Retraction

Retraction: Mutational analysis of the phosphorylation sites of the *Leishmania mexicana* kinesin homologue LmxKin29 (*J. Phys.: Conf. Ser.* 1664 012067)

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This article has been retracted by IOP Publishing at the request of the authors, for the following reasons:

- Some of the DNA constructs (plasmids) were created by people not listed as co-authors, nor acknowledged as contributors. Permission was not sought by the author to publish work relating to these plasmids.
- Figure 1c was reproduced by the author without them getting permission from the owner.
- Figure 4b was used by the author without them getting permission from the creator.
- Errors in calculations in figures 3d, 3e and 3f, which display the wrong values and are labelled incorrectly. A corrected figure is shown below.

IOP Publishing also confirms the removal of co-author Martin Wiese, who did not agree to this publication.

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Mutational analysis of the phosphorylation sites of the *Leishmania mexicana* kinesin homologue LmxKin29

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Abstracts

Kinesins are motor proteins, have been identified in a wide diversity of eukaryotes, from protists to mammals. The kinetoplastids’ genome sequences have also shown the presence of a large number of kinesins. The current study presents characterise a novel Lmexicana kinesin LmxM29.3.050, thought to be associated with flagellum formation. It was found express in both the amastigote and promastigote life stages. In this study we assigned this protein as a kinesin homologue to the unknown or orphan kinesins superfamily (1). The biochemical analysis showed the MAP kinase homologue LmxMPK3 can phosphorylate full length of LmxKin29 at serine 554. Localisation studies using GFP-tagged LmxKin29 revealed that it is predominantly found in between the nucleus and the flagellar pocket, while in dividing cells LmxKin29 was found at the anterior and posterior ends of the cells. Hence, LmxKin29 might play a role in cytokinesis. Double allele deletion was successfully generated. Morphological analysis of promastigotes with LmxKin29 tagged with GFP displayed no obvious phenotypic differences comparing the mutants with wild type cells.

Keyword: Kinesin, kinetoplast Kinesin, kinetoplast MAP kinase, phosphoprotein, Kinesin function, intercellular transport, Motor protein

1. Introduction

Kinesins constitute a superfamily of molecular motor proteins that convert the energy from ATP hydrolysis into mechanical work to transport a wide variety of cargo along microtubules which essential for diver cellular processes(1, 2). For instance, intracellular transport vesicles, organelles, chromosomes, and cell division (3, 4). Intracellular transport is essential and highly regulated for morphogenesis and functioning of the cell (5). Biological functions in kinesins also play an important role in cell division by regulating the mitotic spindle filament formation polymerising and depolymerising spindle microtubules, attachment and movement chromosomes, antiparallel spindle movements during mitosis and meiosis (6, 7). Disruption of the normal function of these proteins leads to a group of different diseases dyskinesia diseases PCD which distinct as a group of different diseases like chronic upper and lower
respiratory tract disease and left-right asymmetry defects (8, 9). Kinesin have a significance role as therapeutic targets regarding to the molecular basis of their function(10). The kinesins are considered as the largest group of conserved motors compared with other motor proteins (dyneins and myosins), because they are found in all eukaryotes (1, 11). Several studies confirmed that KIFs possess a highly conserved globular motor domain of ~340 amino acids, which involves an ATP-binding sequence and a microtubule-binding sequence (3, 12, 13). Essentially, a kinesin can be classified based on the position of the motor domain within the molecule. N-kinesins have a motor domain in the NH2-terminal region, M-kinesins have one in the middle region, and C-kinesins have it in the COOH-terminal region of the protein(7). Unlike the motor domain, the tail domains of most kinesins are highly divergent (2, 13, 14). In 2004, Lawrence with other researchers, published a standardised kinesin nomenclature, defining 14 families (kinesin-1–14), as well as some „orphan” kinesins that could not be put in any of the 14 families (15). The kinesin superfamily of motor proteins is now subdivided into 17 families, some of the ungrouped kinesins. The specific existence of some families in a subset of species capable to form cilia and flagella indicates shared biological functions in relation to these structures (1). The kinetoplastids” genome sequences have also shown the presence of a large number of kinesin motor proteins (41 kinesin proteins in T. brucei. (16). Several studies have suggested that kinesin function is regulated by mitogen-activated protein (MAP) kinases which can be involved in several steps including the selection of motors, loading of cargo, control of directionality or movement towards correct locations, velocity and finally, unloading and release of the cargo at its destination(2, 4, 17-19). Very little is known about the kinesin of L. mexicana the causative agent of cutaneous leishmaniasis. According to the transcriptomes study of L. mexicana promastigotes reported by (20) that LmxM.29.0350 has been found in all L. mexicana life stages, however amastigotes in vitro infected macrophages showed the highest level of transcriptomic mRNA LmxKin29 compared with other stages (20).

The current study we report the first attempt to characterise LmxM.29.0350 (LmxKin29). In vivo phosphoproteome analysis has been used on L. mexicana promastigotes and amastigotes revealed that potential phosphorylation sites occurred on serine 548, serine 551 and serine 554 in the LmxKin29 peptide (31). A peptide derived from the kinesin LmxKin29 was shown to be phosphorylated by activated LmxMPK3 (32). The Leishmania MAP kinase LmxMPK3, which has been shown to be important for flagellum length regulation (23). This prompted us to clone the gene for full length LmxKin29 defining the starting point of this project. The full length sequence of LmxKin29 was identified with a tblastn identity search of TriTrypDB (kinetoplastid genomic resources web site).

