within 24 hr of removing Cym with some
332 residual eGFP expression detected the following day (Fig. 4A). Similarly, removal of
333 Cym during RNAi complementation resulted in more rapid reduction of eGFP
334 expression within 24 hr after removing Cym (Fig. 4B). Collectively these data confirm
335 the specificity of three independent inducers, ability to tune the amount of Cym and to
336 control when Cym induction occurs. Together the results establish proof of principle for
337 a triple inducer system in procyclic *T. brucei* that can be used for elegant molecular
338 genetic studies during RNAi complementation.

339

340 **Discussion**

341 The use of inducible expression for reverse genetics and functional genomics screens
342 has been critical for studying gene function in the model kinetoplastid *T. brucei.*
343 Advances include several RNAi library screens, and an overexpression gain-of-function
344 screen (16, 24). However, there are limitations to the single inducer Tet-On system that
345 have prohibited powerful multiplexed screens (25). Here, we have established a highly
346 tractable, triple inducer system capable of independently or simultaneously inducing the
347 expression of three ectopic genes within one cell line. A triple inducer cell line allows for
348 more flexibility to now perform sophisticated genetic screens, and mechanistic studies
349 that could never have been achieved with the single inducer Tet-On system or the
350 recently reported dual inducer system for RNAi complementation (19).

351 We first attempted to engineer a parental cell line for triple induction that only required
352 one additional drug marker to allow for more flexibility in the design of downstream
353 experiments. However, the pCuRO-eGFP construct that contained both the CymR
354 gene, and a cumate inducible reporter gene (eGFP) only produced clones that
355 displayed leaky eGFP expression (Fig S1B). At this time, we are not certain why this
356 arrangement and integration into the 177 bp repeat region resulted in uncontrolled
357 eGFP expression (Fig S1A B).

358 The basis for the triple inducer system is a parental cell line called PHITER (derived
359 from SMUMA) which separated the Cym elements onto two vectors for integration at
360 separate genomic loci. By capitalizing on the previously reported inducible elements
361 using Tet, Van and Cym to control gene expression in *T. brucei* (26, 17, 18), we
362 demonstrated constitutive expression of all the repressor elements CymR, VanR, and
363 TetR (Fig S3), and characterized cumate inducible eGFP in a single cell line (Fig 1B).
364 Longer exposures of up to 2 minutes confirmed that there was no evidence of leaky
365 expression in the uninduced cells. Clonal PHITER cell lines have doubling times (9-10
366 hrs) comparable to the parental strain SMUMA and are available to the field. In contrast,
367 the widely used 29-13 cell line for the single inducer Tet-On system grows more slowly
368 (~12.8 hr), expresses lower levels of TetR, and displays considerable divergence in the
369 minicircle genome (27).

370 As proof of concept, we evaluated the full potential of the triple inducer system using the
371 previously characterized Van/Tet dual inducer cell line for POLIB RNAi
372 complementation, IBComp^{VaT} (19). Consecutive transfections of pCymRHyg then pCuO-
373 eGFP produced IBComp-eGFP^{PHIT}, a cell line that could be used for RNAi and ectopic

374 expression of two genes independently or simultaneously (Fig 1C). Independent
375 induction with Cym, Tet, and Van was demonstrated with no evidence of promiscuous
376 expression (Fig 2A, B). Van induction resulted in *POLIB* mRNA depletion (98-99%
377 decreased) and Tet induction resulted ectopic expression of IBWT (10.1 fold) that
378 closely resembled those in the parental IBComp^{VaT} experiments (Fig 2A, B). In addition
379 to controlling gene expression independently, we also demonstrated that Cym induction
380 of eGFP could be tunable over a range of Cym concentrations (1 - 10 μg/mL) and that
381 there was sustained expression of Cym induced eGFP (Fig S2C, D). Even the highest
382 concentration of Cym do not impact fitness of cells when tested in the parental cell line
383 SMUMA (Fig S2C). Importantly, Van + Tet dual induction for RNAi complementation led
384 to a near complete rescue of the POLIB RNAi defect and sufficient overexpression of
385 ectopic protein (Fig 2C, D).

386 We also confirmed concurrent gene expression with various dual and finally the full
387 triple inducer conditions. Combining Van + Cym still resulted in the characteristic 98%
388 POLIB mRNA depletion and LOF, and Cym +Tet resulted in robust expression of both
389 eGFP and IBWT (Fig 3). Induction with all three inducers allowed for sustained
390 expression of ectopic POLIB (Fig 3D), expression of eGFP, knockdown of endogenous
391 POLIB (Fig 3C), and a near complete rescue of the RNAi fitness defect (Fig 3A). These
392 results demonstrated that all inducer combinations caused the expected expression
393 output.

