Targeting LmxMKKK19 for better understanding of Mitogen-Activated Protein Kinase cascades in *Leishmania mexicana*

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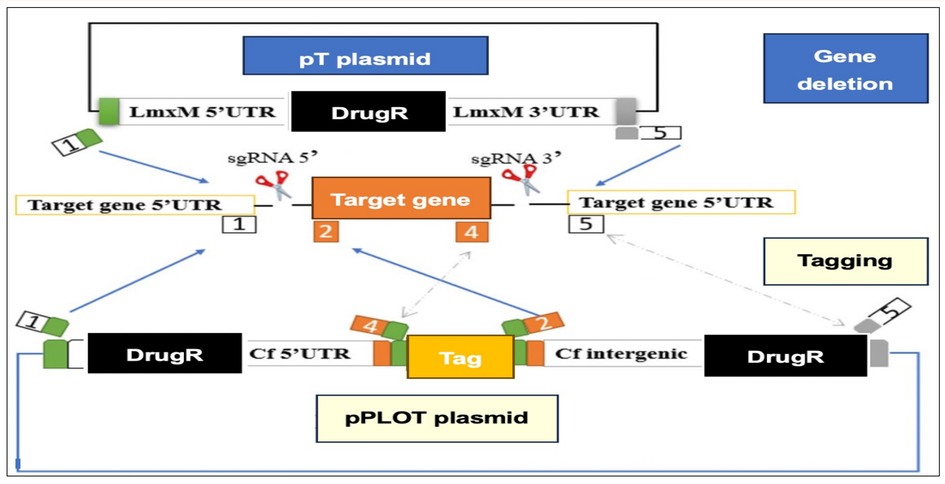
Introduction

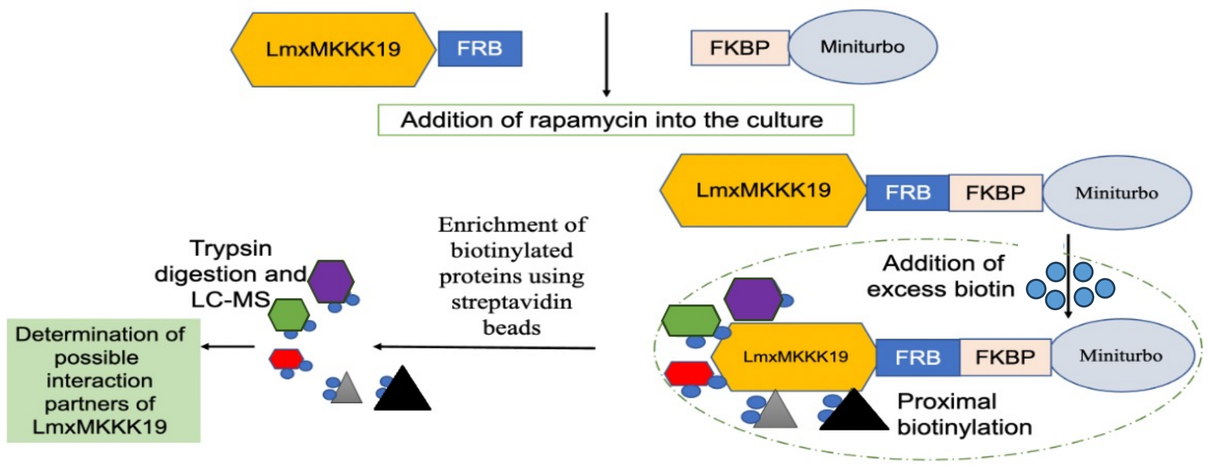
- Little is known about *Leishmania* MAP kinase kinase kinases (MAPKKK). LmxMKKK19 has been annotated in TriTrypDB as a putative MAPKKK in *Leishmania mexicana.* To understand MAP kinase cascades in *Leishmania,* we targeted LmxMKKK19 for deletion and tagging using CRISPR-Cas9.
- Single and double allele knockout clones were generated by electroporation utilising two different resistance markers (figures 1 and 2).
- Add-back clones were obtained implementing homologous recombination of *LmxMKKK19* into the ribosomal RNA gene locus (figures 3 and 4). The generated clones were screened for changes in morphology, growth and their potential to infect Balb/c mice (figures 5, 6 and 7).
- To unveil the localisation of LmxMKKK19 in promastigotes, the protein was tagged with mNeonGreen (mNG) using CRISPR-Cas9 at either its N- or C-terminus (figure 8). A CRISPR-Cas9 cell line, expressing miniTurbo biotin ligase fused to FKBP, a component of the 2C-BioID system, was used to fuse the second component, FRB (figure 9), to LmxMKKK19 for proximity labelling of its interaction partners using biotin, isolation of biotinylated proteins using streptavidin-agarose, tryptic digest and mass spectrometry.
- LmxM.33.2090 was determined as a putative interaction partner of LmxMKKK19. Using CRISPR-Cas9 on a cell line already expressing mNG-tagged LmxMKKK19, monomeric red fluorescent protein (mRFP) fused to LmxM.33.2090 showed co-localisation in fluorescence microscopy (figure 10).

