Supplementary Experimental Procedures

Protein Expression and Purification

The N-terminal 19 amino acid residues of \textit{Tb}RET2, predicted to be a mitochondrial importing signal, were omitted from our constructs. The crystallized full-length \textit{T. brucei} RET2 triple mutant, 94R/122R/207R, construct consists of residues 20–487 with a TEV-cleavable N-terminal 6xHis tag in pPROEX HTa. Protein was expressed in \textit{E. coli} cell strain BL21goldDE3. The cells were grown at 37°C until the \(A_{600}\) reached 0.8, and then cooled to 22°C. The cells were then induced with 1 mM IPTG and incubated for 12 hours at 22°C. SeMet-substituted protein was expressed in M9 minimal medium supplemented with amino acids as described by Van Duyne \textit{et al.} (Duyne et al., 1993). The cells were harvested by centrifugation and resuspended in buffer A (20 mM Tris, 500 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, pH 8.0). Cells were lysed by three cycles in a French press and insoluble material was removed by centrifugation. Subsequent purification steps were carried out at 4°C. The supernatant was passed through a Ni-NTA column, washed with buffer A, and eluted with buffer A containing 250 mM imidazole. TEV protease was added immediately into the collected fractions after Ni-NTA purification and the mixture was incubated at 4 °C overnight. The buffer of the TEV-treated \textit{Tb}RET2 protein mixture was then exchanged with 20 mM Tris, 500 mM NaCl, 10% glycerol, pH 7.8, using a PD-10 desalting column. The protein solution was subsequently passed through a second Ni-NTA column to remove the cleaved His-tag, His-tagged rTEV protease and any uncleaved \textit{Tb}RET2. The flow-through was concentrated to 5 mg/ml using a Centricon spin-column and applied onto a Superdex-75 column for a size-exclusion chromatography step. The protein appeared to
be monomeric with an apparent mass of 55 kDa. After concentrating the peak fraction to 5 mg/ml, 2 mM Na-UTP and 10 mM Mg$^{2+}$ were added, and the solution flash-frozen in liquid nitrogen in 50 µl aliquots and preserved at −80°C (Deng et al., 2004a).

**Trypanosome cell lines and culture**

Bloodstream form trypanosomes (427 strain) were cultivated in HMI-9 medium containing 10% fetal bovine serum at 37°C/5% CO$_2$. The *Tb*RET2 conditional knockout cell lines were generated by the stepwise replacement of the one of the *Tb*RET2 alleles with a cassette encoding T7 RNA polymerase and neomycin resistance genes, followed by introduction of a tetracycline-regulated ectopic *Tb*RET2 allele into the ribosomal DNA spacer region, and then replacement of the second *Tb*RET2 allele with the tetracycline repressor and hygromycin resistance genes in the presence of 1 µg/ml tetracycline as described previously (Schnaufer et al., 2001). The construct for targeted replacement of the first *Tb*RET2 allele, *Tb*RET2KO1, was generated by replacement of the NotI-MluI and Xba-StuI fragments of pLew13 (Wirtz et al., 1999) with the upstream and downstream flanking regions of *Tb*RET2, respectively. The upstream *Tb*RET2 flanking region was PCR amplified from *T. brucei* 427 genomic DNA with 5’ ATAGCGGCCGCCATCACCCTTTTAGTCGG 3’ and 5’ ATACGCGTCTCGAGCCAATCAACAGCTGTG 3’ and downstream flanking region with 5’ GTATCTAGATTAAATGTGAATAGGGAGCATGGG 3’ and 5’ ATAAGGCCGTGCGGCCGCCATTTACCATGTGATG 3’, with the restriction sites in italics. The downstream flanking region contained an XhoI site that was removed prior to cloning into pLew13 by linearizing at this site, repairing the gap with T4 DNA
polymerase and religating the vector. The construct for replacement of the second
*Tb*RET2 allele, *Tb*RET2KO2, was made by digesting *Tb*RET2KO1 with XhoI and Swal
and isolating the vector backbone containing the *Tb*RET2 targeting regions and inserting
the XhoI-StuI fragment from pLew90 (Wirtz et al., 1999). The full length *Tb*RET2 ORF
was amplified with 5’ GCAAGCTTATGGATGCATACCGCGCC 3’ and 5’
GCAGATCTCATTACGCGTGTCGTCGACGC 3’, cut with HindIII and BglII, and used
to replace the luciferase gene in pLew79 (Wirtz et al., 1999) cut with HindIII and BamHI
to yield the tetracycline-regulated *Tb*RET2 construct, reg*Tb*RET2.

Bloodstream form *T. brucei* were transfected sequentially with 10 µg of NotI-
linearized *Tb*RET2KO1, reg*Tb*RET2 and *Tb*RET2KO2 as previously described
(Schnaufer et al., 2001). The resultant cell clones were selected by resistance to 2.5
µg/ml G418, 5 µg/ml hygromycin and 2.5 µg/ml phleomycin, respectively. Tetracycline
(1 µg/ml) was added to the cells prior to knockout of the second endogenous *Tb*RET
allele with *Tb*RET2KO2 to ensure the expression of *Tb*RET2 during selection. The
genotype of the knockout cell lines was confirmed by both PCR and Southern analysis.

Expression of *Tb*RET2 was inactivated by washing and resuspending the cells in
medium without tetracycline. Growth of the cells was monitored in parallel with a
duplicate culture of cells to which tetracycline was added. The cell densities were kept
between 0.25 X 10^6 and 2 X 10^6 throughout all experiments. The cells were counted
using a Coulter Counter and the cumulative cell number was calculated by multiplying
the cell number by the dilution factor.

**TUTase activity assays**
Purified recombinant TbRET2 proteins were assayed for nucleotide addition to 5’ labeled-5’CL18 alone or in combination with 3’CL13pp and gPCA6-2A to form a trimeric, precleaved insertion editing substrate as previously described (Ernst et al., 2003). Each reaction was performed in buffer containing 25 mM HEPES, pH 7.9, 10 mM magnesium acetate, 5 mM calcium chloride, 0.5 mM dithiothreitol and 1 mM EDTA with 25 mg of protein and 100 µM of each nucleotide and was incubated for 3 hours at 27°C. Reaction products were phenol-chloroform extracted, ethanol precipitated and run on 11% polyacrylamide-7M urea gels and visualized by phosphorimaging (Storm PhosphorImager, Molecular Dynamics).