Chapter 2

Validation Of Glutathione Synthetase as a Therapeutic Target Through Gene Knockout Using CRISPR-Cas9 *

Abstract

Genetic validation provides a strategic advantage in identifying and prioritizing drug targets in the rational drug discovery process against infectious diseases like leishmaniasis. Visceral leishmaniasis, caused by *Leishmania donovani,* continues to be a significant health concern, necessitating the development of novel therapeutic strategies. Targeting the parasite's redox metabolism has emerged as a promising approach due to its essentiality and the differences in the host's redox pathways. In this chapter, we investigated the essentiality and functional role of the glutathione synthetase enzyme by employing a CRISPR-Cas9-based gene knockout approach to generate glutathione synthetase-deficient *Leishmania donovani* strains. Complete deletion of the glutathione synthetase gene could not be achieved due to copy number variation or aneuploidy in the 14^{th} chromosome. However, deletion in a single locus impaired the parasite's growth and infectivity, emphasizing its essential role in *Leishmania* pathogenesis. Our findings thus highlight glutathione synthetase as a crucial enzyme for parasite virulence, making it a promising target for the development of novel antileishmanial therapeutics.

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* Part of the work is communicated for publications

2.1 Introduction

Targeted gene replacement has been an effective strategy for elucidating the functional roles of genes that are involved in core cellular pathways. In parasitology research, such as leishmaniasis, malaria, and trypanosomiasis, identification of essential genes of the pathogenic organism is crucial for understanding the parasite's biology and developing therapeutic strategies against it. However, *Leishmania* parasites naturally possess biological characteristics that complicate genetic manipulation; they are very susceptible to aneuploidy, rendering the alteration of critical genes difficult and unstable (Dumetz et al., 2017a; Rogers et al., 2011a; Sterkers et al., 2011). RNA interference is also ineffective in the majority of *Leishmania* species(Robinson & Beverley, 2003). In some *Leishmania* species, although RNA interference is effective, it has not been utilized to the same degree as *Trypanosoma brucei*, primarily because of the absence of an inducible mechanism to examine the role of key genes (Lye et al., 2010). Although many attempts at earlier gene replacement approaches were based on homologous recombination, which often fails and was a time-consuming technique, the recent introduction of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based gene editing in eukaryotic cells had become a precise, effective, and high-throughput genetic tool to enable the study of a larger set of genes. Different strategies have been developed to deliver or express the Cas9 protein of *Streptococcus pyogenes* or *Staphylococcus aureus* origin and single guide RNA (sgRNA) into *Leishmania* cells, facilitating precise genome editing (Abdi Ghavidel et al., 2024; Beneke et al., 2017b; Zhang et al., 2020a; Zhang & Matlashewski, 2015).

The process of novel drug discovery and development requires significant time and investment, often leading pharmaceutical companies to neglect diseases like

leishmaniasis due to their low market potential, which primarily affects people from low and middle-income countries(Choi et al., 2021; Santos et al., 2020). Because of these reasons, modern drug discovery efforts for leishmaniasis remain inadequate despite posing a significant global health challenge to a large number of people. With a few chemotherapeutic options that are also limited by factors like toxicity, drug resistance, and high cost, it becomes important to search for a new therapeutic target for the development of an effective antileishmanial compound. As we discussed in the previous chapter, the dependence of *Leishmania* parasites on the trypanothione-based redox system underscores the therapeutic importance of each enzyme of the trypanothione biosynthesis pathway. Different enzymes of this pathway have already been studied by either genetic or biochemical methods. For instance, genetic and chemical analysis on *Leishmania infantum* revealed that the parasites could not survive upon complete deletion of the trypanothione synthetase, unless previously complemented with an episomal copy of the gene, thereby rendering it a potential therapeutic target (Sousa et al., 2014). The same study also showed that, unlike trypanothione synthetase, glutathionylspermidine synthetase, responsible for synthesizing glutathionylspermidine (Gsp) from glutathione (GSH) and spermidine, is not essential for the parasite's survival. The essentiality of the glutathione synthetase has been already validated in *Trypanosoma brucei,* where the depletion of the enzyme resulted in the loss of intracellular trypanothione and subsequent parasitic death, demonstrating its fundamental function in redox metabolism (Pratt et al., 2014). The study also showed that exogenous glutathione could partially rescue the parasites, indicating the ability of these parasites to transport exogenous glutathione. Similarly, gamma-glutamylcysteine synthetase, a rate-limiting enzyme in glutathione and trypanothione biosynthesis, was found to be critical in *Leishmania infantum,* as the null

mutant could only be produced through episomal expression. Even the heterozygous mutants exhibited decreased thiol levels, increased susceptibility to oxidative stress and antimonials, and compromised intracellular survival, thereby emphasizing the essential function of thiol metabolism in parasite defense and pharmacological response(A. Mukherjee et al., 2009b). These findings underscore these enzymes' potential as pharmacological targets in controlling trypanosomatids. Although these significant insights have been obtained from genetic and biochemical studies in related organisms, suggesting the importance of glutathione synthetase enzyme in trypanothione biosynthesis, a direct genetic validation in *Leishmania,* to support its essentiality, is still lacking. In this chapter, we aim to address this gap by producing glutathione synthetase-deficient *Leishmania donovani* parasites and thereby assessing the role of glutathione synthetase in the parasite survival and virulence.

The *glutathione synthetase* gene is present as a single exon on the 14th chromosome of *L. donovani,* as reported in a whole genome sequencing study from multiple clinical isolates of *Leishmania donovani* (Downing et al., 2011a). Therefore, complete disruption of the *glutathione synthetase* gene requires successful replacement of the open reading frames (ORFs) with two selection marker genes or insertion of a stop codon in both alleles of a homologous pair of chromosomes (Bryant et al., 2019;Engstler & Beneke, 2023a). Until the development of the CRISPR Cas9-based gene editing system, gene disruptions in unicellular eukaryotes like *Leishmania* were mainly achieved by homologous recombination, in which a number of recombination cassettes are constructed, containing a drug resistance marker gene flanked by two homology arms of the targeted gene (Cruz & Beverley, 1990;Hwang et al., 1996). By using this method, recombination is achieved in one allele at a time, therefore, it requires multiple rounds of transfection to generate a complete knockout strain. These

challenges in the conventional homology recombination method were overcome by the introduction of the CRISPR-based technique for trypanosomatids. In our study, we employed LeishGEdit (Beneke et al., 2017b), a CRISPR Cas9-based toolkit that has been successfully applied in different *Leishmania* species, including *L. braziliensis*, *L. major,* and *L. donovani,* to delete hundreds of genes (Alpizar-Sosa et al., 2022; Espada et al., 2021a; Halliday et al., 2024; Sharma et al., 2022). This method involves the generation of the SpCas9 and T7 RNA polymerase expressing *Leishmania* strain, followed by the transfection of PCR amplified DNA templates for single guide RNAs (sgRNAs) and donor DNAs (Figure 2.5). This enables the *in vivo* transcription of the single guide RNAs, followed by the assembly with the Cas9 nuclease that precisely recognizes and cuts at the target sites. The selectable marker gene is then integrated through the parasite's homology-directed repair mechanism. One major advantage of the LeishGEdit toolkit is that it does not require multiple cloning steps or *in vitro* transcription of sgRNAs, making it suitable for high-throughput gene editing in *Leishmania* parasites. Since the *glutathione synthetase* gene encodes the enzyme glutathione synthetase, a key component in the parasite's redox biology; therefore, deletion of the gene is expected to impair the redox homeostasis, which we can further evaluate through phenotypic and biochemical analysis.