Methods

1) Tagging or deletion of target genes using a CRISPR-Cas9 (Beneke *et al.,* 2017)



2) Determination of interaction partners of LmxMKKK19 by 2C-miniTurboID and LC-MS



Results

1) Generation of *LmxMKKK19* knockout clones using CRISPR-Cas9

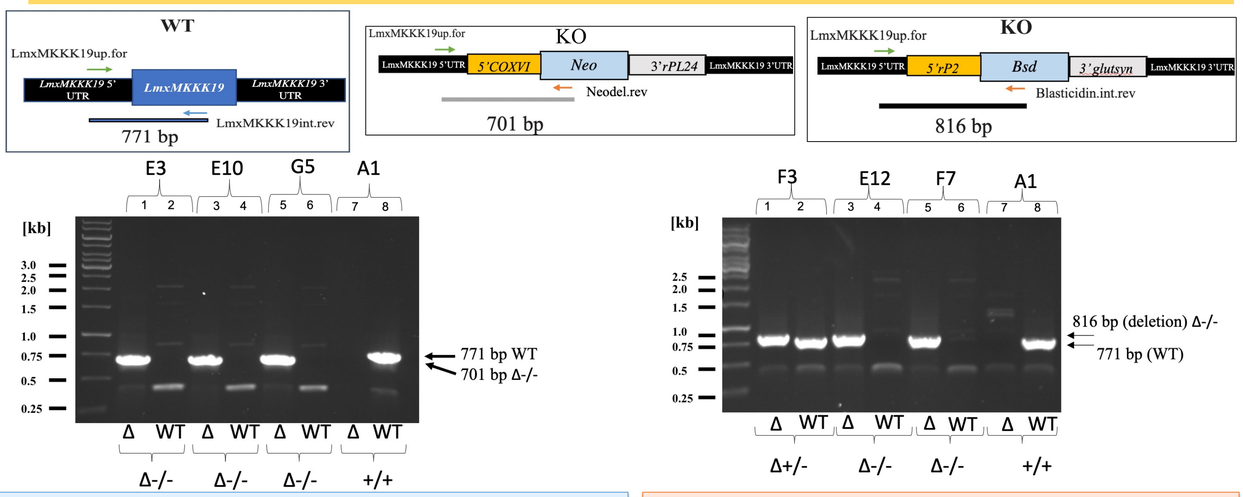


Figure 1. PCR analysis of Δ*MKKK19* cell lines generated using neomycin repair cassette. A1 is the parental cell line. Δ*MKKK19*_E3, Δ*MKKK19*_E10 and Δ*MKKK19*_G5 are Δ*MKKK19* cell lines. Lanes 1, 3, 5, 7, PCRs with LmxMKKK19up.for and Neodel.rev oligomers to detect the gene replacement by the neomycin repair cassette (701 bp fragment). Lanes 2, 4, 6, 8, LmxMKKK19up.for and LmxMKKK19int.rev primers to test the presence of *LmxMKKK19* (771 bp fragment).

Figure 2. PCR analysis of Δ*MKKK19* cell lines produced using a blasticidin repair cassette. A1 is the parental cell line. Δ*MKKK19*_F3 , Δ*MKKK19*_E12 and Δ*MKKK19*_F7 are Δ*MKKK19* cell lines. Lanes 1, 3, 5, 7, PCRs with LmxMKKK19up .for and blasticidin.int.rev to detect the gene replacement by the blasticidin repair cassette (816 bp fragment). Lanes 2, 4, 6, 8, LmxMKKK19up.for and LmxMKKK19int.rev primers to test the presence of *LmxMKKK19* WT allele (771 bp fragment).

2) Generation of gene add-back clones using homologous recombination into rRNA gene locus

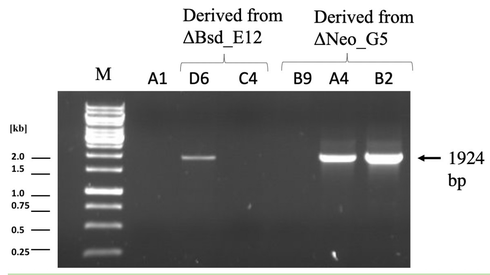


Figure 3. Confirmation of add-back clones by diagnostic PCR. A1 is the parental cell line. The expected PCR fragment size is 1,924 bp.

