Title
Microtubule plus-end binding proteins facilitate intracellular trypanosome infection

Journal
Cellular Microbiology, 15(4)

ISSN
1462-5814

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Publication Date
2013-04-01

DOI
10.1111/cmi.12056

Peer reviewed

Published in final edited form as:
Cell Microbiol. 2013 April ; 15(4): 571–584. doi:10.1111/cmi.12056.

Host microtubule plus-end binding protein CLASP1 influences sequential steps in the *Trypanosoma cruzi* infection process

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Abstract

Mammalian cell invasion by the protozoan parasite *Trypanosoma cruzi* involves host cell microtubule dynamics. Microtubules support kinesin-dependent anterograde trafficking of host lysosomes to the cell periphery where targeted lysosome exocytosis elicits remodeling of the plasma membrane and parasite invasion. Here, a novel role for microtubule plus-end tracking proteins (+TIPs) in the coordination of *T. cruzi* trypomastigote internalization and post-entry events is reported. Acute silencing of CLASP1, a +TIP that participates in microtubule stabilization at the cell periphery, impairs trypomastigote internalization without diminishing the capacity for calcium-regulated lysosome exocytosis. Subsequent fusion of the *T. cruzi* vacuole with host lysosomes and its juxtanuclear positioning are also delayed in CLASP1-depleted cells. These post-entry phenotypes correlate with a generalized impairment of minus-end directed transport of lysosomes in CLASP1 knockdown cells and mimic the effects of dynactin disruption. Consistent with GSK3β acting as a negative regulator of CLASP function, inhibition of GSK3β activity enhances *T. cruzi* entry in a CLASP1-dependent manner and expression of constitutively active GSK3β dampens infection. This study provides novel molecular insights into the *T. cruzi* infection process, emphasizing functional links between parasite-elicited signaling, host microtubule plus-end tracking proteins and dynein-based retrograde transport. Highlighted in this work is a previously unrecognized role for CLASPs in dynamic lysosome positioning, an important aspect of the nutrient sensing response in mammalian cells.

Introduction

Microtubules form polarized dynamic networks in cells that are responsive to extracellular signaling inputs. At the plus-ends, microtubule associated proteins, referred to as plus-end

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The authors have no conflict of interest.

tracking proteins (+TIPs), assemble as heterogeneous complexes, in a dynamic fashion, to influence tubulin polymer assembly and/or disassembly and to regulate the interactions of microtubules with the cell cortex and other cellular structures (recently reviewed in (Galjart, 2010, Jiang *et al.*, 2011, Kumar *et al.*, 2012). The core components of microtubule plus-ends are members of an evolutionarily conserved 'end-binding' (EB) protein family. EB1 and EB3 accumulate uniformly at the growing tip of microtubules (Tirnauer *et al.*, 2000) and mediate the binding of other plus-end tracking proteins such as CLIP-170 (Bieling *et al.*, 2008, Wittmann, 2008), cytoplasmic linker-associated proteins (CLASPs) (Akhmanova *et al.*, 2001, Komarova *et al.*, 2005, Mimori-Kiyosue *et al.*, 2005) and the large subunit of dynactin ($p150^{Glued}$) (Berrueta *et al.*, 1999). Mammalian CLASPs bind to microtubule plus-ends and play a key role in stabilizing microtubules, impacting mitotic spindle formation (Mimori-Kiyosue *et al.*, 2006) and dynamic processes at the cell periphery (Akhmanova *et al.*, 2001, Mimori-Kiyosue *et al.*, 2005). In epithelial cells, CLASPs undergo asymmetric distribution to accumulate at the leading edge where their interactions with components of the cell cortex regulate cytoskeletal dynamics (Wittmann *et al.*, 2005, Watanabe *et al.*, 2009). EB1 and CLIP-170 localize exclusively to microtubule plus ends, but the interaction of CLASPs with microtubules is spatially regulated. In the main body of migrating cells CLASP binding to microtubules is plus end-restricted whereas CLASPs bind along the length of microtubules in the lamella (Wittmann *et al.*, 2005). CLASPs regulate peripheral microtubule and actin dynamics via their interactions with actin (Tsvetkov *et al.*, 2007) and regulatory proteins, such as IQGAP (Watanabe *et al.*, 2009) and cell cortex associated proteins LL5β and ELKS (Lansbergen *et al.*, 2006b). Both the microtubule and IQGAP-binding activities of CLASPs are negatively regulated by phosphorylation at multiple sites by glycogen synthase kinase 3β (GSK-3β) (Akhmanova *et al.*, 2001, Watanabe *et al.*, 2009, Kumar *et al.*, 2009). Phosphorylation of GSK3β on Ser9 by the serine/threonine kinase, Akt inhibits its kinase activity (Pandey *et al.*, 1998). As such, CLASP-mediated microtubule dynamics at the plus-ends are likely to be responsive to signals that activate PI-3K/Akt signaling in mammalian cells (Akhmanova *et al.*, 2001, Lansbergen *et al.*, 2006b).

The microtubule network in mammalian cells is exploited by a variety of intracellular pathogens to facilitate their uptake and for formation, stabilization and maintenance of their intracellular vacuoles (Clausen *et al.*, 1997, Aiastui *et al.*, 2010, Oelschlaeger *et al.*, 1993, Schwan *et al.*, 2009). The protozoan parasite, *Trypanosoma cruzi* invades a wide variety of non-professional phagocytic cell types where cell invasive trypomastigotes subvert a universal calcium-dependent plasma membrane repair process for entry (Reddy *et al.*, 2001). Ca^{2+}-dependent exocytosis of host lysosomes in the vicinity of the parasite attachment site primes the host plasma membrane for invagination and parasite entry (Tardieux *et al.*, 1992, Rodriguez *et al.*, 1996, Rodriguez *et al.*, 1997, Reddy *et al.*, 2001, Fernandes *et al.*, 2011). Lysosome fusion with the parasite vacuole can coincide with its formation during the *T. cruzi* internalization process (Tardieux *et al.*, 1992), but more typically, lysosomes fuse with the parasite vacuole a post-entry step in the infection process (Woolsey *et al.*, 2003). Irrespective of differences in timing, lysosome fusion with the *T. cruzi* vacuole is essential for retention of internalized parasites and thus, for successful establishment of intracellular infection (Woolsey *et al.*, 2004, Andrade *et al.*, 2004). Host microtubule dynamics are important for facilitating T. cruzi entry into non-professional phagocytes where they have

