Extracellular-signal regulated kinase 8 of Trypanosoma brucei uniquely phosphorylates its proliferating cell nuclear antigen homolog and reveals exploitable properties

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ABSTRACT

The Trypanosoma brucei subspecies T. brucei gambiense and T. brucei rhodesiense are vector-borne pathogens that cause sleeping sickness also known as Human African Trypanosomiasis (HAT), which is fatal if left untreated. The drugs that treat HAT are ineffective and cause toxic side effects. One strategy for identifying safer and more effective HAT drugs is to therapeutically exploit essential gene targets in T. brucei. Genes that make up a basic mitogen-activated protein kinase (MAPK) network are present in T. brucei. Tb927.10.5140 encodes an essential MAPK that is homologous to the human extracellular-signal regulated kinase 8 (HsERK8) which forms a tight complex with the replication factor proliferating cell nuclear antigen (PCNA) to stabilize intracellular PCNA levels. Here we demonstrate that (TbPCNA) is uniquely phosphorylated on serine (S) and threonine (T) residues in T. brucei and that TbERK8 phosphorylates TbPCNA at each of these residues. The ability of an ERK8 homolog to phosphorylate a PCNA homolog is a novel biochemical property that is first demonstrated here in T. brucei and may be unique to this pathogen. We demonstrate that the potent HsERK8 inhibitor Ro318220, has an IC\textsubscript{50} for TbERK8 that is several hundred times higher than its reported IC\textsubscript{50} for HsERK8. This indicated that the active sites of TbERK8 and HsERK8 can be selectively inhibited, which provides a rational basis for discovering inhibitors that specifically target this essential parasite MAPK to kill the parasite.

Introduction

Two Trypanosoma brucei subspecies, T. brucei gambiense and T. brucei rhodesiense, are the causative agents of Human African Trypanosomiasis (HAT) or sleeping sickness, a meningo-encephalitic disease. This parasite is endemic in the sub-Saharan region of Africa where it is transmitted by the bite of infected tsetse fly from the Glossina species and is most often fatal if left untreated.\textsuperscript{1} The available HAT treatments suffer from toxic side effects, difficulty in administration, and a lack of targets that can be selectively inhibited in the parasite. The T. brucei genome possesses the basic components of a mitogen-activated protein kinase (MAPK) signaling network\textsuperscript{2,3} found in eukaryotic cells. Such networks are generally regulated by sequential phosphorylation of a 3-tiered superfamily of serine/threonine protein kinases consisting of MAPK kinase kinases, MAPK kinases, and MAPKs.\textsuperscript{4-6} Once activated, MAPKs phosphorylate downstream effectors that include transcription factors, protein kinases, and phosphatases.\textsuperscript{7} In T. brucei, MAPKs have been shown to regulate differentiation, stress response, and transmission in both the insect and mammalian stages of this heteroxenous parasite.\textsuperscript{8,11} The important role that MAPKs have in regulating many critical processes in T. brucei makes them attractive for therapeutic exploitation. We previously demonstrated that depleting Tb297.10.5140 expression in bloodstream form T. brucei arrests proliferation and leads to its death, discovering that this MAPK is essential for the pathogenic form of the parasite.\textsuperscript{12} We demonstrate in this study that the Tb927.10.5140 gene, which encodes an extracellular-signal-regulated kinase 8 (TbERK8) homolog uniquely phosphorylates the essential replication factor proliferating cell nuclear antigen of T. brucei in vitro and in the parasite. We discuss the distinct biochemical properties of TbERK8, which opens opportunities for discovering new small molecules that can kill T. brucei by selectively inhibiting TbERK8.

Results

Phylogenetic analysis of TbERK8

The amino acid sequence of Tb927.10.5140 was analyzed with BLAST on the Orthologous group tool\textsuperscript{13} to retrieve a non-biased group of orthologous sequences. Redundant sequences were filtered out of the group while TbMAPK1 (Tb927.10.7780) and TbMAPK3 (Tb927.8.3550) were added in to optimize the ClustalW alignment among the orthologous groups. Table 1 lists the...
Table 1. ERK8 orthologs. The Tb927.10.5140 ORF was searched using BLASTO on the Orthologous group tool to retrieve an unbiased group of related eukaryotic MAPKs listed below.

| Kinase          | Accession Number |
|-----------------|------------------|
| Tb ERK8         | Tb927.10.5140    |
| Ce ERK7         | Q11179           |
| Dm ERK7         | AAF46481         |
| Hs ERK8/MAPK15  | NP_620590        |
| Sp MAPK Spk1    | NP_594009        |
| Hs MAPK3        | NP_002737        |
| Sc MAPK1/3 fus3 | NP_009537        |
| Hs MAPK7        | NP_000740        |
| Ce MAPK-1       | CE24971          |
| Hs MAPK2        | Hs2096E529       |
| Hs MAPK1        | NP_620407        |
| Hs MAPK13       | NP_002745        |
| Dm p38b         | NP_477363        |
| Dm MAPK2        | NP_477163        |
| Sp Pmk1         | NP_595289        |
| Hs MAPK14       | AAP36304         |
| Hs MAPK11       | NP_002742        |
| Hs MAPK12/p38gamma | AA640118     |
| Sc KSS1p        | NP_011554        |
| Sc Kdx1p        | NP_012761        |
| Hs MAPK4        | CAA4211          |
| Hs MAPK6        | NP_002739        |
| Sc Smk1p        | NP_015379        |
| Ce Protein SMA-5, isoform a | NP_741909 |
| Ce NLK Isoform c | NP_001022807 |
| Ce lit-1        | NP_001022806     |
| Ce lit-1_b      | NP_001022805     |
| Tb MAPK3        | Tb927.8.3550     |
| Tb MAPK1        | Tb927.10.7780    |
| SRC tyrosine_kinase | NP_033297 |

Note. Tb: Trypanosoma brucei, Ce: Caenorhabditis elegans, Dm: Drosophila melanogaster, Sp: Schizosaccharomyces pombe, Sc: Saccharomyces cerevisiae, Hs: Homo sapiens.