2. Material and Methods

2.1 Expression and purification LmxKin29 recombinant protein

LmxKin29 was expressed as a glutathione S-transferase (GST) fusion protein. The protein was expressed from a pGEX vector producing a recombinant fusion protein with the GST-tag (26 kDa) located at the N-terminus followed by LmxKin29. The use of GST as a fusion tag is an appropriate method because in many cases the fusion protein can be purified as a soluble protein rather than from inclusion bodies. We have used a modified pGEX-KGSP (24) which lacks a serine followed by proline close to the fusion site. The only other S/TP motif in the GST-tag is located N-terminally to the thrombin cleavage site and thus could be removed together with thrombin if required.
2.2 Kinase reaction

The eluted and dialysed fusion proteins 2-5 µg/µL of kinase assay solution (5 µL 10 × kinase buffer, 2.5 µL of 500 cpm/pmol [γ-32P] ATP and ddH2O were added to a total volume of 50 µL). As a control substrate 5 µL MBP 4.5 µg/µL was used. All reaction tubes were placed in an end-over-end rotator and incubated at 27°C for one hour. 12.5 µL of 5 × SSB 2% (SDS, 20% glycerol, 0.001% bromphenol blue, 200 mM DTT and 62.5 mM Tris-HCl pH 6.8) were added, the sample heated in a heat block at 95°C for 10 minutes and subsequently resolved on SDS-PAGE. The gel was Coomassie-stained, dried, and exposed to X-ray films at -70°C. The GST-tag by cleavage with thrombin if required. MAP kinase His-LmxMPK3 used in this study was expressed with an N-terminal hexa-histidine tag For in vitro analysis co-expression of hexahistidine-tagged LmxMPK3 along with its activating MAP kinase LmxMKK was used (23). The expression vector pJCduet, contains two multiple cloning sites (MCSs). LmxMPK3 was cloned in the first one and the second MCS was used to clone the gene for the activator kinase LmxMKK (Appendix1). This construct allows purification activated His- LmxMPK3 from bacteria.

2.3 Culturing of L. mexicana promastigotes

Promastigotes were grown in complete SDM medium (25) and at 27°C. Antibiotics were added, if required, at the following concentrations: Blasticidin S deaminase (BSD). (5 µg/mL), phleomycin binding protein or belocin (Phleo) (5 μg/mL), hygromycin B (20 μg/mL). A fresh culture was inoculated every 4-6 days diluting the old culture 1:1,000 into 10 mL fresh medium.

2.4 LmxKin29 deletion constructs

Two independent LmxKin29 null mutants were obtained by replacing both alleles of LmxKin29 with different resistance marker genes conferring resistance to Blasticidin (Bla) and phleomycin (Phleo). In order to generate the deletion construct of Lmxkin29, Initially, the LmxKin29 upstream region (679 bp) and downstream region (627 bp) were amplified by using PCR technique using genomic DNA from L.mexicana. Upstream sequence which functions as a targeting sequence located in front of the start codon of the LmxKin29 gene forward. While the downstream region is the sequence just after the stop codon of LmxKin29 were designed with the restriction sites as shown in below.

LmxKin29upstream Forward: 5`GATATCCGCGACCCATAGGACATGTCATCTCTCC-3`  
LmxKin29upstream Reverse: 5`TTGTGTCGTCCTCATCCGACCAGCTAGGAGATCTCGAG-3`.  
LmxKin29downstream Forward: 5`CCATGACCGTGCAGTGGTATGCGAGCGGACC-3`  
LmxKin29downstream Reverse: 5`CCTAGGGCTAGCTTGTTCGGACACTGCGATATGCCGGG-3`.  

The two PCR fragments were separated on a 0.8% agarose gel and cloned into pGEM®-T Easy (Promega) plasmid resulting in the two constructs pGEMKin29upi and
pGEMKin29dsi. They were cleaved with AvrII and NdeI resulting in two fragments, 706 bp and 2990 bp for pGEMKin29up and 43 bp and 3601 bp for pGEMKin29dsi. To generate pGEMKin29ds the 706 bp and dephosphorylated 3601 bp fragments were isolated, ligated and transformed into E. coli DH5α. The derived plasmid was confirmed by cleavage with NeoI, Nhel and AvrII. The derived plasmid was confirmed by sequence. pGEMKin29ds was cleaved with EcoRV to remove the pGEMT easy plasmid. The purified 1279 bp fragment contained the upstream and downstream regions of LmxKin29. This fragment was inserted into pBSKII(+) which had been cut before with the same restriction enzyme to produce pBupKin29ds. Resistance marker genes were introduced into pBupKin29ds, two antibiotic resistance genes were chosen coding for: Phleo, and BSD. The resistance marker gene for phleo was isolated from a plasmid pCR2.1phleo containing the gene conferring phleomycin resistance by cleaving it with NeoI and AvrII (AvrII overhangs are compatible to those generated by NheI which was used to cut the vector). The phleomycin fragment was ligated with pBupKin29ds to produce pBKin29upPhleods. The Blasticidin S gene was isolated from pEX-A2-BLA-ALA (Unpublished Wiese). The construct was cleaved with NeoI and Nhel, then ligated with r-pBupKin29ds. Finally, the two new constructs pBKin29upBlads and pBKin29upPhleods were confirmed.