2.2 Materials and methods

2.2.1 Parasite Culture and Chemicals

Wild-type *Leishmania donovani* promastigote cells (MHOM/IN/1983/AG83) were cultured at 25 °C in M199 media (pH 7.4) supplemented with 10% FBS (heat-inactivated). The *Cas9* and *T7 RNAP* expressing *Leishmania donovani* promastigote-

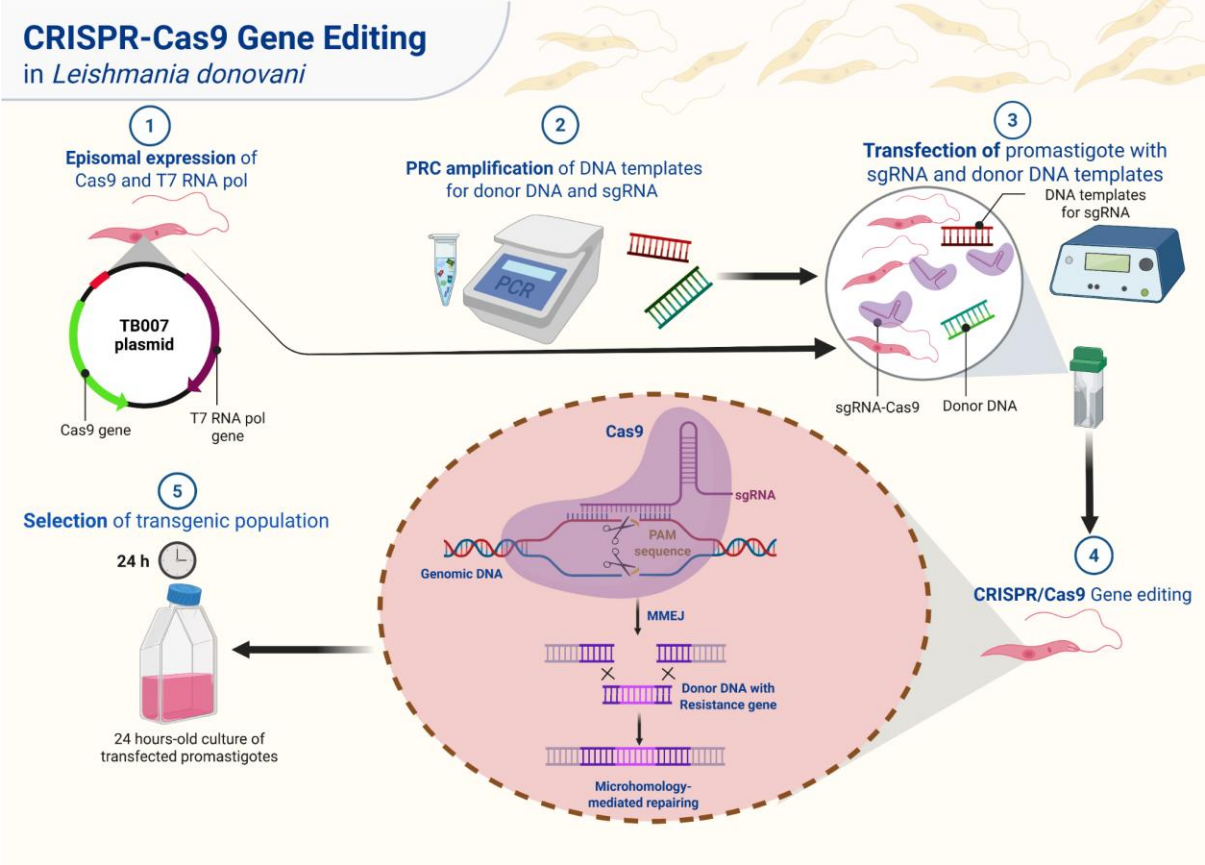

Figure 2.1: Schematic representation of the gene editing steps using the LesihGEdit toolkit. First, the Cas9 and T7 RNA polymerase-expressing *Leishmania donovani* promastigotes are generated through episomal expression of the pTB007 plasmid, followed by transfection with the DNA templates of single guide RNAs 9sgRNAs) and donor DNAs. Finally, successfully edited gene-harbouring parasites are selected by antibiotic-containing media.

cells (*L. dono* cas9 T7) were maintained at 25 °C in complete M199 media containing 100μg/ml hygromycin B. Electroporation buffer was prepared following the protocol (Beneke et al., 2017b). Plasmids- pTB007, pTNeo_v1, pTPuro_v1, and pPLOTv1 neo-mNeonGreen-neo plasmids were kind gifts from Prof. Eva Glunez's lab at the University of Bern. These plasmids were used to transform *E. coli* Top10 cells, then isolated for further use. Antibiotics- Hygromycin-B, Puromycin dihydrochloride, and Neomycin sulfate were purchased from Himedia. For PCR NEB HiFi Phusion polymerase kit was used.

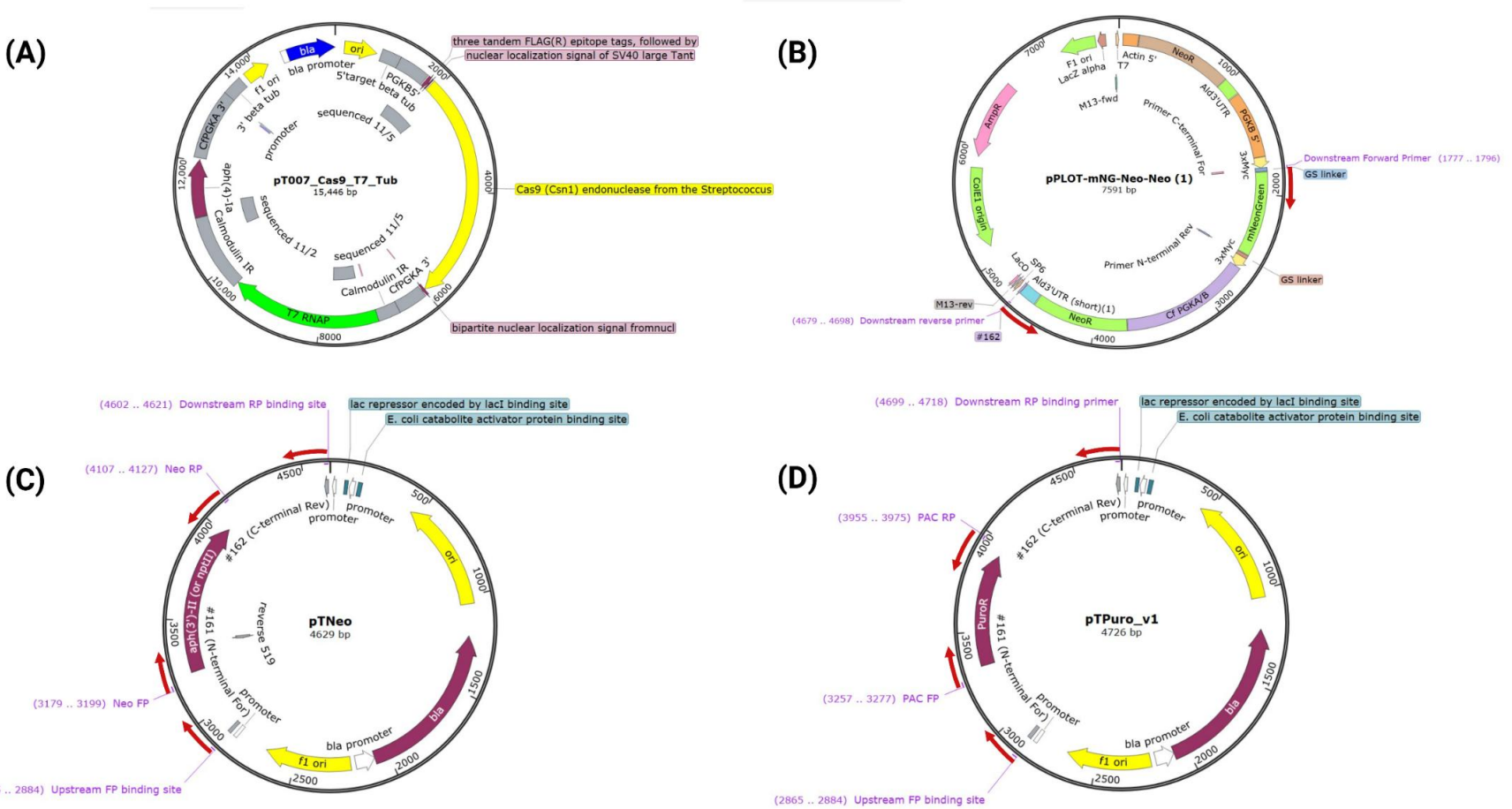

Figure 2.2: SnapGene maps of the plasmids used in the LeishGEdit toolkit. (A) Map of the pTB007 plasmid, it includes the coding sequences for *Sp*Cas9, T7 RNA polymerase, and hygromycin B resistance gene, used for episomal expression of Cas9 and T7 RNA polymerase (B) map of the pPLOT plasmid containing the sequence of mNeonGreen fluorescent protein and Neomycin resistance gene, serving as a template for donor DNA for tagging experiments (C) & (D) pTNeo & pTPuro plasmid, respectively, having the neomycin/ puromycin resistance gene, used as DNA templates for donor DNA amplification.