394 Another important aspect of the triple inducible system is the ability to control timing of
395 independent inductions for more dynamic experimental applications. Control of RNAi
396 (removal of the Van inducer) was previously demonstrated by removing the Van inducer

397 to restore the expression of chromosomally epitope tagged POLIB (19). Here we
398 additionally demonstrate the temporal control of Cym inductions by removing the Cym
399 inducer after 48 hrs of eGFP expression. In single or the full triple induction conditions,
400 removal of Cym inducer resulted a rapid decline in eGFP expression within 24 hrs.
401 During the triple induction, POLIBWT ectopic expression remained consistent
402 throughout the induction demonstrating that cumate inducible protein could be
403 temporally expressed without impacting concurrent RNAi complementation (Fig 4C).

404 When designing the dual and triple inducer systems, relative fold increase in expression
405 levels were taken into consideration. To maximize overexpression of an ectopic protein
406 during RNAi complementation, we placed POLIBWT on a Tet expression vector that had
407 previously reported to give a greater fold increase compared to the Van expression
408 vector (250 fold vs 18 fold increase in fluorescence intensity (17). The POLIB RNAi
409 stemloop construct described previously (28) was then placed on a Van expression
410 vector and efficient RNAi knockdown using this system was previously reported (17,
411 19). Even though fold expression using Van induction was substantially lower, the long
412 dsRNA constructs used in *T. brucei* provide ample siRNAs for efficient knockdown. The
413 cumate inducible system was then used for expression of a third gene independent of
414 RNAi complementation due to the previously demonstrated ability to tune the amount of
415 protein and temporally control expression (18). We have not tried other design
416 combinations of the triple inducible system.

417 However, the Cym, Tet, and Van inducible elements each have limitations that should be
418 considered during experimental design. Tet is a common contaminant in FBS used to
419 culture parasites possibly leading to low levels of background protein expression.

420 Previously, 250 μM Van was reported to cause a slight fitness defect in *T. brucei* (19)
421 and has been demonstrated to exhibit dose-dependent anti-trypanosomal activity in *T.*
422 *congolensi* with a reported IC50 of 768 μg/ml. (29). Lastly, Cym and Tet inducible protein
423 expression is reduced in concurrent inductions with Van (Fig 3D), thus the concentration
424 of Cym used in combination with Van may need to be titrated to circumvent any
425 observed reduction in protein expression.

426 Our study provides proof of concept for the first dynamic triple control system that
427 allows for robust RNAi complementation and control of a third element based on Tet,
428 Van and Cym inducible gene expression, and expands the toolbox available for *T.*
429 *brucei* functional genomics. Similar to the dual induction system, the triple system
430 allows for independent and tunable gene expression to control multiple processes.
431 Additionally, the three elements can also be temporally modulated for simultaneous or
432 more elegant staggered (or contemporaneous) experimental designs such as
433 overexpressing proteins that specifically modulate kDNA stability prior to performing
434 RNAi complementation.

435

436 **Figure Legends**

437

438 **FIG 1. Triple Inducer Systems. (A)** Diagram of plasmids integrated into the eGFP^{PHITER}
439 cell line. Tetracycline repressor (TetR), vanillic acid repressor (VanR), T7 RNA
440 polymerase (T7RNAP), puromycin-N-acetyl-transferase (PAC), cumate repressor
441 (CymR), hygromycin B phosphotransferase (HPH), neomycin phosphotransferase II

442 (G418), and cumate operator (CuO). **(B)** Western blot detection of eGFP and loading
443 control EF1α1 following a 48 hr induction of eGFP^{PHITER} clones with 25 µg/mL Cym. 2 x
444 10^6 cell equivalents were loaded per lane. **(C)** Diagram of plasmids integrated into the
445 IBComp-eGFP^{PHIT} cell line. Blastacidin S-deaminase (BSD), vanillic acid operator
446 (VanO), tetracycline operators (TetO), bleomycin resistance gene. Other annotation as
447 described in A. **(D)** Northern blot of total RNA from IBComp-eGFP^{PHIT} clonal cell lines.
448 SMUMA, parental cell line as a control. Membrane was probed for individual repressor
449 mRNAs, stripped and then reprobed. *Tubulin*, loading control.

450 **FIG 2. Independent gene expression in IBComp-eGFPPHIT cells. (A)** Western
451 blot detection of POLIB, eGFP and EF1α1 from IBComp-eGFP^{PHIT} cells independently
452 induced with Tet (4 μg/ml), Van (250 μM) Cym (3 μg/ml) over an 8 day induction. SE,
453 single expressor cell line representing endogenous POLIB protein (4 x 10^6 cell
454 equivalents). 2 x 10^6 cell equivalents were loaded per lane. **(B)** Northern blot of total
455 RNA from IBComp-eGFP^{PHIT} cells. U, uninduced; Cym, induced with 3 μg/ml Cym for 48
456 hr; Tet, induced with 4 μg/ml Tet for 48 hr; Van, induced with 250 μM Van for 48 hr; VaT,
457 induced with Van and Tet for 48 hr. Top, probing of *POLIB* mRNA. Bottom, probing of
458 *Tubulin* mRNA as a loading control. **(C)** IBComp-eGFP^{PHIT} was grown in the absence of
459 presence of Tet (4 μg/ml), Van (250 μM) Cym (3 μg/ml), and Tet and Van combined.
460 Error bars represent ± s.d. of the mean from 3 biological replicates. **(D)** Western blot
461 detection of IBWT and loading control EF1α1 from IBComp-eGFP^{PHIT} grown in 250 μM
462 Van and 4 μg/ml Tet for 8 days. SE, single expressor cell line control. 2 x 10^6 cell
463 equivalents were loaded per lane.

