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Eh Klp5 is a divergent member of the kinesin 5 family that regulates genome content and microtubular assembly in *Entamoeba histolytica*

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Summary

Earlier studies have established two unusual features in the cell division cycle of *Entamoeba histolytica*. First, microtubules form a radial assembly instead of a bipolar mitotic spindle, and second, the genome content of *E. histolytica* cells varied from 1× to 6× or more. In this study, Eh Klp5 was identified as a divergent member of the BimC kinesin family that is known to regulate formation and stabilization of the mitotic spindle in other eukaryotes. In contrast to earlier studies, we show here that bipolar microtubular spindles were formed in *E. histolytica* but were visible only in 8–12% of the cells after treatment with taxol. The number of bipolar spindles was significantly increased in Eh Klp5 stable transformants (20–25%) whereas Eh Klp5 double-stranded RNA (dsRNA) transformants did not show any spindles (< 1%). The genome content of Eh Klp5 stable transformants was regulated between 1× and 2× unlike control cells. Binucleated cells accumulated in Eh Klp5 dsRNA transformants and after inhibition of Eh Klp5 with small molecule inhibitors in control cells, suggesting that cytokinesis was delayed in the absence of Eh Klp5. Taken together, our results indicate that Eh Klp5 regulates microtubular assembly, genome content and cell division in *E. histolytica*. Additionally, Eh Klp5 showed alterations in its drug-binding site compared with its human homologue, Hs Eg5 and this was reflected in its reduced sensitivity to Eg5 inhibitors – monastrol and HR22C16 analogues.

Introduction

Entamoeba histolytica is a protozoan pathogen that shows an unusual phenotype for cell cycle progression in axenic cultures. Alternation of genome duplication with mitosis is not tightly coupled so that each nucleus contains heterogeneous genome contents ranging from 1× to 6× or more and multinucleated cells are commonly observed (Gangopadhyay *et al*., 1997; Das and Lohia, 2002). This phenotype indicates that, checkpoints that ensure chromosome segregation and cytokinesis after genome duplication are either absent or inactive in this organism. Analysis of the recently published genome sequence of *E. histolytica* (Loftus *et al*., 2005) shows that sequence homologues of a large number of genes essential for cell cycle progression in other organisms are either absent or significantly divergent in this organism (C. Mukherjee and A. Lohia, unpubl. obs.).

Earlier studies reported an atypical circular assembly where microtubular fibres radiated out from the centre towards the nuclear envelope in *E. histolytica* (Orozco *et al*., 1988; Solis and Barrios, 1991; Vayssie *et al*., 2004). Resistance of *E. histolytica* to antimitotic drugs such as colchicine and benomyl have been attributed to differences in the amino acid sequences of Eh α,β-tubulins compared with other tubulin homologues (Sanchez *et al*., 1994; Katiyar and Edlind, 1996). However, sensitivity to the microtubule-stabilizing drug, taxol was predicted from the conserved taxol-binding site in the modelled tertiary structure of *E. histolytica* tubulin (Roy and Lohia, 2004). It has also been predicted that the microtubular assembly may be unstable due to steric hindrances in tubulin polymerization. However, reasons for the formation of a radial microtubular assembly instead of a typical bipolar spindle are not clear because proteins required for spindle pole or MTOC formation are present in *E. histolytica* (Ray *et al*., 1997). It has been difficult to study microtubule dynamics and chromosome segregation in *E. histolytica* because little is known about proteins regulating microtubular assembly.

Kinesin motor proteins regulate microtubular spindle assembly, chromosome movements, cell division and transport cargo on microtubules (reviewed in Walczak and Mitchison, 1996; Miki *et al*., 2005). Kinesins have been classified into different subfamilies based on the conserved features of their motor domains, because the non-motor domain could be significantly divergent between members of the same family (Miki *et al*., 2005; Wickstead

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and Gull, 2006). The plus end directed BimC kinesins (kinesin 5) are necessary for bipolar mitotic spindle formation and spindle pole body/centrosome separation or the maintenance of pole separation (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt *et al*., 1992; Sawin *et al*., 1992; Heck *et al*., 1993; Blangy *et al*., 1995; Mayer *et al*., 1999). Members of this family, function to polarize microtubular bundles by a relative sliding movement (Kapitein *et al*., 2005).

The Eg5 kinesins form an important subgroup of the BimC family, sensitive to the small molecule inhibitor monastrol. Mammalian cells treated with this drug arrest in mitosis with monopolar microtubular fibres due to specific inhibition of Eg5 activity (Mayer *et al*., 1999). Subsequently other Eg5 inhibitors – HR22C16 analogues were identified through chemical genetic screens (Hotha *et al*., 2003) that were shown to be more efficient than monastrol (Marcus *et al*., 2005).

In this study we have discovered that, in addition to the radial microtubular assembly, bipolar spindles are also formed in *E. histolytica*. Using a functional genomics approach we have demonstrated that Eh Klp5 is a variant of the Eg5/BimC family, which shows reduced sensitivity to Eg5 inhibitors and plays a novel role in regulating microtubular assembly, genome content and cell division in *E. histolytica*.

Results

Identification of kinesin like proteins in E. histolytica

Analysis of the completed genome sequence of *E. histolytica*, showed the presence of six genes encoding kinesin like proteins (Klp). These were identified by similarity to other kinesin like proteins in the non-redundant database and the genes were named as *Eh KlpA1, Eh KlpA2, Eh Klp2, Eh Klp3, Eh Klp4* and *Eh Klp5*. BLAST analysis (Altschul *et al*., 1990) of the motor domains showed that the *E. histolytica* kinesin like proteins were fairly divergent from other homologues. Phylogenetic analysis showed that the C-terminal kinesins Eh KlpA1 and Eh KlpA2 were 99% identical to each other and clustered with the kinesin 14 family (Fig. 1). Two N-terminal kinesins, Eh Klp2 and Eh Klp3 clustered as an outgroup of the kinesin 12 family (Miki *et al*., 2005). Members of this group have been shown to function in organelle transport, neuronal development and mitosis (reviewed in Miki *et al*., 2005). Figure 1 shows that Eh Klp4 did not cluster with any subfamily, similar to results shown recently (Wickstead and Gull, 2006).

The BimC or kinesin 5 family includes *Aspergillus* BimC, *Schizosaccharomyces pombe* CUT7, *Drosophila* KLP61F, *Xenopus* Eg5 (Xl Eg5) and *Homo sapiens* Eg5 (Hs Eg5) (Enos and Morris, 1990; Hagan and Yanagida, 1990; Le Guellec *et al*., 1991; Heck *et al*., 1993; Blangy *et al*., 1995). The closest homologues of Eh Klp5 were *Cryptosporidium hominis* Klp EAL35931 and Hs Eg5 and it clustered with the kinesin 5 family (Fig. 1). However, the relatively low bootstrap value suggested that Eh Klp5 could be significantly divergent. In order to determine whether Eh Klp5 was functionally similar to the BimC family proteins, we examined its role in regulating microtubular assembly during chromosome segregation in *E. histolytica*.