2.2.2 Generation of Cas9 and T7 RNAP expressing *L. donovani*

Since the *Leishmania donovani* parasite does not naturally express the Cas9 nuclease, Cas9-expressing *L. donovani* (*L. dono* cas9 T7) cells were generated by delivering ~20μg of the pT007 plasmids through electroporation into the mid-log phage *L. donovani* promastigotes cells (~1x107 cell/ml) (Image:). The pT007 plasmid encodes *S. pyogenes* origin Cas9 nuclease, T7 RNA polymerase, and hygromycin B phosphotransferase. Transfected cells therefore expressed both Cas9 nuclease and T7 RNA polymerase, and also enabled us to select the transfected cells in 100μg/ml hygromycin B containing M199 media.

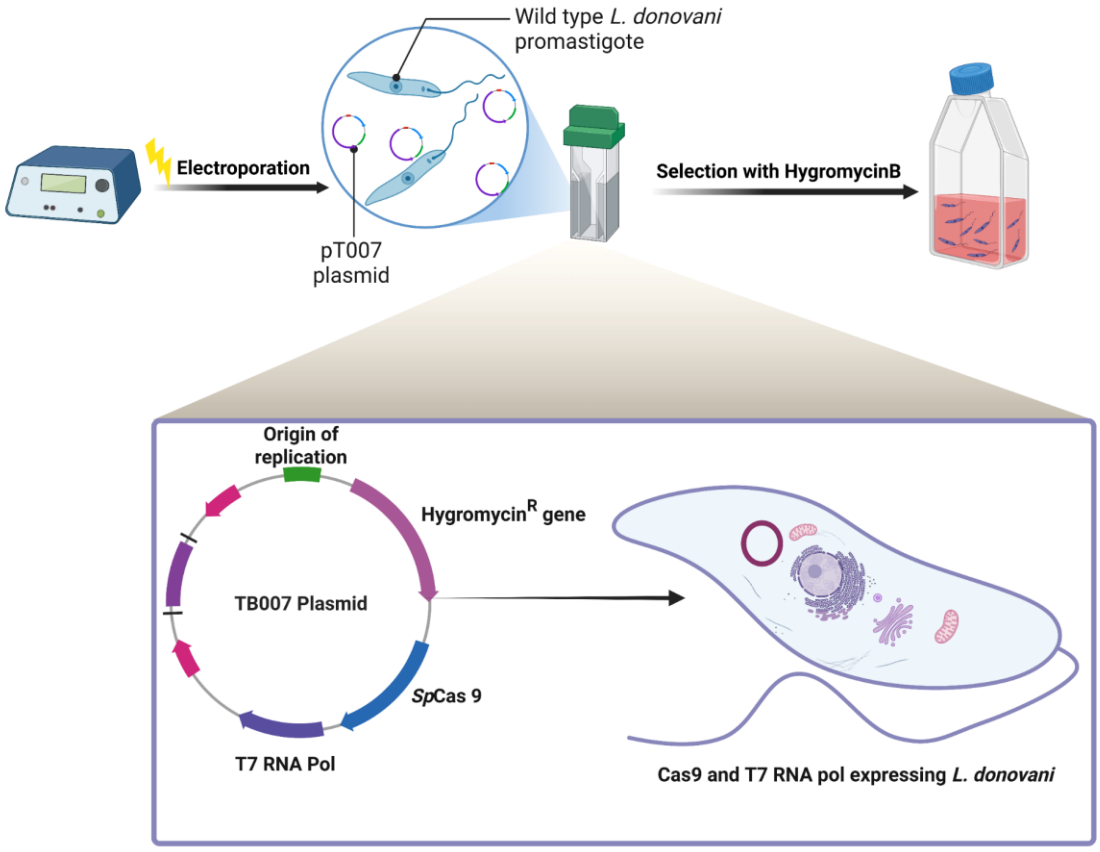

Figure 2.3: Schematic representation of the generation of Cas9-expressing *L. donovani* parasites. The pT007 plasmid, carrying *S. pyogenes* origin *Cas9 nuclease*, *T7 RNA polymerase*, and *hygromycin B phosphotransferase* genes, was delivered into the mid-log-phased *L. donovani* promastigotes through electroporation. Transfectants were subsequently selected in complete M199 media supplemented with hygromycin-B, enabling episomal expression of the transgenes.

2.2.3 Generation of for Glutathione synthetase-heterozygous (-/+) and null mutants (-/-) *L. donovani*

The gene-knockout experiments were performed as previously described (Beneke & Gluenz, 2019; Espada et al., 2021b; Roberts et al., 2022). Briefly, oligonucleotide primers for the 5′ and 3′ specific single guide RNAs (sgRNAs), the G00 RNA scaffold, and donor DNA templates were designed using the LeishGEdit website and synthesized commercially (Table 2.1). Verification of all the sgRNA and donor DNA primer sequences was carried out in the SnapGene software (Figure 2.4), aligning the gene-specific complementary sequences of the primers to the genomic locus of the glutathione synthetase (LdBPK_140970.1) gene, located on the 14th chromosome of *L. donovani*.

Table 2.1: Primer sequences generated by LeishGEdit. Underlined are complementary sequences of the untranslated regions of the target gene**.**

Upstream forward primer	CCTCCCCCATCTCTCATACTCCACGCACCGGTATAATGCAG ACCTGCTGC
Upstream reverse primer	GGCGGTGGCCGTCGTCGTCGTGGCGGGCATACTACCCGAT CCTGATCCAG
Downstream forward primer	GCGGCCTTGGACAGCGTGCAGATAATCAGTGGTTCTGGTA GTGGTTCCGG
Downstream reverse primer	CAGCCACCCTCAGCGGCCACGATGACACCACCAATTTGAG AGACCTGTGC
5' sgRNA primer	GAAATTAATACGACTCACTATAGGTGTCTGTTCTGAAGATGA GAGTTTTAGAGCTAGAAATAGC
3' sgRNA primer	GAAATTAATACGACTCACTATAGGAGGTGCTGCAATTTGTT GTGGTTTTAGAGCTAGAAATAGC
G00 primer (sgRNA scaffold)	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGG ACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

To amplify the 5′ and 3′ sgRNA templates, 10 µl of each 4 µM sgRNA primer was aliquoted into a PCR tube and stored at -80°C. During the freezing process, the master mix was prepared, containing 3.4 µl of ultrapure water, 4 µl of 5x reaction buffer supplemented with 7.5 mM MgCl2, 2 µl of 20 µM G00 primer, and 0.4 µl of 10 mM

