IDENTIFICATION OF A PSEUDOKINASE REGULATOR OF TRANSFERRIN ENDOCYTOSIS IN THE AFRICAN TRYPANOSOME USING THE SMALL-MOLECULE AEE788

by

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(Under the Direction of Kojo Mensa-Wilmot)

ABSTRACT

The protozoan parasite *Trypanosoma brucei* causes the disease Human African Trypanosomiasis (HAT) and the cattle wasting disease, Nagana. To avoid difficulties associated with protein alignments, we used small molecules that perturb trypanosome biology and then identified proteins within the affected biological pathways. The kinase inhibitor AEE788 selectively blocks transferrin endocytosis without affecting uptake of bovine serum albumin. We have used hypothesis-generating phospho-proteomics to identify the protein Tb427tmp.160.4770 as an AEE788-pathway protein. Using RNAi, we establish Tb427tmp.160.4770 as a selective regulator of transferrin endocytosis. We used comparative phospho-proteomics after knockdown of Tb427tmp.160.4770 to identify Tb427tmp.160.4770 pathway proteins, five of which were also identified as AEE788 pathway proteins. We hypothesize that the AEE788 and Tb427tmp.160.4770 pathway proteins are selective modulators of transferrin uptake in the African trypanosome. These studies highlight the power of small molecules as tools for identifying new and regulatory genes involved in transferrin endocytosis pathway in *T. brucei*.

INDEX WORDS: *Trypanosoma brucei*, endocytosis, pseudokinase, small molecule, chemical biology, clathrin mediated endocytosis, trypanosomiasis, transferrin, AEE788

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Trypanosoma brucei

Trypanosoma brucei is a protozoan parasite endemic to regions of rural sub-Saharan Africa (reviewed in Brun, 2009, Malvy, 2011). This parasite is responsible for human disease (human African trypanosomiasis), as well as veterinary disease (Nagana in cattle). *T. brucei* poses significant health and economic burdens to the region; approximately 70 million people are at risk of trypanosomiasis and thousands of people are infected every year, though that number is highly subject to social and political changes throughout the region.

T. brucei has a biphasic life-cycle; the parasite resides in vertebrates and also in the tsetse fly. In vertebrates, the early stage of infection is characterized by infection of blood and lymph fluids. Symptoms of this stage are non-specific and include fever and general malaise (Vincedeau, 2005). Progression to the late stage of disease involves invasion of the central nervous system and crossing of the blood-brain barrier. Late stage symptoms are serious, and can include changes in behaviour, disruption of the circadian rhythm, and coma (Vincedeau, 2005). If left untreated, trypanosomiasis is almost certainly fatal (Steverding, 2008).

The bi-phasic lifecycle of *T. brucei* necessitates metabolic and morphological changes of the parasite in response to nutrient availability and host immune response (Morgan, 2001, Urbaniak, 2012). The metabolic processes of the two life cycle stages are distinct. Insect stage trypanosomes have highly active mitochondria and rely heavily on oxidative respiration for energy production. In the vertebrate stage, however, the primary

energy generation process is glycolysis. This requirement means that the bloodstream form trypanosome needs an effective system to take in host nutrients.

2. Endocytosis and Vesicle Trafficking

2.1 Clathrin-Mediated Endocytosis and Vesicle Trafficking

Endocytosis is a membrane-mediated process through which extracellular materials, like nutrients and signalling molecules, are brought into the cell (reviewed in Doherty, 2009). In *Trypanosoma brucei*, endocytosis is an important factor in determining the virulence of the parasite (Subramanya, Hardin, Steverding, & Mensa-Wilmot, 2009), as it is the means by which the parasite acquires growth factors from host serum (Schell, 1991) and is the primary mechanism of immune evasion, including clearance of trypanolytic factors from the cell surface (Blaber, 1979).

Endocytosis and vesicle trafficking can be broadly characterized in three steps: vesicle formation, endosome sorting, and recycling of endosomes (reviewed in Doherty, 2009). Humans have a well-defined endocytic system; however, *Trypanosoma brucei* has a diverged genome lacking key components of "canonical" endocytic machinery (reviewed in Morgan, 2002*a-b*).

The first step in endocytosis, vesicle formation, is most often mediated through the dynamics and interactions of the coat-protein clathrin (reviewed in Bonifacino & Lippincott-Schwartz, 2003, and (McMahon & Boucrot, 2011)). Clathrin is a hexamer of three clathrin heavy chain proteins and three light chain proteins (Kirchhausen, 2000), and is well conserved across Eukarya (Kirchhausen, 2000). The six subunits of clathrin combine to form a single clathrin "triskelion" unit; this structure polymerizes around sites of endocytosis with the aid of adaptor proteins (Bonifacino & Lippincott-Schwartz, 2003). The most common adaptor protein for clathrin-mediated endocytosis in vertebrates is adaptor protein-2 (AP-2) (Bonifacino & Lippincott-Schwartz, 2003). The adaptor protein binds to

the cytoplasmic domains of endocytic receptors and recruits clathrin to these sites of endocytosis (Ohno, 1995). Clathrin recruitment results in the polymerization of clathrin triskelia around the endocytic site which contributes to membrane curvature and indentation; these clathrin-associated membrane indentations are known as clathrin-coated pits (CCPs) (Bonifacino & Lippincott-Schwartz, 2003). Further clathrin polymerization and membrane curvature results in an invagination of the plasma membrane that continues until clathrin surrounds the entire membranous structure, the clathrin-coated vesicle (CCV) stage.

In *T. brucei*, clathrin is essential for both endocytosis and viability (Allen, Goulding, & Field, 2003). Knockdown of clathrin in bloodstream-form trypanosomes leads to a change in cell morphology known as the "Big Eye" phenotype and eventually trypanosome death. The "Big Eye" phenotype is characterized as a grossly enlarged flagellar pocket or endosomal structure at the posterior of the trypanosome and can also result in cell rounding (Allen, 2003). Interestingly, the *T. brucei* genome does not include a gene for the clathrin adaptor-protein AP-2 (Morgan, 2002), but the genomes of the closely related species *T. cruzi* and *Leishmania major* encode genes for AP-2 (Kalb, 2016; Denny, 2004). This raises the question of how clathrin is recruited to sites of endocytosis in *T. brucei* in the absence of AP-2.

Vesicle formation is completed by pinching of vesicles from the plasma membrane. In most systems, the GTPase dynamin contributes to this step (reviewed in (Ferguson & De Camilli, 2012)). Dynamin is the founding member of the dynamin-like protein (DLP) family of GTPases, a family responsible for membrane remodelling (Ferguson & De Camilli, 2012). Dynamin polymerizes in a helical shape around the necks of budding CCVs, and a conformational change in the dynamin multimer separates the endocytic vesicle from the cell surface (Chappie, Acharya, Leonard, Schmid, & Dyda, 2010). When

dynamin is knocked down in vertebrates, vesicle formation is unable to be completed and leads to a build-up of tubular clathrin-coated structures at the cell surface (Ferguson et al., 2009; Marks et al., 2001). However, in *T. brucei*, knockdown of the dynamin homologue has no effect on clathrin-mediated endocytosis (Morgan, Goulding, & Field, 2004), indicating another point of divergence in the mechanism of clathrin-mediated endocytosis in *T. brucei*, as compared to humans.

As endocytosis is used to bring in wide varieties of extracellular materials, sorting mechanisms are needed to ensure that the endocytosed materials are delivered to the correct areas of the cell. In vertebrates, sorting begins at the cell surface upon ligand binding to a receptor (Leonard, 2008). Ligand binding promotes clustering of receptors and is thought to promote recruitment of clathrin-mediated endocytosis (CME) machinery to these sites. Sorting also occurs at the early endosome stage (Lakadamyali, 2006). Rab proteins are a class of small GTPases and are essential for proper endosome trafficking and sorting (Field, 1998). In humans and trypanosomes, Rab proteins localize to different subsets of membranous organelles, forming a network of targeting molecules to influence directionality of endosome traffic (Ackers, Dhir, & Field, 2005; Mayle, Le, & Kamei, 2012). These Rabs are responsible for coordinating vesicle fusion proteins, like SNARE's, and their nucleotide binding state (GTP/GDP) determines the ability of the associated vesicle to be trafficked along microtubules by motor proteins, or to fuse with an acceptor membrane (Zerial & McBride, 2001). Rab5 regulates the fusion of early endosomes with the primary endosomes that have just bud off of the plasma membrane (Lakadamyali, 2006; Rink, 2005). In humans, early endosomes can be classified in two ways: dynamic and static. Dynamic early endosomes mature rapidly to late endosomes and are both Rab5 and Rab7 (a marker for the late endosome) positive. Static early endosomes make up the majority of early endosomes and mature slower (remain only Rab5 positive for

longer). Rab4 is associated with recycling endosomes and appears to regulate some dynamics of fast receptor recycling (Sluijs, 1991). Early endosomes in both the dynamic and static pools eventually mature to late endosomes, marked by Rab7 (Rink, 2005). The late endosome is the stage in which some ligands are released from their receptors, as is the case with low density lipoprotein (LDL) (Lakadamyali, 2006).

Endosome and receptor recycling is a key step in the overall membrane homeostasis and regulation of endocytosis (Wang, 2018). In vertebrates, endosome recycling occurs through two pools: fast and slow recycling (Ghosh, 1994). The fast recycling system shuttles endocytosed materials directly from early endosomes back to the cell surface. Slow recycling cargo is trafficked from early endosomes to the endocytic recycling compartment (ERC) before being sent back to the cell surface. Both static and dynamic early endosomes can utilize either recycling pathway. Rab4 and Rab11 are important for endosome recycling in trypanosomes (Hall, 2005; Umaer, 2018).

2.2 Transferrin Endocytosis

Transferrin is a protein in vertebrates that binds to free iron in the blood (Crichton, 1987). Transferrin acts as a growth factor to *Trypanosoma brucei* and is the major source of iron for the parasite (Schell, 1991). Without transferrin, trypanosomes cannot proliferate (Schell, 1991). Transferrin is brought into the trypanosome by clathrin-mediated endocytosis (Allen et al., 2003). The trypanosome transferrin receptor (TbTfR) is a heterodimer of ESAG6 and ESAG7 (Steverding, 1995). The TbTfR is anchored to the membrane with a single glycophosphatidylinositol (GPI) anchor on the C-terminus of ESAG6 (Steverding, 1995). In vertebrates, transferrin is also endocytosed by a receptor through clathrin-mediated endocytosis (reviewed in Mayle et al., 2012). Vertebrate TfR has the advantage of being a transmembrane protein and has a cytoplasmic domain that can be used in adaptor protein recruitment and sorting (Rothenberger, 1987). The

trypanosome TfR, with its single GPI-anchor, does not share this feature. Although a means through which the trypanosome can sort GPI-anchored endocytic cargo is not known as of yet, different recycling kinetics and endosomal localization of variant surface glycoprotein (VSG) and TbTfR indicates that a pathway exists and has yet to be discovered (Overath & Engstler, 2004).

The transferrin receptor in vertebrates binds transferrin, and the receptor: ligand complex is brought into the cell by clathrin-mediated endocytosis (Mayle, 2011). Once internalized, the transferrin cargo non-preferentially enters into either static or dynamic early endosomes (Lakadamyali, 2006). In the late endosome, transferrin remains bound to the TfR, but releases its bound iron. The TfR: apo-Tf (transferrin with no iron bound) complex is then recycled to the plasma membrane in either fast or slow recycling pathways (Mayle et al., 2012). The process in trypanosomes is not as well defined (reviewed in Reyes-López, 2015). The TbTfR: Tf complex is trafficked to early endosomes, but it is not known if there are different pools of early endosomes, like in vertebrates. The transferrin complex is then shuttled to late endosomes. At the late endosome stage, transferrin is released from TbTfR and is trafficked to lysosomes for degradation (Grab, 1992). The empty TbTfR is trafficked to recycling endosomes before being sent back to the flagellar pocket (Kabiri & Steverding, 2000).

In vertebrates, Rab11 is necessary to recycle the TfR (a transmembrane protein) back to the plasma membrane in vertebrates (Eggers, 2009). In trypanosomes, Rab11 was initially found to colocalize with recycling receptors, including the TbTfR, and was therefore believed to be responsible for directing all receptor recycling (Jefferies, 2001). However, later studies found that loss of Rab11 had no effect on the recycling of GPI-anchored receptors, like the TbTfR and VSG (Umaer, 2018). Knockdown of Rab11 did, however, affect the recycling of transmembrane proteins like ISG65 (invariant surface

glycoprotein 65) (Umaer, 2018), indicating two possible pathways that control receptor recycling, one that is specific for GPI-anchored receptors, and one that is specific for transmembrane receptors.

3. Phospho-signalling

Protein phosphorylation is a well-studied post-translational modification of proteins and is one of the most important signalling methods a cell has in its tool kit (Reiterer, Eyers, & Farhan, 2014). The catalysis of transferring a phosphate group onto a substrate is performed by kinases; the reverse reaction, removing a phosphate group, is performed by phosphatases (Reiterer et al., 2014). Kinases and phosphatases work in tandem to control the delicate homeostasis of the phospho-proteome of the cell and respond to external and internal stimuli (Eyers & Murphy, 2013). In the human genome there are about 500 genes encoding kinases, just under 2% of the human genome (Eyers & Murphy, 2013). Kinases are known regulators of almost every biological process in the cell, including cell cycle, migration, morphology, endocytosis, and metabolism (Reiterer et al., 2014).

Structurally, kinase domains tend to be very similar (Eyers & Murphy, 2013). The kinase domain is generally made of an N-lobe and a C-lobe. Where these two lobes meet is the active site of the enzyme- both ATP and the peptide substrate bind in this pocket. Two well-conserved domains within the C-lobe are the A-loop and C-loop (Taylor, Zhang, Steichen, Keshwani, & Kornev, 2013). The A-loop, or activation-loop, is a key regulatory domain of kinases and its conformation- “in” or “out”- often determines if a kinase is “on” (active) or not. The C-loop, catalytic-loop, is responsible for the catalytic activity of the kinase and contains the active site motif- HRD- which is responsible for the transfer of the ɣ-phosphate of ATP to the substrate. Numerous amino acid residues in both the N-lobe and C-lobe bind and stabilize ATP and the substrate in the active site of the kinase.

Pseudokinases are classified as proteins with kinase-like domains but are missing one or more canonical kinase characteristics- like ATP binding, the HRD active site responsible for transferrin the gamma-phosphate of ATP to its substrate (often replaced with an HRN motif), or activation loop (A-loop; sometimes due to missing DFG or APE motifs which flank the loop and influence its confirmation). The phosphotransfer activity of pseudokinases varies; some pseudokinases cannot carry out this reaction at all, while others still can, but at rates much lower than those of canonical active kinases (Reiterer et al., 2014). The roles of pseudokinases in signalling pathways involve modulating signalling through promoting or blocking protein-protein interactions, moderating the localization of binding partners, or through binding competition with signalling proteins like active kinases or phosphatases to prevent modification to the substrate peptide's phosphorylation state (Reiterer et al., 2014).