464

465 **FIG 3. Concurrent Gene Expression in IBComp-eGFP^{PHIT} cells. (A)** IBComp-
466 eGFP^{PHIT} was grown in the absence or presence of Van (250 μM), or inducer
467 combinations; Cym (3 μg/ml) and Tet (4 μg/ml), Cym and Van, and Cym, Tet, and Van
468 for 8 days. Error bars represent ± s.d. of the mean from 3 biological replicates. **(B)**
469 Northern blot of total RNA from IBComp-eGFP^{PHIT} cultured for 48 hours in the presence
470 of inducer combinations. U, uninduced; TC, Tet and Cym; VC, Van and Cym; VTC, Van,
471 Tet and Cym; V, Van. Top, probing of *POLIB* mRNA. Bottom, probing of *Tubulin* mRNA
472 as a loading control. **(C)** Western blot of POLIB, eGFP and EF1α1 loading control from
473 IBComp-eGFP^{PHIT} cells concurrently induced with Cym, Tet, and Van for 8 days. SE,
474 single expressor cell line control. 2 x 10^6 cell equivalents were loaded per lane. **(D)**
475 Western blot of eGFP, POLIB and EF1α1 loading control from IBComp-eGFP^{PHIT} cells
476 concurrently induced with Cym, Tet, and Van for 8 days. SE, single expressor cell line
477 control. 2 x 10^6 cell equivalents were loaded per lane. **(E)** Representative images of
478 eGFP expression for IBComp-eGFP^{PHIT} in the presence or absence of Cym+Tet+Van for
479 48 hours. DAPI staining (yellow); eGFP expression (green). Size bar, 10 μm. **(F)**
480 Representative images of POLIB IBComp-eGFP^{PHIT} in the presence or absence of
481 Cym+Tet+Van for 48 hours. DAPI staining (yellow); anti-protein A (red). Size bar, 10 μm.

482 **FIG 4. Temporal Expression of eGFP in the Triple Inducer System. (A)** Western blot
483 detection of eGFP and EF1α1 loading control from IBComp-eGFP^{PHIT} cells induced with
484 Cym (3 μg/ml), for 2 days then in the absence of Cym for subsequent days. D2*, sample
485 taken from cell before Cym inducer was removed. 2 x 10^6 cell equivalents were loaded
486 per lane. **(B)** Western blot detection of eGFP, POLIB and EF1α1 loading control from

487 IBComp-eGFP^{PHIT} cells induced with Van, Tet and Cym for 2 days then in the absence
488 of Cym for subsequent days. D2*, sample taken from cell before Cym inducer was
489 removed. SE, single expressor cell line control. 2 x 10^6 cell equivalents were loaded
490 per lane. **(C)** IBComp-eGFP^{PHIT} was grown in the absence or presence of Cym (3 μg/ml)
491 or all inducers for 5 days. Red triangle, Cym inducer was removed and grown in
492 absence of Cym. Graphs represent averaged data from two biological replicates.

493

494 **Supplemental Fig 1. Optimization of the Cumate Inducer System. (A)**

495 Diagram of plasmids integrated into the CuRO-eGFP cell line. Cumate repressor
496 (CymR), (HPH), neomycin phosphotransferase II (G418), cumate operator (CuO),
497 tetracycline repressor (TetR), vanillic acid repressor (VanR), T7 RNA polymerase
498 (T7RNAP), puromycin-N-acetyl-transferase (PAC). **(B)** Western blot detection of eGFP
499 and EF1α1 loading control from CuRO-eGFP clonal cell lines. 2 x 10^6 cell equivalents
500 were loaded per lane. **(C)** Western blot detection of eGFP and EF1α1 loading control
501 from eGFP^{PHITER} clonal cell lines. 2 x 10^6 cell equivalents were loaded per lane. Image
502 represents a longer exposure (90 seconds) of the data presented in Figure 1B.