Many proteins form stable interaction with PCNA through the conserved PIP-box motif. TbERK8 contains a putative PIP-box motif that is 75% identical to the functional PIP-box of HsERK8 (Fig. 1B arrows, and 3A). One of the proposed functions for the PIP-box motif of HsERK8 is to mediate its interaction with HsPCNA and form a stable complex that prevents PCNA from being degraded by the proteasome.13 We generated the GST-TbERK8-PIP-box fusion protein (GSTtbE8-PIP) depicted in Figure 3B to test the ability of this motif to interact with TbPCNA. Figure 3C shows a representative immunoblot depicting in vivo protein interaction between TbERK8 and TbPCNA using the PY20 antibody (Fig. 2C). The tryptic peptides of phosphorylated TbERK8 were analyzed by LC-MS/MS to further investigate if the tyrosine residue in the activation loop was phosphorylated. This analysis revealed the phosphorylated residues pT174 and pY176, which correspond to the activation loop motif of TbERK8 (Fig. 2D). Based on these observations, we conclude that TbERK8 has dual-specific activity that allows it to autophosphorylate the T and Y residues that make up its activation loop.

Examination of the putative TbERK8 PIP-box

We tested TbERK8 purified from baculovirus (TbERK8\textsubscript{BV}) for its ability to autophosphorylate by incubating it in buffer K that contained \(^{32}\text{P}-\gamma\text{-ATP.\ Autophosphorylation was determined in TbERK8 by using autoradiography to detect its incorporation of} \(^{32}\text{P},\ indicating that recombinant TbERK8 purified as an active protein (Fig. 2A). We further designed a C-terminal truncated form of TbERK8 that expressed only the kinase domain residues (1-343 of TbERK8) fused to GST (GST-TbERK8\textsubscript{KD}) and re-tested its ability to autophosphorylate. The GST-TbERK8\textsubscript{KD} also incorporated \(^{32}\text{P}\) when incubated in kinase buffer with \(^{32}\text{P}-\gamma\text{-ATP\ substrate, indicating that it was able to autophosphorylate (Fig. 2B). Typically, ERKs become fully activated by upstream dual-specific MAPK-kinases that phosphorylate a conserved T-X-Y motif in the activation loop to pT-X-pY.}^{7,15} \text{ERK8 family members are atypical because full activation is catalyzed by autophosphorylation of the T-X-Y residues that make up the activation loop instead of by a dual-specific upstream MAPK-kinase.}^{7,14-16} \text{We demonstrated that TbERK8 could autophosphorylate tyrosine residues by using the PY20 antibody (Fig. 2C). The tryptic peptides of phosphorylated TbERK8 were analyzed by LC-MS/MS to further investigate if the tyrosine residue in the activation loop was phosphorylated. This analysis revealed the phosphorylated residues pT_{174} and pY_{176}, which correspond to the activation loop motif of TbERK8 (Fig. 2D). Based on these observations, we conclude that TbERK8 has dual-specific activity that allows it to autophosphorylate the T and Y residues that make up its activation loop.\)
strategy was not successful at detecting stable interactions between TbERK8 and TbPCNA (data not shown). Based on these observations, we conclude that the putative PIP-box of TbERK8 is very poor at forming a stable complex with TbPCNA in comparison with functional canonical PIP-box motifs from other *T. brucei* proteins.

**Examination of TbPCNA from in vivo pull-down assays**

The reciprocal *in vivo* pulldowns were also done with hemagglutinin-tagged TbPCNA (TbPCNA_{HA}). We immunoprecipitated TbPCNA from stably transfected *T. brucei* strains that express wt-TbPCNA_{HA} at endogenous or overexpressed levels and subjected it to LC-MS/MS analysis. This pulldown strategy was also ineffective at detecting interactions between TbERK8 and TbPCNA (data not shown). In addition we examined the phosphorylation status of TbPCNA_{HA} pulled down from *T. brucei* by LC-MS/MS. This analysis showed that TbPCNA_{HA} pulled down from *T. brucei* was phosphorylated at residues T202 (Fig. 4A) and S216 (Fig. 4B) whether it was isolated from *T. brucei* that expressed it at endogenous or overexpressed levels. These phosphorylated residues are
situated in the unique insertion region of TbPCNA (Fig. 4C). Furthermore, this observation indicates that an unknown kinase activity within *T. brucei* is able to phosphorylate TbPCNA at these residues.

**TbERK8 phosphorylates TbPCNA in its unique insertion**

Both wt-TbERK8HA and K42A-TbERK8HA mutant was overexpressed in stable *T. brucei* transfectants by tetracycline induction (Fig. 5A). We demonstrated that the K42A-TbERK8HA mutant was defective in 32P-γ-ATP kinase autophosphorylation assays (Fig. S1). These overexpressed TbERK8 proteins were adsorbed to anti-HA affinity beads for use in kinase assays with buffer K and 32P-γ-ATP to verify their activity. The generic kinase substrate myelin basic protein (MBP) and recombinant TbPCNA6H purified from *E. coli* were tested as substrates in the kinase assay. The autoradiographs from the kinase assays demonstrated that TbPCNA was phosphorylated with a greater efficiency than MBP by TbERK8 immunoprecipitated from *T. brucei* (Fig. 5B). As a mock control, the affinity beads were incubated in lysates from non-transgenic *T. brucei* 221 and only background labeling of both MBP and TbPCNA was detected (Fig. 5C). When anti-HA affinity beads adsorbed with the K42A-TbERK8HA mutant were used in kinase reactions, only background levels of phosphorylation similar to those of the mock control were observed (Fig. 5D). The wt-TbERK8HA phosphorylated TbPCNA6H about 20-fold above the mock control and about 7-fold above the K42A-TbERK8HA (Fig. 5E and F). The ability to label TbPCNA6H in this kinase assay clearly depended on the K42 residue of TbERK8 being intact. We conclude from these results that the kinase activity of TbERK8 was responsible for phosphorylating TbPCNA in the kinase assay. We repeated the kinase assays with affinity beads adsorbed with wt-TbERK8HA in buffer K that had cold ATP to analyze the phosphorylated TbPCNA6H substrate by mass spectrometry. LC-MS/MS analysis of tryptic TbPCNA6H peptides from the kinase assay identified peptides that were phosphorylated at residues T202 and S211 (Fig. 5G). The pT202 residue matched the position phosphorylated in TbPCNAHA immunoprecipitated from *T. brucei* and the pS211 residues is also within the unique insertion region of TbPCNA.