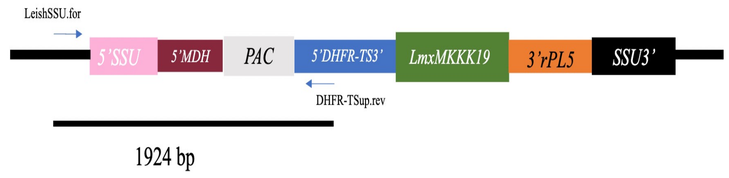


Figure 4. Schematic representation of genomic situation after homologous recombination.

3) Unveiling the role of LmxMKKK19 in *Leishmania mexicana* promastigotes

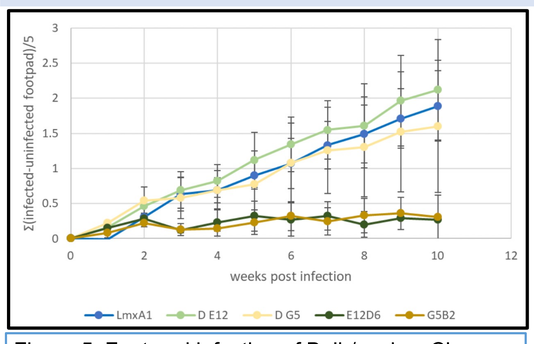


Figure 5. Foot pad infection of Balb/c mice. Clones E12 (ΔBsd) and G5 (ΔNeo) are deletion clones while E12D6 and and G5B2 are add back clones. LmxA1 is the parental cell line.

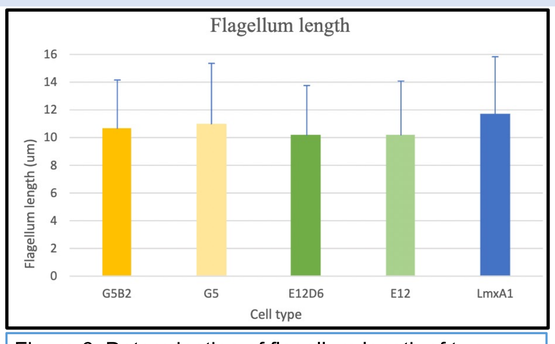


Figure 6. Determination of flagellum length of two deletion clones (E12 and G5), two add-back clones (E12D6, G5B2) and the parental cell line (LmxA1). 200 cells were randomly measured.

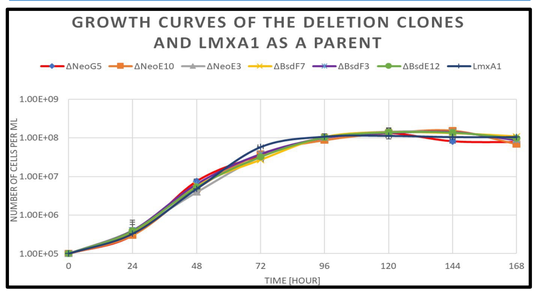
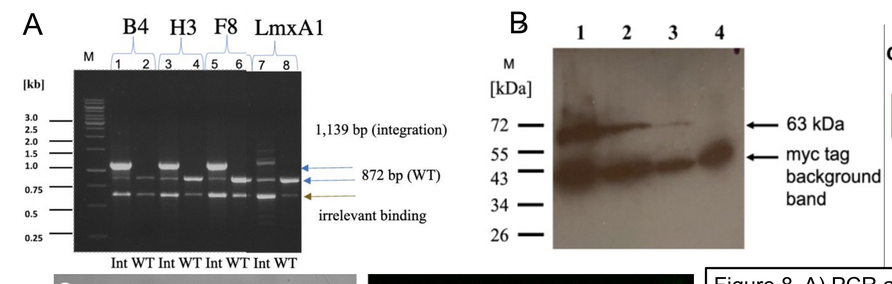


Figure 7. Proliferation experiment on deletion clones of LmxMKKK19 (G5, E10 and E3 clones for ΔNeo , F7, F3 and E12 for ΔBsd) and LmxA1 as a parental cell line. The experiment was repeated three times.