503

504 **Supplemental Fig 2. Cumate Inducible eGFP Expression in IBComp-eGFP^{PHIT}.**

505 **(A)** Western blot detection of eGFP and EF1α1 loading control from IBComp-eGFP^{PHIT}
506 cells induced with varying concentration of Cym for 48 hr. 2 x 10^6 cell equivalents were
507 loaded per lane**. (B)** Western blot detection of eGFP and EF1α1 loading control from
508 IBComp-eGFP^{PHIT} cells induced with varying concentration of Cym for 8 days. **(C)**

509 IBComp-eGFP^{PHIT} was grown in the absence of presence of varying concentrations of
510 Cym. Graphs represent averaged data from two biological replicates.

511

512 **Supplemental Fig 3. Characterization of PHITER clonal cell lines. (A)** Western blot
513 detection of TetR and EF1α1 loading control from PHITER clonal cell lines. 2 x 10^6 cell
514 equivalents were loaded per lane**. (B)** Northern blot of total RNA from PHITER clonal
515 cell lines. Left, probing for *CymR* mRNA. Right, probing for *VanR* mRNA. Bottom for
516 each, rRNA as a loading control.

517

518 **Funding**

519 This work was financially supported by Bridge Funding from the College of Natural
520 Sciences, University of Massachusetts, Amherst, the Donald P. Reed Legacy Fund and
521 R21AI183196-01. The authors declare no competing financial interests.

522

523 **References**

524 1. Morrison LJ, Steketee PC, Tettey MD, Matthews KR. Pathogenicity and virulence of
525 African trypanosomes: From laboratory models to clinically relevant hosts. Virulence.
526 2023 Dec 31;14(1):2150445.

527 2. Matthews KR. 25 years of African trypanosome research: From description to
528 molecular dissection and new drug discovery. Molecular and Biochemical
529 Parasitology. 2015 Mar;200(1–2):30–40.

530 3. Lukeš J, Speijer D, Zíková A, Alfonzo JD, Hashimi H, Field MC. Trypanosomes as a
531 magnifying glass for cell and molecular biology. Trends in Parasitology. 2023
532 Nov;39(11):902–12.

533 4. Dewar CE, Casas-Sanchez A, Dieme C, Crouzols A, Haines LR, Acosta-Serrano Á,
534 et al. Oxidative Phosphorylation Is Required for Powering Motility and Development
535 of the Sleeping Sickness Parasite Trypanosoma brucei in the Tsetse Fly Vector.
536 Hajduk SL, editor. mBio. 2022 Feb 22;13(1):e02357-21.

537 5. Shapiro TA, Englund PT. THE STRUCTURE AND REPLICATION OF
538 KINETOPLAST DNA. Annu Rev Microbiol. 1995 Oct;49(1):117–43.

539 6. Jensen RE, Englund PT. Network News: The Replication of Kinetoplast DNA. Annu
540 Rev Microbiol. 2012 Oct 13;66(1):473–91.

541 7. Amodeo S, Bregy I, Ochsenreiter T. Mitochondrial genome maintenance—the
542 kinetoplast story. FEMS Microbiology Reviews. 2023 Nov 1;47(6):fuac047.

543 8. Kitchin PA, Klein VA, Fein BI, Englund PT. Gapped Minicircles. A novel replication
544 intermediate of kinetoplast DNA. Journal of Biological Chemistry. 1984
545 Dec;259(24):15532–9.

546 9. Hines JC, Ray DS. Structure of discontinuities in kinetoplast DNA-associated
547 minicircles during S phase in Crithidia fasciculata. Nucleic Acids Research. 2007
548 Nov 26;36(2):444–50.

549 10. Schneider A, Ochsenreiter T. Failure is not an option – mitochondrial genome
550 segregation in trypanosomes. Journal of Cell Science. 2018 Sep
551 15;131(18):jcs221820.

552 11. Aeschlimann S, Stettler P, Schneider A. DNA segregation in mitochondria and
553 beyond: insights from the trypanosomal tripartite attachment complex. Trends in
554 Biochemical Sciences. 2023 Dec;48(12):1058–70.

555 12. Klingbeil MM, Motyka SA, Englund PT. Multiple Mitochondrial DNA Polymerases in
556 Trypanosoma brucei. Molecular Cell. 2002 Jul;10(1):175–86.

557 13. Chandler J, Vandoros AV, Mozeleski B, Klingbeil MM. Stem-Loop Silencing Reveals
558 that a Third Mitochondrial DNA Polymerase, POLID, Is Required for Kinetoplast DNA
559 Replication in Trypanosomes. Eukaryot Cell. 2008 Dec;7(12):2141–6.

560 14. Bruhn DF, Sammartino MP, Klingbeil MM. Three Mitochondrial DNA Polymerases
561 Are Essential for Kinetoplast DNA Replication and Survival of Bloodstream Form
562 Trypanosoma brucei. Eukaryot Cell. 2011 Jun;10(6):734–43.

563 15. Miller JC, Delzell SB, Concepción-Acevedo J, Boucher MJ, Klingbeil MM. A DNA
564 polymerization-independent role for mitochondrial DNA polymerase I-like protein C
565 in African trypanosomes. Journal of Cell Science. 2020 May 1;133(9):jcs233072.