been implicated in the targeting of lysosomes to the plasma membrane during *T. cruzi* entry (Rodriguez *et al.*, 1996, Tyler *et al.*, 2005). Live cell imaging reveals transient enrichment of GFP-α-tubulin at the parasite attachment site (Tyler *et al.*, 2005) and the directed movement of peripheral lysosomes toward the invading parasite (Rodriguez *et al.*, 1996). Disruption of anterograde lysosome transport in host cells with microinjected antibodies to kinesin heavy chain impedes cell entry by *T. cruzi*, as does chemical inhibition of host microtubule dynamics (Rodriguez *et al.*, 1996). Together, these observations support a model in which *T. cruzi* trypomastigotes exploit kinesin-based motility to transport host lysosomes to the cell periphery, where they undergo regulated exocytosis at the parasite attachment site to facilitate entry and vacuole biogenesis. Cytosolic free calcium transients triggered in host cells by invasive *T. cruzi* trypomastigotes (Tardieux *et al.*, 1994, Caler *et al.*, 2000) are associated with transient depolymerization of cortical actin microfilaments (Rodriguez *et al.*, 1995) and lysosome exocytosis (Rodriguez *et al.*, 1997, Fernandes *et al.*, 2011). *T. cruzi* also stimulates the localized production of $PI(3,4,5)P_3$ at the host cell plasma membrane in a class I PI-3K-dependent manner prior to entry, where chemical and genetic targeting of host PI-3K inhibited parasite uptake and lysosome fusion with the nascent parasite vacuole (Woolsey *et al.*, 2003). Despite this basic knowledge, the mechanisms by which *T. cruzi*-triggered signaling pathways integrate with host membrane and cytoskeletal dynamics to promote parasite entry remain largely unknown. Given the highly responsive nature of the cortical cytoskeleton to extracellular signals (Saarikangas *et al.*, 2010) and the key role played by microtubule plus-end binding proteins in cellular responses to these cues (Akhmanova *et al.*, 2001, Zhou *et al.*, 2004, Lansbergen *et al.*, 2006b), we sought to determine whether microtubule plus-end binding proteins participate in the *T. cruzi* infection process.

Results

T. cruzi trypomastigote invasion is oriented with respect to host microtubules

Previous studies have indicated that host microtubule dynamics contribute to the entry of non-professional phagocytes by *T. cruzi* tryopmastigotes (Rodriguez *et al.*, 1996, Tyler *et al.*, 2005). It has also been appreciated that *T. cruzi* trypomastigotes exhibit a strong preference for invasion at cell edges (Schenkman *et al.*, 1988). Using EB3-GFP to decorate microtubule plus-ends in polarized human foreskin fibroblasts (HFF), we observe that *T. cruzi* trypomastigotes display uniaxial entry at the elongated ends of fibroblasts, where they enter in a ‘head-on’ orientation to the direction of microtubule elongation (Fig. 1A-C). Examination of 200 random cells with invading or recently internalized parasites reveals that >80% *T. cruzi* trypomastigotes are oriented with host microtubules in a parallel fashion (Fig. 1D). Following invasion of host cells, *T. cruzi* transiently reside in tight-fitting vacuoles that accumulate host lysosomal markers within the first 60-90 minutes (Woolsey *et al.*, 2003). Parasite-containing vacuoles remain LAMP-positive for at least 8 hours (Chessler *et al.*, 2008) before their disruption (Ley *et al.*, 1990, Andrews *et al.*, 1990) and asynchronous release of parasites into the host cytosol (Chessler *et al.*, 2008). We noted here that internalized *T. cruzi* parasites move inward toward the host cell nucleus (Fig. 1E) where approximately half of the internalized parasites exhibit juxtanuclear localization 90 minutes post-entry (Fig. 1E). Although this number rapidly plateaus early in the infection (Fig. 1E),

all of the internalized parasites exhibit juxtanuclear positioning by 24 hours (data not shown). The biphasic nature of this process is curious and may reflect heterogeneity in the molecular composition of the parasite vacuole and its ability to interact with host machinery required for trafficking. The significance of juxtanuclear positioning of the *T. cruzi* vacuole is currently not known, however, the minus-end directed movement of internalized parasites suggests a role for microtubules and dynein-dynactin motor complexes.

CLASP1 depletion impairs *T. cruzi* internalization and delays lysosome fusion with the vacuole

Given the central role for microtubule plus-end tracking proteins (+TIPs) in regulating microtubule dynamics and their responsiveness to cellular signaling cues known to regulate *T. cruzi* entry, we sought to determine whether host microtubule +TIPs function in the establishment of intracellular *T. cruzi* infection. We focused on CLASPs as peripheral +TIPs that bridge interactions between microtubule plus-ends and the cell cortex (Akhmanova *et al.*, 2001). Enrichment of GFP-CLASP1/2 at the site of *T. cruzi* trypomastigote entry and surrounding recently internalized parasites in time-lapse imaging of live cells (Fig. 2; Fig. S1) is consistent with previous observations of transient localization of GFP-α-tubulin at the parasite attachment site (Tyler *et al.*, 2005). The relative enrichment of CLASP-GFP with invading or recently internalized *T. cruzi* trypomastigotes as compared to cytosolic GFP was quantified (Fig. 2E; Fig. S1).

Acute siRNA-mediated silencing of CLASP1 in HeLa cells (Fig. 3A) significantly compromised the capacity for *T. cruzi* trypomastigote invasion (Fig. 3B) without affecting Ca^{2+}-triggered lysosome exocytosis, as measured by the release of β-hexosaminidase in the presence of Ca^{2+} in streptolysin-O permeabilized cells (Fig. S2). This reduced capacity for parasite invasion in CLASP1-depleted cells was replicated with all 4 of the individual siRNA duplexes represented in the original siRNA CLASP1-targeting pool and with an independent pool with different targeting sequences (Fig. S3), providing confidence that the phenotypic effects of CLASP1 depletion are specific (ie. not owing to an ‘off-target’ effect). In addition to the impact of CLASP1-depletion on *T. cruzi* trypomastigote entry, the subsequent delivery of the lysosomal marker, LAMP-1, to the parasite vacuole was significantly delayed (Fig. 3C). Despite observations that CLASP1-GFP and CLASP2-GFP are enriched at the site of parasite entry and around recently internalized parasites (Fig. 2), acute silencing of endogenous CLASP2 (Fig. S4A), which shares a high degree of homology with CLASP1 with reported overlapping/redundant functions (Akhmanova *et al.*, 2001, Mimori-Kiyosue *et al.*, 2005), failed to impact *T. cruzi* invasion and LAMP1-delivery to the parasite-containing vacuole (Fig. S4B,C). A notable difference between CLASP1- and CLASP2-silenced cells is the reduction of actin stress fibers that is consistently associated with CLASP1 knockdown (Fig. S4D and data not shown). As CLASP1 transcript levels are ~3-fold higher than CLASP2 transcripts in siControl-transfected HeLa cells (Fig. S4A) it is possible that the phenotypic effects associated with CLASP1-silencing are only observed when a critical threshold of CLASP is reached in the cell and that targeting CLASP2 fails to lower the total CLASP to this threshold. The observation that cell infection by *Toxoplasma gondii*, a protozoan parasite with a completely different mode of entry, was not impaired in CLASP1-depleted cells (Fig. S5) suggests that cellular phenotypes associated with CLASP1

knockdown are related to the specific mode of host cell entry exploited by *T. cruzi* trypomastigotes (Caradonna *et al.*, 2011).