Expression of Eh Klp5 in E. histolytica

We used a twofold strategy to characterize the function of *Eh Klp5* – (i) to study gain of function by over-expressing this gene and (ii) to study loss of function by inhibiting expression of *Eh Klp5* in *E. histolytica* cells. To study gain of function, *Eh Klp5* was cloned under the *Eh* actin promoter in the *E. histolytica* expression vector pJST4 (Ghosh *et al*., 1996) (Fig. 2A). A multiaffinity epitope tag – CHH (Honey *et al*., 2001) was fused at the C-terminal for detection of Eh Klp5 in stable transformants. Increased expression of the recombinant gene in stable transformants was favoured by increasing the concentration of G418 (40 $\mu g\ ml^{-1}$) in culture and a concomitant increase in the copy number of the transfected plasmid (Hamann *et al*., 1995). A comparison of mRNA levels of stable transformants of Eh Klp5 CHH and wild-type cells showed no significant difference in the expression of Eh Klp5 (Fig. 3a), while the protein expression was increased about 1.3- to 1.5-fold in Eh Klp5 CHH transformants (Fig. 3b). It was also observed that Eh Klp5 was localized primarily in the nuclear fraction (Fig. 3c).

Secondly, to study the effect of loss of function of Eh Klp5, we made double-stranded RNA (dsRNA) constructs (Fig. 2B) as described earlier (Kaur and Lohia, 2004) and G418 resistant stable transformants were obtained for further analysis. Figure 3a and b shows that *Eh Klp5* mRNA and protein expression were significantly lowered (< 95%) in the dsRNA transformants compared with wild-type cells. Wild-type untransformed cells and empty vector (Fig. 2C) transformed cells showed no difference in expression levels of Eh Klp5 (data not shown).

Role of Eh Klp5 in microtubular assembly at mitosis

In order to visualize the microtubular assembly during mitosis, we treated *E. histolytica* cells with taxol, which is known to arrest cells in metaphase by preventing depolymerization of microtubules. Growth of *E. histolytica* was monitored at different taxol concentrations (Table S1) and 10 μM taxol was used for 10 h to ensure mitotic arrest in subsequent experiments. The microtubular assembly was localized with polyclonal anti Eh β-tubulin antibody.

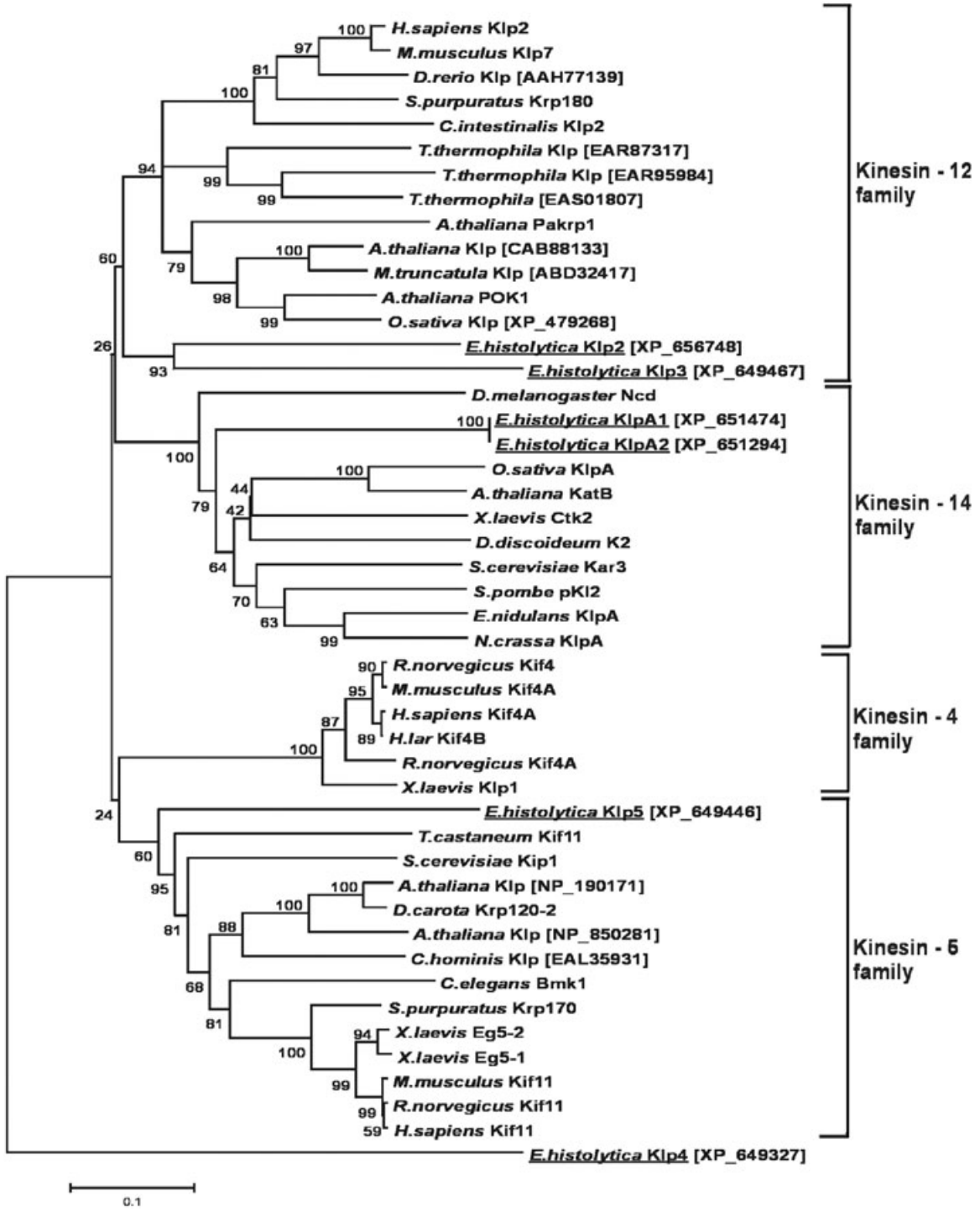

Fig. 1. Phylogenetic analysis of kinesins from *E. histolytica.* The motor domain sequences of *E. histolytica* kinesin like proteins and their closest homologues from non-redundant database were aligned using ClustalW and bootstrapped 1000 times using Neighbor Joining method of MEGA v3.1. Protein distances were calculated using the algorithm of p-distance. Bar represents the scale length.

Figure 4 shows the different microtubular structures seen in wild-type *E. histolytica* cells. In untreated cells, tubulin is primarily concentrated at the centre of the nucleus (Fig. 4a). The most common structure (approximately 90% of the cells) seen after taxol treatment was a circular distribution of tubulin (Fig. 4b) at the periphery of the nucleus. Both these structures have been described earlier (Vayssie *et al.*, 2004). We could not detect well-