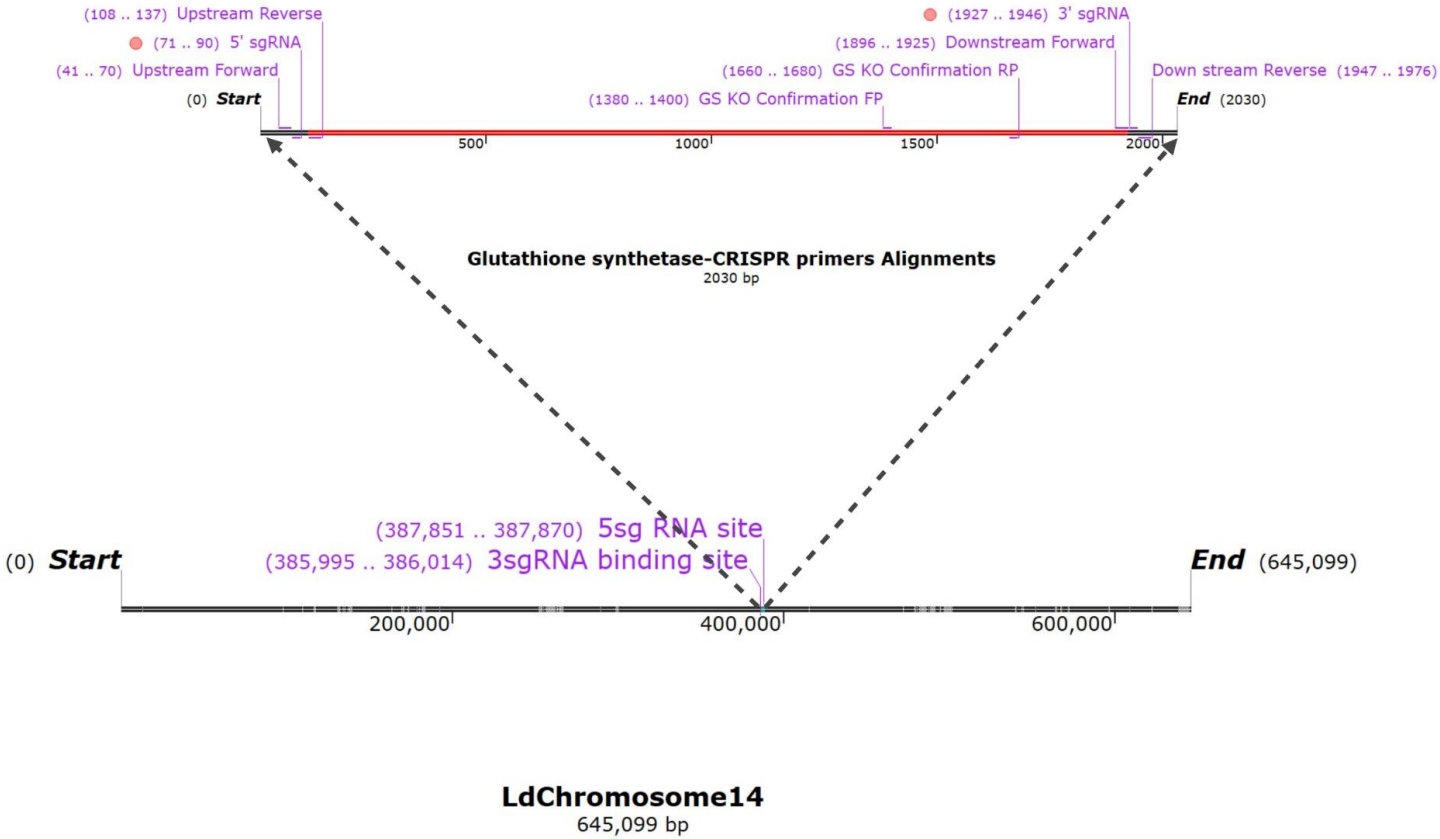

Figure 2.4: Schematic representation of the CRISPR design for knockout of *glutathione synthetase* gene in *Leishmania donovani.* The gene ID LdBPK_140970.1 was used to design primers (in purple) that were aligned with the genomic DNA sequence of *L. donovani* (red and black) using the SnapGene software.

dNTP mix. Finally, 0.2 µl of Phusion high-fidelity polymerase was added. Hot-start PCR was performed under the program: 98°C for 30 s (1 cycle); 98°C for 10 s, 60°C for 30 s, 72°C for 15 s (35 cycles); 72°C for 10 min (1 cycle). As soon as the temperature reached 98°C, the program was paused, and 10 µl of the master mix was added to the frozen primer tubes before resuming the PCR program.

For donor DNA amplification, 4 µl of each 20 µM upstream forward and downstream reverse primers were aliquoted into a PCR tube and frozen at -80°C. While the primer mix was being frozen, the master mix for the PCR reaction was prepared which contained 18.8 µl of ultrapure water, 8 µl of 5x reaction buffer supplemented with 7.5 mM MgCl2, 0.75 µl of 25 mM MgCl2 solution, 0.6 µl of 100% (v/v) DMSO, 0.8 µl of 10 mM dNTP mix, and 0.5 µl of ~50ng/µl pTNeo plasmids. Finally, 0.4 µl of the Phusion high-fidelity polymerase was added. As soon as the temperature reached 94°C, the program was paused, 32 µl of the master mix was added quickly to the frozen primer tube and resumed the PCR program. The PCR products were precipitated by isopropanol and sodium acetate, washed twice with 70% ethanol, and dissolved in ultra-purified water after overnight drying.

For generating the heterozygous mutants, 5µg of each 5′ and 3′sgRNA templates and donor DNA templates amplified from pTNeo were electroporated with ~5x10^6/ml mid-log phase *L. dono* cas9 T7 promastigote cells. Transfectants were selected and maintained using 50 µg/ml neomycin and 100 µg/ml hygromycin B containing M199 media. For complete knockout (*GS -/-)* of the *glutathione synthetase* gene in *L. donovani*, the heterozygous (*GS* -/+) mutant cells were obtained sequentially after 3 to 4 rounds of subculturing. To disrupt the second allele of the *glutathione synthetase* gene, donor DNA having a puromycin resistance gene was amplified from the pTPuro plasmid using the upstream forward and downstream reverse primers (Table 2.1). The

PCR, DNA purification, and electroporation steps are consistent with those in the generation of heterozygous mutants. The positive knockout cells were selected and maintained in M199 media containing 50μg/ml neomycin and 10 μg/ml puromycin.