To relate the CLASP1 phenotype to microtubule dynamics at the plus-ends we examined the impact of silencing the end-binding protein, EB1 on *T. cruzi* entry and kinetics of LAMP1-delivery to the parasite vacuole. EB1 is considered a 'core' component of the +TIPs, since virtually all other +TIPs, including CLASP1, bind EB1 directly and require EB1 for plus-end localization, whereas EB1 itself can track microtubule ends autonomously. Unlike CLASP1, acute silencing of EB1 (Fig. 3D) failed to affect *T. cruzi* entry (Fig. 3E) and did not significantly impact LAMP1 accumulation in the parasite vacuole (Fig. 3F). Similar findings were observed when EB2 and EB3 (homologues of EB1; (Lansbergen *et al.*, 2006a)) were targeted in HeLa cells, singly or in combination with EB1 (data not shown). We next tested the role for dynactin, a large multi-protein complex that interacts with microtubules and dynein motors, which has been implicated in microtubule-dependent vesicular trafficking (Burkhardt *et al.*, 1997, Habermann *et al.*, 2001, Valetti *et al.*, 1999, Vaughan, 2005). A core component of dynactin, $p150^{Glued}$ (DCTN1), colocalizes with EB1 at microtubule plus-ends and facilitates loading of vesicular cargo onto dynein (Vaughan *et al.*, 2002). While acute silencing of DCTN1 (Fig. 3G) failed to inhibit *T. cruzi* entry (Fig. 3H) a marked delay in LAMP1 delivery to the parasite vacuole was observed (Fig. 3I) similar to the effect of CLASP1 depletion (Fig. 3C).

+TIPs modulate the juxtanuclear positioning of the *T. cruzi* vacuole

Following host cell entry, internalized *T. cruzi* migrate toward the host nucleus where they undergo a developmental switch and eventually lyse the vacuole to become cytoplasmically localized (Andrews *et al.*, 1990). To assess the impact of +TIP depletion on the inward movement of the *T. cruzi* vacuole, HFF transfected with control non-targeting siRNA or siRNAs targeting CLASP1 or EB1, were pulsed with *T. cruzi* trypomastigotes for 15 minutes and the juxtanuclear localization of parasite-containing vacuoles was evaluated at 2 hours post-infection, a time point at which approximately half of the internalized parasites have migrated to the nucleus (Fig. 1E). Fewer internalized parasites achieved a juxtanuclear position by 2 hrs in EB1- or CLASP1-silenced cells (Fig. 4A) or in cells overexpressing p50 dynamitin, a subunit of the dynactin complex which disrupts dynein-dependent cargo transport (Burkhardt *et al.*, 1997) (Fig. 4B). In agreement with other phenotypic differences observed between CLASP1- and CLASP2-depleted cells, acute silencing of CLASP2 fails to delay juxtanclear positioning of internalized *T. cruzi* (Fig. S4E). Together, these data highlight the critical influence of CLASP1 expression on *T. cruzi* trypomastigote entry as well as the post-entry steps of lysosome-vacuole fusion and juxtanuclear positioning of the parasite vacuole and suggest a potential coupling of entry and post-entry events in the parasite infection process. Unlike CLASP1, the core microtubule end-binding protein EB1 and dynactin were dispensable for *T. cruzi* internalization. However, dynactin influenced the post-entry steps of lysosome-vacuole fusion and the inward migration of the parasite-containing vacuole as did EB1.

CLASPs influence dynamic positioning of lysosomes in mammalian cells

Our observation that depletion of CLASP1 or dynactin (DCTN1) in mammalian cells exerts similar effects on the post-entry processes of lysosome marker delivery to the *T. cruzi* vacuole and its retrograde transport toward the host cell nucleus, lead us to investigate whether CLASP1 expression influences minus-end directed lysosome transport, a dynein-dependent process (Jordens *et al.*, 2001). To test this, we exploited the ability to induce rapid redistribution of lysosomes by altering cytosolic pH (Heuser, 1989). At neutral pH, lysosomes in control and CLASP1-depleted cells display similar distributions (Fig. 5; pH 7.2). However, the rapid perinuclear clustering of lysosomes triggered by a shift to alkaline pH conditions is significantly impaired in CLASP1 knockdown cells (Fig. 5; pH 7.6). Similar results were obtained when starvation was used as the stimulus to promote perinuclear localization of lysosomes (data not shown) (Korolchuk *et al.*, 2011). Here we show that lysosome redistribution under alkaline conditions is also compromised in CLASP2-depleted cells as compared to control cells (Fig. S6) however the impairment appears to be less pronounced than in CLASP1-silenced cells. These novel findings indicate that in addition to its role in microtubule plus-end stabilization, CLASPs plays an important role in microtubule-dependent lysosome positioning in cells similar to that observed for dynein or $p150^{Glued}$ (Caviston *et al.*, 2011, Tan *et al.*, 2011).