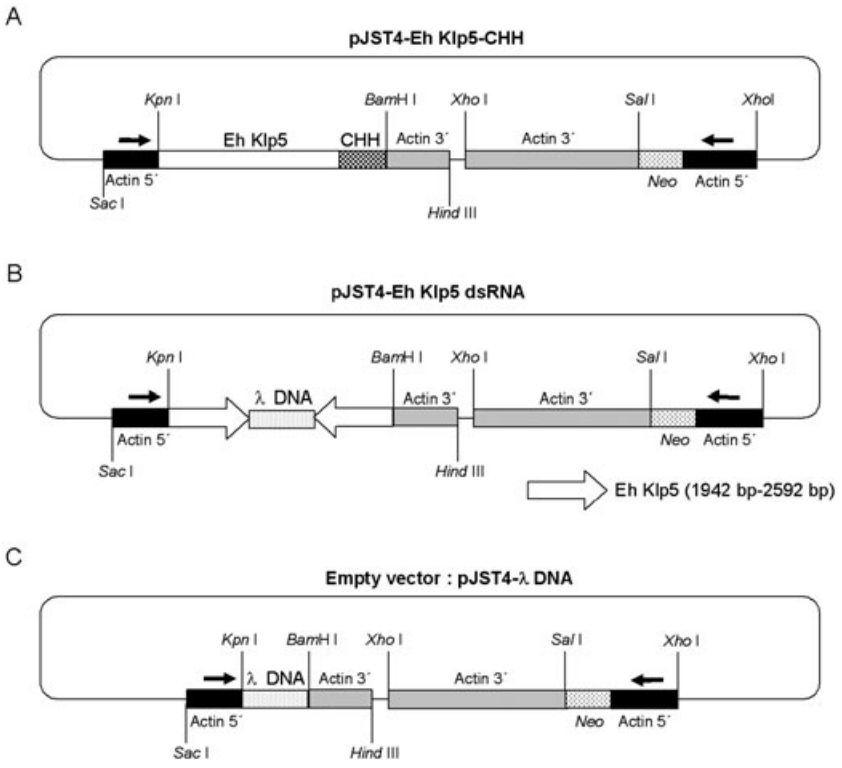

Fig. 2. Plasmid constructs for the expression of Eh Klp5 in *E. histolytica*. Schematic diagram of the constructs in pJST4 (Ghosh *et al*., 1996) expressing (A) multiaffinity epitope (CHH) tagged Eh Klp5 (2592 bp); (B) 3′ end of Eh Klp5 (1942–2592 bp) cloned in a head to head orientation with an intervening λ stuffer (560 bp); (C) Empty vector was constructed by cloning the λ stuffer fragment downstream of the actin promoter.

defined microtubular fibres in these circular assemblies possibly due to the low-resolution microscopy used here. In the remaining 8–10% of the cells we could distinctly identify either a single bipolar spindle (Fig. 4c and d), or cells with two spindles (Fig. 4e). Sometimes, in binucleated cells we could identify a spindle and a circular assembly simultaneously suggesting that both structures were used in mitosis (Fig. 4f).

Like the wild-type cells, taxol treated Eh Klp5-CHH transformant (Fig. 5 row a and b) showed both the circular (Fig. 5 row a) and spindle microtubular assemblies (Fig. 5 row b) after staining with Eh β-tubulin antisera. Eh Klp5 was localized in Eh Klp5 CHH transformants, with anti-HA monoclonal antibody. It may be seen that Eh Klp5 localized on the circular microtubular assembly (Fig. 5 row a) and spindle assembly (Fig. 5 row b). Importantly, it was observed that the frequency of bipolar spindle assembly in Eh Klp5-CHH transformant was much higher (20–25%) than control cells (8–12%). In contrast, taxol treated Eh Klp5 dsRNA transformants did not show any bipolar spindles (<1%). Circular microtubular assembly was seen in these cells after staining with Eh β-tubulin antisera (Fig. 5 row c). Eh Klp5 could not be detected in Eh Klp5 dsRNA transformants (Fig. 5 row d).

Our results suggest (i) *E. histolytica* cells can form bipolar microtubular spindles in addition to the commonly occurring radial microtubular assembly, (ii) a single cell may form more than one spindle or simultaneously a bipolar spindle and a radial microtubular assembly, (iii) increase in Eh Klp5 leads to an increased number of cells with bipolar spindles at mitosis, and (iv) genome partitioning can be carried out with significantly lowered amounts of Eh Klp5 and without a bipolar spindle assembly.

Eh Klp5 regulates the genome content of E. histolytica

To study the role of Eh Klp5 in regulating genome content in axenic cultures we have analysed the DNA content of Eh Klp5 CHH, Eh Klp5 dsRNA and control transformants by flow cytometry. It was seen that there was no significant difference in the generation time of the different cell types (data not shown). Flow cytometric analysis of *E. histolytica* cells (Gangopadhyay *et al*., 1997; Das and Lohia, 2002) showed a unimodal and overlapping distribution of heterogenous DNA content (1–6×) rather than discrete populations of G1 (1×) and G2 (2×) cells. We have therefore used electronic gates M1–M4 (Fig. 6A) to demarcate populations of cells with 1×, 2×, 4× and greater than 4× genome contents as described earlier (Gangopadhyay *et al*., 1997; Das and Lohia, 2002).

Flow cytometric analysis of 48 h cultures showed a decrease in heterogeneity of genome content in Eh Klp5 CHH transformants compared with control cells (Fig. 6A). The percentage of cells in each of the gates (M1–M4 corresponding to 1×, 2×, 4× or greater than 4× genome

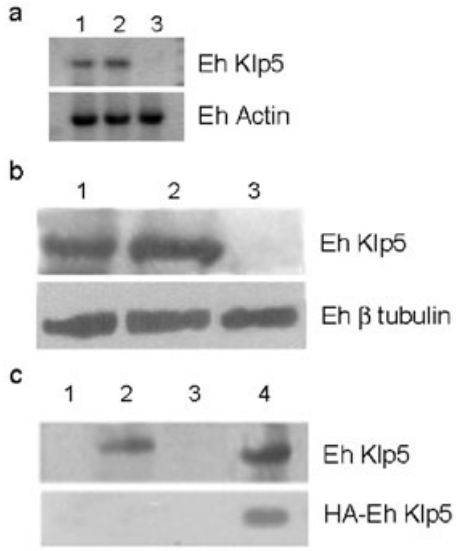

Fig. 3. Expression of Eh Klp5 in *E. histolytica*. Analysis of Eh Klp5 expression was carried out on wild-type cells and different transformants.
a. RT-PCR analysis of RNA from untransformed *E. histolytica* (lane 1), Eh Klp5 CHH transformant (lane 2) and Eh Klp5 dsRNA transformant (lane 3) using oligonucleotide primers from Eh Klp5 and Eh Actin. Actin was used as a control to demonstrate RNA quality and expression levels in different cell types.
b. Western analysis of Eh Klp5 expression. Anti-Eh Klp5 and anti-Eh β-tubulin polyclonal antisera were hybridized to Western blots of untransformed *E. histolytica* (lane 1), Eh Klp5-CHH transformant (lane 2) and Eh Klp5 dsRNA transformant (lane 3). Eh β-tubulin was a control for expression levels in different cell types.
c. Localization of Eh Klp5. Cytoplasmic and nuclear fractions of wild-type *E. histolytica* (lanes 1 and 2) and Eh Klp5 CHH transformants (lanes 3 and 4) were separated by SDS-PAGE and Western blotted. Blots were hybridized with anti-Eh Klp5 antibody and anti-HA monoclonal antibody.

contents) was analysed for these transformants and the average of three independent experimental sets was determined (Fig. 6B). The results show an overall reduction in the heterogeneity of the genome content in Eh Klp5 CHH transformant suggesting better regulation of the cell division cycle. Specifically, the fraction of cells containing 4× genome content was greatly reduced in Eh Klp5 CHH transformants (6%) and increased in Eh Klp5 dsRNA transformants (44%) compared with control (28%). Thus Eh Klp5 possibly facilitates efficient chromosome segregation so that cells with greater than 4× genome contents do not accumulate in cultures of Eh Klp5 CHH transformants. The reverse effect was seen when Eh Klp5 was depleted in dsRNA transformants.