In trypanosomes, phosphorylation pathways are different than those in humans. Despite having known tyrosine phosphorylation (Nett, Davidson, Lamont, & Ferguson, 2009; Nett, Martin, et al., 2009), trypanosomes have no known genes encoding receptor or non-receptor tyrosine kinases (RTK and NRTK, respectively) (Parsons, Worthey, Ward, & Mottram, 2005); in humans, RTKs are important signalling molecules, and control many key pathways in the cell, like cell proliferation with the epidermal growth factor receptor (EGFR) (Oda, Matsuoka, Funahashi, & Kitano, 2005). Trypanosomes also lack genes encoding SH2 domains which are important in substrate interactions with tyrosine kinases (Lim & Pawson, 2010). It is thought that trypanosomes are able to carry out tyrosine phosphorylation through dual-specificity kinases that are able to phosphorylate both serine and threonine as well as tyrosine residues (Lochhead et al., 2003; Parsons et al., 2005). Despite these absences, small-molecule tyrosine kinase inhibitors are still able to perturb the trypanosome phospho-proteome, disrupt cellular processes, and kill the

trypanosome (Behera, Thomas, & Mensa-Wilmot, 2014; Subramanya & Mensa-Wilmot, 2010; Wheeler-Alm & Shapiro, 1992), offering an interesting tool to study interesting trypanosome biology and for potential drug development to treat trypanosomiasis.

4. Small Molecules

Organisms that have divergent genomes from well-studied model organisms, like *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and humans, are difficult to study using methods like genome sequence alignment. These difficulties can be avoided by using small molecules to perturb biology, and insights into divergent biological processes can be made more accessible (Mayer, 2003). Trypanosomes are an excellent candidate for this type of work. Tyrosine phosphorylation is an important process in trypanosomes (Subramanya & Mensa-Wilmot, 2010), but trypanosomes lack key aspects of human tyrosine kinase phospho-signalling (Katiyar et al., 2013; Parsons et al., 2005). Previous work with the pan-tyrosine kinase inhibitor Tyrphostin A47 identified the diacylglycerol (DAG) stimulated transferrin endocytic pathway as tyrosine kinase regulated (Subramanya & Mensa-Wilmot, 2010). In humans, DAG signalling, and regulation of DAG stimulated endocytosis, is largely carried out by serine/threonine (ser/thr) kinases in the PKC family (Huang, 1989). In trypanosomes, inhibition of ser/thr kinases was not able to block transferrin endocytosis after DAG stimulation, indicating that the DAG-stimulated transferrin- endocytosis pathway is ser/thr kinase independent (Subramanya & Mensa-Wilmot, 2010). Further work identified the human receptor tyrosine kinase (RTK) inhibitor Lapatinib (Novartis) as a selective inhibitor of transferrin endocytosis, despite the trypanosome's lack of RTKs (Guyett, Behera, Ogata, Pollastri, & Mensa-Wilmot, 2017). Affinity chromatography with Lapatinib identified multiple trypanosome proteins, including Glycogen Synthase Kinase 3β, GSK3β (Katiyar et al., 2013). Further genetic work verified

GSK3β as a selective regulator of transferrin endocytosis in the African trypanosome (Guyett, Xia, Swinney, Pollastri, & Mensa-Wilmot, 2016).

This work continues the strategy of using small molecules as tools for biological discovery. Here we will describe the small molecule AEE788, a selective inhibitor of transferrin endocytosis (Sullenberger, Pique, Ogata, & Mensa-Wilmot, 2017) in *T. brucei* and identify Tb427.tmp.160.4770 as a protein within AEE788 affected pathways, and a selective regulator of transferrin endocytosis in the trypanosome- making Tb427.tmp.160.4770 the first pseudokinase to be assigned a biological role in the African trypanosome.

CHAPTER 2

IDENTIFICATION OF A PSEUDOKINASE REGULATOR OF TRANSFERRIN ENDOCYTOSIS IN THE AFRICAN TRYPANOSOME USING THE SMALL-MOLECULE AEE788[1]

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Abstract

The protozoan parasite *Trypanosoma brucei* causes the disease Human African Trypanosomiasis (HAT) and the cattle wasting disease, Nagana. *T. brucei* has a highly divergent genome compared to humans and yeasts, making it difficult to study the biological pathways of the parasite based on identification of proteins by alignment. To avoid these difficulties, we used small molecules that perturb trypanosome biology and then identified proteins within the affected biological pathways. The kinase inhibitor AEE788 (Novartis) selectively blocks transferrin endocytosis. We have used hypothesis-generating phospho-proteomics to identify the protein Tb427tmp.160.4770 as an AEE788-pathway protein. Using RNAi, we established Tb427tmp.160.4770 as a selective regulator of transferrin endocytosis, similar to AEE788. Intracellularly, the protein was detected in cytoplasmic regions of the trypanosome, including physiologically relevant areas for endocytosis, proximal to the kinetoplast and flagellar pocket and vesicles containing endocytosed cargo. Finally, we used comparative phospho-proteomics after knockdown of Tb427tmp.160.4770 to identify Tb427tmp.160.4770 pathway proteins, five of which were also identified as AEE788 pathway proteins. We hypothesize that the AEE788 and Tb427tmp.160.4770 pathway proteins are selective modulators of transferrin uptake in the African trypanosome. These studies highlight the power of small molecules as tools for identifying new and regulatory genes involved in transferrin endocytosis pathway in *T. brucei*. Additionally, Tb427tmp.160.4770 is also the first pseudokinase to be assigned a biological function in a trypanosome.

Introduction

Trypanosoma brucei is a protozoan parasite endemic to regions of rural Sub-Saharan Africa (Brun, 2010). This parasite is responsible for human African trypanosomiasis (HAT), and the cattle wasting disease, nagana. The majority of the

genome of *T. brucei* is annotated as “hypothetical” for proteins of unknown function (TriTrypDB). Trypanosomes offer an interesting system to study cell biology and have contributed to major findings in eukaryotic biology, like the discovery of GPI-anchors (Ferguson, Low et al. 1985), trans-RNA splicing (reviewed in (Liang, Haritan, Uliel, & Michaeli, 2003)), RNA editing (reviewed in (Koslowsky, 2004)), and glycosomes (Opperdoes & Borst, 1977). However, popular methods to study trypanosome biology like protein sequence alignment, have been unreliable due to divergence of the *T. brucei* genome sequence compared to humans.

Transferrin endocytosis is an essential pathway for *Trypanosoma brucei* (Schell, Borowy, & Overath, 1991). Transferrin is an iron chelator in vertebrate blood and is an essential growth factor for *T. brucei* (Schell, 1991). In humans, the pathway of transferrin endocytosis is well understood (excellent review in (Mayle et al., 2012). However, *T. brucei* lacks key components of the vertebrate transferrin endocytic pathway (reviewed in (G. W. Morgan, Hall, B., Denny, P., Field, M., Carrington, M., 2002)). In humans, transferrin is endocytosed through clathrin-mediated endocytosis on a transmembrane receptor, the transferrin receptor (TfR) (Schneider, Owen, Banville, Williams, 1984; McClelland, Kühn, Ruddle, 1984). The human TfR interacts with adaptor protein-2 (AP-2) to recruit clathrin to sites of endocytosis. AP-2 binds the cytoplasmic domain of TfR (Jing, Spencer, Miller, Hopkins, & Trowbridge, 1990) and interacts with PIP_2 (phosphatidylinositol 4,5-bisphosphate) molecules in the plasma membrane (Gaidarov & Keen, 1999; Rohde, Wenzel, & Haucke, 2002), and facilitates the assembly of a clathrin lattice on the plasma membrane (Shih, Gallusser, & Kirchhausen, 1995). Clathrin-coated vesicles (CCVs) are “pinched” from the plasma membrane in a process involving the GTPase dynamin (Marks

et al., 2001). The internalized vesicles move through the endomembrane system of the cell.

The *T. brucei* system for transferrin endocytosis is different. First, the transferrin receptor is not a transmembrane protein; it is a heterodimer of the gene products ESAG6 and ESAG7 and is anchored to the outer leaflet of the plasma membrane with a a single glycophosphatidylinositol (GPI) anchor on ESAG6 (Salmon, 1994; Steverding, 1995). Therefore, there is no cytoplasmic domain to facilitate recruitment of endocytic machinery to the receptor. *T. brucei* also lacks the clathrin adaptor protein AP-2 (G. W. Morgan, Hall, B., Denny, P., Carrington, M., Field, M., 2002). In humans, AP-2 is essential for endocytosis of transferrin (Boucrot, Saffarian, Zhang, & Kirchhausen, 2010). Another point of divergence can be found in fission of the CCV from the plasma membrane. Dynamin is essential for this step in human systems, but knockdown of a dynamin homologue in *T. brucei* has no effect on transferrin endocytosis (Morgan et al., 2004). These differences highlight a need for new approaches for studying the trypanosome endocytosis system, because alignment searches of commonly studied systems, like humans and yeast, are unlikely to identify homologs of vertebrate proteins that are crucial for transferrin endocytosis in trypanosomes.

Small molecules can be useful tools for studying biology. Small molecules can perturb biological pathways that can be identified in phenotypic screens (Mayer, 2003). Analysis of proteins perturbed by treatment of cells with small molecules can identify novel proteins associated with the perturbed biological pathways. Confirmation of these results with genetic approaches can validate the identified protein's role in the biological pathway. This approach allows for discoveries of new pathway proteins without relying on knowledge of other species' biological systems. This strategy was used to identify

glycogen synthase kinase isoform 3β, GSK3β, as a regulator of transferrin endocytosis, using the small molecule, Lapatinib (Guyett, Behera et al. 2017; Guyett, Xia et al. 2016).

AEE788 is a small-molecule receptor tyrosine kinase inhibitor in humans; AEE788 inhibits transferrin endocytosis and S-phase entry (Sullenberger et al., 2017). Disruptions in the phospho-proteome of *T. brucei* after treatment with AEE788, revealed possible mediators of the biological effects of AEE788 (Sullenberger et al., 2017).

Of the proteins affected after treatment of *T. brucei* with AEE788, the protein Tb427tmp.160.4770 was an interesting candidate for follow-up. Tb427tmp.160.4770 was hyper-phosphorylated after nine-hour treatment with AEE788, the same time point where transferrin endocytosis is inhibited 90% (Sullenberger et al., 2017). There was no effect on the phosphorylation of Tb427tmp.160.4770 after four-hour treatment with AEE788, when there was no defect in transferrin endocytosis. Bioinformatic analysis revealed the human protein with the closest identity (30%) to Tb427tmp.160.4770, was AP-2 Associated Kinase 1 (AAK1). AAK1 is a regulator of clathrin-mediated endocytosis through phosphorylation of AP-2 (Conner & Schmid, 2002; Conner, Schröter, & Schmid, 2003). Genetic knockdown of Tb427tmp.160.4770 confirmed it to be essential for trypanosome proliferation and endocytosis of transferrin. The selective effect of Tb427tmp.160.4770 knockdown on endocytosis of different ligands was the same trend observed after treatment of trypanosomes with AEE788. Phospho-proteomic analysis also revealed five proteins whose phosphorylation was altered by both AEE788 and knockdown of Tb427tmp.160.4770. Together, these tools identified novel proteins that might mediate transferrin endocytosis in the African trypanosome.

Methods and Materials

Cell Cultures

Cultures of bloodstream form *Trypanosoma brucei brucei* Single Marker (SM) (Wirtz, 1999) lines were maintained in HMI-9 medium in logarithmic growth ($<10^6$ /mL) (Hirumi, 1989) containing G418 (6.5 *μ*g/mL). SM trypanosomes co-express a T7 RNA polymerase and Tet repressor for conditional expression of ectopic gene products under tetracycline regulation. The RNAi line for Tb427tmp.160.4770 knockdown was maintained with G418 (6.5 *μ*g/mL) and hygromycin (5 *μ*g/mL). The V5- tagged Tb427tmp.160.4770 line was maintained in G418 (6.5 *μ*g/mL), hygromycin (5 *μ*g/mL), and blasticidin (10 *μ*g/mL).

Generation of p2T7-Tb427tmp.160.4770 RNAi Vector

A gene fragment for Tb427tmp.160.4770 was obtained by identifying a 527 bp unique sequence within the protein using the program RNAit (Redmond, Vadivelu, & Field, 2003). Primers for amplification of the double-stranded DNA fragment were:

Forward: 5'-GGATCCTTCTGCTTCTCGCAGACTGAGCGG-3',

Reverse: 5'- CTCGAGAAGAGGTCATCCGTTGTTGGTTTTTGAGGCT-3'

on a template of *T. brucei* genomic DNA (100 ng), purified as described (Medina-Acosta, 1993). To facilitate cloning into RNAi vectors, a 5' *BamH1* cut site, 5'-GGATCC-3' and a 3' *Xho1* cut site, 5'-CTCGAG-3' were included in the primers (underlined in respective primer sequences, above). Following PCR clean-up (Qiagen) and addition of 3'- A overhangs (Invitrogen pCR™8/GW/TOPO® TA Cloning® Kit), the Tb427tmp.160.4770 fragment was cloned into pCR®8/GW/TOPO (Kalidas, 2011). The TOPO-Tb427tmp.160.4770 fragment plasmid was digested using *BamH1* and *Xho1* restriction enzymes (New England Biolabs). The Tb427tmp.160.4770 gene fragment in the TOPO-Tb427tmp.160.4770 vector and the destination p2T7^{TABlue} RNAi vector (Alibu, 2004) were

digested, separated, and extracted from a 1% agarose gel (Qiagen). Following an Antarctic phosphatase treatment of the p2T7^{TABlue} vector (New England Biolabs), the gene fragment and linearized vector were ligated together with T4 DNA ligase (New England Biolabs) to generate the final p2T7-Tb427tmp.160.4770 RNAi construct.

Trypanosome Transfection with p2T7-Tb427tmp.160.4770 RNAi Vector

Single-marker trypanosomes were grown in HMI-9 medium to a density of $8x10^5$ cells/mL. For transfection, $4x10^7$ cells were pelleted (3000 x*g* for five minutes), washed in 10 mL of 10 mM glucose in phosphate buffered saline (PBSG), and resuspended in 100 μL of Lonza Nucleofector solution (Lonza, Basel, Switzerland) with 20 μg of linearized p2T7-Tb427tmp.160.4770. Linearization of p2T7-Tb427tmp.160.4770 vector was carried out by incubating 20 μg of recombinant construct for three hours at 37 °C with Not1-HF (New England Biolabs), then heat inactivating the restriction enzyme for 30 minutes at 67 °C. Cells were transfected using a Lonza Nucleofector 2b device (single pulse, X-001 program), transferred to 30 mL of pre-warmed HMI-9 medium, and incubated for 18 hours at 37°C, 5% CO_2. Cells were serially diluted (1:10, 1:100, 1:1000) in HMI-9 medium, seeded in 24-well plates, and selection drugs added (G418 (6.5 μg/mL) and Hygromycin (5 μg/mL)). After four days, clonal populations were identified by microscopic evaluation, and population densities established with a Coulter Counter (Beckman).

Cell Proliferation and DNA Organelle Duplication

Trypanosomes (p2T7- Tb427tmp.160.4770 RNAi) were seeded in a 24-well plate at $5x10^4$ cells/mL, and RNAi induced with tetracycline (1 μg/mL). Cell density was determined every 12 hours over a 48-hour period with a haemocytometer. Two technical replicates were included for all three independent, biological replicates.

To track DNA organelle duplication, RNAi was induced 24 hours (see above). Cells were pelleted (3000 x*g* for five minutes) and fixed in 4% paraformaldehyde (PFA) in

phosphate-buffered saline (PBS) for 15 minutes at room temperature. Fixed trypanosomes were adhered to poly-L-Lysine coated coverslips for 15 minutes and mounted on a slide with DAPI (1.5 μM) in Vectashield mounting medium (Vector Laboratories, Burlingame, Ca). Slides were visualized (approximately 120 trypanosomes counted per sample). Statistical significance of differences in kinetoplast and nuclei number per cell was determined by chi-square analysis using GraphPad Prism software (version 7.0c).