566 16. Horn D. Genome-scale RNAi screens in African trypanosomes. Trends in
567 Parasitology. 2022 Feb;38(2):160–73.

17. Sunter JD. A vanillic acid inducible expression system for Trypanosoma brucei. Molecular and Biochemical Parasitology. 2016 May;207(1):45–8.

18. Li FJ, Xu ZS, Aye HM, Brasseur A, Lun ZR, Tan KSW, et al. An efficient cumate-inducible system for procyclic and bloodstream form Trypanosoma brucei. Molecular and Biochemical Parasitology. 2017 Jun;214:101–4.

19. Armstrong R, Romprey MJ, Raughley HM, Delzell SB, Frost MP, Chambers J, et al. An independently tunable dual control system for RNAi complementation in Trypanosoma brucei. Yurchenko V, editor. PLoS One. 2025 May 12;20(5):e0321334.

20. Lamb JR, Fu V, Wirtz E, Bangs JD. Functional Analysis of the Trypanosomal AAA ProteinTbVCP with trans-Dominant ATP Hydrolysis Mutants. Journal of Biological Chemistry. 2001 Jun;276(24):21512–20.

21. Wang Z, Morris JC, Drew ME, Englund PT. Inhibition of Trypanosoma brucei Gene Expression by RNA Interference Using an Integratable Vector with Opposing T7 Promoters. Journal of Biological Chemistry. 2000 Dec;275(51):40174–9.

22. Wirtz E, Leal S, Ochatt C, Cross GeorgeAM. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei. Molecular and Biochemical Parasitology. 1999 Mar;99(1):89–101.

23. Huang Y, Dong X, Sun SY, Lim TK, Lin Q, He CY. ARL3 GTPases facilitate ODA16 unloading from IFT in motile cilia. Sci Adv. 2024 Sep 6;10(36):eadq2950.

24. Carter M, Gomez S, Gritz S, Larson S, Silva-Herzog E, Kim HS, et al. A Trypanosoma brucei ORFeome-Based Gain-of-Function Library Identifies Genes That Promote Survival during Melarsoprol Treatment. Phillips M, editor. mSphere. 2020 Oct 28;5(5):e00769-20.

25. Budzak J, Siegel TN. Next generation genetic screens in kinetoplastids. Nucleic Acids Research. 2025 Jun 6;53(11):gkaf515.

26. Wirtz E, Clayton C. Inducible Gene Expression in Trypanosomes Mediated by a Prokaryotic Repressor. Science. 1995 May 26;268(5214):1179–83.

27. Zhao X, He Y, Zhang F, Aphasizheva I, Aphasizhev R, Zhang L. Comparative mitochondrial genome and transcriptome analyses reveal strain-specific features of RNA editing in *Trypanosoma brucei*. Nucleic Acids Research. 2025 Jul 8;53(13):gkaf661.

28. Bruhn DF, Mozeleski B, Falkin L, Klingbeil MM. Mitochondrial DNA polymerase POLIB is essential for minicircle DNA replication in African trypanosomes. Molecular Microbiology. 2010 Mar;75(6):1414–25.

603 29. Sadiq AY, Bilbis LS, Andrew O, Yusuf H. Synthesis and in vitro anti-trypanosomal
604 activity of Vanillic Acid and Para- Hydroxybenzoic Acid on T. congolense. 2021;