We also tested TbERK8BV for activity against TbPCNA using kinase buffer K with 32P-γ-ATP. The autoradiographs from kinase reactions showed that both TbERK8BV and the 36-kDa TbPCNA6H were phosphorylated (Fig. 6A). We repeated the kinase reactions in buffer containing cold ATP and resolved the phosphorylated TbPCNA by SDS-PAGE for examination by LC-MS/MS. More than 96% of the TbPCNA sequence was covered by the peptides detected by LC-MS/MS. Phosphorylated residues corresponding to S211 (Fig. 6B) and S216 (Fig. 6C) were identified on 2 of the peptides.

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**Figure 2.** Autophosphorylation of TbERK8. Top panels: (A) Autoradiograph of purified recombinant TbERK8BV incubated in kinase reaction buffer without (−) or with (+) 10 μCi 32P-γ-ATP; (B) Autoradiograph showing the autophosphorylation of the GST-TbERK8 kinase domain (GST-TbERK8KD) that lacks the C-terminus. GST-TbERK8KD was tested for autophosphorylation in a kinase reaction without (−) or with (+) 10 μCi 32P-γ-ATP; (C) Immunoblot using PY20 antibodies to detect phosphotyrosine residues associated with autophosphorylation of TbERK8BV. Bottom panels are StainFree™ gels showing the loading controls for TbERK8 samples. (D) LC-MS/MS mass spectrum of 166EQVARPVT(phospho)DY(phospho)IMTR180 peptide from TbERK8 containing the phosphorylated T174 and Y176 residues, with m/z = 651.30 (3+), 0.52 ppm error.
phosphorylated S\textsubscript{216} residue matched the phosphorylated serine residue identified from TbPCNA that was immunoprecipitated from \textit{T. brucei}. The phosphorylated S\textsubscript{211} identified by this analysis is also located within the unique TbPCNA insertion.

**In vivo phosphorylation of TbPCNA reduced upon TbERK8 depletion**—We examined the effect of TbERK8 depletion on TbPCNA phosphorylation in \textit{T. brucei} using the plasmid, pHAR (Fig. 7A). \textit{T. brucei} stably transfected with this plasmid simultaneously expressed HA-tagged TbPCNA under the control of the endogenous TbPCNA allele and tetracycline-inducible RNAi expression against TbERK8 mRNA. Tetracycline induction of \textit{T. brucei} stably expressing pHAR resulted in a decrease of TbERK8 mRNA levels as well as decreased proliferation (Fig 7B). This result was consistent with our previous study of depleting TbERK8 in bloodstream form \textit{T. brucei}.\textsuperscript{12} We also observed that the \textit{in vivo} levels of endogenously-tagged

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**Figure 3.** Testing putative TbERK8 PIP-box in TbPCNA pull-down assays. (A) Alignment of putative PIP-box motifs from ERK8 homologs of \textit{T. brucei} (Tb), \textit{T. congolense} (Tco), \textit{T. cruzi} (Tc), and \textit{H. sapiens} (Hs). The putative PIP-box motifs are 100% identical in related trypanosome species and are 75% identical to the functional PIP-box of HuERK8. (B) Schematic presentation of GST-T. brucei PIP-box fusion proteins. The PIP-box in the schematic represents the putative TbERK8 PIP-box or canonical PIP-box sequences from TbAUK1 or TbPFC19. (C) Immunoblot analysis of wt-TbPCNA\textsubscript{6H} in control (Tet −) or tetracycline-induced (Tet +) bloodstream form \textit{T. brucei} with antisera to hemagglutinin (α-HA). Bottom panel shows Coomassie-stained gel image of variant surface antigen (VSG) loading control. (D) Examination of wt-TbPCNA\textsubscript{6H} pulled down by GST-control (GST-Ctrl) or GST-TbERK8-PIP-box (GSTtbE8-PIP-box) glutathione agarose beads. Top panel shows the Coomassie-stained gel image of GST fusion proteins, middle panel shows the immunoblot analysis using α-HA antibodies to detect wt-TbPCNA\textsubscript{6H}, and lower panel shows Coomassie-stained gel image of VSG loading control. (E) Quantitation of wt-TbPCNA\textsubscript{6H} pull down, Bar graph showing results from densitometry trace comparing the amount of TbPCNA pulled down by GST-Ctrl beads to that pulled down by GST-tbERK8-PIP beads. Values are mean arbitrary units (AU) with standard deviation for 3 independent experiments. (F) Pull down of recombinant TbPCNA\textsubscript{6H} by GST-bound TbERK8 PIP-box (GSTtbE8-PIP) and GST-bound canonical PIP-boxes from TbAUK1 (GSTtbAUK1-PIP) and TbPFC19 (GSTtbPFC-PIP). The top 2 panels show StainFree\textsuperscript{™} gels of TbPCNA\textsubscript{6H} bound to the beads in comparison to the loading controls. The third StainFree\textsuperscript{™} gels of the GST-PIP-box proteins used in pull-downs. The bottom 2 panels show the immunoblot analysis of TbPCNA\textsubscript{6H} bound to the beads in comparison to the loading controls. (G) Mean quantitation values from 3 independent experiments of TbPCNA\textsubscript{6H} pulled down by various GST-PIP-boxes. Error bars show standard deviation (*** indicate p-value < 0.05). (H) TbPCNA calibration curve from StainFree\textsuperscript{™} gels showing average RFU intensity values with standard deviations from replicate experiments. (Relative Fluorescent Units, RFU), (Arbitrary Units, AU).
TbPCNA decrease after 48 h of TbERK8 depletion (Fig. 7C). Neither T202,S211 nor S216 residues were phosphorylated in TbPCNAHA immunoprecipitated from *T. brucei* after 48 h of TbERK8 depletion (data not shown). The mass spectrometry analysis of the TbPCNATSS triple mutant control expressed and immunoprecipitated in *T. brucei* did not detect any phosphorylated residues. Table 2 summarizes the phosphorylated TbPCNA residues that we have identified in vivo and in vitro.