Endocytosis Assays

Effects of treatment with AEE788 or knockdown of Tb427tmp.160.4770 on endocytosis of various ligands was determined by flow cytometry (Guyett et al., 2016; Sullenberger et al., 2017). In AEE788 treatment, trypanosomes were treated with 5 μM AEE788 or 0.1% DMSO vehicle control for four or nine-hours (Sullenberger et al., 2017). RNAi was induced for 24 hours as mentioned above. Cells were pelleted (3000 x*g*, five-minutes), washed in 1 mL serum-free HMI-9 medium, and resuspended in 1 mL of serum-free HMI-9 medium. The fluorescent ligands transferrin-AlexaFluor488 (Tf-488) or bovine serum albumin-AlexaFluor647 (BSA-647), 5 μL each of 5 mg/mL Tf-488 and BSA-647, were added. Trypanosomes were incubated for fifteen minutes at 37°C, 5% CO_2, with tube caps open. Cells were pelleted at 4°C (3000 x*g*, five minutes), resuspended in 1 mL of cold PBSG, and 2 μL of 1.5 mM propidium iodide (PI) added. Cells were analysed by flow cytometry (12,000- 15,000 events per sample) in three independent replicates. Standard deviation and statistical significance of differences between treatment groups was carried out with student's t-test using GraphPad Prism (version 7.0c).

Endogenous N-terminus V5 tagging of Tb427tmp.160.4770

V5 epitope tagging of Tb427tmp.160.4770 was carried by homologous recombination, following an established protocol (S. Shen, Arhun, G., Ullu, E., Tschudi, C., 2001). The PCR primers used were:

Forward: 5'- TACTTAGGTCAGTACCATAGTTTGGCGAGGTCACTGA AGTATTCCTCACACGAAGAAAGGAAGAAACAGAGAAGAAAAGTGAAGGAATAACCC GGGATGGCCAAGCCTTTGTCTCAAGAAG -3'

Reverse: 5'-GGTACCATCCTTGTTATACTTCTCAATTTTACCCTTTGA TGAGAAGTTCTTGTCCTTGGACTTACTAGATTTACTATCACTGCCCCCGGGCGTAGA ATCGAGACCGAGGAGAGGGTTAG -3'.

PCR was carried out with iProof high-fidelity PCR polymerase master-mix (Bio-rad, Hercules, Ca), yielding a product with a sequence identical to the 5' UTR of Tb427tmp.160.4770, followed by a blasticidin resistance gene, then a fusion of the V5-epitope tag to the beginning of the 5' coding sequence of Tb427tmp.160.4770. The entire PCR product was transfected into p2T7- Tb427tmp.160.4770 RNAi line trypanosomes as described earlier, and drug-resistant clones selected following the same protocol as described earlier.

Western blot analysis

Knockdown of Tb427tmp.160.4770 in a p2T7- Tb427tmp.160.4770 RNAi line and chromosomal integration of V5-epitope tag into an allele of Tb427tmp.160.4770 (V5-Tb427tmp.160.4770/RNAi) was monitored with western blot analysis. V5-Tb427tmp.160.4770/RNAi and p2T7- Tb427tmp.160.4770/SM trypanosomes were incubated for 24 hours with tetracycline (1 μg/mL) to induce knockdown of Tb427tmp.160.4770. Trypanosomes were pelleted (3000 x*g*, five minutes), washed in PBSG, resuspended in 15 μL of SDS sample buffer, and heated at 95 °C for five minutes.

Proteins were separated by SDS-PAGE (12% acrylamide) and labelled with Stain-Free probe to detect total protein load (Bio-Rad, Hercules, Ca). The stain-free gel was UV activated for five minutes (best sensitivity protocol) to determine total protein loaded before transfer to a PVDF membrane (semi-dry, rapid transfer; Bio-Rad, Hercules, Ca). The PVDF membrane was blocked in 5% milk in TBS-T (20 mM Tris buffered saline with 0.1% Tween-20) for one hour. Membranes were probed with primary antibody dilutions (rabbit anti-peptide Tb427tmp.160.4770 (KMW lab): 1:1000, or rabbit anti-V5 (Cell Signalling Technology): 1:2000) in 5% bovine serum albumin (BSA) in TBS-T for one hour. PVDF membranes were washed to remove excess primary antibody three times, five minutes each with TBS-T. The membrane was then incubated for one hour with alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad, Hercules, Ca) diluted 1:2000 in 5% BSA in TBS-T. Excess secondary antibody was washed off as above, and the membrane developed for three minutes with Immune-Star AP substrate (Bio-Rad, Hercules, Ca). Digital images of the membrane were captured on a ChemiDoc MP system (Bio-Rad). Normalization of western blots was performed against the total protein loaded per-lane using Image Lab software (Bio-Rad).

Fluorescence Microscopy

Intracellular localization of V5-Tb427tmp.160.4770 was determined by immunofluorescence staining. For methanol fixation/permeabilization, V5-Tb427tmp.160.4770/RNAi trypanosomes (10^6) were pelleted (3000 x*g*, five minutes), washed in PBSG, adhered to poly-L-lysine coated coverslips for 2 minutes, air dried briefly, and incubated in pre-chilled 100% methanol for 20 minutes at -20 ºC. Coverslips were washed briefly in PBS before blocking with 10% BSA and 0.1% Triton X-100 in PBS for one hour. Primary antibody staining was done with rabbit anti-V5 (1:500, Cell Signalling Technologies) in blocking buffer for one hour. Coverslips were washed in PBS three times

for five minutes, each time, then incubated with goat anti-rabbit AlexaFluor-594 fluorescent secondary antibody (1:1000, Invitrogen) in blocking buffer for one hour. Coverslips were then washed again, as above, and mounted on microscope slides with 1.5 μM DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, Ca).

For detection of endocytic cargo, V5-Tb427tmp.160.4770/RNAi trypanosomes (10^6) were pelleted (3000 x*g*, five minutes), washed in 1 mL serum-free medium, and resuspended in 1 mL fresh serum-free medium. Fluorescent transferrin (Tf-AlexaFluor-488, 25 μg, Life Technologies) or tomato lectin (TL- DyLight-488, 20 μg, Vector Laboratories) was added to trypanosomes on an ice-water bath. Trypanosomes were incubated for 2 minutes on ice with fluorescent cargo, then washed and resuspended in 1 mL of fresh serum-free medium on ice. Trypanosomes were incubated for five minutes at 37 °C, then pelleted at 4°C (3000 x*g*, five minutes) and fixed in 4% PFA in PBS for 15 minutes at 4 °C. Fixed trypanosomes were adhered to poly-L-lysine coated coverslips before fixative was quenched with 0.15 M glycine in PBS for ten minutes at room temperature. Trypanosomes were then permeabilized with 0.2% Triton X-100 in PBS (500 μL per coverslip) for twenty minutes at room temperature and stained for the V5-epitope as described earlier.

SILAC Phospho-proteomics

Proteins in Tb427tmp.160.4770 pathways were investigated using phospho-proteomics involving stable isotope labelling of amino acids in cell culture (SILAC) (Guyett et al., 2017). For this, p2T7-Tb427tmp.160.4770/SM trypanosomes were cultured in heavy (H) medium ($^{13}C_6$-L-Arginine, $^{2}H_4$-L-Lysine), and control (uninduced) trypanosomes were grown in light (L) medium (L-Arginine, L-Lysine) for five days (Ong, 2002). Trypanosomes grown in heavy medium were induced with tetracycline to knock down protein expression of Tb427tmp.160.4770. Twenty-four hours after induction of knockdown, trypanosomes

($4x10^7$/ sample) were pelleted (3000 x*g*, five minutes) and washed in 10 mL of PBSG containing 1 mM sodium orthovanadate (Na_3VO_4). The entire Tet+ (H) sample was then combined with the entire control Tet- (L) sample, resuspended in 500 μL lysis buffer (50 mM HEPES (pH 7.6), 8 M urea, 1 mM Na_3VO_4), and lysed by sonication (Q Sonica Q55, Newtown, Ct) (performed in three, 15 second intervals at 60 amps with resting on ice for 30 seconds between intervals). Alkylation of protein was carried out with 9 mM iodoacetamide for 20 minutes, protected from light. The sample was diluted five-fold into: 50 mM HEPES (pH 7.6) and 1 mM Na_3VO_4. Proteins were then digested with 100 μL of trypsin immobilized agarose bead slurry (Thermo Scientific, Waltham, Ma) for 48-hours at room temperature.

To isolate digested peptides, the mixture was diluted 10-fold in 0.1% trifluoroacetic acid (TFA) in preparation for reverse-phase chromatography. The entire mixture (22 mL) was loaded onto a SepPak C_{18} reverse-phase column and washed with 1) 0.1% TFA and 2) 1% acetonitrile in 0.1% TFA, 3 mL each. Peptides were eluted from the column with 25% and 50% acetonitrile in 0.1% TFA, 6 mL total. The eluate was dried under N_2 gas. Phospho-peptides were enriched and analyzed as described previously (Guyett et al., 2017). Phospho-peptide enrichment was done on an $FeCl_3$ -charged immobilized metal affinity column (IMAC; Andersson and Porath, 1985) made in house at Fred Hutchinson Cancer Research Center (Seattle, Wa). LC-MS/MS of phospho-peptide samples was done by dividing each sample into two technical replicates for analysis. Each replicate was diluted in 2% acetonitrile and 0.1% formic acid in water and resolved on an Easy-nLC 1000 (Thermo Scientific). Liquid chromatography was coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific) which was operated in a data dependent MS/MS mode over the m/z range of 400- 1500 (Guyett et al., 2017).

Results

1. Discovery of Tb427tmp.160.4770 as an AEE788-pathway protein

1.1 AEE788 Selectively Inhibits Transferrin Endocytosis in T. brucei

AEE788 associates with TbGSK3β (Katiyar et al., 2013), a regulator of transferrin endocytosis in the trypanosome (Guyett et al., 2016). So the effect of AEE788 on the endocytosis of transferrin in *T. brucei* was tested (Sullenberger et al., 2017). Uptake of fluorescent tomato lectin (TL- 488), bovine serum albumin (BSA- 647), and transferrin (Tf-488) was determined by flow cytometry (Sullenberger et al., 2017). After four hours of treatment with AEE788, there was no defect in transferrin endocytosis (Sullenberger et al., 2017). However, after nine-hour treatment of trypanosomes wih AEE788, transferrin endocytosis was selectively inhibited (Sullenberger et al., 2017). After nine-hour treatment, transferrin uptake decreased by 87% ($p= 2.8 \times 10^{-3}$)(Fig 1C). In contrast, BSA uptake increased 40% after nine-hour treatment of trypanosomes with AEE788 ($p= 3.1 \times 10^{3}$)(Fig 1C). Tomato lectin uptake was not affected by treatment with AEE788 (p= 0.9, data not shown). These data demonstrate the utility of AEE788 as a small-molecule tool to selectively study the transferrin endocytic pathway in *T. brucei*.

1.2 Treatment of Trypanosomes with AEE788 increases phosphorylation of Tb427tmp.160.4770

Sullenberger et al. showed that treatment of trypanosomes with AEE788 for 9-hours inhibits transferrin endocytosis (Sullenberger et al. 2017). Phosphoproteomics was used to identify trypanosome proteins potentially within pathways that AEE788 affected (Sullenberger et al., 2017). Among the identified proteins, Tb427tmp.160.4770 was notable. The protein was hyper-phosphorylated after trypanosomes were treated for nine-hours with AEE788 (Table 1). In TriTrypdb.org (Aslett et al., 2010), Tb427tmp.160.4770 is annotated as a hypothetical serine/threonine protein kinase, while its *T. brucei* 927-

strain homologue, Tb927.9.6560, is described as a NAK-like pseudokinase. The most closely related human protein to Tb427tmp.160.4770 is human AP2-associated kinase 1 (AAK1). AAK1 shares only 30% identity in the kinase domain (Figure 2). AAK1, a serine/threonine kinase, regulates clathrin-mediated endocytosis in human cells (Conner and Schmid 2002). Considering the role of AAK1 in human systems and the selective inhibition of transferrin endocytosis in trypanosomes by AEE788, we hypothesized that Tb427tmp.160.4770 was involved in the AEE788 affected transferrin endocytosis pathway in *T. brucei*.

2. Genetic Knockdown of Tb427tmp.160.4770

2.1. Tb427tmp.160.4770 is essential for proliferation of T. brucei.

To determine necessity of Tb427tmp.160.4770 for trypanosome proliferation, *Trypanosoma brucei brucei* single marker line (SM) (Wirtz, 1999) was stably transfected with a p2T7^{TABlue} RNAi construct (Alibu, Storm, Haile, Clayton, & Horn, 2005) containing a fragment of Tb427tmp.160.4770 (Fig. 3A). To induce knockdown of Tb427tmp.160.4770, tetracycline (1 μg/mL) was added to trypanosomes at a starting density of $1x10^4$ cells/mL. Cell density was measured every 12 hours over 48 hours (Fig. 3B). At 24 hours, trypanosome proliferation ceased in Tet+ induced cells; they reached a density of $7.8x10^5$ cells/ mL and maintained it for 48 hours post induction (Fig. 3B). Knockdown of Tb427tmp.160.4770 was confirmed by western blotting after 24 hours of tetracycline induction using an anti-Tb427tmp.160.4770 antibody (Fig. 3B). The signal for Tb427tmp.160.4770 in induced (Tet+) cells was 71% (std dev ± 13.7, n=3) less than that of uninduced (Tet-) cells, after normalizing for total protein load.

2.2. Tb427tmp.160.4770 is important for efficient transferrin endocytosis.

AEE788 inhibits transferrin endocytosis in *T. brucei* concurrent with hyper-phophorylation of Tb427tmp.160.4470 (Sullenberger et al. 2017). To determine if Tb427tmp.160.4770 acts in the same pathway as AEE788, the gene was knocked down by RNAi, and endocytosis of transferrin (Tf), bovine serum albumin (BSA), tomato lectin (TL), and haptoglobin/haemoglobin (H/H) were studied (Fig. 4). Uptake of tomato lectin (TL-488) and haptoglobin/ haemoglobin (H/H-488) were not affected after knockdown of Tb427tmp.160.4770 (Supplemental Figure 1). The difference in uptake of either tomato lectin or haptoglobin/ hemoglobin after knockdown of Tb427tmp.160.4770 was not statistically significant ($p= 0.85$, 0.41 ($n= 3$, 2), TL and H/H, respectively). Endocytosis of transferrin (Tf-488) and BSA (BSA-647) was affected after loss of Tb427tmp.160.4770; transferrin endocytosis decreased by 60%, while BSA uptake increased 31%. Differences in BSA and transferrin uptake were statistically significant ($p= 0.038$, and 0.0058 ($n=3$), for BSA and transferrin, respectively). Because of the similarities in endocytosis after treatment of trypanosomes with AEE788 and knockdown of Tb427tmp.160.4770, we concluded that Tb427tmp.160.4770 works within the pathway effected by AEE788.