2.5 Fluorescence microscopy with live cell imaging Cooling slide preparation

50-500 μL of a log-phase Leishmania culture were centrifuged at 5,600 × g for 2 min, cells were washed with 1 mL ice-cold 1 × PBS and subsequently resuspended in 200 μL ice-cold 1 × PBS. The tube was placed on ice for one hour. 4 μL of the live parasite suspension was examined using fluorescence microscopy. GFP fluorescence was observed with the FITC filter (λ=540 nm) and pictures were typically taken with an exposure time of 100 – 200 ms, depending on the intensity of the protein.

2.6 Microscopy techniques and flagellar length determination

Light microscopic examination is a simple technique to determine flagellar length. 50 μL – 100 μL of log-phase Leishmania promastigote culture were centrifuged at 5,600 × g for 2 min, the supernatant was removed and the pellet resuspended in 50 μL fixation solution. 4 μL of the suspension was used on a slide and it covered with a cover slip without trapping air bubbles. Cells were examined under light microscope with magnification (40×1000), 5-15 fields view were captured by GXCAM camera, and GXCapture Software was used. Flagellar lengths were measured for 200 cells of each clone, from the cell surface to the flagellar tip exactly tracing the flagellum by using the freehand tool of the Image J software Version 1.51p.

2.7 Expression constructs for complementation of LmxKin29

Here, LmxKin29 fused to GFP was expressed in null mutant promastigotes. Two strategies were used to generate the add-back clones. Initially, episomal complementation the null mutants were transfected with a plasmid containing LmxKin29 fused with GFP. Plasmids can independently replicate in Leishmania and replication occurs even in the absence of any
leishmanial sequence (26). pTH6nGFPc and pTH6cGFPn (27) were used to tag LmxKin29 with GFP either at the N-terminus or C-terminus of the protein. The protein was expressed in the two null mutant clones by introducing pTHGFPgLmxKin29 where GFP is fused to the N-terminus of LmxKin29 and pTHLmxKin29GFP with a C-terminal fusion. Both transfections were successful and two clones were selected from each transfection and grown in culture with hygromycin B (20 μg/mL). Secondly, complementation of LmxKin29 by reintegrating into its genomic DNA locus, a DNA cassette had to be constructed which contained the puromycin N-acetyltransferase gene (PAC) for selection of recombinant clones besides the two flanking regions and the ORF of LmxKin29. The fragment cloned into the BupKin29ds knockout construct to produce pBupGFPKin29ds and pBupKin29GFPds. To prepare the fragments for electroporation the plasmids pBupGFPKin29 and pBupKin29GFP were cleaved with EcoRV to isolate the 5885 bp and 5880 bp, respectively, under sterile conditions.

2.8 Transfection of L. mexicana by Electroporation (Amaxa)

Transfections were performed using a human T-cell Amaxa nucleofector kit following manufacturer’s instructions. A dense cell culture of $4 \times 10^7$ cells was used. Promastigotes were harvested by centrifugation for 15 min at 2,500 × g at 4°C. The supernatant was removed by careful pipetting and the pellet resuspended in 100 μL supplemented electroporation buffer containing 1–5 μg of DNA fragment or plasmid. The cell suspension was transferred to a pre-cooled electroporation cuvette. The solution was then electroporated, using the programme V-033 on an Amaxa Nucleofector, followed by incubation on ice for 10 min. The solution was then transferred into 10 mL SDM medium and incubated for 24h at 27°C. Following the overnight culture, two dilutions were prepared. (1:2) and (1:40). The relevant antibiotics were added (5 μg/mL phleomycin, 20 μg/mL hygromycin, blasticidin 5 μg/mL, and 40 μM puromycin) and the solution was distributed across a 96-well plate, using 200 μL per well. The plates were sealed with parafilm and incubated at 27°C for 10-14 days. Wells where significant growth occurred were identified using an inverted microscope and the content of these wells was transferred into 2 mL SDM and expanded to 10 mL upon successful growth.

2.11 Immunoblot analysis

Cell lysates were prepared using RAPA buffer [25mM Tris-HCl pH 7.6,150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS], then resolved by electrophoresis on SDS-PAGE then blotted to a Polyvinylidene Difluoride membrane using an XCell II™ Blot Module at a current of 100 mA for 90 minutes. The membrane was then incubated for one hour at 37°C in blocking solution; this was then replaced with blocking solution [1 × PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4), 5% (w/v) low-fat dried milk powder, 20 mM Tris-HCl pH 7.5, 0.2% Tween 20] containing the primary antibody (Mouse IgG (HRP conjugated) diluted 1:1000 in blocking buffer) and incubated overnight at 4°C or at 37°C. The membrane was then washed four times for 5 min with 1 × PBST at room temperature. It was then incubated for one hour at 37°C with the incubated with the respective rabbit antiserum diluted 1:1000 in blocking buffer secondary antibody,
washed three times for 5 min in 1 × PBST then twice for 5 min in 1 × PBS. The blot was developed by incubating the membrane with an ECL substrate (Equal volumes peroxide solution and a luminol/enhancer solution for five minutes. Then exposure to the film.