We conclude that TbERK8 phosphorylates TbPCNA in vivo.

**Small molecule inhibitors distinguish between active sites of HsERK8 and TbERK8**

We probed the active site of TbERK8 with the inhibitor SCYX5070 to test for selectivity. This 2,4-diaminopyrimidine was a logical first choice because it kills *T. brucei* at submicromolar concentrations and TbERK8 (formerly annotated as Tb10.70.2070) was one of the many kinases that its immobilized version pulled down from parasite lysates. We tested SCYX5070 by HTRF and determined its IC$_{50}$ for TbERK8 to be about 1.6 mM. At a concentration of 1 mM, SCYX5070 inhibited HsERK8 activity to 37% ± 7% versus 59% ± 11% inhibition for TbERK8 which is significantly different with a *p*-value $= 0.02$ (Fig. S2). Ro318220 is an HsERK8 inhibitor with reported IC$_{50}$ values that ranges from 5-10 nM. We tested this inhibitor at 1 μM and 10 μM against TbERK8 as shown in the representative kinase assay shown in Figure 8A. This inhibitor was able to reduce the activity of TbERK8 to 49% and 27% at 1 μM and at 10 μM respectively (Fig. 8B). Eight-point dose response curves done with Ro318220 against TbERK8 were used to calculate an IC$_{50}$ of 1.4 μM (Fig. 8C). This IC$_{50}$ of Ro318220 for TbERK8 ranges between 100 -to- 300 times higher than the IC$_{50}$ values of this compound reported for HsERK8.

**Discussion**

TbERK8 is a member of the ERK8 family of MAPKs first identified in human cells. These family members have
characteristically long C-terminal extensions that are not conserved. The ERK8 MAPKs are atypical because they become fully activated by autophosphorylation instead of by an upstream MAPK kinase. This study reveals that TbERK8 has some biochemical properties that distinguish it from its HsERK8 homolog.

We conclude that TbERK8 phosphorylates TbPCNA in T. brucei. By using this parasite system, we also present the first example of a PCNA homolog being phosphorylated on serine and threonine residues. All of the TbPCNA residues phosphorylated by TbERK8 in this study were located in an insert that is predicted to form an extended backside loop (Fig. 9A). The presence of such a loop is a feature that has only been identified in the PCNA homologs of kinetoplastid parasites. The in vitro kinase assays clearly demonstrated that TbERK8 was able to phosphorylate TbPCNA at the same residues that we identified from TbPCNA directly immunoprecipitated from T. brucei. Interestingly, the phosphorylation patterns of TbPCNA that was immunoprecipitated from the parasite did not exactly match those obtained from recombinant TbPCNA that was phosphorylated by recombinant TbERK8 in vitro. One possible explanation for such discrepancies is that an unidentified factor co-purifies with immunoprecipitated TbERK8 and regulates the way that this kinase phosphorylates TbPCNA. Our inability to phosphorylate TbPCNA with HsERK8 (Fig. S3) or to phosphorylate HsPCNA with TbERK8 (data not shown) and results from a previous study strengthen the hypothesis that this mechanism of PCNA serine/threonine phosphorylation is unique to T. brucei. However, this mechanism of PCNA phosphorylation might also be possible in other kinetoplastid parasites.

Figure 5. Activity assay with immunoprecipitated TbERK8. (A) Immunoblot verifying the expression of wt-TbERK8 and K42A-TbERK8 in the parasite after tetracycline induction (+). Arrow points to the 52-kDa HA-tagged TbERK8 band recognized by α-HA antibodies. T. brucei variant surface antigen (VSG arrow) was used for the loading control. 32P-Kinase assay with wt-TbERK8 immunoprecipitated from T. brucei. Top panel shows the autoradiograph of kinase immunoprecipitation assays done with immunoadfinity beads pre-adsorbed with (B) wt-TbERK8, (C) T. brucei 221 lysate and (D) K42A-TbERK8. Each of the 3 sets of beads were tested with either no substrate (first lane of each experiment in the top row), 100 μmol of MBP (second lane of each top panel), or 100 μmol of TbPCNA (third lane of each panel). The second panel shows the StainFree loading controls for MPB and TbPCNA. The third panel shows the α-HA immunoblot detecting the HA-tagged TbERK8 bound to the immunoadfinity beads (absent in beads pre-incubated with T. brucei 221 lysates). The bottom panel is a α-IgG immuno-blot as a loading control for the affinity beads. (E) LC-MS/MS mass spectrum of SKGESDKT(phospho)EDDEADAC(cam)S(phosphor)VR213 peptide from TbPCNA containing phosphorylated T202 and S211 residues, with m/z = 791.31 (3+), 1.9 ppm error.
One perceived weakness in our discovery is the observation that none of the TbPCNA residues phosphorylated by TbERK8 had a proline at the C1 position. ERKs are proline-directed serine/threonine \((S/T)P\) kinases in general and such activity has been demonstrated in HsERK8. However, the presence of a Pro at the C1 position is not an absolute substrate requirement for ERKs and other MAPKs. We have identified only one potential site with a \((S/T)P\) motif within TbPCNA (263AEKSP267), but no phosphorylated Ser266 residue was detected by our mass spectrometry analysis of phosphorylated TbPCNA from either \textit{in vitro} or \textit{in vivo} preparations. We speculate that the residues that we identified by mass spectrometry represent TbPCNA sites that are the most accessible to TbERK8 and make up the majority of phosphorylation events. The other sites might represent ones that are phosphorylated at low frequencies below the detection limit of mass spectrometry analysis. The notion that TbERK8 can phosphorylate TbPCNA at other residues is strengthened by \textit{in vitro} kinase assays using the TbPCNA\textsubscript{TSS} triple mutant (Fig. S3).