2.3. A fraction of Tb427tmp.160.4770 co-localizes with endocytic vesicles

A possible explaination for how Tb427tmp.160.4770 effects endocytosis is through association with endocytic vesicles. To do this, we investigated the location of Tb427tmp.160.4770 in the trypanosome. To determine the intracellular location of Tb427tmp.160.4770, p2T7-Tb427tmp.160.4770 RNAi cells were transfected with an N-terminal V5-tagged Tb427tmp.160.4770 (S. Shen, Arhin, Ullu, & Tschudi, 2001), and the presence of the tagged protein was confirmed by Western blot analysis (Fig. 2C). Immunofluorescence staining of V5-Tb427tmp.160.4770 showed dispersed, cytoplasmic puncta. A fraction of V5-Tb427tmp.160.4770 co-localized with endocytosed transferrin-

AlexaFluor-488 and tomato lectin-AlexaFluor-488 (Fig. 2A). Using 100% methanol fixation, which removes most cytoplasmic proteins, V5-Tb160.4770 was detected exclusively near the kinetoplast (Fig. 2C).

2.4. Loss of Tb427tmp.160.4770 Interferes with DNA Organelle Copy Number

Effect of Tb427tmp.160.4770 on DNA containing organelle duplication was tested by staining mitochondrial and nuclear DNA. Stages of the trypanosome division cycle can be determined by the number of kinetoplasts (mitochondrial nucleoids, K) and nuclei (N) per cell (Sherwin & Gull, 1989). In G1 and S-phase, a trypanosome has one kinetoplast and one nucleus (1K1N). In G2, the trypanosome has two kinetoplasts, but one nucleus (2K1N) (Woodward & Gull, 1990). Post-mitotic cells are 2K2N until the completion of cytokinesis, when two daughter trypanosomes will be 1K1N (Sherwin & Gull, 1989).

After knockdown of Tb427tmp.160.4770 for 12 hours, there was a decrease in 1K1N cells (77.2% to 63.7%) and increases in 2K2N (10% to 15%) and abnormal cells (>2K >2N) (2.4% to 8%). The difference in the distribution of K/N populations between control and RNAi induced trypanosomes was not statistically significant at 12 hours ($p= 0.11$, χ^2 5.965, d.f.= 3). Twenty-four hours post induction, the proportions of 2K1N cells were 10% and 8% in Tet- and Tet+ samples, while 2K2N cells were 7% and 10%, respectively. However, there was a 30% decrease of 1K1N cells (81% to 51%), and an increase in abnormal K/N cells (2.2% (Tet-) to 30% (Tet+)). In the “abnormal” population, 15% of all Tet+ cells were either 3KxN or xK3N (Supplemental Figure 2). Trypanosomes with more than 4 kinetoplasts or nuclei (xKxN) made up 10% of the total Tet+ population. At 24 hours of knockdown, the difference in K/N distribution of Tet- and Tet+ K/N populations was statistically significant ($p<0.0001$, χ^2 32.07, d.f.= 3).

2.5. Knockdown of Tb427tmp.160.4770 alters the phospho-proteome of T. brucei

Treatment of trypanosomes with AEE788 disrupted phosphorylation homeostasis (Table 1). We therefore investigated effects of Tb427tmp.160.4770 knockdown on the phospho-proteome of *T. brucei* in order to identify potential pathway proteins of Tb427tmp.160.4770 (Fig. 6, Table 2). Knockdown was induced in trypanosomes labelled with heavy isotopes in a SILAC phospho-proteomics approach (Ong, 2002). Control (H (Tet-)/ L (Tet-)) phospho-peptide analysis shows a tight grouping of phospho-peptide abundance ratios centered on one, between Heavy (H) and Light (L) samples (Supplemental Fig 3).

Analysis of phospho-peptides whose abundance changed at least two-fold in at least two of three independent biological replicates, revealed that knockdown of Tb427tmp.160.4770 affected 202 proteins, 62 decreased in phosphorylation, while 140 increased in phosphorylation (Fig. 6). Select Tb427tmp.160.4770-pathway proteins are presented in Table 1. Pathway-proteins known to be involved in endocytosis and vesicle trafficking included kinesins (Tb427.06.4390, Tb427.07.3000, and Tb427.05.2410), TbEpsinR (Tb427tmp.50.0006), and VAMP (Tb427.05.3560). Identification of these endocytosis pathway proteins suggests a possible mechanism by which Tb427tmp.160.4770 may selectively regulate endocytosis of transferrin and BSA.

Discussion

Small molecules may be useful tools in studying biology, especially in systems that are poorly characterized and have many species-specific proteins not found in more widely-studied cells, like vertebrates and yeasts. Transferrin endocytosis in trypanosomes is very different from the process in vertebrates (G. W. Morgan, Hall, B., Denny, P., Field, M., Carrington, M., 2002), and that is of particular interest. Uptake of transferrin is an essential process for trypanosomes (Schell, 1991), but its route of internalization is distinct from that of humans. In humans, transferrin is endocytosed on a transmembrane

transferrin receptor and is dependent on adaptor protein-2 (AP-2) to facilitate the recruitment of the coat protein clathrin to sites and vesicle budding. Dynamin is critical for the removal of clathrin-coated vesicles from the plasma membrane. In trypanosomes, the transferrin receptor is GPI-anchored (Salmon, 1994) and has no cytoplasmic domain to facilitate adaptor recruitment or sorting. *T. brucei* also lacks AP-2 based on sequence alignment searches (G. W. Morgan, Hall, B., Denny, P., Carrington, M., Field, M., 2002), and the dynamin orthologue is not essential for clathrin-mediated endocytosis (Morgan et al., 2004). These differences in the transferrin endocytosis pathways highlight the importance of studying this pathway using methodology not based on protein sequence similarity.

In this study we utilized the small-molecule AEE788 to discover Tb427tmp.160.4770 as a possible component of endocytic pathways perturbed by AEE788 in the trypanosome. AEE788 inhibits transferrin uptake after nine-hour treatment and increased BSA uptake (Sullenberger et al., 2017). Knockdown of Tb427tmp.160.4770 resulted in a decrease of transferrin uptake and an increase in BSA uptake (Fig 4). This trend was similar to that observed after treatment of trypanosomes with AEE788 (Sullenberger et al., 2017). Thus, AEE788 phenocopies knockdown of Tb427tmp.160.4770 in its effect on both transferrin and BSA endocytosis. These data point to differential regulation of endocytosis of transferrin and BSA in *T. brucei*.

A fraction of Tb427tmp.160.4770 co-localizes with both endocytosed transferrin and tomato lectin (Fig. 5B). This was an interesting observation as loss of Tb427tmp.160.4770 inhibits transferrin endocytosis but has no effect on the uptake of tomato lectin (Supplemental Figure 1). Data from previous investigations of transferrin and VSG uptake offers possible explanation of our findings (reviewed in (Field & Carrington, 2009; Overath & Engstler, 2004)). Although both VSG and transferrin are GPI-anchored,

they are trafficked differently through the endomembrane system, indicating the presence of specialized systems for sorting GPI-anchored proteins. Transferrin moves from early endosome to the late endosome where transferrin is released from the transferrin receptor (TfR) (Maier & Steverding, 1996). Transferrin is transported to the lysosome for degradation, but the TfR moves to the recycling endosome, from where it can return to the flagellar pocket. VSG, on the other hand, moves directly from the early endosome to the recycling endosome, without entering the late endosome compartment. We hypothesize that Tb427tmp.160.4770 only affects vesicles containing GPI-anchored proteins moving between the early and late endosomes or late endosomes and the lysosome.

Bovine serum albumin (BSA) is an endocytosed ligand that may not have a receptor in *T. brucei* (Coppens, Opperdoes, Courtoy, & Baudhuin, 1987). Loss of Tb427tmp.160.4770 increases BSA uptake (Fig. 4) by mechanisms which are not currently understood. Knockdown of GSK3β led to a loss of transferrin endocytosis but did not significantly affect BSA uptake (Guyett et al., 2016).

Phosphorylation of proteins is altered after knockdown of Tb427tmp.160.4770 (Table 2). It is significant that five proteins, aside from Tb427tmp.160.4770, that were affected by loss of Tb427tmp.160.4770 were also affected by treatment of trypanosomes with AEE788 (Table 3). The proteins include: hypothetical proteins Tb427.08.8000, Tb427.10.15040, and Tb427tmp.01.1960, as well as Tb427.06.640 (Kinetoplastid specific protein phosphatase) and Tb427.05.2820 (NEK6, putative). Proteins affected by both treatment with AEE788 and loss of Tb427tmp.160.4770 may be regulators of endocytosis. A direct experimental test of this hypothesis may help assign roles to proteins which currently have no known function in the trypanosome. The interaction between Tb427tmp.160.4770 and TbEpsinR is especially interesting. TbEpsinR is hyper-phosphorylated after knockdown of Tb427tmp.160.4770 (Table 2). TbEpsinR is one of the

few clathrin adaptor proteins in *T. brucei* and is essential for transferrin endocytosis, but is not essential for all endocytosis (Gabernet-Castello, Dacks, & Field, 2009). TbEpsinR and Tb427tmp.160.4770 associate in some way with clathrin (Manna, 2017). Given these facts, it will be interesting to test whether knockdown of TbEpsinR has the same effect on endocytosis as Tb427tmp.160.4770; reduction in transferrin uptake, but increase in BSA uptake.

Tb427tmp.160.4770 is a pseudokinase; it has a kinase-like domain, but lacks features of enzymatically active kinases (reviewed in (Reiterer et al., 2014)). First, the canonical HRD catalysis motif found in most active kinases (reviewed in (Hanks & Hunter, 1995)), is replaced in Tb427tmp.160.4770 with HRN, which is found in some pseudokinases (Shi, Telesco, Liu, Radhakrishnan, & Lemmon, 2010). Second, Tb427tmp.160.4770 lacks the DFG motif, which is involved in magnesium binding and the structure of the activation loop (A-loop). Despite these changes, the loss of Tb427tmp.160.4770 perturbs phospho-proteome homeostasis (Figure 6). This effect could be the result of disrupted interactions between Tb427tmp.160.4770 pathway proteins and a trypanosome kinase or phosphatase. In human cells, the pseudokinase HER3 modulates signaling pathways of active kinases HER2 and HER1 by blocking the homodimerization of HER1 and HER2, and instead forming a heterodimer with decreased phospho-transfer activity (Shi et al., 2010). We hypothesize that Tb427tmp.160.4770 interacts with trypanosome kinases or phosphotases to elicit it's physiological responses (Table 5).

In this study, we show the value of using a small-molecule to study biological pathways in *T. brucei*. AEE788 inhibits transferrin endocytosis, and treatment of *T. brucei* with the small-molecule leads to hyper-phosphorylation of Tb427tmp.160.4770 (Sullenberger et al., 2017). These results led to the hypothesis that Tb427tmp.160.4770

is involved in the regulation of transferrin endocytosis in *T. brucei*. We confirm here that Tb427tmp.160.4770 is involved in the selective regulation of transferrin endocytosis (Fig. 4) and identify TbEpsinR as a Tb427tmp.160.4770-pathway protein (Table 2). Further investigation into Tb427tmp.160.4770 pathway proteins and the hypothetical proteins affected by both AEE788 treatment and knockdown of Tb427tmp.160.4770 could shed light on novel regulators of trypanosome transferrin endocytosis as well as the pathway and interactome of the first pseudokinase to be assigned a biological function in *T. brucei*.

Figures

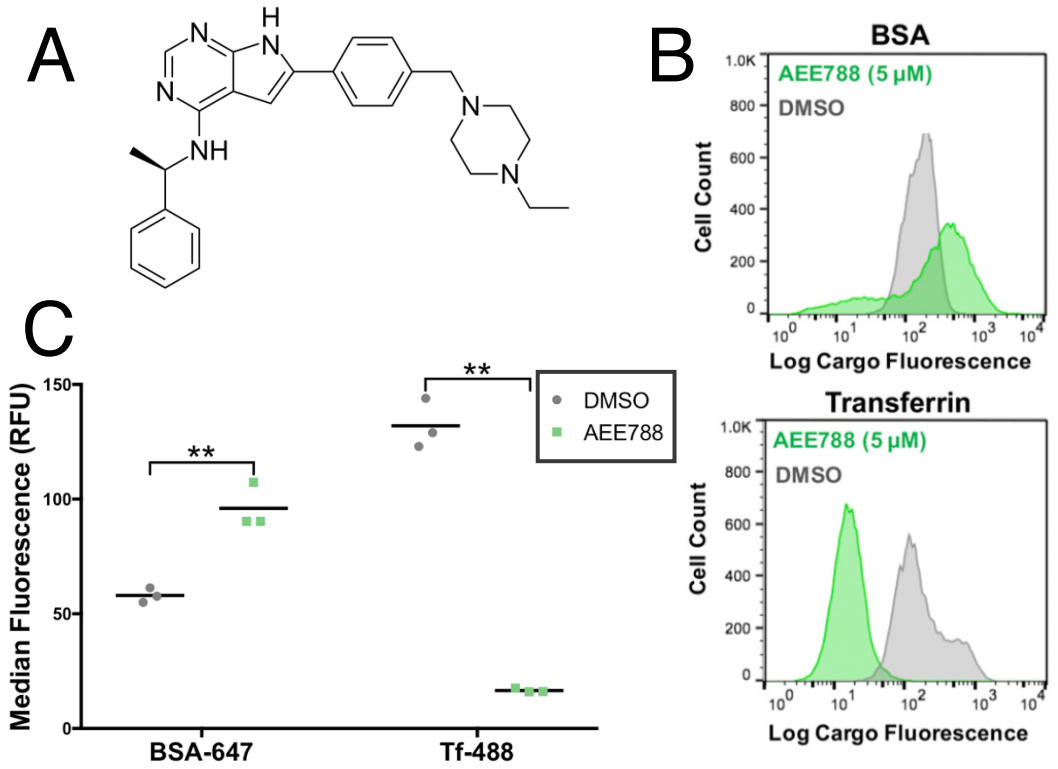


Figure 1. AEE788 Selectively Inhibits Transferrin Endocytosis. Cells were incubated with 5 μM AEE788 (or DMSO, the drug vehicle) for nine hours. Cells were then washed in serum free medium, incubated with fluorescently labelled transferrin (AlexaFluor 488) and bovine serum album (BSA, AlexaFluor-647) for 15 minutes at 37 °C. Following a wash with PBSG and addition of 3 μM (final) propidium iodide, the median fluorescence of cells was measured and analysed by flow cytometry. A) Chemical structure of AEE788 (Novartis) B) Representative histograms of median fluorescence with and without addition of AEE788. C) Quantitation of median fluorescence of transferrin-AlexaFluor488 and BSA-AlexaFluor647 in cells incubated in presence of AEE788, and those that incubated with DMSO (Adapted from Sullenberger et al., 2017). Bar indicates mean of three independent trials. **$p < 0.01$

Table 1. Treatment with AEE788 increases phosphorylation of Tb427tmp.160.4770. Following a nine-hour treatment with either 5 μM AEE788 or DMSO (0.1%), trypanosomes collected and phospho-peptide enrichment using an IMAC column. Abundance of phospho-peptides was determined by LC-MS/MS. Proteins identified with an (*) altered phosphorylation after knockdown of Tb427tmp.160.4770 (Table 3). Phosphosites with phosphoRS score > 80% are highlighted in red (Adapted from Sullenberger et al., 2017).