The high proportion of 4× genome containing cells in control and Eh Klp5 dsRNA transformant may be due to either the presence of multiple genome contents in a single nucleus-reflecting a defect in chromosome segregation or the formation of multinucleated cells suggesting a defect in cytokinesis. As flow cytometric analysis does not distinguish between multinucleated cells and uninucleated cells containing multiple genome contents, we used scanning cytometry to analyse the genome content in individual nuclei of Eh Klp5 transformants. Results obtained from scanning cytometry (Fig. 6C) showed that the average distribution of genome content in individual nuclei of Eh Klp5-CHH transformants ranged from 1–2×, while in control cells and Eh Klp5 dsRNA transformants, it was much higher (1–4×). Thus depletion of Eh Klp5 likely inhibits chromosome segregation so that nuclei containing 2–4× genome contents accumulate in Eh Klp5 dsRNA transformants.

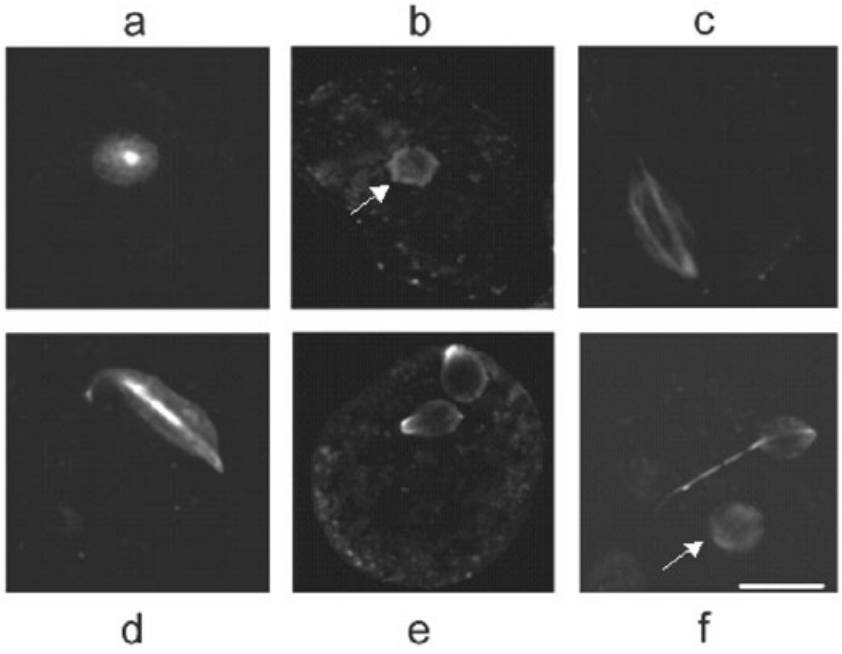

Fig. 4. Microtubular assembly in *E. histolytica*. Wild-type *E. histolytica* cells were stained with anti-β-tubulin antibody before (a) and (b–f) after treatment with 10 μM taxol for 10 h. Cells arrested in mitosis show both radial microtubular assembly (b and f) and bipolar spindles (c–f). Arrows indicate radial assembly in a cell with a single nucleus (b) and a binucleated cell (f) that shows both a radial and spindle assembly. Bar represents 10 μm.

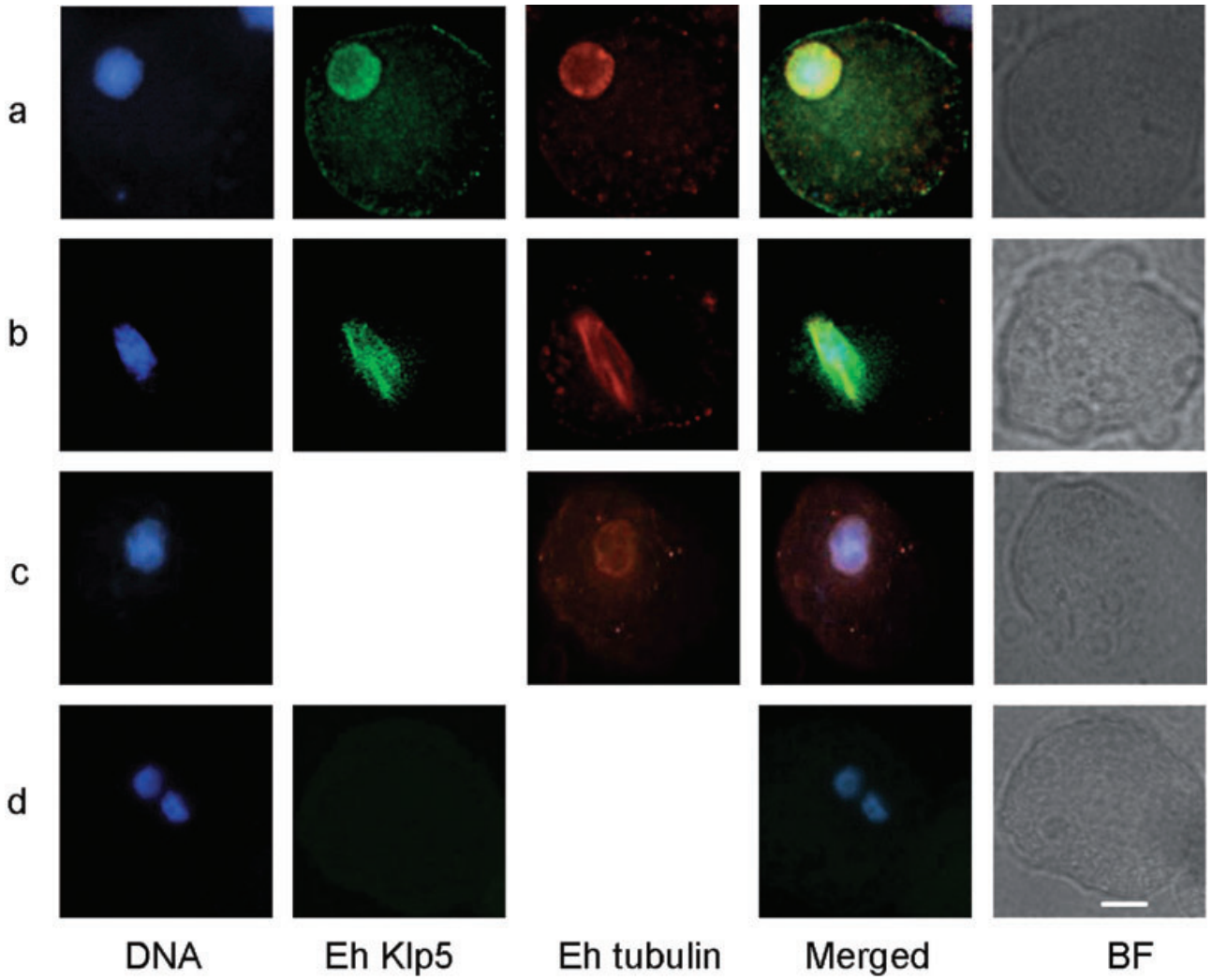

Fig. 5. Immuno-localization of Eh Klp5 with tubulin in *E. histolytica*. Trophozoites were allowed to grow on coverslips at 37°C and treated with 10 μM taxol for 10 h at 37°C and fixed directly.
a and b. Eh Klp5-CHH transformant was hybridized with monoclonal anti-HA antibody and polyclonal anti Eh β-tubulin antibody.
c. Eh Klp5 dsRNA transformant was stained with polyclonal anti Eh β-tubulin antibody.
d. Eh Klp5 dsRNA transformant was stained with polyclonal anti-Eh Klp5 antibody. DNA was stained with DAPI and visualized under 40× oil objective. Bright field (BF) images are shown on the side. Bar represents 10 μm. Controls were performed with each fluorochrome separately and shown that there was negligible cross-over of fluorescence between the three filters used here (data not shown).