Inactivation of GSK-3β increases *T. cruzi* entry

CLASP recruitment to microtubule plus-ends and its interaction with EB1 and IQGAP are negatively regulated by GSK3β-dependent phosphorylation at multiple sites (Watanabe *et al.*, 2009, Kumar *et al.*, 2009). While the majority of the work characterizing these interactions have been focused on CLASP2, homology between CLASP1 and CLASP2 and the conservation of putative GSK3β phosphorylation sites, suggests that CLASP1 is also negatively regulated by GSK3β. The activity of GSK3β is inhibited when phosphorylated on Ser9 by the serine/threonine kinase Akt (Jope *et al.*, 2004) which becomes activated in a PIP_3-dependent manner downstream of class I PI-3Ks (Coffer *et al.*, 1998). The elevated levels of phospho-Akt^{Ser473} and phospho-$GSK3\beta^{Ser9}$ triggered in response to *T. cruzi* (Fig. 6A), is consistent with the ability of these parasites to exploit host PI-3K-dependent signaling for invasion (Woolsey *et al.*, 2003). Consistent with a role for CLASP1 in *T. cruzi* entry, chemical inhibition of GSK3β predicted to increase CLASP1 functionality, enhanced trypomastigote invasion (Fig. 6B) in a CLASP1-dependent manner (Fig. 6C). Conversely, expression of constitutively active GSK3β-S9A, which is known to inhibit CLASP binding of microtubule plus-ends (Wittmann *et al.*, 2005, Akhmanova *et al.*, 2001), hinders *T. cruzi* invasion (Fig. 6D). Thus, with its clear influence on *T. cruzi* internalization and functional regulation downstream of the Akt-GSK3β signaling axis, CLASP1 is well positioned to integrate parasite-elicited signals with host microtubule dynamics to influence *T. cruzi* internalization. Moreover, the extended influence of CLASP1 on post-entry steps in the *T. cruzi* infection process suggests that cellular processes involved in the establishment of intracellular *T. cruzi* infection, normally considered as distinct (internalization, vacuole biogenesis/maturation, vacuole trafficking), are likely to exploit overlapping molecular mechanisms.

Discussion

Establishment of intracellular infection by *Trypanosoma cruzi* trypomastigotes is critical to the pathogenesis of human Chagas' disease, where the importance of host microtubule dynamics in the process of *T. cruzi* entry into non-professional phagocytic cells has been documented (Tardieux *et al.*, 1992, Rodriguez *et al.*, 1996, Tyler *et al.*, 2005). Host microtubules associate with invading *T. cruzi* trypomastigotes and their nascent vacuoles (Tyler *et al.*, 2005). Microtubules function as a conduit for the kinesin-dependent, anterograde transport of lysosomes to the parasite attachment site (Rodriguez *et al.*, 1996) where regulated lysosome exocytosis facilitates *T. cruzi* internalization (Tardieux *et al.*, 1992, Rodriguez *et al.*, 1996, Rodriguez *et al.*, 1997, Reddy *et al.*, 2001, Fernandes *et al.*, 2011). Results from the present study expand our view of the role of host microtubules in the *T. cruzi* infection process, revealing the marked influence of microtubule plus-end tracking proteins on trypomastigote internalization, vacuole biogenesis and retrograde transport of the parasite vacuole (Fig. 7). Accompanying these findings is the demonstration of a previously unrecognized role for CLASPs in dynamic lysosome positioning in mammalian cells, which has important implications for nutrient sensing, autophagy and innate immunity (Korolchuk *et al.*, 2011).

Acute silencing of CLASP1 results in significant impairment of *T. cruzi* trypomastigote invasion of mammalian cells without perturbing anterograde movement of lysosomes (data not shown) or the capacity for Ca^{2+}-regulated lysosome exocytosis (Rodriguez *et al.*, 1997). These findings suggest that CLASP1 expression influences events in the *T. cruzi* invasion process downstream of the kinesin-based delivery of lysosomes to the cell periphery (Rodriguez *et al.*, 1996). Initially puzzling was the observation that silencing of CLASP2, a closely related functional homologue of CLASP1 (Mimori-Kiyosue *et al.*, 2005), fails to replicate the parasite infection phenotypes observed in CLASP1-depleted cells. However, the low level of endogenous CLASP2 mRNA expression in HeLa cells suggests that silencing of CLASP2 does not sufficiently reduce the functional pool of CLASPs in the cell, whereas silencing of the more abundant CLASP1 does. The observation that CLASP2 silencing resulted in a weaker impairment of pH-dependent lysosome distribution in mammalian cells than CLASP1 depletion supports this idea. Alternatively, CLASP1 may exhibit functions that impact the establishment of intracellular *T. cruzi* infection that are independent of those of CLASP2.

By analyzing the consequences of depleting host microtubule plus-end tracking proteins on the *T. cruzi* infection process, a general hierarchy emerged in which CLASP1 expression impacts both parasite entry and post-entry events, whereas dynactin and EB1 function at steps downstream of parasite internalization (Fig. 7). Disruption of dynactin function, which has a well-established role in the maturation of phagosomes and other pathogen vacuoles (Blocker *et al.*, 1997, Jordens *et al.*, 2001, Harrison *et al.*, 2003) delays lysosome fusion with the *T. cruzi* vacuole as well as retrograde transport of internalized parasites, similar to that observed for CLASP1-depletion. In contrast, silencing of EB1, which is required for the plus-end localization of dynactin $p150^{Glued}$ via CLIP-170 (Watson *et al.*, 2006) fails to impact the kinetics of lysosome marker delivery to the *T. cruzi* vacuole. While this observation is consistent with previous reports that dynactin can function independently of

microtubule plus-ends (Watson *et al.*, 2006), the involvement of CLASP1, dynactin and EB1 in the juxtanuclear positioning of the parasite vacuole supports a role for microtubule plus-end stabilization in facilitating interaction of the *T. cruzi* vacuole with dynactin-dynein motor complexes to drive the inward movement of the vacuole. Our finding that CLASP1 depletion impairs the dynein and microtubule-dependent process of perinuclear lysosome aggregation in response to increased cytosolic pH (Heuser, 1989, Caviston *et al.*, 2011), suggests a similar mechanism of organelle/vacuole 'capture' and transport (Vaughan *et al.*, 2002). involving the early participation of CLASP1

The enrichment of CLASP-GFP at the *T. cruzi* entry site is consistent with its role in the early stages of the parasite internalization process. Given that the role of CLASP1 in *T. cruzi* invasion can be uncoupled from other microtubule plus-end binding proteins (EB1 and dynactin) points to unique molecular interactions between CLASP1 and other cellular proteins as critical for influencing the parasite internalization process. Dynamic interactions between CLASPs and the actin regulatory protein IQGAP (Watanabe *et al.*, 2009) or cell cortex-associated proteins (LL5beta and ELKS) (Lansbergen *et al.*, 2006b) may impact *T. cruzi* internalization, directly or indirectly, by modulating cortical actin filament dynamics. Although the *T. cruzi* entry process is frequently referred to as 'actin-independent' because cortical actin polymerization is not a requirement for trypomastigote invasion of non-professional phagocytic cells (Schenkman *et al.*, 1991, Tardieux *et al.*, 1992), this description is misleading given that cortical actin depolymerization, which is associated with parasite-elicited Ca^{2+}-transients, is known to facilitate parasite entry (Tardieux *et al.*, 1994, Rodriguez *et al.*, 1995). Moreover, while cytochalasin D-mediated inhibition of actin polymerization initially enhances *T. cruzi* trypomastigote internalization in many mammalian cell types (Tardieux *et al.*, 1992), recovery from cytochalasin D treatment is essential to promote fusion of early endosomes and lysosomes with the parasite vacuole (Woolsey *et al.*, 2004). Moreover, with prolonged impairment of actin microfilament dynamics there is significant loss of internalized parasites (Woolsey *et al.*, 2004). Thus, host actin dynamics are important for *T. cruzi* trypomastigote invasion and cellular retention (Tardieux *et al.*, 1992)Rodriguez et al., 1995, (Woolsey *et al.*, 2004). As CLASP1 depletion results in loss of actin stress fibers (Fig. S4), we cannot rule out the possibility that the influence of CLASP1 on *T. cruzi* invasion is related to altered actin microfilament dynamics independent of the host microtubule network.