3. Results and discussion

Very little is known about the putative kinesin LmxM.29.0350 of *L. mexicana*. mRNA of LmxM.29.0350 has been found in all *L. mexicana* life stages, however amastigotes in in vitro infected macrophages showed the highest level compared promastigotes and axenic amastigotes (20). The current study is the first attempt to characterise LmxKin29, functional analysis, to confirm that MAP kinase LmMAPK3 that phosphorylates LmxKin29, and to identify the phosphorylation site used by this kinase. LmxKin29 was thought to be a flagellar kinesin because LmxMPK3 a MAP kinase involved in flagellum length regulation can phosphorylate a peptide derived from LmxKin29 (22).

3.1 Characterisation the kinesin LmxKin29 (LmxM.29.0350)

The full length sequence of LmxKin29 was identified with a tblastn identity search of TriTrypDB. Using the sequence of the phosphorylated peptide to search the database resulted in 100% identity with a putative kinesin located on chromosome 29 in *L. mexicana* MHOM/GT/2001/U1103, which was therefore designated LmxKin29 (Fig.1A). LmxM.29.0350 open reading frame (ORF) comprises 1,830 bp encoding a protein of 610 amino acids with a molecular mass of 68.5 kDa (Fig.1E) and an isoelectric point of 8.17 (28). The primary structure contains the typical kinesin three domains. N-terminus, coiled-coil sequence (neck) and the C-terminus (tail) (The Universal Protein Resource UniProt database) (Fig.1B). Determination of potential C-terminal phosphorylation sites of LmxKin29. The full length sequence of LmxKin29 shows that the kinesin contains four serine residues followed by a proline and one threonine residue followed by a proline constituting potential MAP kinase phosphorylation sites (Fig. 1A). Serine 333 is part of the motor domain. LmxKin29 is a homologue to the putative kinesin (Tb927.6.1770) which has been identified by (29) as an orphan kinesin. According to TriTrypDB the predicted function of Lmj.30.0350 and Tb927.6.1770 is a motor-driven movement along microtubules via polymerisation or depolymerisation of microtubules raising the possibility that LmxKin29 may have a similar cellular function.

3.2 LmxKin29 superfamily

Kinesin superfamly is the first step to study a new kinesin by using BLAST database search (15). Depend on the a phylogenetic tree have been generated to organise 486 kinesin-like sequences from 19 eukaryotes (1), we assigned LmxM.29.0350 as a kinesin homologue of LmxE.30.0350 which belongs to the unknown or orphan family of kinesins as displayed in (Fig.1C). Sequence alignment of LmxKin29 homologues in *Leishmania* shows that the phosphorylation sites S551 and S554 are conserved in all of them (Fig1D). Also, LmxKin29 shows high levels of amino acid identity with LmxKin29 homologues in other *Leishmania* species such as, 86% with the LTRL590_300009000 *L. tropica* kinesin, 87% with the LdBPK_30.0350 *L. donovani* kinesin, 87% with the LinJ.30.0350 *L. infantum* kinesin, with LtaP30.0410 *L. tarentolae* 78% and LbrM.30.0390 *L. braziliensis* 87 % with conserved of serines 551 and 554 (Fig1.D). The closest human kinesins showed low similarities to...
LmxKin29, e.g. KIF11 shows 36% amino acid sequence identity. KIF3A shows 38% identity (152/397) and 53% positives (211/397). Contrary to expectations, LmxKin29 did not show significant sequence identity with the two types of flagellar kinesin-2, the flagellar heterotrimeric Kinesin-II and the homodimeric OSM3 kinesin in different organisms. However, LmxKin29 might be a kinesin specific for L. mexicana and hence functional analysis was pursued in this project. The low percentage of identical amino acids seen in most protein kinesins of unicellular parasites observed in phylogenetic studies suggests that these proteins are potential targets for specific inhibitors, which can developed into new drugs against the pathogen.