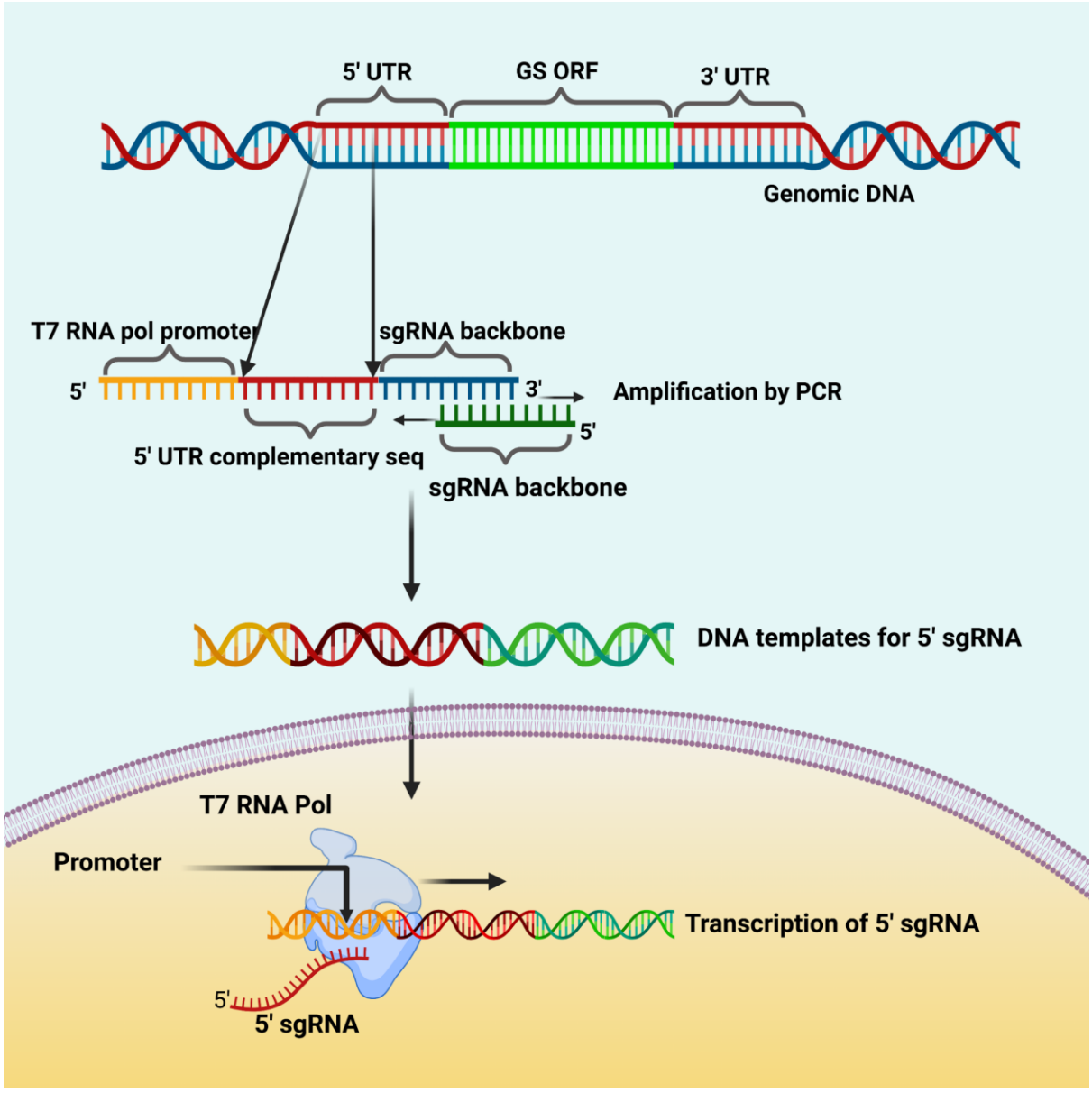

Figure 2.5: Schematic illustration of *invivo* transcription of single guide RNAs (sgRNAs) in *Leishmania donovani*. Gene-specific primers, containing a T7RNA promoter sequence (yellow), a complementary sequence of the target (red), and a sgRNA scaffold sequence (blue) were designed and PCR-amplified using the G00 primer. The resulting DNA templates were then delivered *into Leishmania donovani* parasites through electroporation, enabling the *in vivo* transcription of sgRNA by T7 RNA polymerase.

2.2.4 Confirmation of glutathione synthetase-heterozygous disruption (-/+) and null mutant *L. donovani* (-/-)

To confirm the successful integration of the neomycin resistance gene and verify the heterozygous disruption of the *glutathione synthetase* (*GS)* gene, PCR amplification was carried out using the genomic DNA of the mutant *L. donovani* cells. Primers designed from the internal sequences of the neomycin resistance gene were used to confirm its insertion using PCR, while the presence of the second allele of the *GS* gene was also verified by *GS* gene specific primers.

The reaction reactions were set up where each mixture mix was prepared with 9.8 µl of ultrapure water, 4 µl of 5x reaction buffer supplemented with 7.5 mM MgCl2, 0.75 µl of 25 mM MgCl2 solution, 0.6 µl of 100% (v/v) DMSO, 0.4 µl of 10 mM dNTP mix, 0.2 µl of the Phusion high-fidelity polymerase and 0.25 µl of ~50ng of isolated DNA from heterozygous mutant *L. donovani* cells. Finally, the frozen mixtures of each forward and reverse primer set were added to the master mix, and the PCR amplification program was started.

2.3.5 Generation of GS-tagged mNeoGreen Expressing *L. donovani*

For generating the mNeoGreen-glutathione synthetase tagged *L. donovani* parasites, the donor DNA was amplified from the pPLOT-mNG-Neo plasmid. 4 µl of each 20 µM downstream forward and downstream reverse primers were frozen at -80°C. During the frizzing process, the master mix for the PCR reaction was prepared, which contained 18.8 µl of ultrapure water, 8 µl of 5x reaction buffer supplemented with 7.5 mM MgCl2, 0.75 µl of 25 mM MgCl2 solution, 0.6 µl of 100% (v/v) DMSO, 0.8 µl of 10

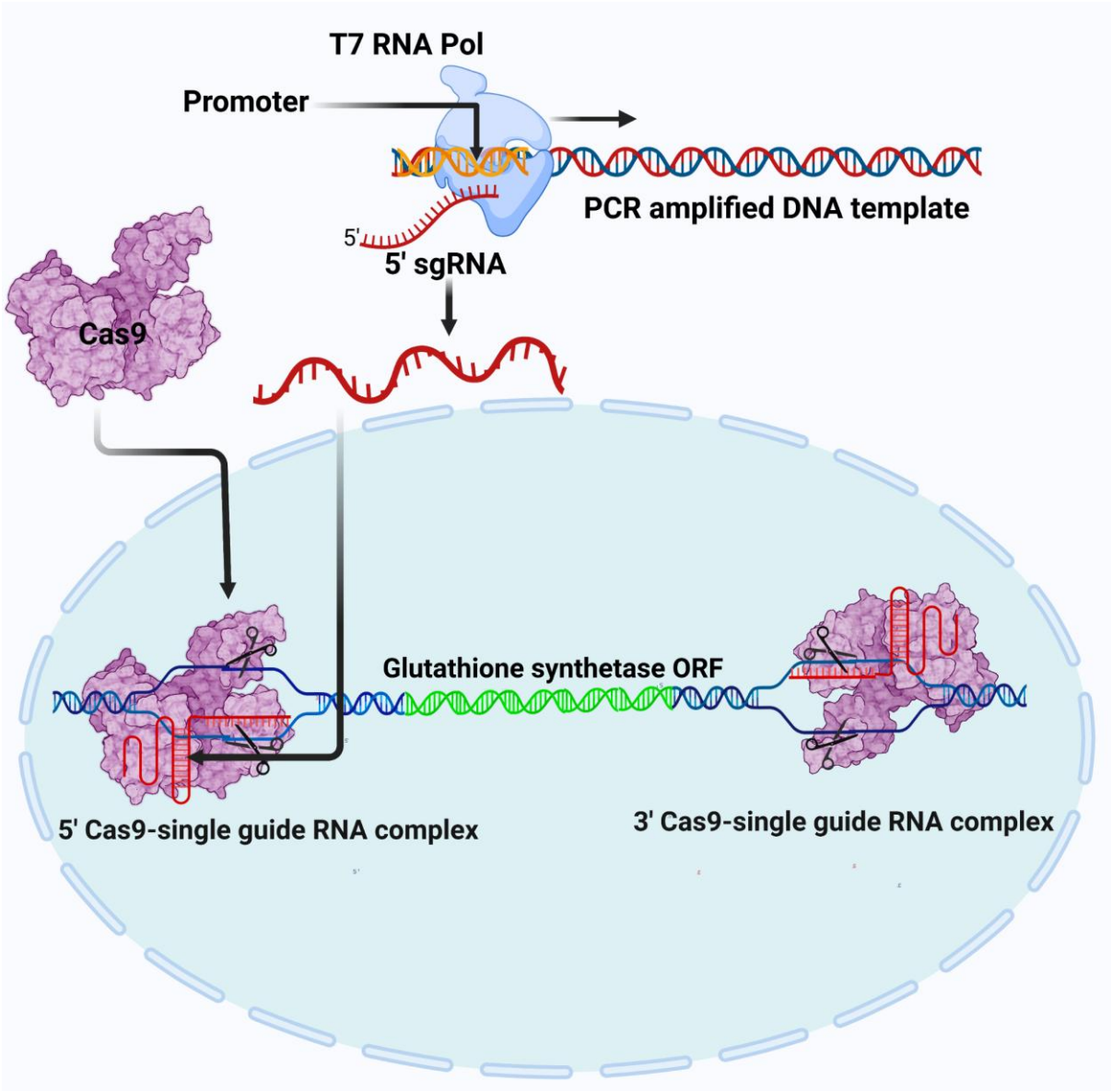

Figure 2.6: Schematic illustration of the assembly and gene editing by Cas9 and sgRNAs. Transcribed single guide RNAs (sgRNAs) assemble with Cas9 nuclease, forming ribonucleoprotein complexes that recognize the upstream and downstream target sites flanking the *glutathione synthetase* coding sequence. Upon binding to the specified region, the Cas9 introduces double-stranded breaks, resulting in the excision of the open reading frame (ORF).

mM dNTP mix, and 0.5 µl of ~50ng/µl of the pPLOT-mNG-Neo plasmid. The donor DNA that contains a mNeonGreen coding sequence and neomycin resistance gene was PCR amplified from the pPLOT-mNG-Neo plasmid and electroporated into *Ld*T7Cas9 cells with DNA templates for 3′ sgRNA. Positive transfectants with the fluorescent-tagged coding sequence successfully inserted downstream of the

glutathione synthetase gene were subsequently selected using M199 media containing 50μg/μl Neomycin.