CLASP binding to microtubules and to IQGAP is negatively regulated by the serine/threonine kinase GSK3β where chemical inhibition of GSK3β enhances CLASP localization to microtubules and stabilizes microtubules at the leading edge of migrating cells (Kumar *et al.*, 2009, Watanabe *et al.*, 2009). Consistent with a role for GSK3β-dependent inhibition of CLASP1, inhibition of GSK3β activity enhances *T. cruzi* entry in a CLASP1-dependent manner, whereas expression of constitutively active GSK3β dampens infection. Thus, localized activation of class I_A PI-3 kinases and PIP_3 accumulation at the *T. cruzi* invasion site (Woolsey *et al.*, 2003) may influence the microtubule and IQGAP-binding activity of CLASPs following Akt-dependent phosphorylation of GSK3β. While we were able to demonstrate that exposure of mammalian cells to *T. cruzi* trypomastigotes results in increased phosphorylation of GSK3β on Ser9, which would lead to inactivation of the kinase

(Jope *et al.*, 2004), we have not been able to detect changes in the phosphorylation state of CLASPs in response to *T. cruzi*-triggered signaling at a population level despite several attempts (data not shown). Given the transient and asynchronous nature of *T. cruzi*-elicited signaling in mammalian host cells (Caler *et al.*, 2000, Woolsey *et al.*, 2003), more sensitive methods will be needed to detect changes in the phosphorylation state of CLASP at the single cell level. However, coupled with the fact that IQGAP activities are modulated by Ca^{2+}-calmodulin (Atcheson *et al.*, 2011), and CLASP-binding to IQGAP and EB1 is regulated by GSK3β, CLASPs are well-positioned both spatially and biochemically to integrate PI-3K and Ca^{2+}-dependent signaling pathways triggered in mammalian host cells early in the *T. cruzi* entry process. Based on current knowledge, both CLASP1 and CLASP2 should be equally competent in this regard.

Overall, this study provides novel information regarding the role of host microtubule plus-end binding proteins in the establishment of intracellular infection by the protozoan parasite, *Trypanosoma cruzi*. CLASP1 plays an important role in parasite internalization and in the dyactin-dependent events of vacuole maturation and its minus-end directed movement. These findings suggest that mammalian CLASPs serve as a critical point of integration for *T. cruzi*-dependent signaling to the actin and microtubule networks in host cells and couple parasite entry with lysosome fusion and juxtanuclear localization of the parasite-containing vacuole. A novel finding of general interest to cell biologists is the role of CLASPs in dynamic positioning of lysosomes in the cell where their minus-end directed movement is impaired in the absence of CLASP1 and to a lesser degree CLASP2. While the role of CLASP1 in the regulation of actin dynamics is not yet understood, recent studies that have identified several new CLASP-interacting proteins in *Drosophila* are certain to pave the way for more mechanistic studies (Lowery *et al.*, 2010). Exploiting pathogens as perturbagens of the CLASP interactome (Schwan *et al.*, 2009) has the potential to illuminate novel biology and will create opportunities to enhance our understanding of the molecular and cellular basis for establishment of intracellular infection by this important human pathogen.

Experimental Procedures

siRNAs, plasmids, and adenovirus

Gene-specific siGENOME SMARTpools containing 4 individual siRNAs targeting human CLASP1, CLASP2, EB1, EB3, DCTN1, a non-targeting control pool siRNA2 (siRNA#2) and individual siRNAs targeting CLASP1 were purchased from Dharmacon (ThermoScientific). siRNAs targeting CLASP1 were: siGENOME SMARTpool M-00683-01-0005 containing 4 siRNA duplexes, the individual duplexes deconvoluted from this pool: D-006831-01-005, D-006831-02-005, D-006831-04-005, D-006831-17-005 and ON-TARGETplus SMARTpool L-006831-00-0005. EB3-GFP, CLASP1-GFP, CLASP2-GFP plasmids were characterized as described (Akhmanova *et al.*, 2001). p50-GFP plasmid was kindly provided by Dr. Vaughan (University of Notre Dame). Constitutively active GSK3β-S9A adenovirus and GFP adenovirus were tested previously (Kumar *et al.*, 2009).

Cell culture, transfection and *T. cruzi* infection

HeLa and human foreskin fibroblasts (HFF), purchased from the American Type Culture Collection (ATCC), were maintained as sub-confluent monolayers in Dulbecco's modified Eagle's Medium (DMEM) containing 4.5g/l glucose (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen). Gene silencing in HeLa cells was achieved using a reverse transfection protocol where 3.5×10^4 HeLa cells were suspended in 115 μl Opti-MEM (Invitrogen) with 1.7 μ l Oligofectamine (Invitrogen) and 25 or 50 nM of a gene-specific or non-targeting siRNA pool. Cells were seeded on $12mm^2$ glass coverslips in 24-well plates (Corning), adjusted to 10% FBS in DMEM and incubated at 37°C, 5% CO_2 for 48 hours prior to infection with *T. cruzi*. Transient transfection of HFF was achieved by nucleofection using an Amaxa Nucleofector™II (Lonza) according to the manufacturer's specifications. Briefly, 5×10^5 HFF suspended in transfection buffer (Amaxa human dermal fibroblast nucleofector kit, Lonza) were mixed with 50nM SMARTpool siRNA or 1μ g plasmid DNA and immediately seeded onto glass coverslips in 6-well plates. To identify transfected cells siRNAs were co-transfected with red fluorescent siRNA tracker (Dharmacon D-001630-02-05). Transfected cells were grown for 48 hr prior to *T. cruzi* infection or cell treatments. To determine knockdown efficiency, western blots of total cell lysates were probed with specific antibodies.