4) C- terminal mNG tagging of LmxMKKK19 for determination of the localisation



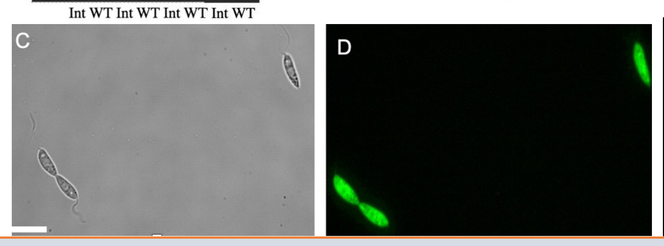


Figure 8. A) PCR analysis to confirm the correct mNG fusion. LmxMKKK19int.for and pPLOTmNG.rev primers used in lanes 1,3,5,7. LmxMKKK19int.for and LmxMKKK19ds.rev used in lanes 2,4,6,8.
B) Immunoblot analysis of C- terminally mNG tagged LmxMKKK19. lane 1, B4; lane 2, H3; lane 3, F8; lane 4, LmxA1; C)MKKK19::mNG_H3 brightfield image; D) MKKK19::mNG_H3 FITC filter.

5) C- terminal FRB tagging of LmxMKKK19

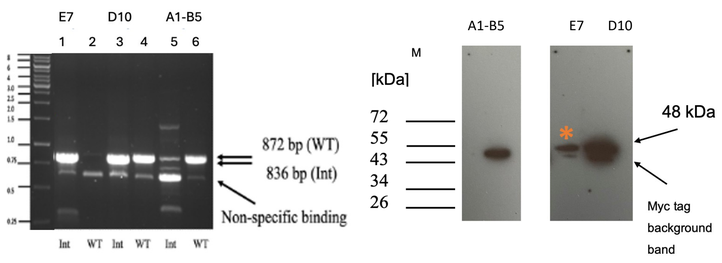


Figure 9. PCR analysis to confirm *LmxMKKK19::FRB* integration into the genome. LmxMKKK19int.for and pPLOTFRB.rev primers were used for diagnostic PCRs in lanes 1, 3, 5 for determination of *LmxMKKK19::FRB* integration. LmxMKKK19int.for and LmxMKKK19ds.rev primers were used for PCRs in lanes 2, 4, 6 to assess the presence of the WT *LmxMKKK19* allele.

6) C- terminal mRFP tagging of LmxM.33.2090 and co-localisation of LmxMKKK19 with LmxM.33.2090

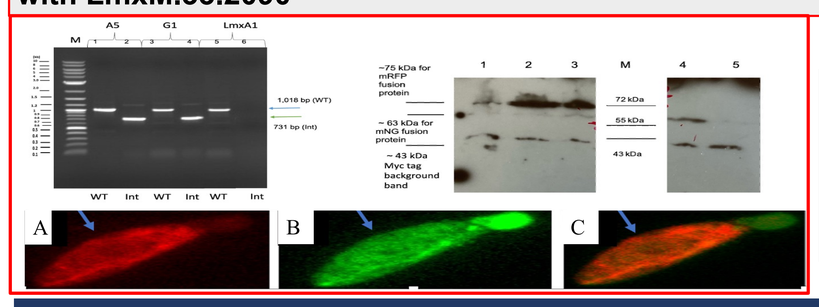


Figure 10. Fluorescence imaging of LmxA1MKKK19::mNG_H3-LmxM.33.2090::mRFP_A5 cells showed by the blue arrows. Left panel (A), TRITC filter; middle panel (B), FITC; right panel (C), mRFP/mNG merge.

Conclusions

- LmxMKKK19 is not essential in culture for *Leishmania* promastigotes.
- LmxMKKK19 localisation is throughout the cytosol with some accumulation close to the flagellar pocket.
- LmxM.33.2090, a putative MAPKK, has been determined as a promising interaction partner of LmxMKKK19 and co-localisation of LmxM.33.2090 with LmxMKKK19 was shown.

References

Beneke, T., Madden, R., Makin, L., Valli, J., Sunter, J., & Gluenz, E. (2017). A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids. *Royal Society open science*, *4*(5), 170095.

Chojnowski, A., Sobota, R. M., Ong, P. F., Xie, W., Wong, X., Dreesen, O., Burke, B., & Stewart, C. L. (2018). 2C-BioID: an advanced two component BioID system for precision mapping of protein interactomes. *Science*, *10*, 40-52

Wiese, M. (2007). Leishmania MAP kinases–familiar proteins in an unusual context. *International journal for parasitology*, *37*(10), 1053-1062.

Wiese, M., Wang, Q., & Görcke, I. (2003). Identification of mitogen-activated protein kinase homologues from Leishmania mexicana. *International journal for parasitology*, *33*(14), 1577-1587.

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