605

Figure 1: Triple Inducer Systems

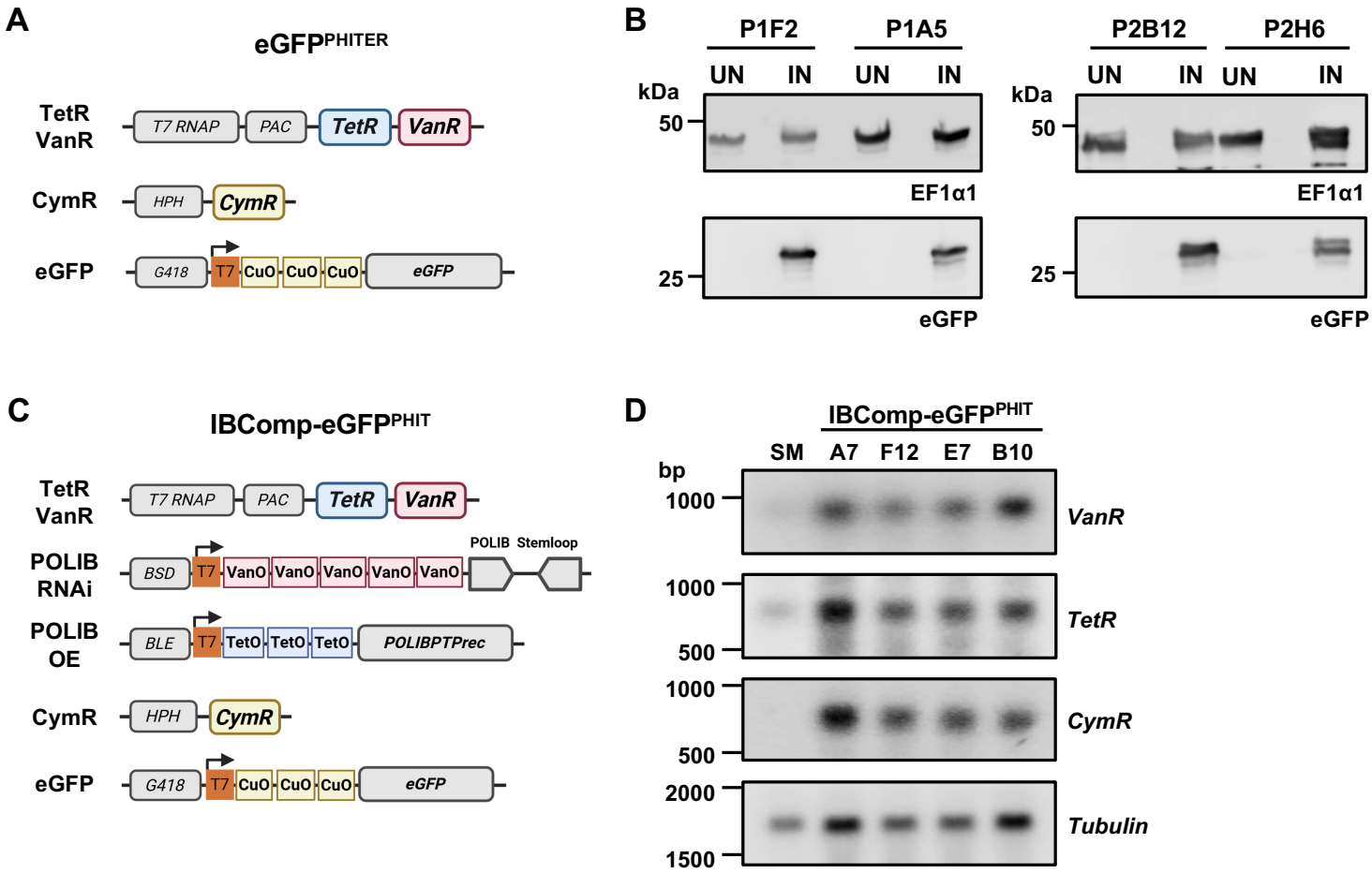

Figure 2: Independent gene expression in IBComp-eGFP^{PHIT} cells