2.3.6 Growth Analysis

The promastigotes from each mutant and wild-type strain were seeded in 12-well plates at a density of $2x10^5$ cells/ml without any antibiotic and incubated at 25° C for 7 days. The number of parasites was calculated daily using a haemocytometer and the growth curves were generated using GraphPad Prism (Version 5).

2.3.7 Fluorescence Microscopy: The expressing *L. donovani* promastigotes, expressing GS-tagged mNeoGreen were harvested after 3-4 subcultures, washed with 1xPBS, and fixed with paraformaldehyde (4%). Fixed cells were then stained with DAPI (2μg/ml) and mounted on glass slides for fluorescent microscopy imaging. For background control, *Ld*T7Cas9 cells (lacking mNeoGreen tag) were also prepared using the same protocol. Images are captured using a Zeiss fluorescence microscope. Excitation/emission settings were 358 mm/461 mm for DAPI and 495 nm/525 nm for mNeonGreen. Micrographs were merged for. Micrographs were merged for visualization.

2.3.8 *In vitro* Infectivity Test

The infectivity test of mutant *L. donovani* parasites, including Cas9-expressing, heterozygous mutant, and null mutants in comparison with the wild type, was performed using THP-1-derived macrophages. THP-1 monocytes were cultured in

complete RPMI-1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a 5% CO_2 incubator. Cells were seeded into a 12-well plate at a density of 2 × 10^4 cells/mL and differentiated into adherent macrophages by treatment with 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) for 12 hours. Subsequently, after differentiation, the PMA-containing media were replaced with fresh media, and the cells were incubated for an additional 24 hours.

Metacyclic promastigotes from each *L. donovani* culture were introduced to infect macrophages at a Parasite: macrophage ratio of 10:1 for 6 hours. Following infection, non-internalized parasites were removed by rinsing with 1xPBS, added fresh media, and continued incubation for 24, 48, and 72 hours. Following incubation, cells were fixed with cold methanol for 15 minutes and subsequently stained with Giemsa for 1 hour.

To calculate the infectivity index, 100 randomly selected macrophages were evaluated per well. The percentage of infected macrophages, the average number of amastigotes per infected macrophage, and the infectivity index (calculated as % infected macrophages × average number of amastigotes per infected macrophage) were calculated from the selected macrophages. All experiments were carried out in triplicate and with technical duplicates.

2.3 Results

2.3.1 Expression of Cas9 and T7 RNA polymerase Does Not Affect the Growth of *Leishmania donovani*

To facilitate CRISPR Cas9-based genome editing in *L. donovani* parasites, we first generated a transgenic parasite line co-expressing *Streptococcus pyogenes* Cas9 and

T7 RNA polymerase through episomal plasmid pTB007 (Figure 2.3). The positive transfectants were selected using hygromycin B-containing media and maintained for further applications. To check whether this expression impacted the parasite viability, we performed a comparative growth analysis of wild-type *L. donovani*, and the Cas9 and T7RNA polymerase expressing strains (*Ld*cas9T7RNAP) for 72 hours [(Figure 2.9 (A)]. The growth curves showed that the episomal expression of the cas9 and T7 RNA polymerase did not impair the parasitic growth and fitness in the promastigote stage.

2.3.2 The Cas9/T7RNA-Pol System Successfully Tagged mNeonGreen to Endogenous GS

To validate the functionality of the Cas9/T7 RNA polymerase system in *Leishmania donovani,* a fluorescent protein-mNeonGreen coding sequence was inserted downstream of the endogenous *glutathione synthetase* (GS) gene. The expression of the mNeonGreen was confirmed in the fluorescent microscopy images (Figure 2.7). With strong cytoplasmic fluorescence, consistent with the ubiquitous expression of GS (Atwal et al., 2016; Lu, 2009) in *Leishmania*, it was confirmed that the fusion of a 235 amino acid fluorescence tag did not interfere with the GS protein expression or the parasitic growth. These outcomes also validated the effectiveness and functionality of the Cas9 nuclease and T7 RNA polymerase in *L. donovani*, supporting the utilization of this system for gene editing purposes in *Leishmania.*

2.3.3 *Glutathione Synthetase* Gene in *L. donovanii* Is Present in Multiple Genomic Loci

Using CRISPR Cas9-mediated genome editing, we successfully replaced both alleles of the *glutathione synthetase (GS)* gene present on chromosome 14 of *Leishmania donovani* with the neomycin and puromycin resistance genes. The resulting parasites exhibited resistance to neomycin (40μg/ml) and puromycin (20μg/ml), confirming the successful integration of these marker genes. PCR amplification using gene-specific primers for neomycin and puromycin further validated the presence of the resistance genes at the expected loci. However, PCR analysis of genomic DNA from the knockout *Leishmania* strain (*Ld*GS-/-) unexpectedly revealed the continued presence of the *GS* gene [Figure 2.8(A)]. These findings suggest that the presence of the *GS* gene in additional copies, likely due to aneuploidy or gene copy number variation. Such genomic plasticity is extremely common in *L. donovani*, making it difficult to achieve complete knockouts for a gene (Dumetz et al., 2017b; Negreira et al., 2022, 2023). Genomic plasticity in *Leishmania* parasites, such as copy number variation of a particular gene or aneuploidy in a specific chromosome, is well characterized and often linked with adaptive response under stress conditions (Laffitte et al., 2016;Lypaczewski et al., 2018), underscoring the challenges of genetic manipulations in these parasites.

2.3.4 Disruption of a Single Locus of the *Glutathione Synthetase* Gene Reduces the Promastigote Growth in *L. donovani*

A complete null mutation of the *glutathione synthetase* (*GS)* gene could not be achieved in *Leishmania donovani,* due to the presence of the *GS* gene in multiple