In addition to uninucleated cells with multiple genome contents, cells with two nuclei were also found in significantly high numbers in Eh Klp5 dsRNA transformants (discussed below, Fig. 9) compared with control and Eh Klp5 CHH transformants, suggesting that cell division was delayed in Eh Klp5 dsRNA transformants. These results indicate that depletion of Eh Klp5 affects both chromosome segregation and cytokinesis in *E. histolytica*.

Expression of other Eh Klps in Eh Klp5 dsRNA transformant

In order to identify whether the expression of other kinesin like proteins was altered in Eh Klp5 dsRNA transformants, we compared their mRNA levels in Eh Klp5 dsRNA transformants and control cells (Fig. 7). While Eh KlpA1/2 and Eh Klp2 did not show any change in expression levels, Eh Klp3 expression was at least threefold higher and Eh Klp4 expression was increased sixfold compared with control cells. It is likely that increased levels of Eh Klp4 and Eh Klp3 compensate the function of Eh Klp5 in promoting growth because no significant change was seen in the generation time of Eh Klp5 dsRNA transformants compared with control cells. However this increased expression of Eh Klp3 and Eh Klp4 did not support bipolar spindle assembly in Eh Klp5 dsRNA transformants, suggesting that of all the kinesin like proteins, Eh Klp5 alone is required for bipolar spindle formation or stabilization in *E. histolytica*.

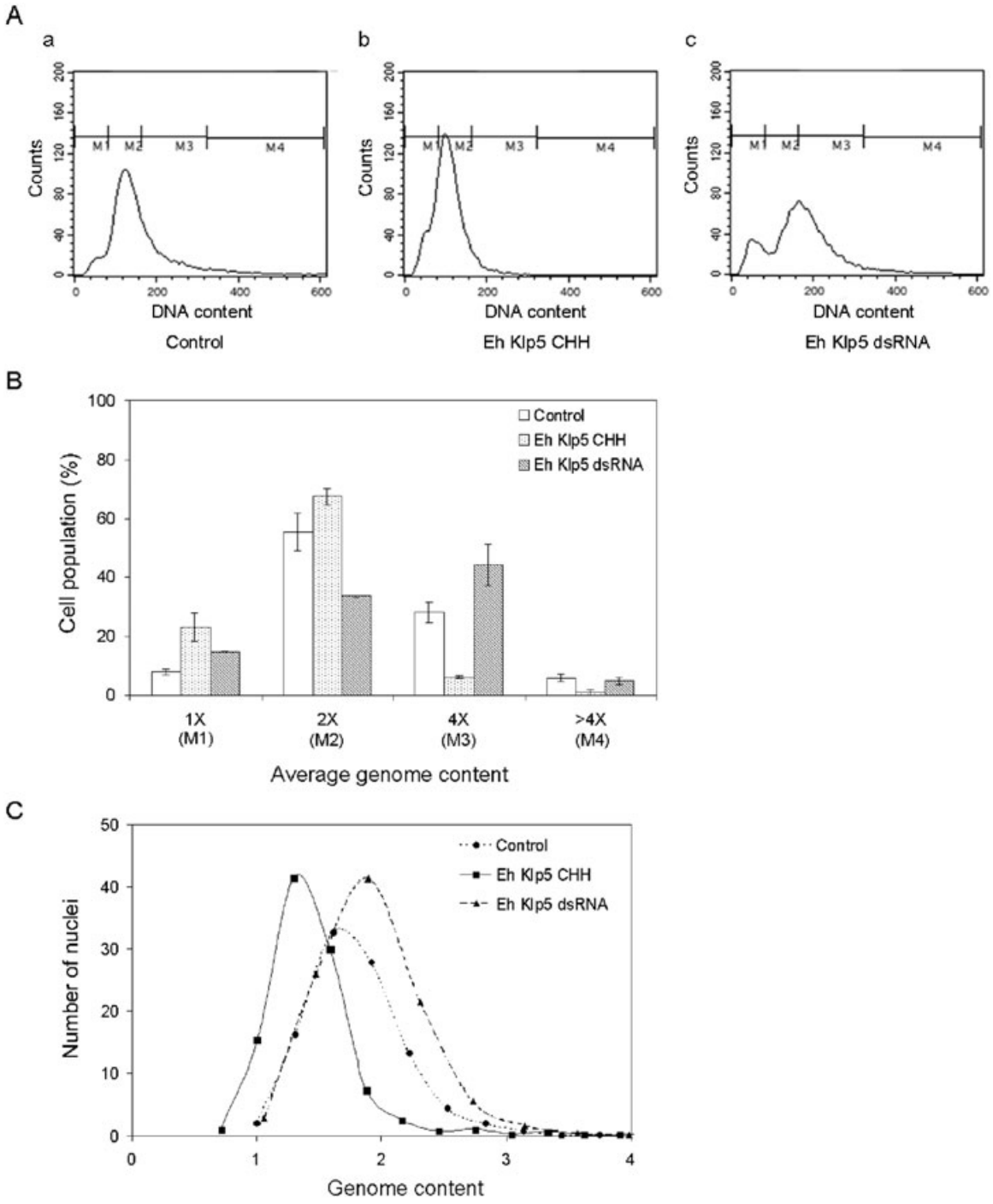

Fig. 6. Effect of Eh Klp5 expression on the genome content of *E. histolytica* cells.
A. Forty-eight hours grown cells of (a) control transformants (with empty vector) of *E. histolytica*; (b) Eh Klp5-CHH transformant and (c) Eh Klp5 dsRNA transformant were analysed by flow cytometry. The cells were initially subcultured with 3×10^4 cells per ml. After 48 h the cell count was approximately 5×10^5 cells per ml in control and 4×10^5 cells per ml in Eh Klp5 transformants. *X*-axis shows the DNA content and *Y*-axis shows the number of cells. Electronic gates M1, M2, M3 and M4 were set to demarcate cells with 1×, 2×, 4× and greater than 4× DNA content respectively.
B. Percentage of cells in each electronic gate (from A) was calculated and the average from three independent sets of experiments is shown. Error bars represent ± SD ($n = 3$).
C. The fixed cells were stained with DAPI and individual nuclei were scanned for DNA content in a MetaCyte scanning cytometer using Metafer4. A minimum of 2000 nuclei were scanned for each of the transformants and the values were normalized with respect to the empty vector control transformant and represented as histograms.

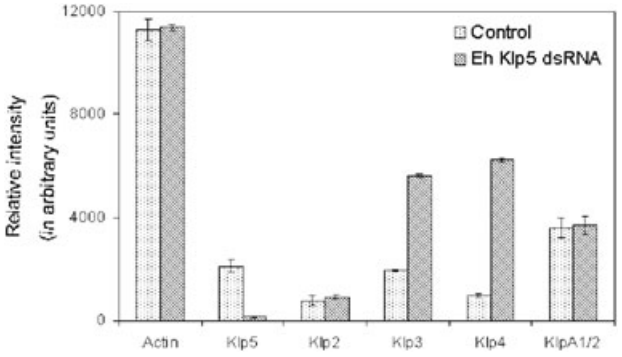

Fig. 7. Expression of different *E. histolytica* Klps in control and Eh Klp5 dsRNA transformant. RT-PCR was carried out on total RNA from control and Eh Klp5 dsRNA transformant using *Eh Klp2–5, KlpA1/2* and *actin* primers and analysed by agarose gel electrophoresis. The gel images were scanned in a densitometer and ImageQuant 5.2 quantified the relative band intensities of *Eh actin* and *Eh Klp 2–5, A1/2*. Average levels of expression from three independent sets were represented as a bar diagram. Error bars indicate ± SD ($n = 3$). *Eh actin* was used as an internal control to demonstrate RNA quality and expression levels in the two cell types.