All reported cases of HsPCNA phosphorylation occur on tyrosine residues. The phosphorylation of HsPCNA is mediated by tyrosine kinases such as epidermal growth factor receptor (EGFR) and the Recepteur d’Origine Nantais (RON)/c-ABL pathway. Receptor tyrosine kinase-mediated phosphorylation of HsPCNA occurs at Y211 which correlates with aggressive cancer either by increased DNA synthesis activity or inhibition of mismatch repair. Such tyrosine kinase-mediated mechanisms of phosphorylation are not possible in \textit{T. brucei} because the parasite lacks genes for any tyrosine kinases and because TbPCNA has a phenylalanine residue at the position that corresponds to Y211 in HsPCNA (Fig. 9B).

The strengths of this study are that it reveals that the replication factor TbPCNA is the first known biological substrate for TbERK8, which is an essential MAPK in \textit{T. brucei}. Further studies are needed to elucidate the biological role of serine/threonine phosphorylation in TbPCNA. Overall, very few substrates have been identified for ERK8 homologs. The only known cellular substrate of HsERK8 to

**Figure 6.** Recombinant TbERK8\textsubscript{BV} phosphorylates TbPCNA\textsubscript{ATF} \textit{in vitro}. (A) LC-MS/MS mass spectrum of \textit{v}SKGESVDKTEDDEADACS(phospho)\textit{VR}213 peptide from TbPCNA containing a phosphorylated S211 residue, with \textit{m/z} = 1146.47 (2+), 5.1 ppm error. (B) LC-MS/MS mass spectrum of \textit{j}S(phospho)\textit{AKGKDGPLGIGVDVR}231 peptide from TbPCNA containing a phosphorylated S216 residue, with \textit{m/z} = 629.65 (2+), 1.9 ppm error.
date is the RNA binding protein HuR,\textsuperscript{28} which is encoded by the \textit{ELAVL1} gene. HuR binds to the 3'UTR of the PDC4 tumor suppressor to protect it from miRNA 21 mediated gene silencing.\textsuperscript{29-31} Ro318220 is a potent inhibitor of human ERK8 and several other human protein kinases.\textsuperscript{19,32,33} The ability of Ro318220 to inhibit HsERK8 with such a low IC\textsubscript{50} in comparison to TbERK8 presents evidence that the active sites of HsERK8 and TbERK8 can be selectively inhibited. This creates a rational basis for discovering and developing small molecules that selectively inhibit TbERK8 to kill \textit{T. brucei} and have fewer off-target effects in the host. TbERK8 selective inhibitors can also be used to chemically map out its biological function in \textit{T. brucei}. 

**Materials and methods**

**Culturing of \textit{T. brucei}**

Bloodstream form parasites (Lister 427 VSG 221 strain) were incubated in 5% CO\textsubscript{2} at 37 °C in complete HMI-9 medium.
purchased from Axenia Biologix (http://www.axeniabio.com/) using the formulation of Hirumi and Hirumi.

**Primers for TbERK8 and TbPCNA constructs**

The coding region for wild-type TbERK8 (wt-TbERK8) was amplified with the forward primer 5′-CGCGCCAAGCTTATGTCATCAGAAATAGAGCC-3′ and the reverse primer 5′-CTTAAGCTTTGTGCAACACGCAGACGG-3′. The K42A point mutation (K42A-TbERK8) was made in TbERK8 cDNA using the K42A forward primer 5′-GGTTGTAGCGTTAGCAGAGATATACGAGC-3′ and reverse primer 5′-GCCGCGTATATCTT-CGCTAAGCGCTAACAACC-3′ by the annealing overlapping PCR method.

The wild-type TbPCNA coding region (wt-TbPCNA) was amplified by PCR from *T. brucei* genomic DNA using the forward primer 5′-AAGCTTATGCTTGAGGCTCAG-3′ and the reverse primer 5′-CTTAAGCTTGCGTGTCCTACCTTGG-3′. Primers to generate the PIP-box from TbERK8 were 5′-CGATATAGTTGAGTGGAAAGCCG-3′ and reverse primer 5′-GCCGAGA-GTCGACTCAGTGGAAAGCCG-3′. Primers to generate the PIP-box from TbAUK1 were forward primer 5′-GATCCACGTCTTTTCTGAGTAATATG-3′ and reverse primer 5′-TCGACATAATAATACCTTTGGAAG-3′. Primers to generate the PIP-box from TbPFC19 were forward primer 5′-GATCCAGGCGACCTTTATG-3′.

![Figure 8](image-url)
tor tyrosine kinases. Note that TbPCNA has a phenylalanine residue at the corre-
HsPCNA with black arrow showing tyrosine 211, which is phosphorylated by recep-
GST fusion protein (GST-TbERK8KD) in
extension, the nucleotides from 1-1019 of its coding region were
the manufacturer.