3.3 Purification of recombinant LmxKin29 Leishmania proteins

In order determine the potential phosphorylation sites, full- length GST-fusion protein of wild type LmxKin29 and four LmxKin29 mutants constructs had been generated by cloning and site-specific mutagenesis into pGEX-KGSP, by replacing the serine by either alanine or aspartate (aspartate residue, thus mimicking a phosphorylation). pGEX-KGSPKin29, pGEX-KGSPKin29A & pGEX-KGSPKin29SD (SD) replacing the 551 serine by either alanine or aspartatep GEX-KGSPKin29A2 (SD) replacing the 551 serine by alanine, pGEX-KGSPKin29A4 (four phosphorylation sites replaced by alanine: threonine 440 and serine 548, 551, 554) (Fig.3A). In addition, the activated MAP kinase LmxMPK3, was expressed. The hypothesis to be tested was, that only LmxKin29 WT and not any of the mutants in the phosphorylation site will be phosphorylated by activated LmxMPK3 in an in vitro kinase assay using purified recombinant proteins. The recombinant LmxKin29 proteins (GST-LmxKin29WT, GST-LmxKin29SA, GST-LmxKin29SD, GST-LmxKin29A2, and GST-LmxKin29A4 ) were successfully expressed and their predicted sizes are 95.7 kDa (68.7 kDa LmxKin29 + 27.4 kDa GST) which matches to the size of the band on the gel (Fig. 2C). Additionally, there was a very faint double band probably corresponding to the GST-tag (27.4 kDa). The predicted size of His-LmxMPK3 is 49.7 kDa (43.7+ 6 kDa), and this agrees with the position of the band in line 4 of the Coomassie-stained gel (Fig.2C). The purified proteins were used in protein kinase assays in order to determine the ability of LmxMPK3 to phosphorylate LmxKin29 proteins. The four kinesins were subjected to kinase assays with activated LmxMPK3, the reactions was stopped by boiling in SDS sample buffer, proteins were separated on a 14% SDS-PAGE and stained by Coomassie (Fig.2 Da). His-LmxMPK3 with WT,GST-LmxKin29SA, SD A2,A4 in lanes 1, 2,3,4 and 5, respectively. (Fig. Db) displays an autoradiograph after 72 hours of five kinase assays. GST-LmxKin29 showed a strong phosphorylation signal with LmxMPK3. Surprisingly, the activated His-LmxMPK3 still phosphorylated the mutant proteins LmxKin29SA and LmxKin29SD. However the two mutants LmxKin29A2 and LmxKin29A4. GST-LmxKin29SA and GST-LmxKin29SD only lack serine 551 and can still be phosphorylated indicating the serine 551 is not the site used by His-LmxMPK3. GST-LmxKin29A2 carries two mutations in serine 551 and serine 554. The absence of a phosphorylation signal indicates that serine 554 is the phosphorylation site for LmxMPK3.
Fig. 1. Amino acid sequence of LmxKin29 and protein expression. A, Sequence amino acids of LmxKin29 and putative MAP kinase phosphorylation sites are highlighted in red (serine 548, serine 551 and serine 554 (previously found to be phosphorylated in promastigotes and axenic amastigotes in vivo)), motor domain of LmxKin29 is shown in blue. B, Graphical view shows the motor domain (14-355; blue box), coiled-coil (387-534; dark blue) and the tail domain (534-610) (from UniPro data base). C, Phylogenetic tree of kinesin motor domains for about 400 non-redundant sequences from 19 different species including L. major. Orphan kinesins are enlarged in the right panel and the protein from L. major strain Friedlin LmjF.30.0350 corresponding to LmxM.29.0350 is highlighted (yellow box) (Wickstead et al., 2006). D, Partial amino acid sequence alignment of LmxKin29 from L. mexicana with the amino acid sequences of various kinesins. Ldon, LdBPK_300350.1.1 Leishmania donovani BPK282A1, kinesin, putative, length=610 aa; Linf, LinJ.30.0350 Leishmania infantum JPCM5, kinesin, putative, length=610 aa; Ltr, LTRL590_300009000.1 Leishmania tropica L590, kinesin, putative, length=610 aa; Lmj, LmjF.30.0350 Leishmania major strain Friedlin, kinesin, putative, length=607 aa; Lmex, LmxM.29.0350.1 Leishmania mexicana MHOM/GT/2001/U1103, kinesin, putative, length=610 aa; Ltar, LtaP30.0410 Leishmania tarentolae Parrot-Tarl, kinesin, putative, length=615 aa; Cfa, CFAC1_260021700.1 Crithidia fasciculata strain Cf-Cl, kinesin, putative, length=620 aa; Lbr, LbrM.30.0390 Leishmania braziliensis MHOM/BR/75/M2904, kinesin, putative, length=607 aa. The asterisk (*) indicates a conserved amino acid in all homologues (Alignment was achieved by Clustal Omega). (https://www.ebi.ac.uk/Tools/msa/clustalo/). E, Purified recombinant GST-LmxKin29 on Coomassie-stained 14% SDS-PAGE. Lane 1, elution 1; lane 2, elution 2; lane 3, beads; M, marker in kDa.

3.4 Final step to assign phosphorylation site

pGEXKSPS554A was successfully generated as a recombinant LmxKi29 protein construct to produce the final version of mutant protein. Fig2 Ea. shows a comparative of the four purified bands of four recombinant proteins of the GST-LmxKin29 (WT, SA, A2, S554A) on
a Coomassie-stained 14% SDS-PAGE. Kinase assays (Fig. 2Eb) showed phosphorylation of LmxKin29WT and the mutant LmxKin29SA. No phosphorylation was found in LmxKin29A2 and LmxKin29S554A. According to the previous kinase assays, this result proves that serine554 is the phosphorylation site used by activated His-LmxMPK3. The \textit{in vivo} phosphoproteome analysis on \textit{L. mexicana} promastigotes and amastigotes which revealed that the potential phosphorylation sites are serine 551 and serine 554 in the LmxKin29 peptide (21). The mutant LmxKin29A2 (serine 551 and serine 554 replaced by alanine) was also not phosphorylated by LmxMPK3 excluding threonine 440 and serine 548 as phosphorylation sites used by LmxMPK3. This observations support the idea, that serine 440 and 548 are not the target phosphorylation sites leaving serine 554 as the probable phosphorylation site used by His-LmxMPK3. Therefore, pGEX-KGSPKin29S554A was generated and tested. Indeed, LmxMPK3 did not phosphorylate GST-LmxKin29S554A confirming that serine 554 is the phosphorylation site used by activated LmxMPK3.