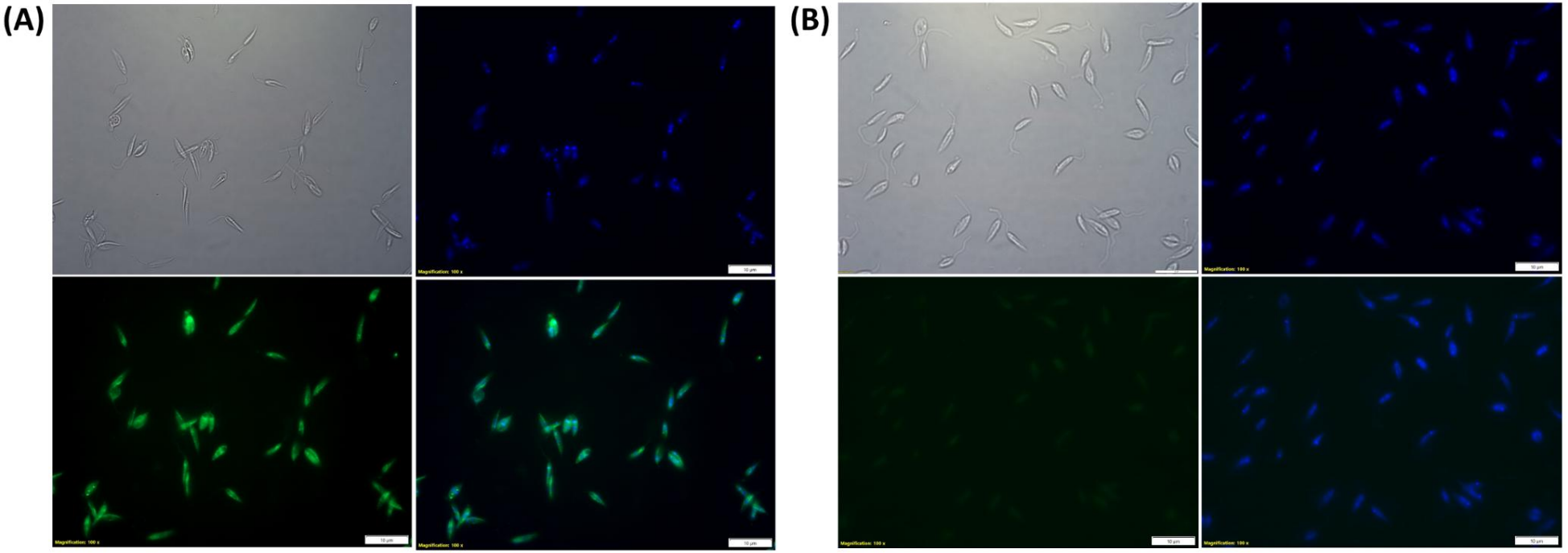

Figure 2.7: Fluorescent microscopy images of mNeonGreen-tagged *glutathione synthetase* gene expression in *Leishmania donovani* at 100X. (A) The ubiquitous expression of green fluorescence (mNeonGreen) tagged the glutathione synthetase protein was observed. Bright-field (upper left), DAPI-stained DNA (upper right), fluorescence-tagged-GS expression (lower left), and merged image (lower right). (B) Fluorescent microscopy images of wild-type *Leishmania donovani* at 100X: Bright-field (upper left), DAPI-stained DNA (upper right), no green fluorescence (lower left), and merged image (lower right).

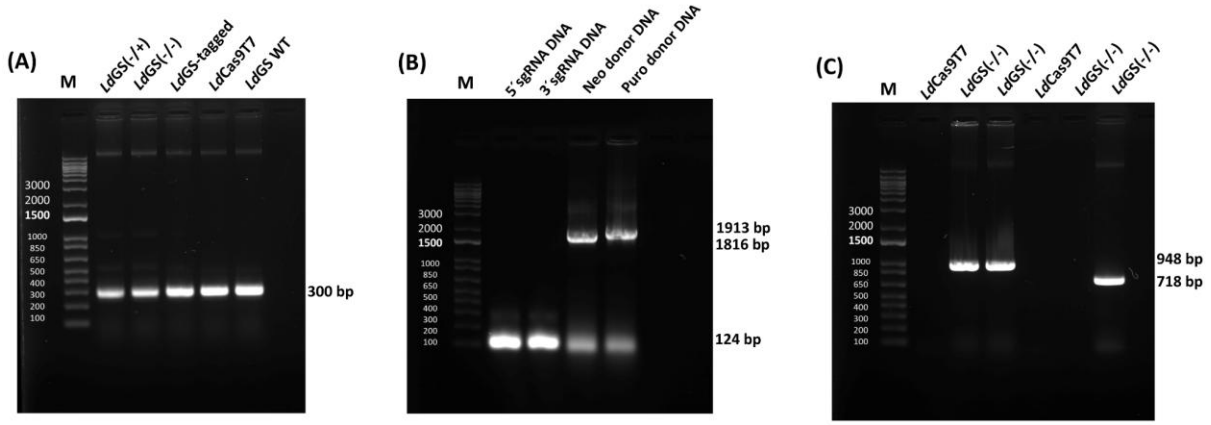

Figure 2.8: PCR confirmation of the CRISPR-Cas9-mediated gene disruption in *Leishmania donovani*. (A) Detection of an extra copy of the *glutathione synthetase* gene in the knockout *L. donovani* (-/-) strain by PCR using gene-specific internal primers. (B) PCR amplification of sgRNA templates (124 bp) and donor DNA sequences containing antibiotic resistance cassettes, amplified from pT Neo and pT Puro plasmids. (C) Verification of successful integration of the Neomycin and Puromycin resistance genes into one allele (heterozygous knockout, *L. donovani GS* -/+) and both alleles (homozygous knockout, *L. donovani GS* -/-). PCR was performed from the genomic DNA using gene-specific primers to confirm the correct insertions.

genomic loci. However, the disruption in a single genomic locus significantly affects the growth of *L. donovani* promastigotes. Growth rates of the parasites after the heterozygous (*Ld*GS-/+) and homozygous (*Ld*GS-/-) knockouts, in comparison to wild-type and Cas9-expressing strains, were assessed [Figure 2.9(A)]. The heterozygous mutant parasites showed a similar growth rate to the wild type, and the Cas9-expressing *L. donovani* promastigotes, indicating that loss of the single *GS* allele did not compromise the parasitic growth and fitness in *in vitro* cultures. In contrast, parasites with complete disruption of the *GS* gene in a single locus exhibited significantly reduced growth, as demonstrated by prolonged proliferation phase, morphological alteration, and formation of cellular aggregates, especially during the initial phase. Upon maturation, these parasites had a smaller cell body size compared to wild-type promastigotes. These results suggest that partial depletion of glutathione synthetase enzyme is sufficient to affect parasitic growth, highlighting the critical role of glutathione biosynthesis in *Leishmania* parasites' viability and development.