Protein ID	Description	Phospho-peptide	Phospho-peptide Spectral Counts (All peptides of parent protein)		P-value
			DMSO	AEE788	
Tb427tmp.160.4770	NAK-family pseudokinase	AVTALssDTASTDPEVLAYR	0 (9)	12 (33)	0.184
Tb427tmp.160.4770	NAK-family pseudokinase	DEAAASsVKscTAAQESGDNDQmVLK	1 (9)	11 (33)	0.087
Tb427.03.3080	Nek1, putative	ADTsDIsLSHEDLsR	12 (16)	0 (7)	0.02
Tb427.08.8000	Hypothetical	GVDTRDSLFADGGELDsFYAK	8 (11)	4 (5)	0.52
Tb427.10.15040	Hypothetical	NVEFPVVGsDEGNKsR	6 (8)	0 (0)	0.03
Tb427tmp.01.1960	Hypothetical	KRPSsIGRPSSR	9 (31)	2 (5)	0.12
Tb427tmp.01.1960	Hypothetical	KTsSAPsLLPQIK	20 (31)	2 (5)	0.03
Tb427.06.640	Protein phosphatase	HSSNNSSTNsGNDKPIETQAPHR	17 (27)	3 (15)	0.26
Tb427.05.2820	NEK6, putative	NPSVTRSPSVLsNSPAPDNLR	8 (9)	16 (20)	0.289

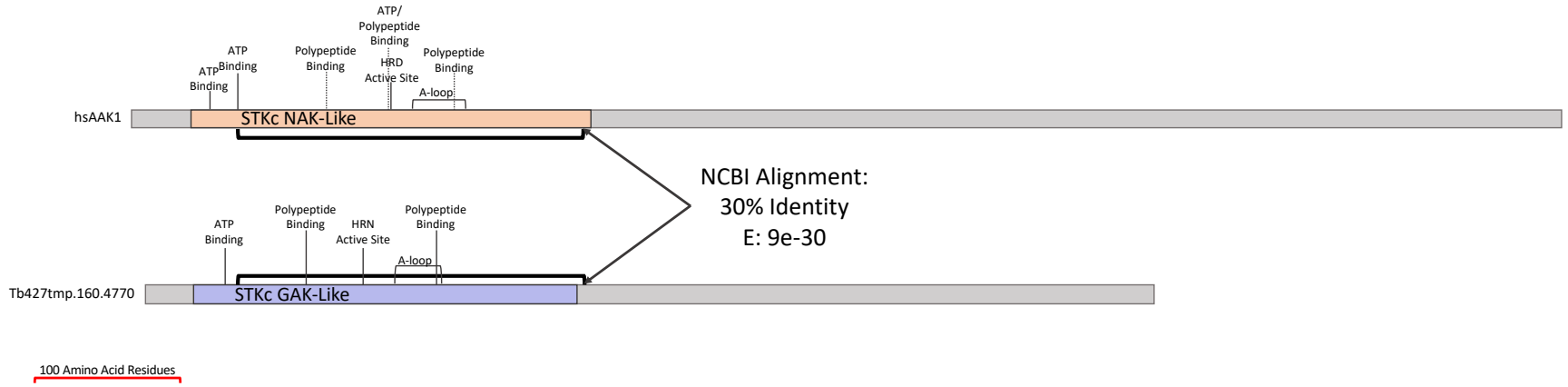


Figure 2. Domains common to Tb427tmp.160.4770 and AP-2 Associated Kinase 1 (hsAAK1). Domains of trypanosome protein Tb427tmp.160.4770 and human AAK1 (UniProtKB: Q2M2I8). Protein alignment was carried out on NCBI Protein Blast. Sequences for Tb427tmp.160.477 and hsAAK1 were obtained from TriTrypDB and UniProtKB, respectively. Feature and domain annotation was determined from NCBI Conserved Domain search. Black brackets show conserved kinase and kinase-like domain of hsAAK1 and Tb427tmp.160.4770, respectively.

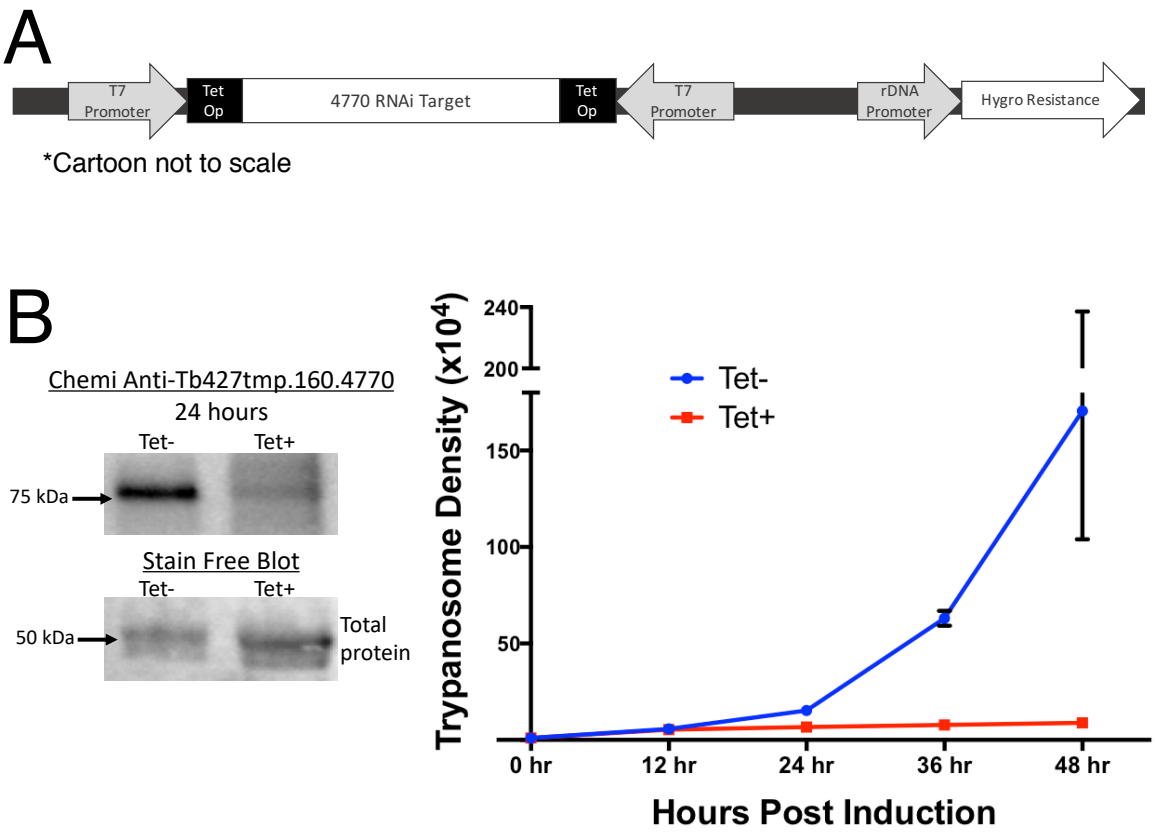


Figure 3. RNAi knockdown of Tb427tmp.160.4770 in *T. brucei*. (A) Cartoon of p2T7^{TABlue} derived RNAi construct transfected into Single Marker (SM) cells. Dual, opposing T7 promoters drive expression of Tb427tmp.160.4770 fragment under tetracycline regulation. Antibiotic (hygromycin) resistance expression driven by constitutively active rDNA promoter. (B) Cell proliferation assay after knockdown of 4770. Cells were seeded at 1x10^4 cells/ mL, knockdown induced with 1 μg/mL tetracycline and cell density determined via haemocytometer every 12 hours for 48 hours total. Error bars represent standard deviation of three trials. Western blot insert: Tb427tmp.160.4770 protein level 24 hours after induction of knockdown. Cells were collected, and the blot probed with anti-4770 polyclonal antibody. Stain-Free blot shows total protein load; stain free technology uses a trihalo compound that is covalently linked to tryptophan residues upon exposure to UV light, allowing the residues to be visualized (Bio-Rad).

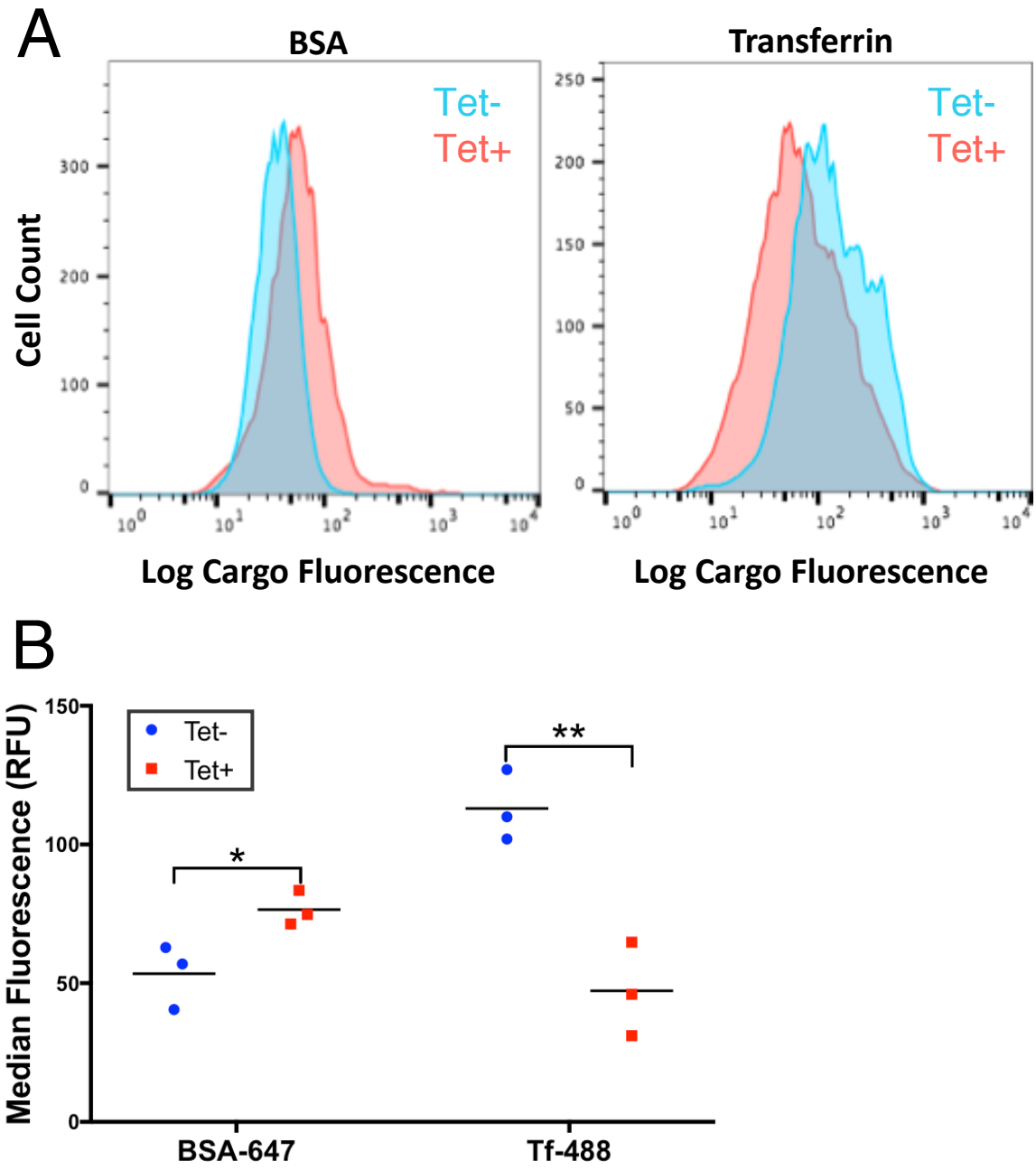


Figure 4. Tb427tmp.160.4770 selectively regulates transferrin endocytosis. Knockdown of Tb427tmp.160.4770 was induced for 24 hours with 1 μg/mL tetracycline. Cells were incubated with 25 μg/mL BSA-AlexaFluor-647 or 25 μg/mL Transferrin-AlexaFluor-488 (Tf-488) for 15 minutes at 37°C. Fluorescence was analysed with flow cytometry (15,000 events per sample). A) Representative histograms of fluorescence intensity per cell in uninduced (Tet-) and knockdown induced (Tet+) cells for BSA-647 and transferrin-488, respectively. B) Quantitation of median fluorescence of BSA-647 and Tf-488 in uninduced (Tet-) and knockdown induced (Tet+) cells (BSA and Tf, n= 3). Bars represent mean. *$p < 0.05$, **$p < 0.01$

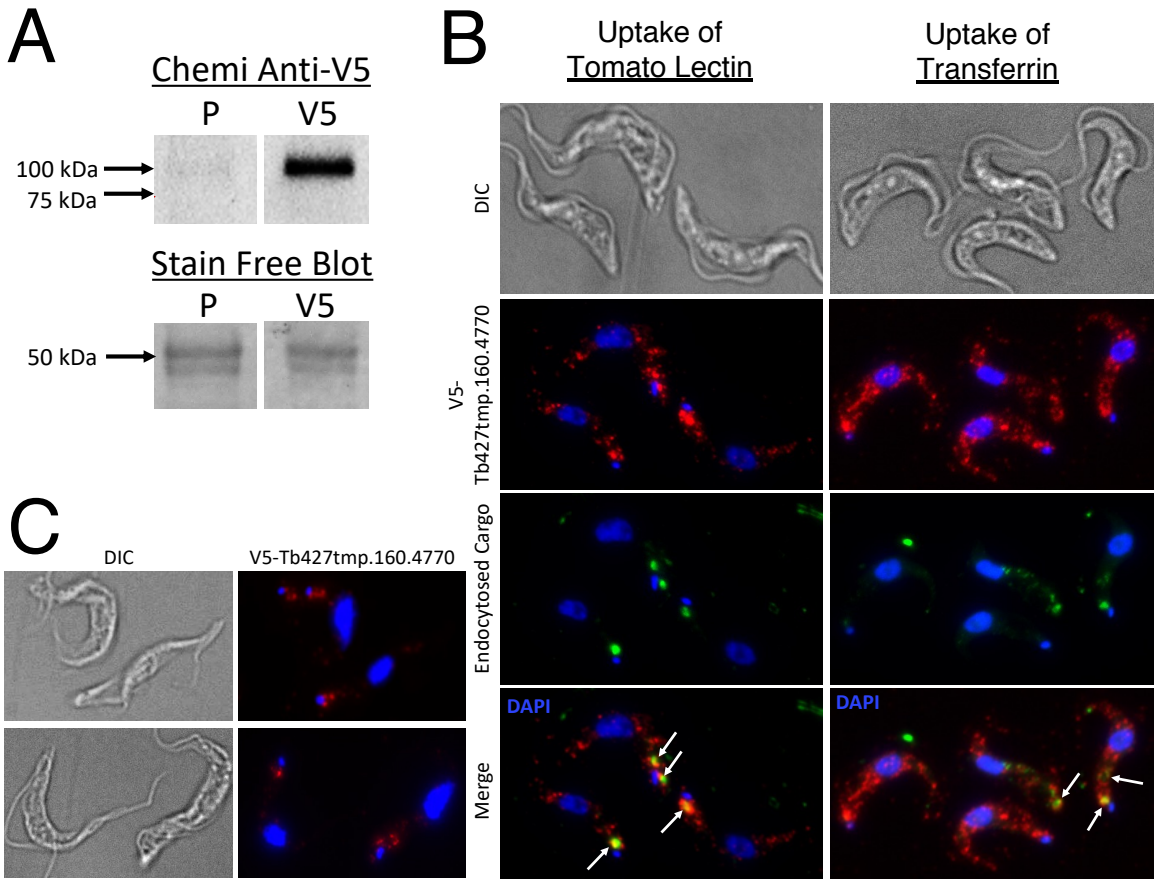


Figure 5. Intracellular location of endogenously tagged V5-Tb427tmp.160.4770. (A) Western blot of Tb427tmp.160.4770 with V5 epitope. P= parental cell line, V5= tagged cell line. Stain-free technology described in materials and methods. (B) Detection of V5-Tb427tmp.160.4770 and internalized tomato lectin-488 (TL) or transferrin-488 (Tf). Cells were incubated on ice for 2 minutes with either 20 μg/mL tomato lectin-488, or 25 μg/mL transferrin-488, washed, then incubated at 37°C for 5 minutes. Cells were fixed in 4% paraformaldehyde at 4°C, then stained for V5 epitope tag. White arrows indicate co-localization of V5-Tb427tmp.160.4770 and internalized cargo. (C) V5-Tb427tmp.160.4770 tagged trypanosomes fixed and permeabilized in 100% methanol. Trypanosomes were stained with monoclonal rabbit anti-V5 (Cell Signalling Technologies) antibody in blocking buffer.