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TbPCNA6H, was puri
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pTrcHis A (Invitrogen, https://www.thermo
wt-TbPCNA was subcloned into the BamHI/HindIII sites of

expression plasmid (Cat# 17-0719-01, GE Healthcare, http://www.gelifesciences.com/webapp/wcs/stores/servlet/Home/en/GELifeSciences-us/). Protein was eluted from the CM column with buffer A (150 mM NaCl in 20 mM Tris, pH 7.4, 10% Glycerol and 1 mM EDTA). These fractions were pooled and loaded onto hydroxyapatite (HAP) chromatography resin (Cat# 130-0151, BioRad, http://www.bio-rad.com/en-us/category/cht-ceramic-hydroxyapatite-crys
talline-hydroxyapatite-resins). TbERK8BV was eluted from the HAP column in buffer B (HEPES, pH 7.4 10% glycerol, 75 mM NaCl, 75 mM phosphate ions). Fractions from the HAP column were pooled and loaded onto an S-Fast Flow column (Cat# 17-0511-01, GE Healthcare Life Sciences, http://www.gelifesciences.com/webapp/wcs/stores/servlet/Home/en/GELifeSciences-us/) and eluted with buffer A containing 250 mM NaCl.

**Purification of TbERK8bv from SF9 insect cells**

A recombinant baculovirus encoding the full coding region of TbERK8 was constructed using the pFastBac1 baculovirus expression system (ThermoFisher Scientific, https://www.thermofisher.com/order/catalog/product/1071204). Sf9 insect cells (4 × 250 ml) were grown to a density of 2.0 × 10^6 cells/ml and then infected with the recombinant virus at a multiplicity of infection of 3-5. After rotation at 150 rpm for 72 h at 28 °C, the cells were harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at −80 °C. The frozen pellet was thawed on ice and then resuspended in 200 ml of 50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonfonyl fluoride, 1 mM benzamidine-HCl, 1 μg/ml leupeptin, 2 μg/ml aproti
in, and 1 μg/ml pepstatin. After 30 min on ice, the lysate was cleared by centrifugation at 35,000 rpm for 30 min in a Beckm
an 45 Ti rotor at 4 °C. The clarified lysate was loaded on a carboxymethyl (CM) chromatography column (Cat# 17-0719-01, GE Healthcare, http://www.gelifesciences.com/webapp/wcs/stores/servlet/ProductDisplay?categoryId=11438&catalogId=10101&productId=17628&storeId=11787&langId=-1) as recommended by the manufacturer.

**Expression and purification of recombinant proteins in E. coli**

wt-TbPCNA was subcloned into the BamHI/HindIII sites of pTrcHis A (Invitrogen, https://www.thermofisher.com/order/catalog/product/V36520?ICID=search-product) and expressed with a 6×His tag in BL21-DE3. The recombinant protein, wt-
TbPCNAΔC11, was purified by nickel agarose affinity chromatog
raphy (Cat# 88222, Lot# PL208040, Thermo Scientific, https://www.thermofisher.com/us/en/home.html) as recommended by the manufacturer.

To express the TbERK8 kinase domain without the C-terminal extension, the nucleotides from 1-1019 of its coding region were subcloned into the BamHI/EcoRI sites of pGSTag to express it as a GST fusion protein (GST-TbERK8KD) in E. coli. The full-length TbERK8 (nucleotides 1-1329) was subcloned into BamHI/HindIII site of the pGSTag to express it as a GST fusion protein (GST-
TbERK8). The pGEX-2T-HsERK8 expression plasmid was kindly provided by the laboratory of Deborah Lannigan (Vanderbilt University). E. coli expressing either GST-TbERK8, GST-TbERK8KD, or GST-HsERK8 fusion protein were grown at 37 °C until they reached an A600 of 1.0, then chilled on ice before inducing with 0.2 mM isopropyl β-D-1-thiogalactopyranoside at 16 °C for 12 h. The E. coli were pelleted by centrifugation at 7,000 rpm in a Beckm
an JLA 10.5 rotor at 10 °C for 15 minutes. Pellets were lysed by

**Autophosphorylation of recombinant TbERK8bv and GST-TbERK8KD**

We incubated 0.1 μg of purified TbERK8bv or GST-TbERK8KD protein in 30 μl of buffer K (30 mM Tris pH 7.4, 10 mM MgCl2, 1 mM DTT, 5% glycerol, [1mM cold ATP for mass spectrometry assays or 10-100 μM cold ATP for 32P incorporations assays] and 0.1 mg/ml bovine serum albumin). Ten μCi of 32P-γ-ATP were added for detections by autoradiography in labeling experiments. The kinase reactions were incubated at 30 °C for 30 min. SDS loading buffer was added to the reaction mixtures and boiled for 1 min to stop the reaction. TbERK8 autophosphorylation by this method was detected by SDS-PAGE and autoradiography. Alternatively, TbERK8 autophosphorylation was done using 1 mM of cold ATP. In this case, TbERK8 autophosphorylation was detected by immunoblot analysis using the anti-phosphotyrosine antibody PY20
Kinase activity assays

Recombinant full-length TbERK8_{BV} purified from Sf9 insect cells (100 nmol) was incubated with 100 μmol TbPCNA_{6H}. For activity assays, 10 μCi of ³²P-γ-ATP (Cat# BLU502H250UC, Perkin Elmer, http://www.perkinelmer.com/product/atp-g-32p-blu002250uc?searchTerm=32p&pushBackUrl=?searchName=32p&No=0&Ns=Best%20Match) were added to 30 μL reactions that used buffer K. The samples were incubated for 5 min at 30 °C and stopped as described above. Reactions were resolved in an SDS-PAGE gel, and bands were visualized by autoradiography. TbPCNA phosphorylation was quantified with a Typhoon FLA 7000 PhosphorImager (GE Healthcare). Autoradiography. TbPCNA phosphorylation was quantified with a Typhoon FLA 7000 PhosphorImager (GE Healthcare).