Parasite infections

T. cruzi trypomastigotes (Tulahuén) isolated from LLcMK2 monolayers as described (Woolsey *et al.*, 2003) were washed in Ringers/BSA or DMEM and incubated with transfected cells for 15 minutes at 37°C. Cells were washed five times with 2% FBS DMEM and incubated in 2% FBS DMEM for indicated times. To inhibit mammalian GSK3β, HeLa cells were pretreated with 1 μM of the GSK3β inhibitor BIO (Tocris Bioscience) for 30 min, washed three times with 2% FBS DMEM and infected with *T. cruzi* for 15 min. Infected cells were fixed in 2% paraformaldehyde/PBS and relative *T. cruzi* invasion was determined by anti-*T. cruzi* antibodies and fluorescence dye followed by staining with 1.25 μ g/ml 4′, 6-diamidino-2-phenylindole (DAPI) to visualize host and parasite nuclei as described (Woolsey *et al.*, 2003). To determine the impact of gene depletion on lysosome distribution, transfected HeLa cells were incubated for 15 minutes in Ringers/BSA solution at pH 7.2 or 7.6 (Heuser, 1989), fixed and lysosomes were visualized following staining with anti-human LAMP1 (The monoclonal antibody H4A3 developed by J.T. August and J.E.K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242). Fluorescence images were obtained with a Nikon TE-300 inverted epifluorescence microscope equipped with an Orca-100 CCD camera (Hamamatsu) and MetaMorph imaging software (Universal Imaging Corporation). For infections with *Toxoplasma gondii*, negative control or CLASP1 siRNA-transfected HeLa cells were infected with 4,000 ß-galactosidase-expressing RH strain *Toxoplasma* tachyzoites. After 72 h, the medium was removed and 100 μl of Z-buffer (10 mM $NaPO_4$ (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 9 mM $MgCl_2$, 0.125% NP-40, 100 mM β-mercaptoethanol) containing 20 μM CPRG was added to each well. Plates were incubated at 37°C for 15′ and absorbance

measured at 570 nm. Numbers of parasites in each well were determined by linear regression analysis from a standard curve prepared in each plate.

Western blot analysis

Cells were lysed in Laemmli sample buffer and total protein separated on 10% SDS-PAGE gels (BioRad) and electrophoretically transferred to PVDF membranes (Millipore). Blots were blocked with 5% non-fat dried milk in TBST (10mM Tris, 150mM NaCl, 0.05% Tween-20) for 1 hour at room temperature. CLASP1 antibody (Epitomics 1:5000) and EB1 antibody (Millipore 1:2000) were diluted in 5% BSA in TBST (10mM Tris, 150mM NaCl, 0.05% Tween-20) and incubated with blots overnight at 4°C. ECL peroxidase-linked secondary antibody (Invitrogen) 1:2000 in blocking buffer was used to detect the signal. To detect phosphorylation Akt (Cell Signaling), endogenous AKT (Cell Signaling) and GSK3β (Cell Signaling), antibodies were diluted 1:1000 in 5% BSA in TBST and incubated with blots overnight at 4°C. Blots were stripped and re-probed with antibodies to α-actin (Sigma; 1:10,000) for normalization of signals. Blots were developed with Supersignal™ substrate (Thermo Scientific).

Lysosome Exocytosis

HFF monolayers (60% confluence) were washed at 4°C with Ca^{2+}-free buffer (containing Mg^{2+} and 10 mM EGTA), followed by two more washes in Ca^{2+}-free buffer. SLO (Sigma-Aldrich) was bound to target cells in Ca^{2+}-free buffer for 5 min at 4°C, and pore formation was triggered by replacing the medium with 37°C buffer containing or not 1.8 mM Ca^{2+}. After 10 min at 37°C, cell supernatants were stained for 15 min with 2.3 μg/ml β-hexosaminidase substrate (Sigma-Aldrich) and analyzed by spectrofluorimeter as described in (Rodriguez *et al.*, 1997).

RNA extraction and quantitative reverse transcription-PCR (RT-PCR)

To determine efficiency of CLASP1 and CLASP2 knockdown, RNA was extracted (Qiagen, 74104) and reverse transcripted to cDNA using iScript (Invitrogen, 170-8890). Human CLASP1 (Hs01076541_m1, Taqman, Invitrogen) and CLASP2 (Hs00380556_m1, Taqman, Invitrogen) were amplified in triplicates by using standard amplification conditions for ABI (ABI PRISM 7900 HTA FAST). GAPDH was used to normalize the Ct values of the target genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Authors would like to gratefully acknowledge Dr. Vaughan for providing the p50-GFP construct and helpful suggestions and for D. Ndegwa for excellent technical support. This work was supported by the National Institutes of Health grant R21AI090366 and Harvard Medical School Milton Fund awarded to B.A.B. K.L.C. was supported by the Bayer Fund for Scholars in Infectious Diseases. I.B. was supported by NIH-R21AI087485 and MBC-114461.

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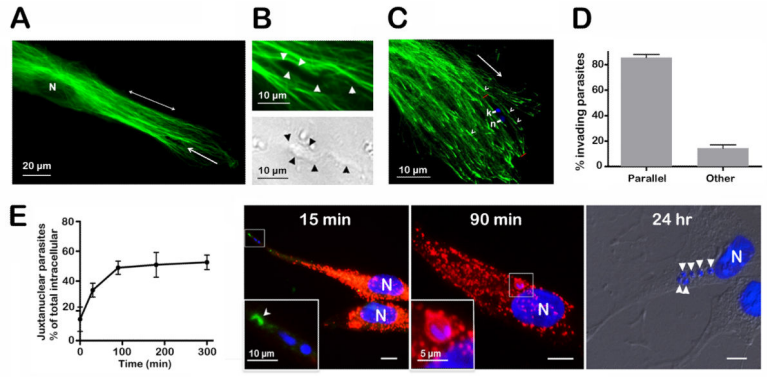

Figure 1. *T. cruzi* trypomastigotes enter cells in a parallel orientation to host microtubules

(A) Representative image of a recently internalized *T. cruzi* trypomastigote in HFF with the microtubules with EB3-GFP (green). Double-headed arrow indicates the orientation of the main microtubules in elongated HFF. Single arrow indicates direction of parasite entry into cell and highlights the intracellular parasite. **(B)** Higher magnification of recently internalized trypomastigote shown in A; outline of the parasite as it displaces microtubules (green) (upper) is seen in the DIC image (lower). **(C)** Image of HFF expressing EB3-GFP (green) enriched at the growing microtubule plus-ends (comet like structures; white arrowheads) with invading *T. cruzi* trypomastigote (DAPI-stained (blue) kinetoplast (k) and nucleus (n) indicated with arrowhead and parasite length bracketed by red bars). Long arrow indicates the orientation of microtubule elongation in HFF. **(D)** The orientation of invading or recently internalized parasites relative to host cell microtubules was scored in 200 infected cells. **(E)** The relative number of intracellular *T. cruzi* exhibiting juxtanuclear localization at indicated time points (graph). HFF were pulsed with trypomastigotes (50 parasites/host cell) for 15 minutes, washed to remove extracellular parasites and incubated for the indicated times prior to aldehyde fixation and immunostaining with anti-*T. cruzi* antibody (green) to detect extracellular parasites and counterstained with anti-LAMP1 (red) and DAPI (blue). Graphical data is expressed as the mean ± S.D. for triplicate samples representative of 3 independent experiments. Representative images are shown to the right.