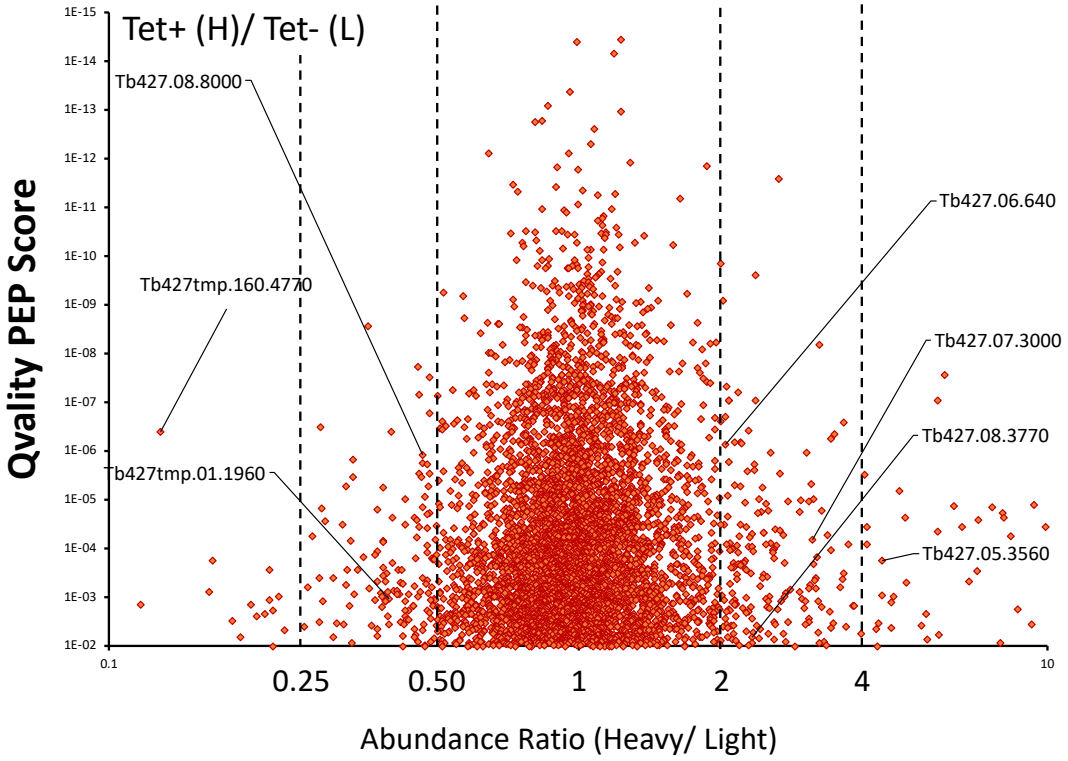


Figure 6. Knockdown of Tb427tmp.160.4770 alters phosphoproteome of *T. brucei*. Bloodstream form trypanosomes stably transfected with Tb427tmp.160.4770 p2T7- RNAi construct were labeled in heavy (H) or light (L) labeled HMI-9 SILAC media prior to induction of knockdown with 1 ug/mL tetracycline (Tet+). Twenty-four hours after additional of tetracycline, $4x10^7$ cells were mixed, lysed, and trypsin digested. Peptides were isolated on a SepPak C18 column. Phosphopeptides were enriched by immobilized metal affinity chromatography (IMAC) and identified by LC-MS/MS. A) Representative proteome profiles of knockdown induced cells (Tet+)/ uninduced cells (Tet-) (heavy/ light relative abundance ratio).

Table 2. Select candidate Tb427tmp.160.4770 pathway proteins. Select peptides that changed in relative abundance by at least 2-fold in at least two of three replicate experiments after knockdown of Tb427tmp.160.4770. Phosphosites with a phosphoRS score of at least 80% are shown in lower case. Proteins in the top, white section of the table all decreased by at least two-fold or greater; proteins in the bottom, grey part of the table increased at least two-fold or greater in abundance.

Gene ID	Description	Phospho-peptide Sequence	H/L Ratio (± std dev)	Qvality PEP Value
Decreased				
Tb247.06.4390	Kinesin heavy chain 5B (KHC1)	DGTPsPNNTQNENLQR	0.34 ± 0.06	$1.9e^{-05}$
Tb427.10.5880	Proteophosphoglycan	AsVSEEANNVSSDRPVGK	0.37 ± 0.07	$5.0e^{-05}$
Tb427.08.8000	Hypothetical	DsLFADGGELDsFYAK	0.38 ± 0.12	$1.2e^{-06}$
Tb427.10.15040	Hypothetical	SGtCVVNLAESTK	0.40 ± 0.06	$4.2e^{-03}$
Tb427tmp.01.1960	Hypothetical	KTsSAPsLLPQIK	0.41 ± 0.02	$1.1e^{-03}$
Tb427tmp.160.4770	NAK-family pseudokinase	DEAAASsVKsCTAAQESGDNDQMVLK	0.07 ± 0.01	$5.0e^{-10}$
Tb427tmp.160.4770	NAK-family pseudokinase	AVTALSSDTAsTDPEVLAYR	0.07 ± 0.09	$7.4e^{-09}$
Tb427tmp.160.4770	NAK-family pseudokinase	sCTAAQESGDNDQMVLK	0.15 ± 0.02	$4.0e^{-07}$
Tb427.08.6810	ANP1	ASSTLKPsPNTSHNPQEPLGtSSTR	0.48 ± 0	$4.4e^{-02}$
Tb427.01.2390	Beta tubulin	INVYFDEAtGGR	0.01 ± 0	$1.5e^{-02}$
Increased				
Tb427.07.5450	Lipin, putative	GLEMSGMsNPSASVAVTNR	2.41 ± 0	$1.8e^{-05}$
Tb427tmp.50.0006	EpsinR	AGITVsEAQR	2.34 ± 0.01	$1.5e^{-03}$
Tb427.07.3000	Kinesin, putative	LSVADSSPSTHSPSPTEsPTVR	2.99 ± 0.25	$6.4e^{-05}$
Tb427tmp.02.1640	Dual specificity phosphatase	IRtPLQQVVEGER	2.14 ± 0.03	$1.1e^{-02}$
Tb427.06.640	Phospho-protein phosphatase	AAPSANVSSVTSPPR	51.03 ± 68.8	$7.2e^{-07}$
Tb427.08.3770	MAP kinase	EDTQDPNKTHYVTHR	2.38 ± 0.03	$6.5e^{-03}$
Tb427.08.3770	MAP kinase	EDTQDPNKtHYVtHR	52.05 ± 67.8	$2.3e^{-02}$
Tb427.08.3770	MAP kinase	LALsNGTTNAK	51.18 ± 69.0	$1.6e^{-02}$
Tb427.08.3770	MAP kinase	EDTQDPNKtHyVTHR	3.62 ± 1.28	$2.3e^{-02}$
Tb427.05.2820	NEK6; protein kinase	MCsPANSPVSPSR	2.46 ± 0.34	$2.2e^{-04}$
Tb427.05.3560	VAMP, synaptobrevin	SAtLSEQAQQFQR	3.84 ± 0.83	$1.8e^{-04}$
Tb427tmp.02.0260	Golgi reassembly stacking protein	VPPPLAFPIIKPAtPSR	2.03 ± 0.04	$1.8e^{-03}$
Tb427.05.2410	Kinesin, putative	IALsGATGDLMK	2.28 ± 0.25	$1.7e^{-05}$
Tb427tmp.02.4140	IP_3 5-phosphatase	FPPTYLCQsPR	51.15 ± 69.1	$5.5e^{-05}$
Tb427.07.5220	Forkhead Kinase	GDICGDFsDAEDGDTSSAVR	2.95 ± 1.03	$2.6e^{-07}$

Table 3. Phospho-peptides whose abundance changed 100-fold in at least two independent SILAC experiments. Phosphosites with a phosphoRS score of at least 80% are shown in lower case. Proteins in the top section of the table all decreased in abundance; proteins in the bottom part of the table increased in abundance.

Gene ID	Description	Phospho-peptide Sequence	H/L Ratio (± std dev)	Qvality PEP Value
Tb427.01.2390	beta tubulin	INVYFDEAtGGR	0.01 ± 0	0.015
Tb427.04.2220	TPR-repeat-containing chaperone protein DNAJ	LSILGDITAEPLsAR	0.01 ± 0	1.4E-05
Tb427.05.1690	hypothetical protein	ELQQQLSStAVAR	0.01 ± 0	0.0012
Tb427.07.3740	hypothetical protein	GMsPEDsNNPESLFVR	0.01 ± 0	1.1E-05
Tb427.08.1300	hypothetical protein	SAAACANtsMETTPEAVNR	0.01 ± 0	0.00025
Tb427.08.7940	receptor-type adenylate cyclase GRESAG 4	GSFIsPMASR	0.01 ± 0	0.037
Tb427tmp.02.1510	hypothetical protein	KRPssQsVGK	0.01 ± 0	0.011
Tb427tmp.160.4710	hypothetical protein	sCEEVGSPDQAVNDSYVQLER	0.01 ± 0	4.97E-09
Tb427tmp.211.4170	hypothetical protein	GAAAGDItPPQDEAEK	0.01 ± 0	1.5E-08
Tb427.01.4310	hypothetical protein	TGAtPLR	100 ± 0	0.0058
Tb427.01.4310	FAZ2; hypothetical protein	sRGPESHSVR	100 ± 0	0.012
Tb427.02.4050	hypothetical protein	GEQDIAVVSSREDDVK	100 ± 0	0.0037
Tb427.03.1920	NOT5 protein	GRPASLVsPPSTTSK	100 ± 0	0.00088
Tb427.03.3300	FAZ19	GAsNDRLPER	100 ± 0	0.027
Tb427.04.3970	hypothetical protein	KHELLLsPPEAEK	100 ± 0	0.0001
Tb427.06.1920	hypothetical protein	LsGTVLK	100 ± 0	0.011
Tb427.06.840	hypothetical protein	HESSSIMGNsPPDSK	100 ± 0	4.2E-09
Tb427.07.1110	asparagine synthetase a	APDYDDWtSPVEASQVVFPR	100 ± 0	1.4E-05
Tb427.07.7240	hypothetical protein	DCATPSAGGYAGSGPsGR	100 ± 0	5.7E-07
Tb427.07.7240	hypothetical protein	DCAtPsAGGYAGSGPSGR	100 ± 0	3.8E-05
Tb427.07.7270	hypothetical protein	FALGEVLAPsPLR	100 ± 0	0.00026
Tb427.10.14410	hypothetical protein	DTAVAtPDAAEAADSQYNR	100 ± 0	1.3E-06
Tb427tmp.01.4480	hypothetical protein	LSKPQQPSNSSGGDsK	100 ± 0	0.0016
Tb427tmp.160.0340	TbMlp-2, Myosin-like protein	EVEELGGSSGPsSAR	100 ± 0	4.8E-07
Tb427tmp.160.4210	hypothetical protein	VsPINDSIPETGQEEQEIGEISPR	100 ± 0	1.4E-05
Tb427tmp.39.0006	eIF-2B GDP-GTP exchange factor	FSADDLFAsLR	100 ± 0	0.00017

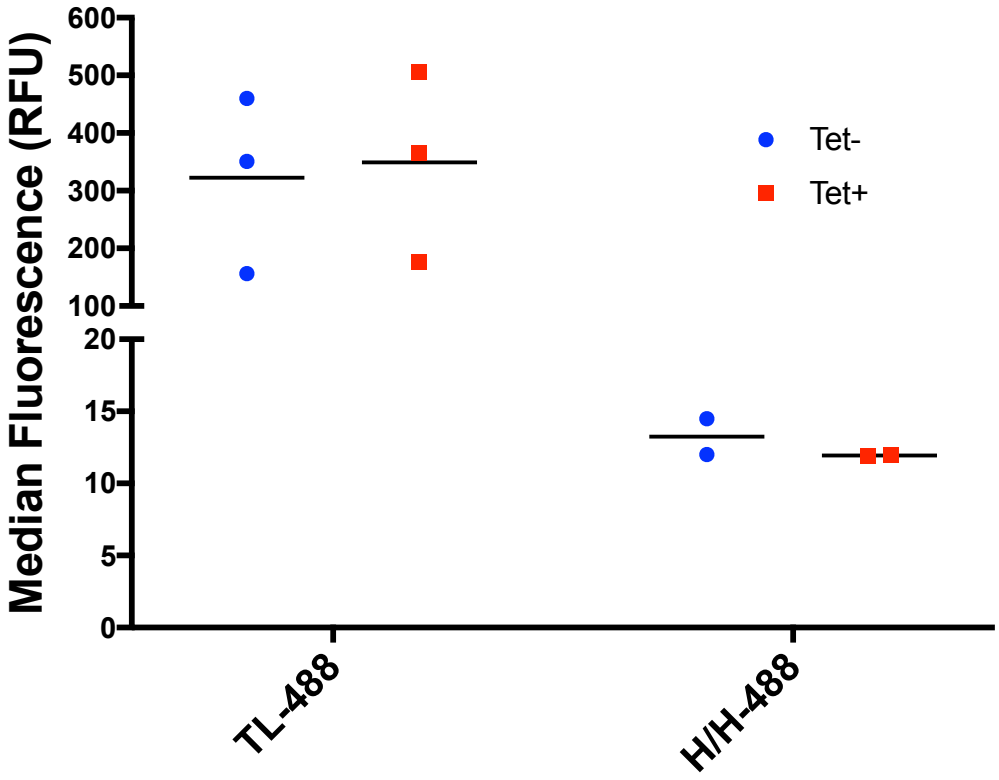
Table 4. Proteins affected by both treatment of trypanosomes with AEE788 and knockdown of Tb427tmp.160.4770. Summary of proteins affected by both AEE788 treatment and knockdown of Tb427tmp.160.4770. Dashes indicate the specific phospho-peptide was not found in relevant experiment. Phosphosites with a phosphoRS score of at least 80% are shown in lower case. Proteins in the top section of the table all decreased in abundance; proteins in the bottom part of the table increased in abundance. Red text shows phospho-peptides that decreased in abundance after treatment with AEE788, green text indicates an increase in abundance. AEE788 data adapted from Sullenberger et al. 2017.