Expression of hemagglutinin tagged (HA-tagged) recombinant proteins in T. brucei

pLEW11-3HA was generated by modifying the plasmid pLEW11,37 which allows for tetracycline inducible protein expression, to include a C-terminal hemagglutinin tag (HA-tag). The coding region for wt-TbERK8, K42A-TbERK8, and wt-TbPCNA were subcloned into the HindIII and AflII restriction sites of pLEW11-3HA. Nucleofections were carried out with 1 μg subcloned into the HindIII and AflII restriction sites of pLEW11-3HA, wt-TbERK8, K42A-TbERK8, and wt-TbPCNA were resolved in an SDS-PAGE gel, and bands were visualized by Coomassie Blue staining or by StainFree gel staining and allowed bands to be visualized by BioRad Chemidoc MP. TbPCNA bands were detected by immunoblotting as described below.

Immunoblot analysis of HA-tagged proteins

Parasites from cultured bloodstream form T. brucei were harvested at 2 × 10^7 cells/ml, lysed with T. lysis buffer (1% Triton X-100, 10 mM Tris pH 7.5, 25 mM KCl, 150 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 20% glycerol), and incubated on ice for 30 min, followed by microcentrifugation at 15,000 rpm for 5 min to produce a clarified lysate. For immunoblotting, 25 μg of clarified lysate was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF). After transferring and blocking, the membrane was incubated with rabbit anti-HA (1: 2,000 dilution) for 1 h and washed 3 times for 5 min with TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.4% Tween 20). After the third wash, horseradish peroxidase-conjugated donkey anti-rabbit IgG (1: 1,000 dilution) (Cat# 32460, Thermo Scientific, https://www.thermofisher.com/antibody/product/Goat-Anti-Rabbit-IgG-H-L-Secondary-Antibody-Polyclonal-32460) was added to the blots for 1 h. The blots were then washed again 3 times for 5 min each and examined by enhanced chemiluminescence (ECL) (Cat# RPN2232 GE Healthcare Life Sciences, http://www.gelifesciences.com/webapp/wcs/stores/servlet/product/ById/en/GELifeSciences-us/28980926).

TbPCNA Pull Down with GST-PIP-box Fusion Constructs—The 3 GST-PIP-box fusion proteins: TbERK8 PIP-box (GSTtbE8-PIP), TbAUK1 PIP-box (GSTtbAUK1-PIP), and TbPFC19 PIP-box (GSTtbPFC-PIP) were overexpressed in E. coli and captured on glutathione agarose beads (Cat# G-250-10) (Gold Bio Inc., https://www.goldbio.com/product/uc1252/glutathione-agarose-resin) following the protocol suggested by the manufacturer. Briefly, the pellets from 250 ml cultures were lysed using 10 mL of buffer 4 (50 mM Tris, 1% DOC, 0.3 M urea, pH 8, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCL) as described by Danilevich et al.40 and clarified by centrifugation (4 °C at 15,000 rpm) using a Beckman JA 25.5 rotor. GST beads (200 μl) were added to the clarified lysate and incubated overnight at 4 °C rotating to saturate the beads. The saturated beads were washed 5 times with PBS. The washed GST agrose beads were incubated with lysates from T. brucei that overexpressed wt-TbPCNA_{6H} for 2 hours at 4 °C. Beads were washed 5 times with PBS, boiled in 5x SDS loading buffer, and resolved by polyacrylamide gel electrophoresis. The loading controls were visualized by Coomasie stain or by StainFree®, (http://www.bio-rad.com/en-us/product/mini-protein-precant-gels/mini-protein-tgx-stain-free-precant-gels), which contains a trihalo compound that produces a fluorescent product when covalently crosslinked to protein tryptophan residues. These gels reduced the need to stain gels and allowed bands to be visualized with a BioRad ChemDoc MP. TbPCNA bands were detected by immunoblots using anti-HA (Cat# MAB3834, Thermo Scientific, http://www.emd millipore.com/US/en/product/Anti-Polyhistidine-Tag-Anti
body, MM_NF-MAB3834) or anti-His antibodies (Cat# H6908, Sigma-Aldrich, http://www.sigmaaldrich.com/catalog/product/sigma/h6908?lang=en&region=US). Standard curves for proteins quantified with StainFree gels and ChemDoc MP were made from 2-fold serial dilutions of TbPCNA.

**Immunoprecipitation of HA-tagged proteins**

Stable *T. brucei* transfectants that expressed either HA-tagged TbERK8 or TbPCNA were induced with tetracycline to a final concentration of 1 μg/ml for 24 hours. Extracts from induced parasites were pelleted by centrifugation and lysed with 1 ml of immunoprecipitation (IP) lysis buffer on ice for 30 min (1% Triton X-100, 10 mM Tris pH 7.9, 25 mM KCl, 150 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, and 1x complete protease inhibitor mix (Cat# 1187358001, Roche, http://www.sigmaaldrich.com/catalog/product/roche/COEDTA_FRO?lang=en&region=US)). Lysates were incubated with 30 μl anti-HA immuno-affinity beads from (Cat# 26181, Thermo Scientific, https://www.thermofisher.com/order/catalog/product/26181) and rotated overnight at 4 °C. The beads were washed 5 times with 1 ml of IP lysis buffer.

**TbPCNA₆H pull down with full length TbERK8_AHA**

wt-TbERK8_AHA prepared from *T. brucei* lysates was adsorbed onto 30 μl of immuno-affinity beads and washed 5 times in 1 ml of immunoprecipitation lysis buffer. Ten microliters of the anti-HA immunoaffinity beads with wt-TbERK8_AHA were incubated with a final concentration of 15 ng/μl of TbPCNA₆H in 300 μl reactions with rotating at 4 °C for 2 hours. Beads were washed 5 times with 1 ml of PBS, resolved by SDS-PAGE, followed by anti-His tag immunoblotting for detection of bound TbPCNA₆H.