Eh Klp5 showed reduced sensitivity to small molecule inhibitors of Eg5 kinesins

The vertebrate Eg5 kinesins form a distinct group of BimC family kinesins that are sensitive to the small molecule inhibitor monastrol and its derivatives (Mayer *et al*., 1999; Maliga *et al*., 2002; Maliga and Mitchison, 2006). As the motor domain of Eh Klp5 showed significant similarity to Hs Eg5, we examined the sensitivity of Eh Klp5 to monastrol in order to establish whether Eh Klp5 was closer in function to Hs Eg5 or to other members of the BimC family.

Alignment of the amino acid sequences of the motor domains of Eh Klp5 and Hs Eg5 (Fig. 8A) show that several amino acid residues like Arg119 (on helix α2), Trp127, Asp130, Leu132, Ala133 (on loop L5) and Thr211 (on helix α3) known to be important in monastrol binding (Yan *et al*., 2004) are absent in Eh Klp5. Homology modelling of the Eh Klp5 motor domain sequence on the crystal structure of Hs Eg5 motor domain (PDB code: 1Q0B) showed that the insertion loop L5 on helix α2 for binding monastrol was much shorter in Eh Klp5 compared with Hs Eg5 (Fig. 8B). Absence of key amino acid residues and shortening of the loop (L5) suggests that Eh Klp5 may be resistant to monastrol compared with Hs Eg5.

Treatment of *E. histolytica* cells with monastrol (Table S1) showed growth arrest at much higher concentrations (200 μM) compared with reports on mammalian cell lines (Kapoor *et al*., 2000). Additionally, it was observed that the ATPase activity of Eh Klp5 motor domain was inhibited up to 25% only by 200 μM monastrol (Fig. S1) – a significantly higher concentration than that required to inhibit Hs Eg5 ATPase activity (Maliga *et al*., 2002). This result suggests that compared with vertebrate Eg5, Eh Klp5 was less sensitive to monastrol. Resistance to monastrol has also been demonstrated in other invertebrate Eg5 homologues (Maliga and Mitchison, 2006).

Monastrol treated mammalian cells accumulate in mitosis with monopolar astral fibres (Kapoor *et al*., 2000) presenting a rosette like structure, instead of bipolar spindles. The distinctive effect of monastrol in forming rosette microtubular structures as seen in vertebrate cells could not be established in *E. histolytica,* because these cells routinely formed radial microtubular assemblies without inhibitors.

HR22C16-A1 and HR22CA16-amine are two analogues (Hotha *et al*., 2003) that have been reported to be more efficient than monastrol in inhibiting Eg5 function (Marcus *et al*., 2005). Similarly, in *E. histolytica* both these inhibitors were more effective than monastrol to arrest growth of cells (Table S1) while A1 was the most effective in inhibiting Eh Klp5 ATPase activity (Fig. S1) albeit at concentrations higher than the reported values for mammalian Eg5 (Marcus *et al*., 2005). Our results support the assumption that structural alterations in Eh Klp5 motor domain may cause reduced sensitivity to Eg5 inhibitors.

Binucleated cells accumulate when Eh Klp5 expression or activity is inhibited

An important effect of Eh Klp5 depletion in Eh Klp5 dsRNA transformants was the significant increase in binucleated cells (12%) in log phase cultures compared with control and Eh Klp5 CHH transformants (3–4%) (Fig. 9). A similar increase in binucleated cells was observed when control cells and Eh Klp5 CHH transformants were treated with monastrol or HR22C16 analogues for 10 h (Fig. 9). Of all the inhibitors, HR22C16-A1 was the most effective. Addition of these compounds to Eh Klp5 dsRNA transformants had little or no effect (Fig. 9). Taxol treated cells did not show a similar increase in the number of binucleated cells in any of the transformants, suggesting that it was the specific depletion or inhibition of Eh Klp5 activity that led to a delay in cell division.

Discussion

In this report we have discovered the role of Eh Klp5 in regulating microtubular spindle assembly and genome content. We have shown that in addition to the radial microtubular assembly described earlier (Orozco *et al*., 1988; Vayssie *et al*., 2004), bipolar spindles are also formed in *E. histolytica*. The spindle assembly may be extremely unstable and possibly eluded earlier methods

A

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Eh Klp5:  2   NIKVYVRCRPGKENEHIAN----IQMDNQNLIV----------SGKKYEFDQVFSSQVN 46
              NI+V VRCRP    E  A+    ++ D     V          S K Y FD VF +
 Hs Eg5:  18  NIQVVVRCRPFNLAERKASAHSIVECDPVRKEVSVRTGGLADKSSRKTYTFDMVFGASTK 77

Eh Klp5:  47  QNQFCDSALEGFIGKVIDGYNCTFFAYGQTGTGKTYTMEGEEE-NE----------GVIP 95
              Q     S +   + +VI GYNCT FAYGQTGTGKT+TMEGE   NE          G+IP
 Hs Eg5:  78  QIDVYRSVVCPILDEVIMGYNCTIFAYGQTGTGKTFTMEGERSPNEEYTWEEDPLAGIIP 137

Eh Klp5:  96  RVINELFMTLEKRGLRYRMRVTHVEIYNEKVYDLL---SDERKELAI-KERKEGNGASPE 151
              R ++++F  L   G  + ++V+ +EIYNE+++DLL   SD  + L +  + +   G   +
 Hs Eg5: 138  RTLHQIFEKLTDNGTEFSVKVSLLEIYNEELFDLLNPSSDVSERLQMFDDPRNKRGVIIK 197

Eh Klp5: 152  SATELTV-TKENVHQILAKSSGQRRTAATDINTNSSRSHCIFTVTVQIIRDSDFEGDFVV 210
                 E+TV  K+ V+QIL K + +R TAAT +N  SSRSH +F+VT+  ++++  +G+ +V
 Hs Eg5: 198  GLEEITVHNKDEVYQILEKGAAKRTTAATLMNAYSSRSHSVFSVTIH-MKETTIDGEELV 256

Eh Klp5: 211  P-GRIHCVDLAGSENSKRAGIIGDKVKQLEGMAINQSLLALNRVIIGVSKGDSYIPFRSS 269
               G+++ VDLAGSEN  R+G +  + +  E   INQSLL L RVI  + +   ++P+R S
 Hs Eg5: 257  KIGKLNLVDLAGSENIGRSGAVDKRAR--EAGNINQSLLTLGRVITALVERTPHVPYRES 314