Gene ID	Description	Phosphopeptide Sequence	Abundance Fold-Change		SILAC Qvaility PEP-value
			SILAC	S.C.	
SILAC Decreased					
Tb427.08.8000	Hypothetical	DsLFADGGELDsFYAK	2.6	--	$1.2e^{-06}$
Tb427.08.8000	Hypothetical	GVDTRDSLFADGGELDsFYAK	--	2	--
Tb427.10.15040	Hypothetical	SGtCVVNLAESTK	2.5	--	$4.2e^{-03}$
Tb427.10.15040	Hypothetical	NVEFPVVGsDEGNKsR	--	100	--
Tb427tmp.01.1960	Hypothetical	KTsSAPsLLPQIK	2.4	10	$1.1e^{-03}$
Tb427tmp.01.1960	Hypothetical	KRPSsIGRPSSR	--	4.5	--
Tb427tmp.160.4770	NAK-family pseudokinase	DEAAASsVKsCTAAQESGDNDQMVLK	14.3	11	$5.0e^{-10}$
Tb427tmp.160.4770	NAK-family pseudokinase	AVTALSSDTAsTDPEVLAYR	14.3	12	$7.4e^{-09}$
Tb427tmp.160.4770	NAK-family pseudokinase	sCTAAQESGDNDQMVLK	6.7	--	$4.0e^{-07}$
SILAC Increased					
Tb427.06.640	Phospho-protein phosphatase	AAPSANVSSVTSPPR	51.03	--	$7.2e^{-07}$
Tb427.06.640	Phospho-protein phosphatase	HSSNNSSTNsGNDKPIETQAPHR	--	5.7	--
Tb427.05.2820	NEK6; protein kinase	MCsPANSPVSPSR	2.5	--	$2.2e^{-04}$
Tb427.05.2820	NEK6; protein kinase	NPSVTRSPSVLsNSPAPDNLR	--	2	--

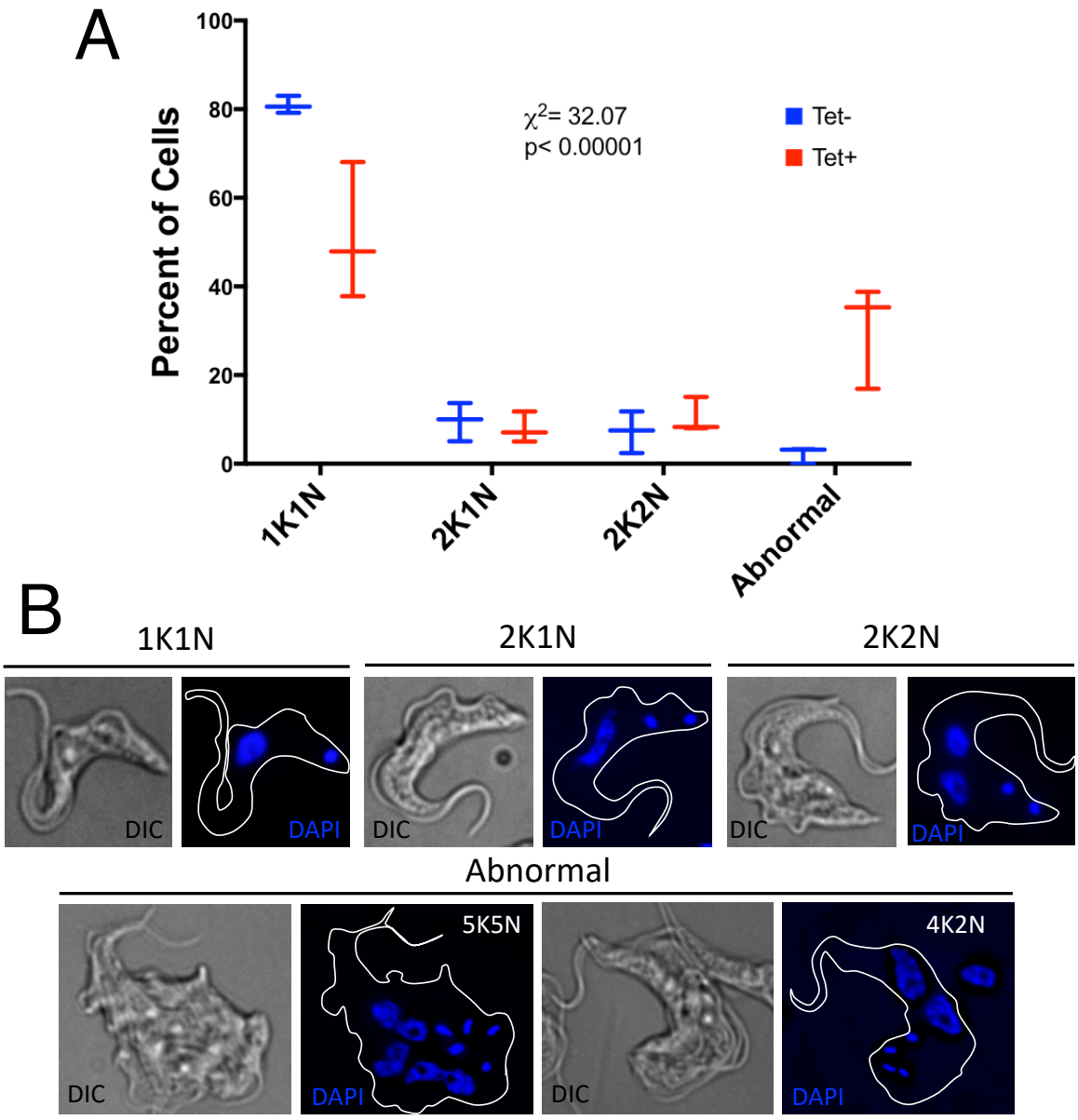
Table 5. Kinases and phosphatases affected after knockdown of Tb427tmp.160.4770. Phosphosites with a phosphoRS score of at least 80% are shown in lower case. Proteins in the top, white section of the table all decreased in abundance; proteins in the bottom, grey part of the table increased in abundance.

Gene ID	Description	Phospho-peptide Sequence	H/L Ratio (± std dev) n=2	Qvality PEP Value
Decreased				
Tb427tmp.160.4770	NAK-family pseudokinase	DEAAASsVKsCTAAQESGDNDQMVLK	0.1 ± 0.01	$5.0e^{-10}$
Tb427tmp.160.4770	NAK-family pseudokinase	AVTALSSDTAsTDPEVLAYR	0.1 ± 0.09	$7.4e^{-09}$
Tb427tmp.160.4770	NAK-family pseudokinase	sCTAAQESGDNDQMVLK	0.2 ± 0.02	$4.0e^{-07}$
Tb427.08.6810	MAP Kinase kinase kinase ANP1	ASSTLKPsPNTSHNPQEPLGtSSTR	0.5 ± 0	$4.4e^{-02}$
Increased				
Tb427.06.640	Kinetoplastid-specific phospho-protein phosphatase	AAPSANVSSVTSPPR	51.0 ± 68.84	$7.2e^{-07}$
Tb427.05.2820	NEK6, protein kinase	MCsPANSPVSPSR	2.5 ± 0.34	$2.2e^{-04}$
Tb427tmp.02.4140	IP_3-5- phosphatase	FPPTYLCQSPR	51.2 ± 69.1	$5.4e^{-05}$
Tb427.07.5220	Forkhead kinase	GDICGDFSDAEDGDTSSAVR	3.0 ± 1.0	$2.6e^{-07}$
Tb427.08.3770	MAP Kinase	EDTQDPNKTHYVTHR	2.4 ± 0.03	$6.5e^{-03}$
Tb427.08.3770	MAP Kinase	LALsNGTTNAK	51.2 ± 69.04	$1.6e^{-02}$
Tb427.08.3770	MAP Kinase	EDTQDPNKtHyVTHR	3.6 ± 1.3	$2.3e^{-02}$
Tb427.08.3770	MAP Kinase	EDTQDPNKtHYVtHR	52.1 ± 69.1	$2.3e^{-02}$
Tb427tmp.02.1640	Kinetoplastid specific, dual-specificity phosphatase	IRtPLQQVVEGER	2.1 ± 0.03	0.01
Tb427.02.4510	CRK9, cdc2-related kinase, putative	SNAESPTADVLR	2.3 ± 0.32	$3.4e^{-04}$

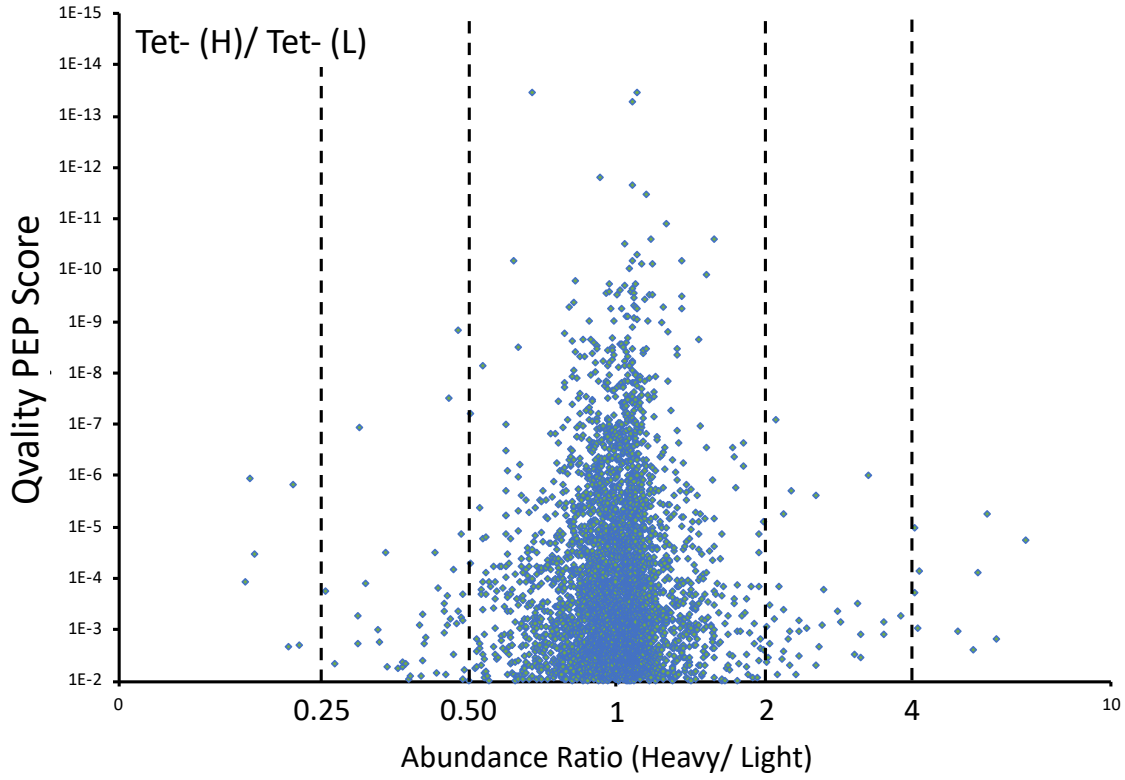
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Tb427tmp.160.4770 is not necessary for endocytosis of tomato lectin or haptoglobin/ hemoglobin. Knockdown of Tb427tmp.160.4770 was induced for 24 hours with 1 μg/mL tetracycline. Cells were incubated with 20 μg/mL Tomato Lectin-AlexaFluor-488 (TL-488) or 38 nM Haptoglobin/Haemoglobin- AlexaFluor-488 (H/H-488) for 15 minutes at 37°C. Fluorescence was analysed with flow cytometry (15,000 events per sample). Quantitation of median fluorescence of TL-488 or H/H-488 in uninduced (Tet-) and knockdown induced (Tet+) cells. Bars represent mean (TL-488 p= 0.85, n=3; H/H-488 p= 0.41, n=2).



Supplemental Figure 2. Knockdown of Tb160.4770 disrupts DNA organelle duplication. Trypanosomes were seeded at 5x10^4 cells/mL and knockdown induced with 1 μg/mL tetracycline for 24 hours. Cells were pelleted, fixed, mounted, and DNA containing organelles stained with 1.5 μM DAPI in Vectashield. A) Box plot shows median and minimum/maximum of three independent biological replicates. Chi square statistical analysis determined significance of difference in population distribution between uninduced (Tet-) and induced (Tet+) cells (d.f.=3). B) Representative images and classification of 1K1N, 2K1N, 2K2N and examples of abnormal trypanosome populations.



Supplemental Figure 3. Steady-State Phosphoproteome of *T. brucei*. Bloodstream form trypanosomes stably transfected with Tb427tmp.160.4770 p2T7- RNAi construct were labeled in heavy (H) or light (L) labeled HMI-9 SILAC media. Cells were collected ($4x10^7$), mixed, lysed, and trypsin digested. Peptides were isolated on a SepPak C18 column. Phosphopeptides were enriched by immobilized metal affinity chromatography (IMAC) and identified by LC-MS/MS. Representative proteome profiles of control knockdown uninduced cells (Tet-)/ uninduced cells (Tet-) (heavy/ light relative abundance ratio).

CHAPTER 3

FUTURE WORK

This work has highlighted the usefulness of small-molecules as tools to study trypanosome biology. We used AEE788 to perturb trypanosome transferrin endocytosis and, in combination with phospho-proteomic analysis, identified a pseudokinase Tb427tmp.160.4770 as a potential regulator of the pathway (Sullenberger et al., 2017). Studies presented in chapter 2, confirmed the role of Tb427tmp.160.4770 as a regulator of transferrin endocytosis, and identified new proteins potentially involved in endocytosis pathways. We discovered six proteins whose phosphorylation status was affected by both AEE788 treatment and knockdown of Tb427tmp.160.4770 (Chapter 2, Table 4). Work on these six unstudied "hypothetical proteins' including Tb427.05.8000, Tb42710.15040, and Tb427tmp.01.1960, offers the opportunity to assign biological function to trypanosome proteins of unknown function. This new information will make contributions to the annotation of the kinetoplastid genome at TriTrypDB.org. Finally, documentation of these proteins as regulators of endocytic pathways will bring us closer to the goal of defining the mechanism of transferrin endocytosis in *T. brucei*. Future work on Tb427tmp.160.4770 is also exciting as it is the first pseudokinase to be assigned to a biological pathway in the African trypanosome.

Several ideas come to mind with regard to completing some of the work reported in this dissertation. To complete the set of studies described in Chapter 2, it will be valuable to generate a control construct for RNAi specificity against Tb427tmp.160.4770. This is to ensure that knockdown phenotypes described in Chapter 2 are the result of decreased selective expression of Tb427tmp.160.4770 alone, and not due to non-specific RNAi

effects (Cullen, 2006; Jackson et al., 2003). To achieve this goal, numerous techniques may be employed (Sharma & Rao, 2009). A common practice involves generating an additional RNAi construct that targets a different DNA sequence within the gene of interest for RNAi degradation (Sharma & Rao, 2009). Reproduction of original phenotypes with the alternate RNAi sequence confirms specificity of RNAi to the desired target. In a second path to verify RNAi specificity, one generates an RNAi resistant version of the target gene (Cullen, 2006; Editorial, 2003) by engineering a version of that maintains the amino acid sequence but has a different nucleotide sequence. This recombinant gene is used to either replace an endogenous copy with or is ectopically expressed. When co-expressed with the RNAi-susceptible version, the resistant gene can rescue the phenotype produced after induction of RNAi. Expression of RNAi resistant genes, however, is limited by efficiency of expression (Cullen, 2006). We advocate use of this second strategy to confirm specificity of RNAi against Tb427tmp.160.4770 (Grewal et al., 2016).

Future work stemming from the results presented here can proceed in two ways. One path could focus on the larger picture of the trypanosome transferrin endocytosis pathway. Many proteins in this biological pathway are still unknown (G. W. Morgan, Hall, B., Denny, P., Carrington, M., Field, M., 2002; G. W. Morgan, Hall, B., Denny, P., Field, M., Carrington, M., 2002), but we have identified promising candidates affected by both loss of Tb427tmp.160.4770 and treatment of trypanosomes with AEE788 for the investigation of their roles in transferrin endocytosis, including three proteins whose functions are not currently known and have not been placed in any biological pathways (chapter two, Table 4). We therefore propose investigating these three hypothetical proteins through genetic knockdown to investigate and characterize their biological function for the first time. Additionally, as described in chapter two, stable isotope labelling of amino acids in cell culture (SILAC) (Ong, 2002) has been a useful tool in identifying

pathway proteins of Tb427tmp.160.4770, but we have thus far only used SILAC to study Tb427tmp.160.4770 phospho-signalling pathways. Expanding the scope of our SILAC experiments to all proteins affected by loss of Tb427tmp.160.4770, not just phospho-proteins, could provide insight into the complete set of pathways influenced by Tb427tmp.160.4770, offering new means by which the pseudokinase might influence transferrin endocytosis.