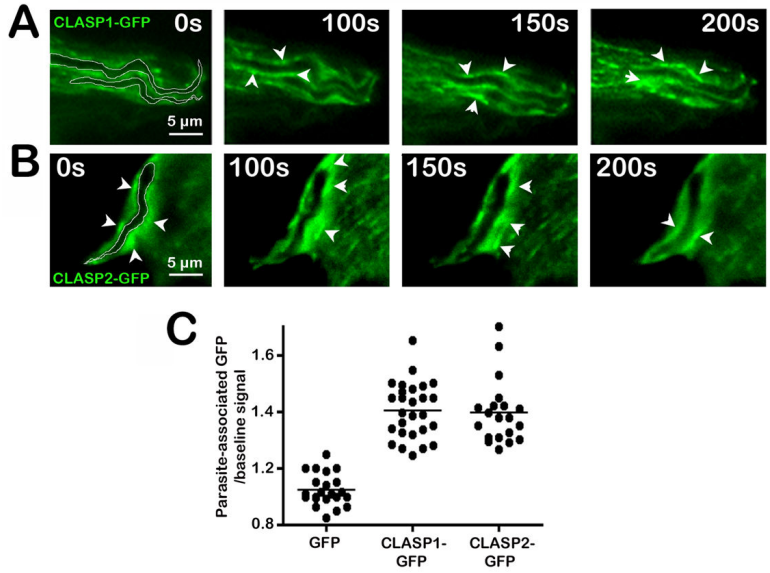

Figure 2. CLASP is transiently enriched at the *T. cruzi* invasion site

Time-lapse fluorescence images of recently internalized *T. cruzi* trypomastigotes (outlined in white in first frames) in HFF expressing CLASP1-GFP **(A)** or CLASP2-GFP **(B).** Image capture was initiated 10 minutes after initial incubation of parasites with host cells (marked as 0 seconds). Arrows indicate sites of enriched CLASP1-GFP or CLASP2-GFP. Scale bar = 5 µm. **(C)** Quantification of CLASP1-GFP enrichment with invading or recently internalized trypomastigotes as determined by the Linescan function in the MetaMorph® imaging software and compared to cytosolic GFP expression. Measurements were made for 25-30 cells each of control (GFP), CLASP1-GFP and CLASP2-GFP and reported as the ratios of pixel intensity for GFP signal proximal to parasites/pixel intensity for non-parasite associated GFP signal along the Linescan measurement (refer to Supporting Fig. S1). Data were analyzed using one way ANOVA; *: $p<0.01$.

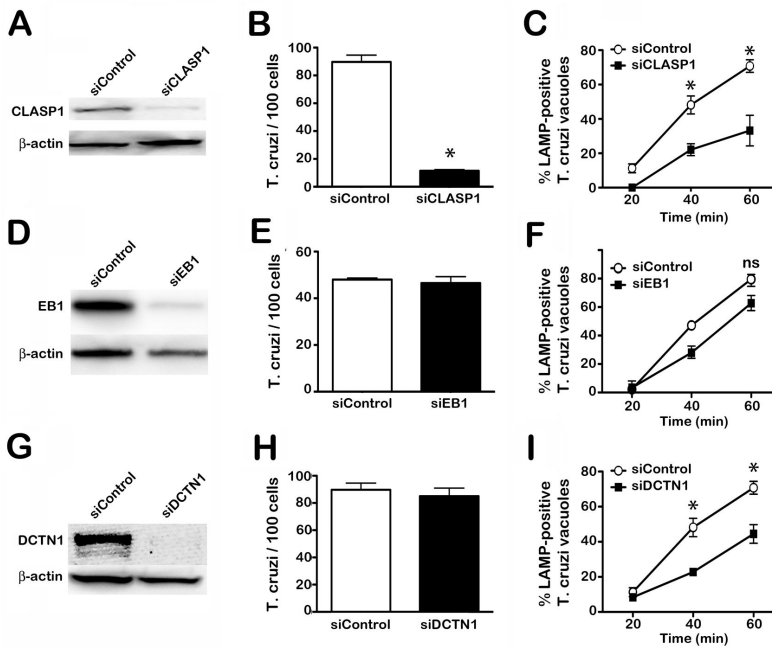

Figure 3. CLASP1 depletion inhibits *T. cruzi* invasion and subsequent fusion with host cell lysosomes

siRNA-mediated silencing of endogenous CLASP1 **(A),** DCTN1 **(D)** and EB1 **(G)** 48 hr post-transfection is demonstrated by western blots to detect protein with specific antibodies. Relative *T. cruzi* invasion after 15 min **(B, F, and H)** or LAMP-1 association with the parasite vacuole (**C, G and I)** in HeLa cells following siRNA-mediated silencing of CLASP1 **(A-C),** DCTN1 **(D-F)** or EB1 **(G-I)** and compared to cells transfected with non-targeting siRNA (siControl). Data are represented as mean ± S.D., n=3 analyzed by Student's T-test. *: $p<0.05$.