Eh Klp5: 270  PLTRILQDALGGASITAMVATISPAQEDLEETLSTLEYAKRVKTIKNTPKQN 321
              LTRILQD+LGG + T++ATISPA  +LEETLSTLEYA R K I N P+ N
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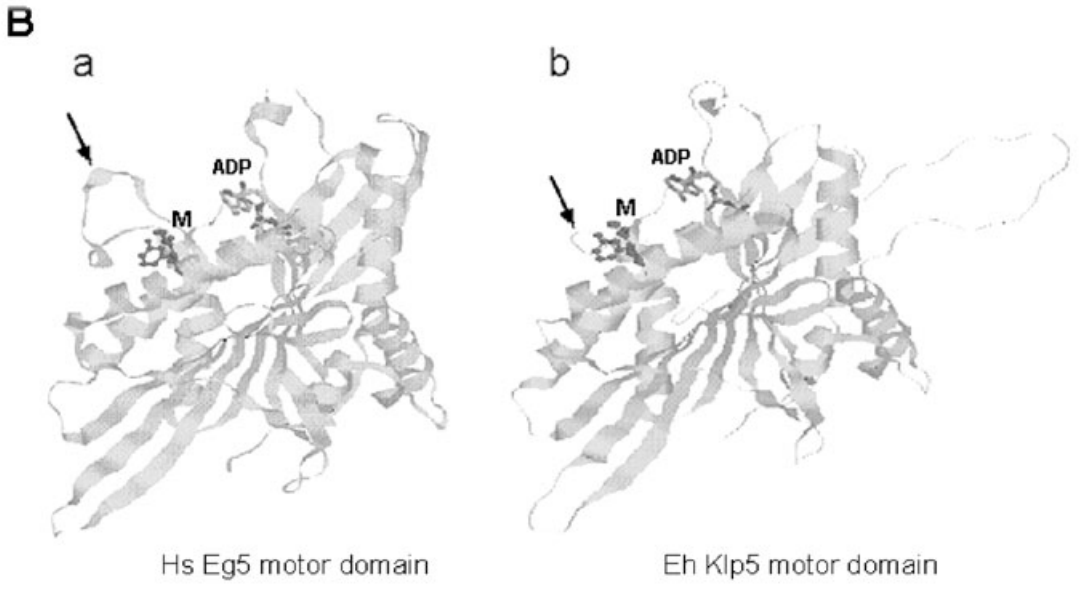

Fig. 8. Comparison of Eh Klp5 and Hs Eg5 motor domains.
A. Amino acid sequence comparison – alignment of amino acid sequences of the motor domains of Eh Klp5 and Hs Eg5 (42% positional identity and 59% similarity). The shaded residues R119, W127, D130, L132, A133 and Y211 important for forming induced fit pocket in Hs Eg5 are absent in Eh Klp5. The amino acid residues within the boxed area are present in the loop L5 on helix α2 of the motor domains.
B. Structural comparison between Eh Klp5 and Hs Eg5 motor domains with respect to monastrol binding. Stereo-view of the motor domains of (a) Hs Eg5 and (b) Eh Klp5 with monastrol (M, ball and stick model) and ADP (stick model) are shown. The loop L5 (indicated with arrow) on helix α2 that accommodates monastrol in Hs Eg5 is shorter in Eh Klp5.

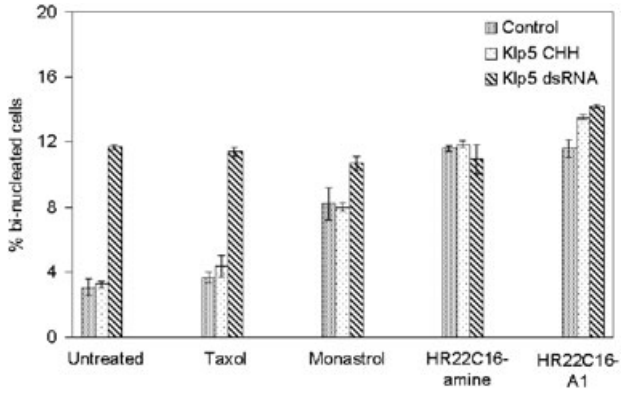

Fig. 9. Accumulation of binucleated cells in *E. histolytica* after inhibition or depletion of Eh Klp5. *E. histolytica* cells were treated with monastrol (200 μM) or HR22C16-A1(50 μM) or HR22C16-amine (50 μM) or taxol (10 μM) for 10 h and the percentage of binucleated cells were calculated in control cells and Eh Klp5 transformants. The results shown are an average of three independent sets of experiments and at least 250 nuclei were counted in each set. Error bars indicate ± SD ($n = 3$).

of visualization. As taxol prevents microtubule depolymerization, we could observe microtubular spindles in this study. However, even after taxol induced stabilization, the frequency of bipolar spindles was relatively low compared with the radial assembly, reiterating the notion that chromosome segregation may occur by atypical methods in *E. histolytica*. Higher frequency of bipolar spindles in stable transformants of Eh Klp5 and absence of spindles in Eh Klp5 dsRNA transformants, suggest that this protein is important for spindle assembly or stability. Reduction in the heterogeneity of genome content was concomitant with increase in bipolar spindles in Eh Klp5 stable transformants. Depletion or inhibition of Eh Klp5 led to accumulation of binucleated cells. All these observations together suggest that Eh Klp5 is a divergent member of the kinesin 5 family that performs novel functions in *E. histolytica* such as regulation of genome content and cell division in addition to the regulation of microtubular spindle assembly. Given the important role of Eh Klp5 in genome transmission and its apparent differences from human Eg5 proteins, this could be an attractive protein for developing parasite specific inhibitors.

Experimental procedures

Cell culture and maintenance

Entamoeba histolytica HM1:IMSS trophozoites were maintained axenically in TYIS 33 (Diamond *et al*., 1978) medium at 37°C. Cells were routinely subcultured for maintenance every 72 h and trophozoites in log phase of growth (24 h–48 h) were used in all experiments. Stable transformants were maintained similarly in the presence of G418.

Cloning and sequencing of E. histolytica *kinesin like proteins*

Oligonucleotide primers specific for *E. histolytica* kinesin like proteins (designed from the genome sequence of *E. histolytica,* TIGR database) were used in polymerase chain reaction (PCR) reactions to isolate the Eh Klps from genomic DNA, cloned into pBlueScript SK(–) plasmid and sequenced by automated DNA sequencing (Applied Biosystems, USA). Homology searches in the database were carried out using BLAST (Altschul *et al*., 1990). Alignment of amino acid sequences was carried out with ClustalW (Thompson *et al*., 1994). The GenBank Accession numbers of *E. histolytica* kinesin like proteins are XP_651474 (EhKlpA1), XP_651294 (Eh KlpA2), XP_656748 (EhKlp2), XP_649467 (EhKlp3), XP_649327 (EhKlp4) and XP_649446 (EhKlp5).

Constructs for expressing epitope tagged Eh Klp5 and Eh Klp5 dsRNA

The DNA fragment encoding the multiaffinity epitope tag CHH (Calmodulin binding domain, HA, His; Honey *et al*., 2001) was cloned in frame with the 3′ end of the *Eh Klp5* gene in pBS SK(–) and checked by DNA sequencing. *Eh Klp5 CHH* was subcloned in the *E. histolytica* expression vector pJST4 (Ghosh *et al*., 1996) for transformation and expression in *E. histolytica* trophozoites.

Double-stranded RNA constructs were made essentially as described in Kaur and Lohia (2004), except that the non-specific stuffer DNA was obtained from bacteriophage λ (560 bp). Six hundred and fifty base pair from the 3′ end (nucleotides 1942–2592) of the *Eh Klp5* gene was subcloned in a head to head orientation with an intervening λ DNA stuffer fragment. This cassette was cloned under the actin promoter in the *E. histolytica* expression vector pJST4 and transformed in *E. histolytica* HM1:IMSS trophozoites. Stable transformants were selected with G418 and maintained at 40 μg ml^{-1} G418. For the empty vector control, the stuffer DNA alone was cloned under the actin promoter in pJST4.