Protein-protein interactions are crucial for the functions of pseudokinases (reviewed in (Reiterer et al., 2014)). Therefore, it is important to investigate the interactome of Tb427tmp.160.4770 to understand its actions. To do this, we propose generating a fusion construct with a promiscuous biotin ligase, such as modified BirA (BirA*) from *E. coli* (Roux, Kim, & Burke, 2013). BioID utilizes biotin ligase to non-specifically label proteins within a radius of ten nanometers from the bait protein, in this case Tb427tmp.160.4770, fused to the biotin ligase (Roux et al., 2013). BioID has been successfully utilized in *T. brucei* previously (Varnaite & MacNeill, 2016). Using this strategy, we hope to determine the interactome of Tb427tmp.160.4770 and identify potential binding partners, which are the means through which pseudokinases like Tb427tmp.160.4770 modulate cellular processes.

Studies of the pseudokinase Tb427tmp.160.4770 (Chapter 2) has provided insight into the regulation of the transferrin endocytosis pathway in the African trypanosome. Our proposed future work may address the mechanism by which the first trypanosome pseudokinase impacts endocytosis pathways in the trypanosome.

Literature Cited

Ackers, J. P., Dhir, V., & Field, M. C. (2005). A bioinformatic analysis of the RAB genes of Trypanosoma brucei. *Mol Biochem Parasitol, 141*(1), 89-97.

Alibu, V. P., et al. (2005). "A doubly inducible system for RNA interference and rapid RNAi plasmid construction in Trypanosoma brucei." Mol Biochem Parasitol **139**(1): 75-82.

Allen, C. L., Goulding, D., & Field, M. C. (2003). Clathrin-mediated endocytosis is essential in Trypanosoma brucei. *EMBO J, 22*(19), 4991-5002.

Aslett, M., et al. (2010). "TriTrypDB: a functional genomic resource for the Trypanosomatidae." Nucleic Acids Res **38**(Database issue): D457-462.

Behera, R., Thomas, S. M., & Mensa-Wilmot, K. (2014). New chemical scaffolds for human african trypanosomiasis lead discovery from a screen of tyrosine kinase inhibitor drugs. *Antimicrob Agents Chemother, 58*(4), 2202-2210.

Bonifacino, J. S., & Lippincott-Schwartz, J. (2003). Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol, 4*(5), 409-414.

Boucrot, E., et al. (2010). "Roles of AP-2 in clathrin-mediated endocytosis." PLoS One **5**(5): e10597.

Brun, R., Blum, J., Chappuis, F., Burri, C. (2010). "Human African trypanosomiasis." Lancet **6736**(09): 60829-60821.

Chappie, J. S., Acharya, S., Leonard, M., Schmid, S. L., & Dyda, F. (2010). G domain dimerization controls dynamin's assembly-stimulated GTPase activity. *Nature, 465*(7297), 435-440.

Conner, S. D. and S. L. Schmid (2002). "Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis." J Cell Biol **156**(5): 921-929.

Conner, S. D., et al. (2003). "AAK1-Mediated *μ2* Phosphorlyation is Stimulated by Assembled Clathrin." Traffic **4**: 885-890.

Coppens, I., et al. (1987). "Receptor-mediated endocytosis in the bloodstream form of Trypanosoma brucei." J Protozool **34**(4): 465-473.

Cullen, B. R. (2006). Enhancing and confirming the specificity of RNAi experiments. *Nat Methods, 3*(9), 677-681.

Editorial. (2003). Whither RNAi? *Nat Cell Biol, 5*(6), 489-490.

Eyers, P. A., & Murphy, J. M. (2013). Dawn of the dead: protein pseudokinases signal new adventures in cell biology. *Biochem Soc Trans, 41*(4), 969-974.

Ferguson, S. M., & De Camilli, P. (2012). Dynamin, a membrane-remodelling GTPase. *Nat Rev Mol Cell Biol, 13*(2), 75-88.

Ferguson, S. M., Raimondi, A., Paradise, S., Shen, H., Mesaki, K., Ferguson, A., et al. (2009). Coordinated actions of actin and BAR proteins upstream of dynamin at endocytic clathrin-coated pits. *Dev Cell, 17*(6), 811-822.

Field, M. C. and M. Carrington (2009). "The trypanosome flagellar pocket." Nat Rev Microbiol **7**(11): 775-786.

Gabernet-Castello, C., et al. (2009). "The single ENTH-domain protein of trypanosomes; endocytic functions and evolutionary relationship with epsin." Traffic **10**(7): 894-911.

Gaidarov, I. and J. H. Keen (1999). "Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits." J Cell Biol **146**(4): 755-764.

Grab, D., Wells, CW, Shaw, MK, Webster, P., Russo, Dc. (1992). Endocytosed transferrin in African trypanosomes is delivered to lysosomes and may not be recycled. *Eur J Cell Biol., 59*(2), 398-404.

Grewal, J. S., McLuskey, K., Das, D., Myburgh, E., Wilkes, J., Brown, E., et al. (2016). PNT1 Is a C11 Cysteine Peptidase Essential for Replication of the Trypanosome Kinetoplast. *J Biol Chem, 291*(18), 9492-9500.

Guyett, P. J., Behera, R., Ogata, Y., Pollastri, M., & Mensa-Wilmot, K. (2017). Novel Effects of Lapatinib Revealed in the African Trypanosome by Using Hypothesis-Generating Proteomics and Chemical Biology Strategies. *Antimicrob Agents Chemother, 61*(2).

Guyett, P. J., Xia, S., Swinney, D. C., Pollastri, M. P., & Mensa-Wilmot, K. (2016). Glycogen Synthase Kinase 3 β Promotes the Endocytosis of Transferrin in the African Trypanosome. *ACS Infect Dis, 2*(7), 518-528.

Hanks, S. K. and T. Hunter (1995). "Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification." FASEB J **9**(8): 576-596.

Hirumi, H., Hirumi, K. (1989). "Continuous cultivation of Trypanosoma brucei blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers." J Parasitol **75**(6): 985-989.

Hu, H., Zhou, Q., & Li, Z. (2015). SAS-4 Protein in Trypanosoma brucei Controls Life Cycle Transitions by Modulating the Length of the Flagellum Attachment Zone Filament. *J Biol Chem, 290*(51), 30453-30463.

Huang, K. P. (1989). The mechanism of protein kinase C activation. *Trends Neurosci, 12*(11), 425-432.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., et al. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol, 21*(6), 635-637.

Jing, S. Q., et al. (1990). "Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization." J Cell Biol **110**(2): 283-294.

Kabiri, M., & Steverding, D. (2000). Studies on the recycling of the transferrin receptor in Trypanosoma brucei using an inducible gene expression system. *Eur J Biochem, 267*(11), 3309-3314.

Katiyar, S., Kufareva, I., Behera, R., Thomas, S. M., Ogata, Y., Pollastri, M., et al. (2013). Lapatinib-binding protein kinases in the African trypanosome: identification of cellular targets for kinase-directed chemical scaffolds. *PLoS One, 8*(2), e56150.

Kirchhausen, T. (2000). Clathrin. *Annu Rev Biochem, 69*, 699-727.

Koslowsky, D. J. (2004). "A historical perspective on RNA editing: how the peculiar and bizarre became mainstream." Methods Mol Biol **265**: 161-197.

Liang, X. H., et al. (2003). "trans and cis splicing in trypanosomatids: mechanism, factors, and regulation." Eukaryot Cell **2**(5): 830-840.

Lim, W. A., & Pawson, T. (2010). Phosphotyrosine signaling: evolving a new cellular communication system. *Cell, 142*(5), 661-667.

Maier, A. and D. Steverding (1996). "Low affinity of Trypanosoma brucei transferrin receptor to apotransferrin at pH 5 explains the fate of the ligand during endocytosis." FEBS Lett **396**(1): 87-89.

Manna, P., Obado, S., Boehm, C., Gadelha, C., Sali, A., Chait, B., Rout, M., Field, MC. (2017). Lineage-specific proteins essential for endocytosis in trypanosomes. *J Cell Sci, 130*(8), 1379-1392.

Marks, B., Stowell, M. H., Vallis, Y., Mills, I. G., Gibson, A., Hopkins, C. R., et al. (2001). GTPase activity of dynamin and resulting conformation change are essential for endocytosis. *Nature, 410*(6825), 231-235.

Mayer, T. U. (2003). Chemical genetics: tailoring tools for cell biology. *Trends Cell Biol, 13*(5), 270-277.

Mayle, K. M., Le, A. M., & Kamei, D. T. (2012). The intracellular trafficking pathway of transferrin. *Biochim Biophys Acta, 1820*(3), 264-281.

McMahon, H. T., & Boucrot, E. (2011). Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol, 12*(8), 517-533.

Medina-Acosta, E., Cross, GA. (1993). "Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure." *Mol Biochem Parasitol, 59*(2): 327-329.

Morgan, G. W., et al. (2004). "The single dynamin-like protein of Trypanosoma brucei regulates mitochondrial division and is not required for endocytosis." *J Biol Chem, 279*(11): 10692-10701.

Morgan, G. W., Hall, B., Denny, P., Carrington, M., Field, M. (2002). The kinetoplastida endocytic apparatus. Part I: a dynamic system for nutrition and evasion of host defences. *Trends in Parasitology, 18*(11), 491-496.

Morgan, G. W., Hall, B., Denny, P., Field, M., Carrington, M. (2002). The endocytic apparatus of the kinetoplastida. Part II: machinery and components of the system. *Trends in Parasitology, 18*(12), 540-546.

Morgan, G. W., Goulding, D., & Field, M. C. (2004). The single dynamin-like protein of Trypanosoma brucei regulates mitochondrial division and is not required for endocytosis. *J Biol Chem, 279*(11), 10692-10701.

Morriswood, B., Havlicek, K., Demmel, L., Yavuz, S., Sealey-Cardona, M., Vidilaseris, K., et al. (2013). Novel Bilobe Components in Trypanosoma brucei Identified Using Proximity-Dependent Biotinylation. *Eukaryot Cell, 12*(2), 356-367.

Oda, K., Matsuoka, Y., Funahashi, A., & Kitano, H. (2005). A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Syst Biol, 1*, 2005 0010.

Ong, S.-E., Blagoev, B., Kratchmarova, I., Kristensen, DB., Steen, H., Pandey, A., Mann, M. (2002). Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Molecular & Cellular Proteomics 1.5, 1*(5), 376-386.

Opperdoes, F. R. and P. Borst (1977). "Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome." FEBS Lett **80**(2): 360-364.

Overath, P., & Engstler, M. (2004). Endocytosis, membrane recycling and sorting of GPI-anchored proteins: Trypanosoma brucei as a model system. *Mol Microbiol, 53*(3), 735-744.

Parsons, M., Worthey, E. A., Ward, P. N., & Mottram, J. C. (2005). Comparative analysis of the kinomes of three pathogenic trypanosomatids: Leishmania major, Trypanosoma brucei and Trypanosoma cruzi. *BMC Genomics, 6*, 127.

Redmond, S., et al. (2003). "RNAit: an automated web-based tool for the selection of RNAi targets in Trypanosoma brucei." Molecular and Biochemical Parasitology **128**(1): 115-118.

Reiterer, V., Eyers, P. A., & Farhan, H. (2014). Day of the dead: pseudokinases and pseudophosphatases in physiology and disease. *Trends Cell Biol, 24*(9), 489-505.

Rohde, G., et al. (2002). "A phosphatidylinositol (4,5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis." J Cell Biol **158**(2): 209-214.

Roux, K. J., Kim, D. I., & Burke, B. (2013). BioID: a screen for protein-protein interactions. *Curr Protoc Protein Sci, 74*, Unit 19 23.

Saharinen, P., Takaluoma, K., & Silvennoinen, O. (2000). Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. *Mol Cell Biol, 20*(10), 3387-3395.

Salmon, D., Geuskens, M., Hanocq-Quertier, J., Nolan, D., Ruben, L., Pays, E. (1994). " A Novel Heterodimeric Transferrin Receptor Encoded by a Pair of VSG Expression Site-Associated Genes in T. brucei." Cell **78**: 75-86.

Schaefer, G., Akita, R. W., & Sliwkowski, M. X. (1999). A discrete three-amino acid segment (LVI) at the C-terminal end of kinase-impaired ErbB3 is required for transactivation of ErbB2. *J Biol Chem, 274*(2), 859-866.

Schell, D., Boroway, N., Overath, P. (1991). Transferrin is a growth factor for the bloodstream form of Trypanosomabrucei. *Parasitology Research, 77*(7), 558-560.

Sharma, S., & Rao, A. (2009). RNAi screening: tips and techniques. *Nat Immunol, 10*(8), 799-804.

Shen, S., Arhun, G., Ullu, E., Tschudi, C. (2001). "In vivo epitope tagging of Trypanosoma brucei genes using a one step PCR-based strategy." Molecular and Biochemical Parasitology **113**: 171-173.

Sherwin, T. and K. Gull (1989). "The cell division cycle of Trypanosoma brucei brucei: timing of event markers and cytoskeletal modulations." Philos Trans R Soc Lond B Biol Sci **323**(1218): 573-588.

Shi, F., et al. (2010). "ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation." Proc Natl Acad Sci U S A **107**(17): 7692-7697.

Shih, W., et al. (1995). "A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes." J Biol Chem **270**(52): 31083-31090.

Steverding, D. (1995). Transferrin-binding protein complex is the receptor for transferrin uptake in Trypanosoma brucei. *The Journal of Cell Biology, 131*(5), 1173-1182.

Subramanya, S., Hardin, C. F., Steverding, D., & Mensa-Wilmot, K. (2009). Glycosylphosphatidylinositol-specific phospholipase C regulates transferrin endocytosis in the African trypanosome. *Biochem J, 417*(3), 685-694.

Subramanya, S., & Mensa-Wilmot, K. (2010). Diacylglycerol-stimulated endocytosis of transferrin in trypanosomatids is dependent on tyrosine kinase activity. *PLoS One, 5*(1), e8538.

Sullenberger, C., Pique, D., Ogata, Y., & Mensa-Wilmot, K. (2017). AEE788 Inhibits Basal Body Assembly and Blocks DNA Replication in the African Trypanosome. *Mol Pharmacol, 91*(5), 482-498.

Taylor, S. S., Zhang, P., Steichen, J. M., Keshwani, M. M., & Kornev, A. P. (2013). PKA: lessons learned after twenty years. *Biochim Biophys Acta, 1834*(7), 1271-1278.

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

Woodward, R. and K. Gull (1990). "Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of Trypanosoma brucei." J Cell Sci **95 (Pt 1)**: 49-57.

Varnaite, R., & MacNeill, S. A. (2016). Meet the neighbors: Mapping local protein interactomes by proximity-dependent labeling with BioID. *Proteomics, 16*(19), 2503-2518.

Zerial, M., & McBride, H. (2001). Rab proteins as membrane organizers. *Nat Rev Mol CellBiol, 2*(2), 107-117.