Overall, LmxMPK3 can phosphorylate LmxKin29 \textit{in vitro} and therefore most likely regulates its activity. However, the phosphoproteome (21) revealed that potential phosphorylation sites occurred on serine 551 and serine 554 in the LmxKin29 peptide \textit{in vivo}. It is possible that His-LmxMPK3 phosphorylates serine 554 first followed by phosphorylation of serine 551. Alternatively, serine 551 is phosphorylated by another kinase. Additionally, amino acid sequence alignments of LmxKin29 with its homologues in other species (Error! Reference source not found.) revealed that serines 551 and 554 are conserved amino acids. They are likely major sites for LmxKin29 regulation by phosphorylation.

Fig. 2. Partial alignment of LmxKin29WT and mutants (SA, SD, A2, A4, 554A). A, LmxKin29SA, replacement of serine 551 with alanine (highlighted in red); LmxKin29SD, replacement of serine 551 with aspartic acid (highlighted in pink); LmxKin29A4, replacement of four residues serine 548, 551, 554, and threonine 440 with alanine (highlighted in blue); LmxKin29A2, replacement of two serine residues, serine 551 and 554 with alanine (highlighted in brown); LmxKin29S554A, replacement of serine 554 with alanine (highlighted in green). The alignment was achieved by https://www.ebi.ac.uk/Tools/msa/clustalo/. B, Graphical, view shows mutational replacement of five LmxKin29 mutants. C, Purified recombinant proteins of GST-LmxKin29 on Coomassie-stained 14%. SDS-PAGE. Lane 1, GST-LmxKin29 WT; lane 2, GST-
LmxKin29SD; lane 3, GST-LmxKin29A4; lane 4, 25 µL of eluted His-LmxMPK3; lane 5, LmxKin29SA; M, marker in kDa. GST protein samples were prepared by using 70 µL suspension of protein with beads (7: 93 v/v) resuspended with 30 µL SDS loading dye and loaded 25 µL for each). D, Radiometric kinase assay of His-LmxMPK3 with GST-LmxKin29, a, Coomassie-stained 14% SDS-PAGE. B, Autoradiograph after 4 hours exposure. Lane 1, His-LmxMPK3 + GST-LmxKin29WT; lane 2, His-LmxMPK3 + GST-LmxKin29SA; lane 3, His-LmxMPK3 + GST-LmxKin29SD; lane 4, His-LmxMPK3 + GST-LmxKin29A4; 5, His-LmxMPK3 + GST-LmxKin29A2; M, marker in kDa. E, Radiometric kinase assay of His-LmxMPK3 with different GST-LmxKin29 proteins, a, Coomassie-stained 14% SDS-PAGE gel, b, autoradiograph after 24 hours of exposure. Lane 1, His-LmxMPK3 + GST-LmxKin29WT; lane 2, His-LmxMPK3 + GST-LmxKin29SA; lane 3, His-LmxMPK3 + GST-LmxKin29A2; lane 4, His-LmxMPK3 + GST-LmxKin29S554A; M, marker in kDa.

3.5 Generation of LmxKin29 single and double knockout clones (ΔLmxKin29+/−) (ΔLmxKin29−/−) and PCR confirmation.

The knockout cassettes were introduced into wild type L. mexicana promastigotes. 3 × 10⁷ late log phase cells were transfected by two consecutive rounds of electroporation. First generate a single allele deletion mutant, ΔLmxKin29+/−, and secondly for a double allele deletion mutant, ΔLmxKin29−/−. Subsequently both electroporation cultures were left to grow for 15-20 days and positive clones were selected selectively by the two antibiotics Phleo and BSD.

To confirm the correct gene replacement in the double allele knockout (Fig. 3A) shows fifteen PCR reactions of the null mutant ΔLmxKin29−/−Phleo/Bla. A single band is visible for phleomycin (766 bp) in lane 9, single knockout, also in lanes 12 and 15 for two double knockout clones, A3 and D11, indicating correct integration of the Phleo gene. While the PCR in lane 5 shows, correct integration of the Bla gene with 937 bp in the single knockout ΔLmxKin29+/−, and lanes 14 and 14 show one band for the two double knockout clones A3 and D11. Lanes 1, 4, and 7 show an 860 bp DNA fragment derived from LmxKin29 that is only present in the wild type and single allele knockout clones. Significantly, null mutants was generated. Several strategies have been successfully applied to investigate the function of LmxKin29 in more detail. Initially by morphological analysis of the LmxKin29 mutant clones comparing them with L. mexicana wild type. Secondly, pathogenesis analysis effect with LmxKin29 absence in L. mexicana.