Raising polyclonal antisera to Eh β-tubulin and Eh Klp5

The 3′ end of Eh Klp5 (nucleotides 1942–2592) and Eh β-tubulin (nucleotides 1–1380) were subcloned in pJC40 and expressed in *Escherichia coli* BL21 (DE3) as 10× His tagged proteins. The recombinant proteins were purified and injected into rabbits for raising polyclonal antisera. Rabbit polyclonal antisera were purified on Protein A-agarose beads (Bangalore Genei, India).

Indirect immunofluorescence

Entamoeba histolytica trophozoites were allowed to grow on coverslips at 37°C and treated with 10 μM taxol (Sigma, USA) for 10 h at 37°C. Cells were fixed and stained with anti-HA antibody, 12CA5 (1:200, Roche, Germany) and FITC-conjugated goat anti-mouse secondary antibody (1:400, Jackson Laboratories, USA) to localize Eh Klp5. This was followed by hybridization with a rabbit polyclonal anti Eh β-tubulin antibody (1:200) and TRITC-conjugated goat anti-rabbit antibody (1:3000, Sigma, USA). DNA was stained with DAPI. All images were acquired with 40× oil objective (numerical aperture 1.3) in a Zeiss Axiovert 200 M Flourescence microscope using Z-stacking (Axiovision 3.1) and analysed by deconvolution.

Isolation of RNA from E. histolytica

Total RNA were isolated from the untransformed *E. histolytica* HM1:IMSS, empty vector transformed control cells, Eh Klp5-CHH and Eh Klp5 dsRNA transformants using Trizol reagent (Invitrogen, USA). Reverse transcriptase (RT)-PCR was performed on 5 μg total RNAs in each case with specific primers and M-MLV reverse transcriptase (Promega, USA).

Cell fractionation and western analysis

Entamoeba histolytica cells were lysed in extraction buffer (10 mM HEPES-KOH, pH 7.2, 24 mM KCl, 10 mM $MgCl_2$) supplemented with 0.1% NP-40 and protease inhibitors. The lysate was layered on a 0.8 M sucrose cushion and centrifuged at 3500 *g* for 20 min to separate the nuclei from the cytosol. Protein samples prepared from cytosolic and nuclear fractions were separated by SDS-PAGE and transferred to nitrocellulose membrane in three sets. The blots were hybridized with (i) rabbit polyclonal Eh-Klp5 antibody (1:200 dilution), (ii) rabbit polyclonal Eh β-tubulin antibody (1:500 dilution), and (iii) mouse monoclonal anti-HA antibody 12CA5 (1:1000 dilution) respectively. This was followed by incubation with HRP conjugated secondary antibodies (1:5000 dilution) and the signals were detected by chemiluminescence using ECL kit (Roche, Germany).

Analysis of DNA content by flow cytometry

To study the DNA content, log phase cells were harvested after 48 h of subculture and fixed in 70% ethanol at –20°C for 15 min. Cells were subsequently washed in PBS and treated with RNAse (0.3 mg ml^{-1} for 5 h) and propidium iodide (PI) (0.5 mg ml^{-1} for 5 min) in the dark. Flow cytometric analysis was carried out using FACSCalibur (Becton Dickinson, USA) equipped with a single laser system (6 W Innova 90-6 argon ion laser). For measurement of DNA content, cells were excited with 488 nm light and emission was measured through 575DF20 (for PI fluorescence; FL2). Data from 10 000 cells were recorded for each experiment and analysed using CELLQUEST software.

Scanning cytometry to determine the genome content of each nucleus

For measuring DNA content of individual nuclei, cells were fixed and stained with DAPI (0.15 μg ml^{-1} for 5 min) and spread on glass slides. Individual nuclei were scanned for DNA content in a MetaCyte scanning cytometer using Metafer4. A minimum of 2000 nuclei were scanned for each strain and the values were normalized with respect to the empty vector transformed HM1:IMSS and represented as histograms.

Treatment of E. histolytica *with small molecule mitotic inhibitors*

Taxol, monastrol, HR22C16 analogues – HR22C16-amine and HR22C16-A1 were added at different concentrations to control and Eh Klp5 transformants. Total cell number (counted after 10 h and 24 h) and number of binucleated cells were counted after 10 h of incubation with drugs and compared with untreated cells. Number of binucleated cells was estimated from counting 250 cells.

Cloning, expression and purification of Eh Klp5 motor domain

The Eh Klp5 motor domain (1–320 aa) was cloned in the *E. coli* expression vector pQE30 and protein expression was induced using 400 μM IPTG in *E. coli* XL1-Blue transformants. 6X-His tagged recombinant Eh Klp5 motor domain was purified by Ni-NTA superflow matrix (Qiagen, Germany). The purified protein was dialysed against ATPase assay buffer containing 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT in 20 mM Tris-HCl (pH 7.5).

In vitro *assay for ATPase activity of Eh Klp5 motor domain*

ATPase activity of Eh Klp5 motor domain was assayed *in vitro* by a colorimetric method using Malachite green (Baykov *et al*., 1988). ATP (100 μM) was added to the assay mixture and ATPase activity was estimated by measuring the released P_i at different times. To determine the effect of monastrol and its derivatives B3 and D1, 5 μM of purified Eh Klp5 motor domain was preincubated with each of these drugs for 30 min at room temperature. For optimal monastrol solubility, the assays were performed in the presence of up to 0.5% DMSO.

Phylogenetic analysis of E. histolytica *kinesins*

For phylogenetic analysis, amino acid sequences of motor domains of Eh Klps and their closest homologues from the non-redundant database were obtained from GenBank. The sequences were aligned in MEGA v3.1 software package (Kumar *et al*., 2004) using ClustalW and automatically edited in the software. Phylogenetic tree was constructed and visualized using p-distance matrix of Neighbor-joining (NJ) method (Saitou and Nei, 1987) through MEGA v3.1. The statistical reliability of individual nodes of the newly constructed tree was assessed by bootstrap analyses with 1000 replications.

Homology modelling of Eh Klp5 motor domain and docking with monastrol

Amino acid sequence of *E. histolytica* Klp5 motor domain (1–300 aa; Accession no.: XP_649446) was used to search protein data bank (PDB, Berman *et al*., 2000) with BLAST (Altschul *et al*., 1990) for finding suitable template for homology modelling. The BLAST search result identified the crystal structure of human Eg5 motor domain with monastrol (PDB code: 1Q0B; Yan *et al*., 2004) with 47% sequence identity with Eh Klp5. Homology modelling was performed using the program HOMOLOGY (MSI, USA). Eh Klp5 motor domain was superimposed on the crystal structure of Hs Eg5 motor domain without altering the co-ordinate system of atomic position in 1Q0B. The root mean square deviation for the superimposition of the two motor domains was 0.7 Å. The model was energy minimized to ensure proper inter-atomic interactions in a given three-dimensional space. Conjugate gradient method was used for minimization with the consistent valence

force field (CVFF) and the program DISCOVER (BIOSYM/MSI). PROCHECK (Laskovski *et al*., 1993) was used to access the geometric quality of the three-dimensional *E. histolytica* Klp5 motor domain and Ramachandran plot (Ramachandran and Sashisekharan, 1968) was drawn.

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Supplementary materials

The following supplementary material is available for this article online:

Fig. S1. Effect of monastrol and HR22C16 analogues on the ATPase activity of Eh Klp5 motor domain.

Table S1. Treatment of *E. histolytica* HM1:IMSS cells with taxol, monastrol and HR22C16 analogues.

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