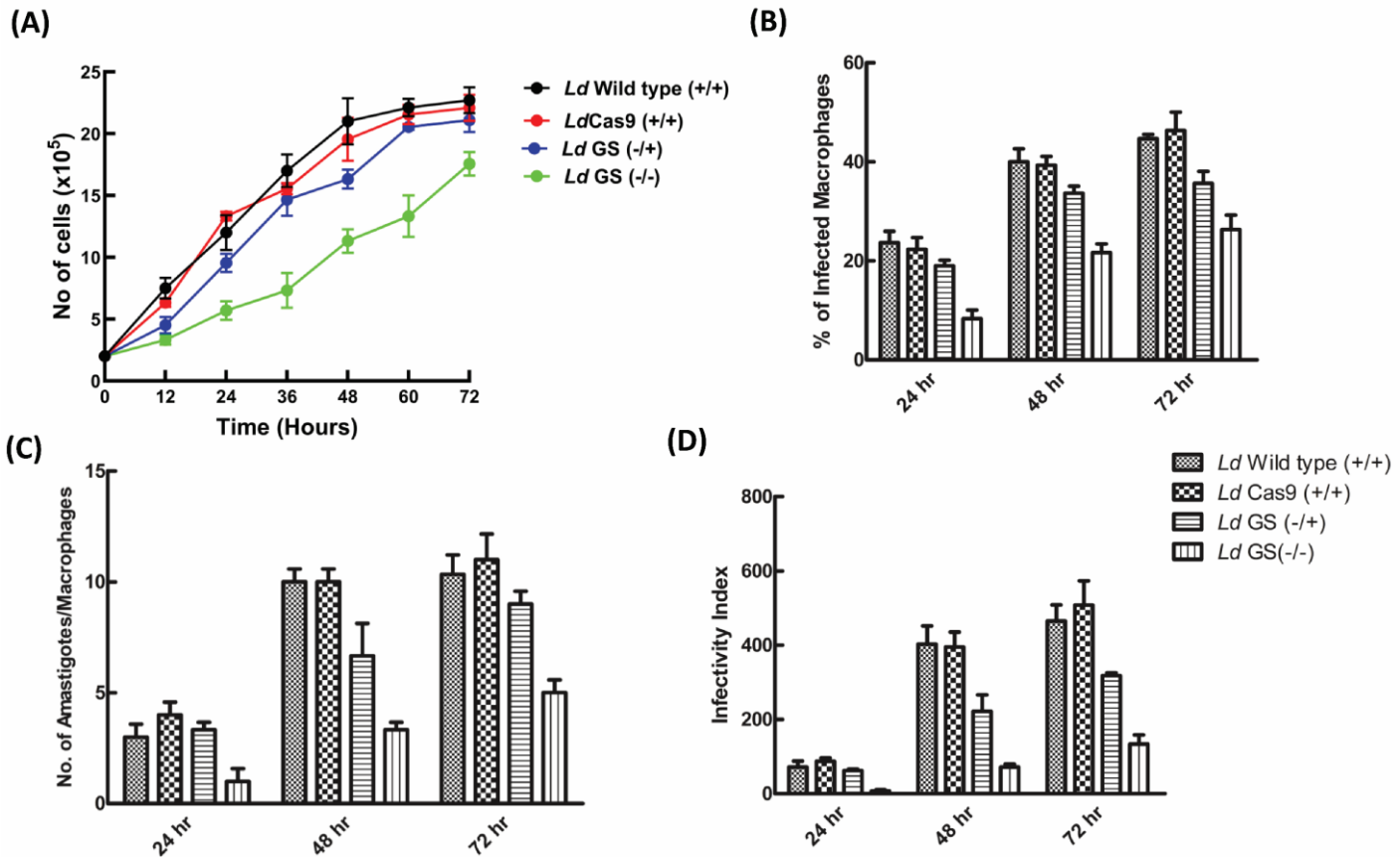

Figure 2.9: Growth and infectivity analysis of mutant *L. donovani* parasites. (A) Growth curve of the *L. donovani* promastigotes seeded at a density of $2x10^5$/ml. Parasites number was counted every 24 hrs of interval using a haemocytometer for 72 hours. The Cas9 and TR RNA Polymerase-expressing and heterozygous mutant *Ld* GS (-/+) showed growth kinetics similar to wild type, whereas the homozygous mutant *Ld* GS (-/-) showed significantly reduced growth kinetics. The data represents the average of three independent experiments (B) Bar graph representing the percentage of infected macrophages, determined by counting 100 randomly selected macrophages for the presence of intracellular amastigotes, (C) Represents average number of intracellular amastigotes in the 100 selected macrophages, and (D) Represents the infectivity index of each *Leishmania* strain, calculated using the formula, infectivity index= (% of infected macrophages) x (average number of amastigotes in 100 macrophages). All the experiments were performed in triplicate.

2.3.5 Disruption of a Single Locus of the *Glutathione Synthetase* Gene Impairs the Infectivity of *L. donovani* in Macrophage Cells

Following the observation of growth reduction and morphological alteration in the promastigote stage with disrupted *glutathione synthetase* (GS) gene, we performed the infectivity test of these parasites in THP-1 monocyte-derived macrophages. After 24, 48, and 72 hours of infection, the infectivity index was determined from the percentage of infected macrophages and the average number of intracellular amastigotes per infected cell. The parasites with disrupted *glutathione synthetase* (GS) gene in a single locus (*LdGS -/-)*, exhibited a significantly lower infectivity index compared to the wild type, Cas9-expressing, and heterozygous mutant parasites (*LdGS -/+)* [Figure 2.9 (C)]. These findings demonstrated that the partial depletion of the GS enzyme might have compromised the parasite's ability to establish infection in the macrophage cells. The decreased infectivity is probably due to reduced glutathione synthetase levels, affecting the parasite's ability to neutralize oxidative stress within the macrophages. These results thus highlight the essentiality of glutathione synthetase enzyme in maintaining the intracellular survival and virulence in *Leishmania donovani.*

2.4 Discussion

Genetic manipulation in *Leishmania* parasites has historically proven as a strategic tool to identify essential genes and their functional roles in the parasite's virulence. In the targeted drug discovery process, the knowledge of the parasite's pathway vulnerabilities is critically important for identifying potential drug targets and subsequently developing specific drugs against the particular targets. This strategy

gives several advantages over blindly testing drugs for infectious diseases (Fellmann et al., 2017; Simon, 2012;H. Li et al., 2020). Although the gene knockdown (RNAi) or gene knockout in *Leishmania* has been challenging due to the complexity of the parasite's biology and high genomic plasticity, frequent aneuploidy, and copy number variation (Sterkers et al., 2014;Rogers et al., 2011b). While the homologous recombination method was the only option for gene replacement, it remains inefficient, time-consuming, and often fails for essential genes, particularly for multicopy genes (Bryant et al., 2019). The advancement of gene editing techniques, like the introduction of CRISPR-Cas9 genome editing in trypanosomatids (Beneke et al., 2017b; Lander & Chiurillo, 2019; Zhang et al., 2020b), has significantly improved the effectiveness of genetic manipulation, enabling high-throughput genetic validation studies in these organisms.

In the current study, we utilized a CRISPR Cas9-based gene editing toolkit, LeishGEdit (Beneke et al., 2017b) to investigate the role of and the essentiality of the *glutathione synthetase* gene for the *L. donovani* parasites. Glutathione synthetase catalyses the synthesis of glutathione (GSH), which serves as a precursor of trypanothione- a unique, powerful antioxidant metabolite of *Leishmania* parasites. The trypanothione-based redox metabolism is proven to be indispensable for the parasites, due to its central role in maintaining the redox homeostasis under the toxic environment inside the immune cells during infections (V. Ali et al., 2022; González-Montero et al., 2024; Irigoín et al., 2008; Krauth-Siegel & Comini, 2008a). While previous studies have indirectly implicated glutathione synthetase enzyme in redox regulation and pathogenicity in *Trypanosoma brucei* and *Leishmania infantum* by genetic and biochemical methods (Pratt et al., 2014; A. Mukherjee et al., 2009b;Sousa et al., 2014), a direct genetic validation in *Leishmania* parasites was missing.

According to the reference genome of *Leishmania donovani*, *glutathione synthetase* gene is located on chromosome 14 as a single exon copy (Downing et al., 2011b). In this study, we attempted to knock out both alleles of the *glutathione synthetase* gene by replacing them with two selectable marker genes. Surprisingly, even after complete replacements, one copy of the *glutathione synthetase* gene was still present, suggesting the aneuploidy in the chromosome 14 or copy number variation of the *GS* gene, which is a common feature in *Leishmania* parasites (Laffitte et al., 2016; Santi & Murta, 2022; Sinha et al., 2018). However, it is well reported that genetic redundancy in *Leishmania*, retention of multiple copies of genes or whole chromosomes, contributes to the parasite's phenotypic robustness and adaptability to challenging environments. This redundancy is mainly achieved through mechanisms like gene copy number variation, duplications, and widespread aneuploidy, rather than classic backup genes as in other eukaryotes. *Leishmania* parasites display high genome plasticity, with frequent changes in chromosome number and structure, gene amplification, and somy (ploidy) alterations. These variations generate genetic redundancy at both the gene and chromosomal level, allowing the parasite to quickly adapt to environmental pressures, including drug exposure. It also ensures that *Leishmania* populations maintain functional capabilities despite genetic disruptions or environmental stressors, providing a buffer against deleterious mutations or loss of specific genes.

In our study, although the complete deletion of the *GS* gene was unsuccessful in our study, likely due to chromosomal aneuploidy and compensatory gene dosage features, we succeeded in generating partial knockouts by disrupting one locus. The resulting parasites exhibited significantly impaired growth in promastigote form in *in vitro* cultures and reduced intracellular infectivity in human monocyte-derived

macrophages. These phenotypic analyses demonstrated the essential role of glutathione synthetase enzyme in the *Leishmania* parasite's survival and virulence. Our findings are therefore consistent with earlier reports that demonstrated trypanothione-based redox pathway perturbations as detrimental to *Leishmania* survival (Castro et al., 2017; A. Mukherjee et al., 2009b). The study thus supports the hypothesis that trypanothione-regulated redox homeostasis is a vital Achilles' heel in these parasites.

For generating complete knockouts in *Leishmania*, where aneuploidy is very frequent, gene-disruption through the insertion of stop codons or base editing will be a suitable approach (Engstler & Beneke, 2023b). Such approaches will also overcome the constraints, like the requirement of more than two sets of donor DNAs and multiple rounds of transfections for generating gene disruptions in multi-copy chromosomes. Moreover, unlike *Trypanosoma spp.*, the key components of RNAi machinery, like argonaute (Ago) proteins, are absent in *Leishmania donovani (Lye et al., 2025),* therefore making gene knockdown approaches inapplicable in this organism. However, conditional knockout systems, the combination of Di-Cre recombinase and CRISPR-Cas9 in *Leishmania donovani,* can be a suitable approach to study stage-specific essentiality of particular genes (Yagoubat et al., 2020).

Nevertheless, our study provides direct genetic validation of the essential role of glutathione synthetase in *Leishmania donovani* survival and pathogenicity. Partial disruption in the *glutathione synthetase* gene. These findings will be instrumental for developing inhibitors targeting glutathione synthetase in *Leishmania donovani*.