3.6 Phenotype Analysis of the LmxKin29 null mutant

Surprisingly, the first microscopic examination of the promastigote culture revealed that no obvious changes could be detected in ΔLmxKin29−/− clones compared to wild type cell size and flagellum length and it was difficult to tell whether the large proportion of normal cells actually reflects cells lacking an abnormal phenotype. The morphological changes were analysed for LmxKin29 mutants versus L. mexicana wild type promastigotes in logarithmic growth (Table1). The data were analysed using a two-tailed, non-paired student’s t-test. The phenotype measurements showed a wide variety of results in body length and flagellum length. Therefore, a correlation test (Pearson correlation coefficient) analysis is used to test the variables between groups. The correlation coefficient, r, ranges from r <1 (Perfect negative or inverse correlation) to r >1 (Perfect correlation).
The (Fig.3D, E and F) displays measurements and statistical analyses of, cell body body width and flagellar length respectively of LmxKin29 mutants. As presented in (Fig. 3D) the body length analysis exhibited a significant increase in body length ($p<0.001$, $t=3.484$) compared between $\Delta LmxKin29+/Bla$ and the wild type and the correlation test showed a significant $r$-value ($r>0$; $r=0.1446$, $p=0.05$). Compared to the wild type the single allele mutant $\Delta LmxKin29+/-Phleo$ D1 and the null mutant A3 displayed a significant decrease in body length ($p<0.001$, $t=5.054$) and ($p<0.001$, $t=5.065$), respectively. However, both analyses have a low correlation value of less than zero. Interestingly, the null mutant D11 showed no significant changes in body length compared to the body length of the wild type. These results are in line with the previous study by (30) which suggested that there is a wide range of changes or a significant variation in body length in a wild type cell culture due to the various cell cycle stages present. Although the comparative analysis of the body width revealed a significant increase for all LmxKin29 mutants ($p<0.001$) versus the wild type, these results displayed no correlation ($r<0$) (Fig. 3E). That could be related to the timing of the measurements with regard to cell density, with regard to cell density, which had a different density.

Flagellum length revealed a significant decrease for $\Delta LmxKin29+/-Phleo$ D1 compared to the wild type but no correlation between them ($r<0$) (Fig. 3F). The single allele $\Delta LmxKin29+/-Bla$ H5 and the null mutant A3 showed no significant difference in flagellum length (Fig. 3F). The double knockout D11 showed a significant increase in flagellum length ($p<0.05$, $t=2.209$) compared to the wild type, but the clones showed no correlation ($r<0$). It was observed that the growth of the flagella extends over multiple cell cycles, growing progressively longer with each cycle, until a certain length when it began to disassemble at the tip. The second possibility to obtain these results can be due to the different cell densities between the mutant clones and the wild type promastigotes (30). A study conducted by (16) found that a knockout of $TbKif13-2$ where both copies of the gene were deleted resulted in no significant elongation of the flagellum and overexpression only slightly decreased flagellar length and the rate of growth of a new flagellum during cell division.

In summary, no morphological changes between cell lines in general. The variations in the three parameters determined in the morphological analysis is likely due to the slightly different culture densities and might represent cells in various stages of the cell cycle.

In conclusion, the morphological analysis for the knockout clones could not prove that LmxKin29 is involved in flagellar formation in promastigotes, which probably means that it has another function. Localisation of the protein in the cells could possibly help to inform about the role of Lmx Kin29 in the parasite. With no apparent function in the promastigote stage LmxKin29 might play a role in the mammalian amastigote stage justifying an in vivo investigation. Why did the loss of LmxKin29 not cause defects in flagellum length in spite of the biochemical analysis which proved that the kinase LmxMPK3 can phosphorylate LmxKin29 LmxKin29 might have another function or there might be other molecules present that may allow through functional redundancy that the morphology of the parasite is not changed in LmxKin29 null mutants (31). It is also possible that LmxMPK3 has additional functions to its role in flagellum length regulation.

Table 1. Cell density for wild type and LmxKin29 mutants at the time of measurements
Fig. 3. Confirmation of LmxKin29 null mutants and basic morphology analysis of transgenic L. mexicana ΔLmxKin29-/-Phleo Bla. A, Agarose gel of PCR and lanes 1, 4, and 7, 860 bp DNA fragment derived from LmxKin29; lanes 5, 11 and 14, 937 bp DNA fragment indicating correct integration of Bla; lanes 9, 12, and 15, 766 bp DNA fragment indicating correct integration of phleo. B, Cartoon showing the properties of each cell measured for analysis; cell body length, flagellum length and body width. C, Table 1 Cell density for wild type and LmxKin29 mutants at the time of measurements. D, Morphological analysis of deletion mutants of LmxKin29 (single and double allele) compared with L. mexicana wild type promastigotes. Means with standard errors of the means are displayed. D, cell body; E, cell body width. F, flagellum length. Stars indicate significant differences (p*<0.05, p**<0.01, p***<0.001) (Student’s t-test and correlation test). Measurements of 200 random cells were taken using Image J.

3.7 Localisation of LmxKin29 null mutant

LmxKin29 fused to GFP was expressed in null mutant promastigotes. The protein was expressed in the two null mutant clones by introducing pTHGFP-LmxKin29 where GFP is fused to the N-terminus of LmxKin29 and pTHLmxKin29GFP with a C-terminal fusion. Both transfections were successful and two clones were selected from each transfection and
grown in culture with hygromycin B (20 μg/mL). Fluorescence microscopy of live or fixed transgenic L. mexicana promastigotes (addback LmxKin29GFP and GFP/LmxKin29 clones exhibited a localisation of LmxKin29 throughout the cytosol with an accumulation next to the flagellar pocket (Fig. 4 A). Although, localisation was similar in most cells, some dividing cells exhibited an accumulation for LmxKin29 at the anterior and posterior ends. Interestingly, a fluorescent spot could be seen in the area where dividing cells were still attached in both LmxKin29GFP and GFP/LmxKin29 (Fig. 4 Ba,b).