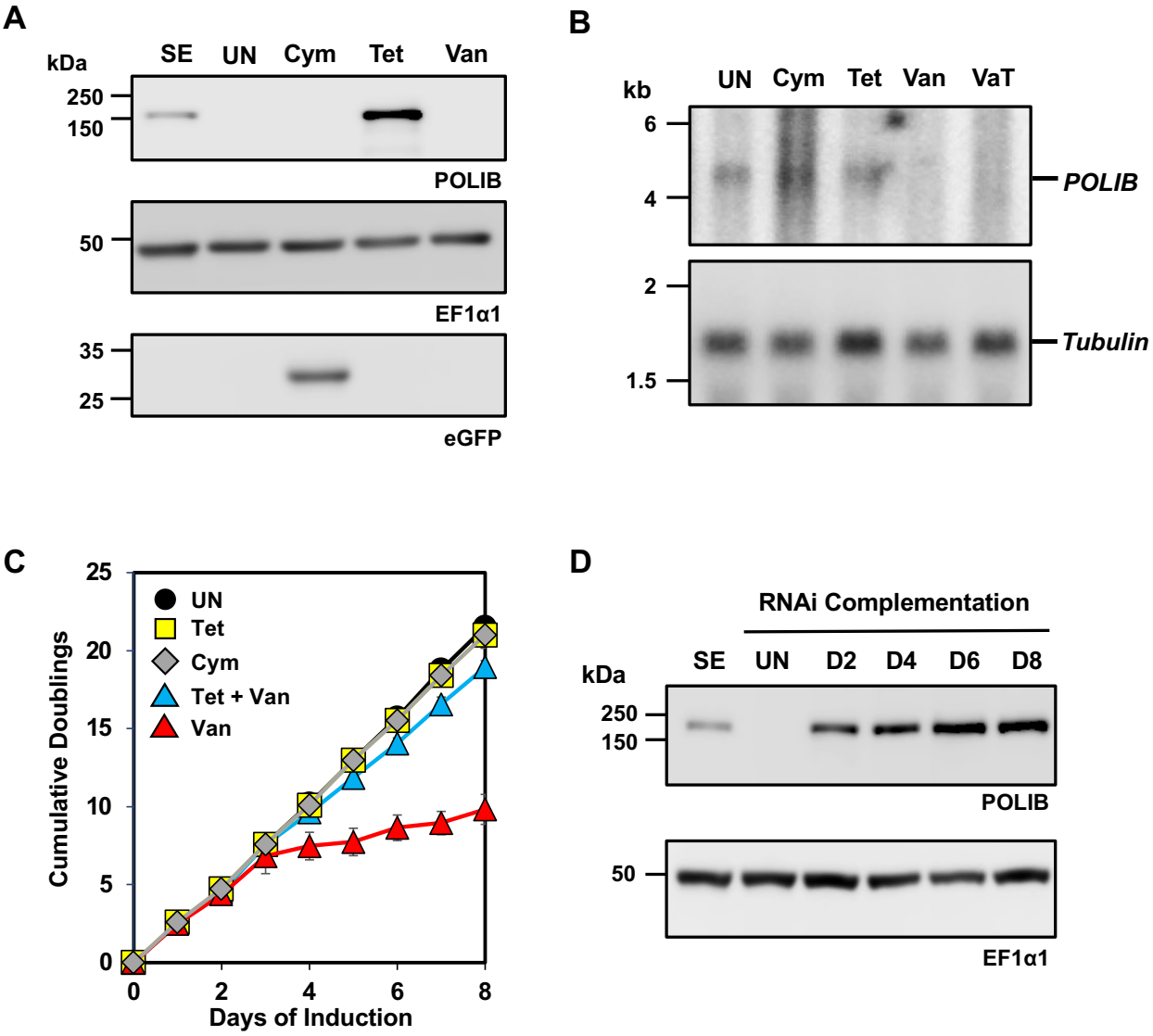

Figure 3: Concurrent Gene Expression in IBComp-eGFP^{PHIT} cells