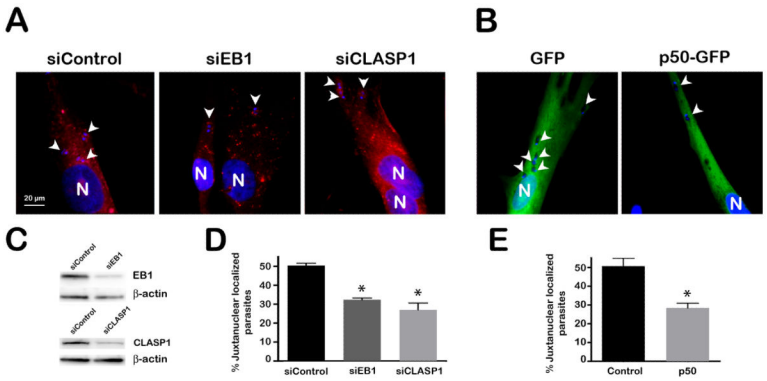

Figure 4. Juxtanuclear positioning of the *T. cruzi* vacuole is facilitated by microtubule plus-end tracking proteins

(A) Positioning of *T. cruzi* vacuoles relative to the host cell nucleus in HFF following siRNA-mediated silencing of EB1 (siEB1) or CLASP1 (siCLASP1). Cells were pulsed with *T. cruzi* trypomastigotes for 15 min followed by a 'chase' in medium for 1.5 hr. Representative images are shown in the top panels where transfected cells were identified with a fluorescent siRNA tracker (red) and intracellular parasites identified by DAPI staining (blue). Graphical representation of quantitative data showing relative number of parasites that exhibit juxtanuclear localization. **(B)** Dynactin p50-GFP expression (green) delays juxtanuclear migration of *T. cruzi* (DAPI: blue). Data are represented as means ± S.D., n=3; and analyzed by Student's T-test. *: $p<0.05$.

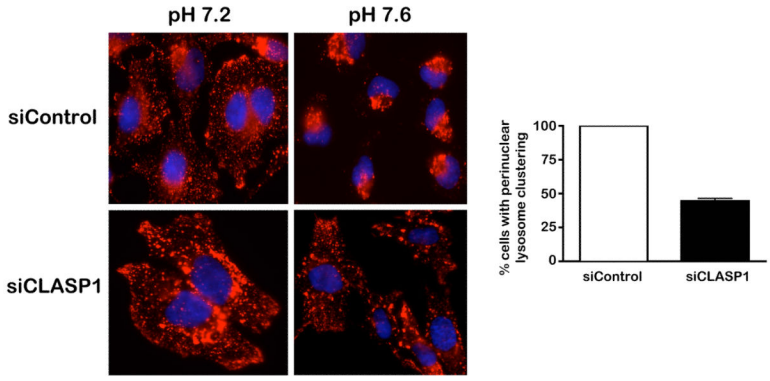

Figure 5. CLASP1 is required for minus-end directed lysosome trafficking

Representative images of HeLa cells transfected with non-targeting siRNA (siControl) or CLASP1-specific siRNA (siCLASP1) following a 15 min exposure to Ringer's-BSA pH 7.2 or pH 7.6 to induce perinuclear localization of lysosomes. Aldehyde-fixed and permeabilized cells were stained with anti-LAMP1 (red) and counterstained with DAPI (blue). Quantitation of perinuclear lysosome clustering for 200 cells (right panel). Data represented as mean ± S.D., n=3; and analyzed by Student's T-test. *: $p<0.01$.

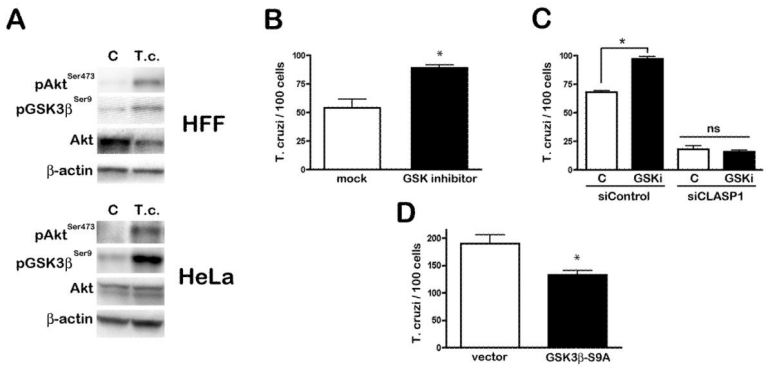

Figure 6. Inactivation of host GSK3β enhances *T. cruzi* entry

(A) Immunoblots demonstrating increased phosphorylation of host cell Akt^{Ser473} and GSK3β^{Ser9} in lysates of mock (*C*) or *T. cruzi*-infected (*T c*.) HeLa or HFF treated for 15 minutes. Blots were stripped and reprobed with antibodies to total Akt and β-actin to normalize signals. **(B)** Pretreatment of HeLa with GSK3β inhibitor (GSKi) for 30 min enhances *T. cruzi* invasion (C) in a CLASP1-dependent manner. (D) *T. cruzi* invasion is reduced in cells expressing constitutively active GSK3β–S9A. The relative number of internalized *T. cruzi* was determined in cells infected with parasites for 15 min. Data is represented as mean ± S.D. n=3; and analyzed by Student's T-test. *: $p<0.05$

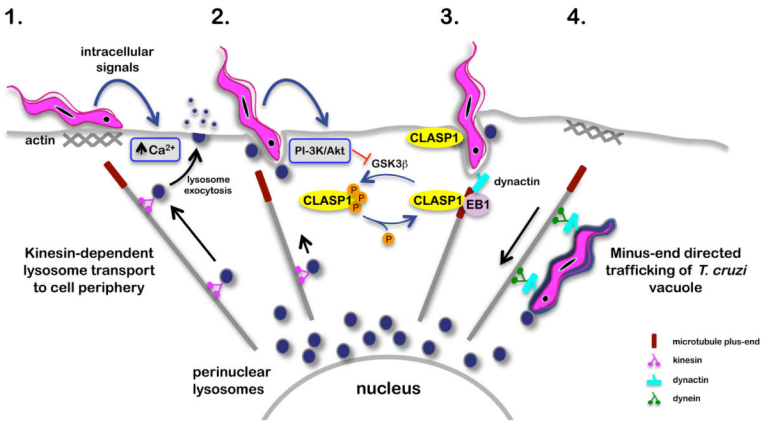

Figure 7. Integrated model of host microtubule function during the establishment of intracellular *T. cruzi* infection

(1) To initiate infection, invasive *T. cruzi* trypomastigotes activate signaling pathways in mammalian host cells including intracellular Ca^{2+}-transients and activation of class I PI-3Ks. Elevated Ca^{2+} results in transient depolymerization of the cortical actin cytoskeleton and promotes lysosome-plasma membrane fusion. Host microtubules transport lysosomes to the cell periphery in a kinesin-dependent manner. **(2)** Trypomastigotes invade at ceramide-rich regions of the plasma membrane generated by the action of released acid sphingomyelinase from exocytosed lysosomes. Parasite-triggered activation of PI-3K/Akt results in phosphorylation and inactivation of GSK3β, and increased CLASP1 association with microtubule plus-ends and cortical actin. **(3)** CLASP1 participates in connecting invading or **(4)** recently internalized parasite vacuole membranes to host microtubules which function to pull the parasite vacuole membrane toward the nucleus and facilitate fusion with lysosomes.