3.8 Verification of GFP-tagged LmxKin29 expression by immunoblotting

The expression of GFP-tagged LmxKin29 in transfectants was validated by immunoblot analysis with an anti-GFP antibody to confirm integrity of the fusion protein (Fig.4C). The cell lysate of four add-back clones, two clones carrying pTHGFPKin29 (A3C10 and D11C2) and two clones with pTHKin29GFP (A3C12 and D11A1) were used. In addition, a cell lysate of L. mexicana expressing GFP only was used as a positive control and the wild type as a negative control. The blot in figure 4C displayed correct band sizes for LmxKin29 fused to GFP at 95.1 kDa (68.3 kDa for LmxKin29 + 26.8 kDa for GFP) for all clones. Free GFP is shown in lane 4 of figure 5C for cells expressing GFP only. No free GFP was detectable in any of the clones expressing GFP-tagged LmxKin29. This confirms that the fluorescence seen in the promastigotes is indeed from the tagged protein and not GFP alone. Fluorescence microscopy localised LmxKin29 fused with GFP next to the flagellar pocket for both N- and C-terminally tagged kinesin. These results are consistent with the phenotype investigations of null mutants for LmxKin29, which proved that LmxKin29 deficiency had no effect on the flagellum length, body width and body length of promastigotes. The proximal flagellar localisation has been shown to be an important regulatory site for different proteins in eukaryotic cells (32) like for instance a NIMA-related kinase, FA2, which is consistent with its role in deflagellation (33). We anticipated that LmxKin29 cellular similarity in localisation with another kinesin could give a clue about its function. Kinesin-13 (32), and Tb927.6.1770 (29) have a similar localisation as LmxKin29 in dividing cells. Hence, this localisation provides evidence that LmxKin29 might be responsible for certain fundamental cellular functions such as microtubule organisation or may mediate cell division. This should be addressed in further work.
Fig.4. Localisation LmxKin29. A, Fluorescence microscopy of methanol-fixed *L. mexicana* promastigotes carrying pTHGFPKin29 (N-terminal), D11C2. Transgenic LmxKin29 variants were visualised with appropriate wavelengths for the indicated fluorophores, using a blue DAPI filter for DAPI, green FITC filter for GFP and white light for bright field (BF) microscopy; merge is a combination of three images using Image J software. Images were taken at 300 ms exposure time for fluorescence and 10 ms exposures for bright field. The arrows point to Kin29 localisation, Bar, 10 μm. B, Fluorescence microscopy of live transgenic *L. mexicana* promastigotes divided cells carrying pTHKin29GFP, a A3C12 (C-terminus GFP), b, carrying pTHGFPKin29, D11C2 (N-terminus GFP). The arrows point to GFPKin29 and Kin29GFP localisation. Bar, 10 μm. C, Identification of expression GFP-tagged LmxKin29 in *L. mexicana* by Immunoblot by using monoclonal anti-GFP (D5.1) XP® Rabbit (concentrated culture supernatants of promastigotes 1×10^8, a, 14% Coomassie-stained SDS-PAGE, b, immunoblot with blotted to nitrocellulose membrane. Lane 1, wild type *L. mexicana* (negative control); lanes 2 and 3, pTHGFPLmxKin29 A3C10 and D11C2, respectively (N-terminal GFP); lane 4, GFP expressed in *L. mexicana* (positive control); lanes 5 and 6, pTHKin29GFP A3C12 and D11A1, respectively (C-terminal GFP), marker in kDa.

**Conclusion**

In summary, LmxKin29 is a typical kinesin motor molecule protein containing the three main parts, motor domain, coiled-coil and the tail domain. LmxKin29 can be bioinformatically classified as members belong to unspecified (anorphan) kinetoplast kinesin.
superfamily. LmxKin29 from *L. mexicana* is a putative kinesin with a high sequence similarity to the *L. major* kinesin LmjF.30.0350 that belongs to the “orphan” kinesins, introduced by (1). LmxKin29 also has a homologue in *T. brucei* (Tb927.5.1870) which was mentioned recently as an orphan kinesin. One of the unanticipated findings was that the LmxKin29 null mutant clones did not show any significant difference compared with the phenotype of the *L. mexicana* wild type. This result proved that LmxKin29 is not involved in the *L. mexicana* flagellum assembly. LmxKin29 plays a significant role in *L. mexicana* pathogenicity in vivo. The most important clinically relevant finding showed that LmxKin29 null mutants are unable to cause lesions in infected Balb/c mice and that no parasites could be detected at the injection site ten weeks post infection. LmxKin29 is not the only kinesin that is essential for parasite survival in an infected host. LmxKin29 and TbKif13-1 have a similar localisation in dividing cells and they both affect the parasite’s pathogenicity. LmxKin29 is essential for survival of the amastigote parasite offering that it would be an ideal drug target.

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