**Autophosphorylation and kinase activity analysis with in vivo expressed TbERK8**

Ten microliters of the HA-immuno-affinity beads with either wt-TbERK8_AHA or the K42A-TbERK8_AHA mutant bound were tested for autophosphorylation as described above. To test the activity of the *in vivo* expressed proteins, we preincubated 10 microliters of the immuno-affinity beads with 10 μM ATP, 10 μCi of ³²P-γ-ATP in 30 μl of buffer K. Subsequently, 100 μmol of either TbPCNA₆H or the generic kinase substrate myelin basic protein (MBP) (Cat# 13-110, Millipore, https://www.emdmillipore.com/US/en/product/MBP,-Dephosphorylated,MM_NF-13-110) were added, and the reactions incubated at 30 °C for 30 min. Reactions were stopped by adding 5x SDS-PAGE loading buffer and boiling for 1 minute. Ten microliters of the kinase IP reaction were resolved by SDS-PAGE and examined by autoradiography. We used a mock reaction (no substrate) for each set of experiments as well as affinity beads incubated in lysates from the non-transfected *T. brucei* 221 wild type strains as negative controls to detect background labeling.

**Peptide sequencing by mass spectrometry**

Peptide sequencing and protein identification was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). For protein identification, we excised and prepared bands from SDS-PAGE gels and subjected them to in-gel trypsin digestion for peptide identification.41

Sequencing was performed using either an LTQ-Orbitrap XL or an LTQ-Orbitrap Velos mass spectrometers (Thermo Scientific, Rockford, IL), each equipped with a 10,000 psi system nanoACUCITY (Waters, City State) UPLC instrument and EZ-Spray source (Thermo Scientific). Reversed phase liquid chromatography was performed using an EZ-Spray C18 column (Thermo, ES800, PepMap, 3 μm bead size, 75 μm × 15 cm). The LC was operated at 600 nL/min flow rate for loading and 300 nL/min for peptide separation over a linear gradient for 60 min from 2% to 30% acetonitrile in 0.1% formic acid. Tandem mass spectrometry experiments using the LTQ OrbitrapXL and the LTQ Orbitrap Velos instruments both used survey scans recorded over a 350-1500 m/z range. The LTQ Orbitrap XL was operated in collision induced dissociation (CID) mode, and MS/MS CID scans were performed on the 6 most intense precursor ions, with the following parameters: a minimum of 1,000 counts precursor, isolation width 2.0 amu, and 35% normalized collision energy. The LTQ Orbitrap Velos was operated in higher energy collision dissociation (HCD) mode, and MS/MS HCD scans were performed on the 6 most intense precursor ions with the following parameters: 3,000 count threshold, isolation width 2.0 amu, and 30% normalized collision energy. Internal recalibration to a polydimethylcyclosiloxane (PCM) ion with m/z = 445.120025 was used for both MS and MS/MS scans on both instruments.22

Mass spectrometry centroid peak lists were generated using in-house software called PAVA, and data were searched using Protein Prospector software v. 5.10.17.42 For protein identification, searches were performed against the curated *T. brucei* 972 genome in the TriTrypDB database v 8.0 (http://tritrypdb.org/tritrypdb/, June, 2014) containing 30,295 entries. This database was concatenated with a fully randomized set of 30,295 entries for estimation of false discovery rate.43 Data were also searched against the SwissProt database (downloaded January 11, 2012) for identification of common contaminant proteins. Peptide sequences were matched as tryptic peptides with up to 2 missed cleavages, and carbamidemethylated cysteines as a fixed modification. Variable modifications included: oxidation of methionine, N-terminal pyroglutamate from glutamine, start methionine processing, protein N-terminal acetylation, and S/T phosphorylation. Mass accuracy tolerance was set to 20 ppm for parent and 0.6 Da fragment for CID experiments, and 20 ppm for parent and 30 ppm for fragment mass error for HCD experiments.

For reporting of protein identifications from this database search, score thresholds were selected that resulted in a protein false discovery rate of less than 1%. The specific Protein Prospector parameters were: minimum protein score of 22, minimum peptide score of 15, and maximum expectation values of 0.02 for protein and 0.05 for peptide matches. Site Localization In Peptide (SLIP) scoring44 was used to score the site assignment for phosphorylation sites, with manual validation of all reported phosphorylation spectra.
Inhibitors

SCYX5070 was a kind gift from William Janzen and Melissa A. Porter, The University of North Carolina at Chapel Hill. Ro318220 (Cat# SC-200619) was purchased from Santa Cruz Biotechnology (http://www.scbt.com/datasheet-200619-ro-31-8220.html). Each inhibitor was pre-incubated with 100 ng of GST-TbERK8 or GST-HsERK8 to a final concentration of 1 μM at 30 °C for 30 minutes before performing a kinase assay using 32P-γ-ATP. The kinase reaction was stopped by boiling in 5x SDS loading buffer then resolved by SDS-PAGE. Standard curves for autoradiography quantitation by PhosphorImager were made from 2-fold serial dilutions of 10 μl Ci of 32P-γ-ATP in 100 μM cold ATP.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to thank Zhijian Jake Tu1 in the Virginia Tech Department of Biochemistry for technical expertise with phylogenetic analyses. Janet Webster and Ling Chen in the Fralin Life Science Institute for critical reading.

Funding

This work was supported in part by Start-up fund NIFA139696, VT Drug Discovery Center VTCDD #119286. MS analysis was performed in the Bio-Organic Biomedical Mass Spectrometry Resource at UCSF (A.L. Burlingame, Director) supported by GM103481.

Notes on contributors

AVL and ZBM performed and analyzed the experiments shown in Figures 1–8 except for mass spectrometry. GMK performed and analyzed mass spectrometry experiments shown in the manuscript. All authors reviewed the results and approved the final version of the manuscript. ZBM conceived and coordinated the study, wrote the paper, and analyzed the experiments.

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