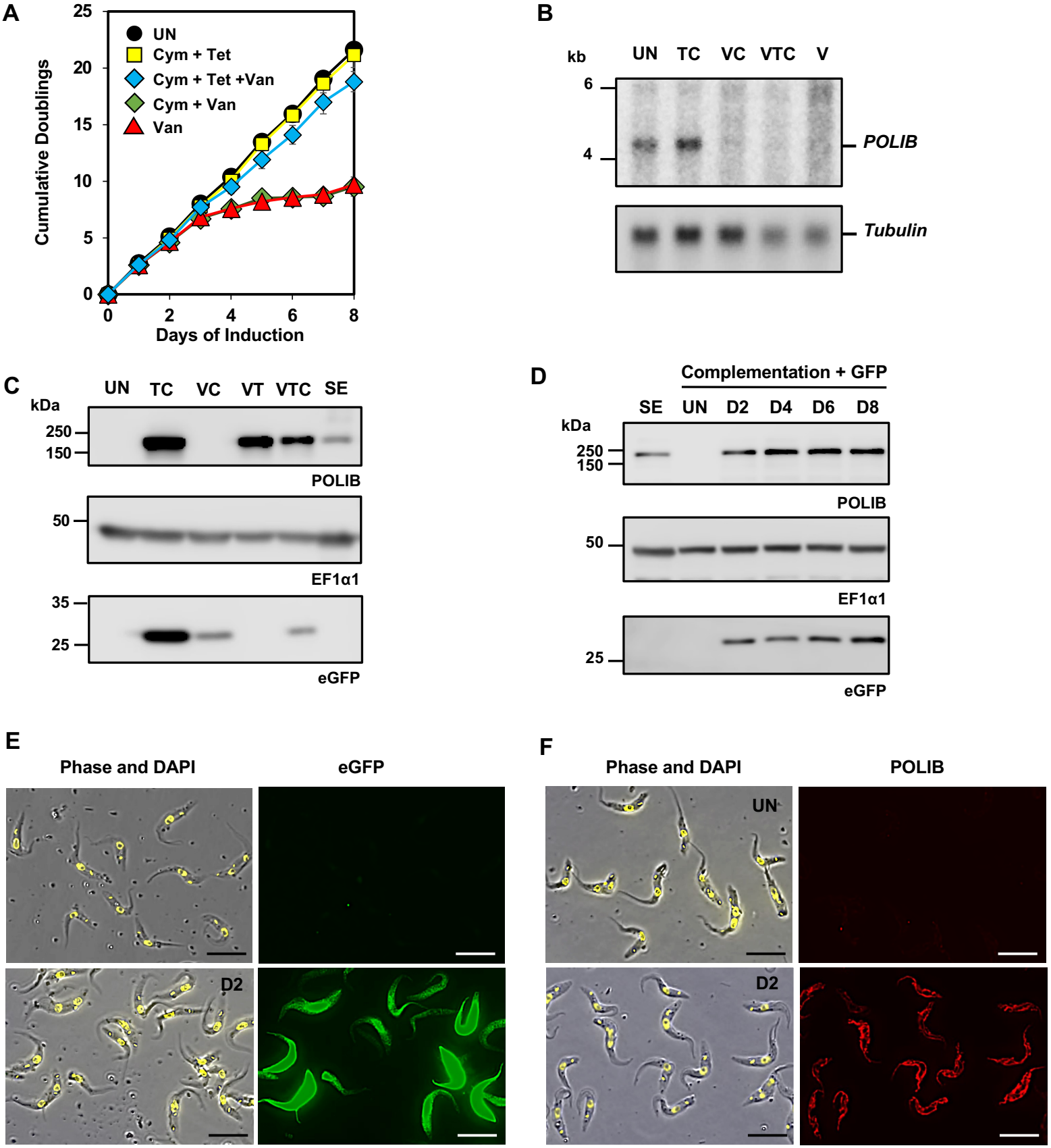
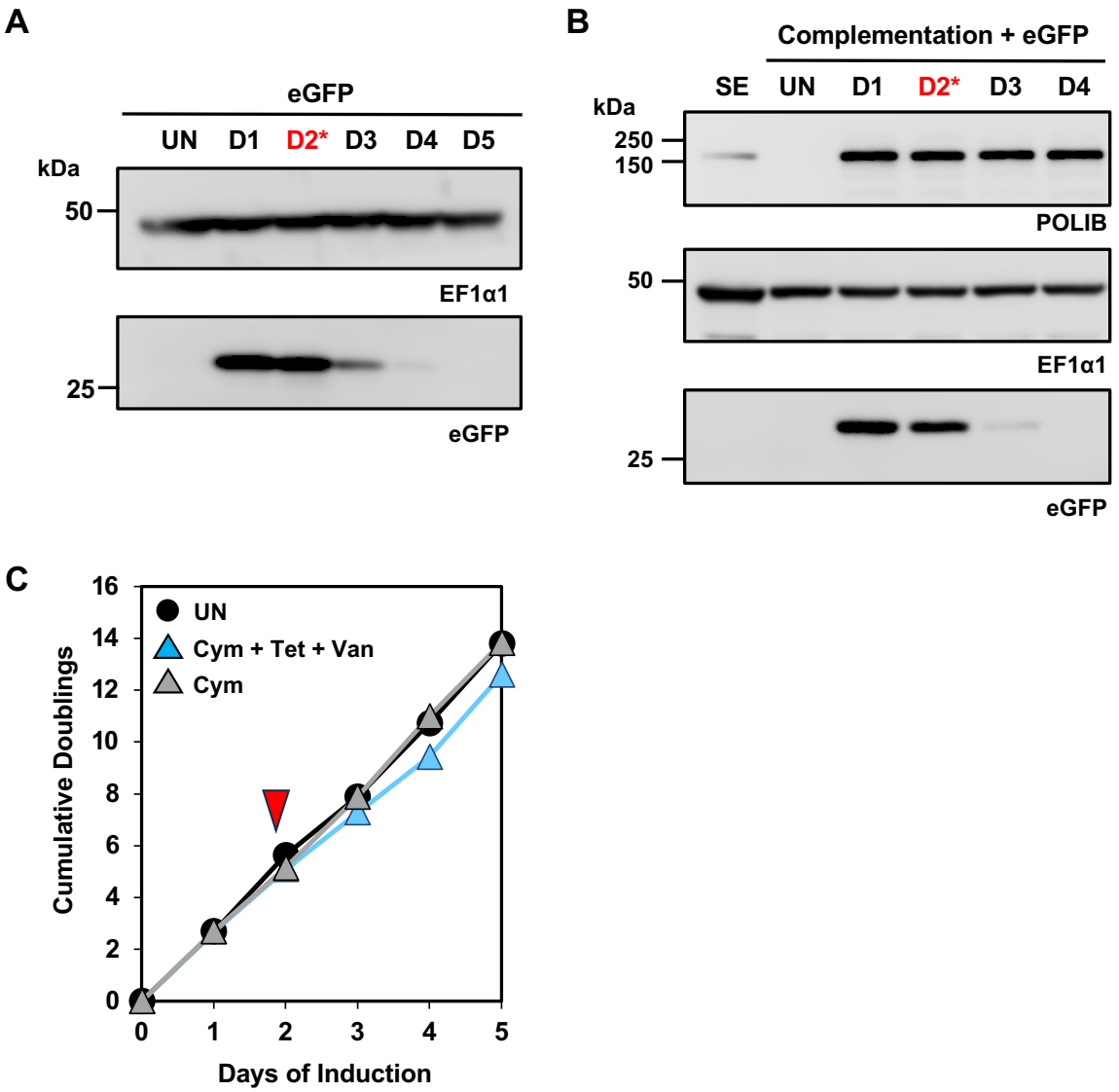
Figure 4: Temporal Expression of eGFP in the Triple Inducer System