The 60 kDa Heat Shock Proteins of *Leishmania donovani* and their impact on viability, stress tolerance, and virulence.

Dissertation

with the aim of achieving the
doctor rerum naturalium (Dr. rer. nat.)

at the Faculty of
Mathematics, Informatics, and Natural Science
Department Biology
University Hamburg

submitted by

Henner Zirpel

from Heppenheim

Hamburg, 2018

Genehmigt vom Fachbereich der Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
der Universität Hamburg
auf Antrag von PD Dr. Joachim Clos
Weiterer Gutachter der Dissertation: Prof. Dr. Tim Gilberger
Tag der Disputation: 24. August 2018
Hamburg, den 29. August 2018

This work has been performed from July 2015 to June 2018 in the research group of PD Dr. Joachim Clos at the Bernhard-Nocht-Institute for Tropical Medicine in Hamburg.

1. Evaluator: PD. Dr. Joachim Clos
Bernhard-Nocht-Institut für Tropenmedizin
Sektion Molekularbiologie und Immunologie
Bernhard-Nocht-Straße 74, 20359 Hamburg

2. Evaluator: Prof. Dr. Tim Gilberger
Bernhard-Nocht-Institut für Tropenmedizin
Sektion Molekularbiologie und Immunologie
Bernhard-Nocht-Straße 74, 20359 Hamburg

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, den 28. Juni 2018

Language Certificate

I as a native speaker have read the present PhD thesis and hereby confirm that it complies with the rules of the English language.

place, date	Marlon Zimmermann
Hamburg, 06.26.18	

Danksagung

An dieser Stelle möchte ich mich besonders bei folgenden Personen bedanken:

PD Dr. Joachim Clos für die Möglichkeit, meine Doktorarbeit in der Sektion Molekularbiologie und Immunologie des Bernhard-Nocht—Instituts anfertigen zu können, sowie für die zahlreichen informativen Diskussionen und Hilfestellungen, welche den Erfolg dieser Doktorarbeit gewährleistet haben.

Dr. Hanna Lotter und Dr. Minka Breloer für die institutsinterne Betreuung meiner Doktorarbeit.

Meiner Familie die mich zu jeder Zeit mit ihnen allen zur Verfügung stehenden Mitteln unterstützt hat.

Der gesamten und ehemaligen Arbeitsgruppe Clos, die mich im vollen Maße unterstützt und maßgeblich dazu beigetragen hat, dass dieses Doktorarbeit gelingt. Im Einzelnen: Dr. Vanessa Adaui-Sicheri, Dr. Katharina Bartsch, Dr. Eugenia Bifeld, Tine Brinker, Michaela Bockelmann, Dr. Julia Eick, Dr. Antje Hombach-Barrigah, Conny Kroeber-Boncardo, Judith Laurentius, Anne MacDonald, Miriam Martens, Henriette Rehn, Elena Riel, Dr. Paloma Tejera Nevada und Dorothea Zander.

Weiterhin möchte ich Fabian Bär, Gregor Deichmann, Michael Ditzel, Julius Funk, Tobias Günther, Elena Riel, David Rosenberger, und Andrea Schulz für die Hilfe außerhalb der Doktorarbeit danken.

1. Abstract

Leishmaniasis is a disease caused by the protozoan parasite *Leishmania spp.* and is a major neglected tropical disease. Individuals infected with *Leishmania* parasites suffer from ulcerating, mostly self healing skin lesion, infections of the mucous membranes, and systemic, visceral infections. The latter are fatal if left untreated. The parasite faces challenges with various stressors during its parasitic life, cycling between sand flies and vertebrate hosts. During these life stages the parasite expresses various Heat Shock Proteins. The Heat Shock Protein 60 kDa (chaperonin 60, CPN60) is present in four different isoforms and at least one is expressed throughout the parasitic life cycle. The major function of chaperones is to assist proper folding of newly synthesised, denatured, miss-folded or un-folded proteins into their correct tertiary structures. However, why the parasite maintains four different CPN60 isoforms, named CPN60.1, CPN60.2, CPN60.3 and CPN60.4, is not known.

Therefore, the aim of this thesis was to analyse the role of the 4 different CPN60s in *Leishmania donovani*, the main causative agent of visceral leishmaniasis. Firstly, double allele gene replacement of the four different CPN60s was performed by homologous recombination and/or by CRISPR/Cas gene editing. For *CPN60.1*, *CPN60.2* and *CPN60.4* viable double allele replacement mutants were obtained while *CPN60.3* is an essential gene in *L. donovani* of which only single allele replacement mutants could be generated. To verify any phenotypes, ectopic copies of the genes of interest (GOI) were introduced into the mutants and the wild type via episomal plasmids, to generate GOI over-expressing mutants. All mutants were analysed for phenotypic changes, such as growth under different conditions, morphology, infectivity and virulence.

It was found that CPN60.1 plays a role in the virulence of the parasite, as a lack of CPN60.1 leads to a 50% reduction of the relative parasite load in murine macrophages. For CPN60.2 a slightly reduced cell body length and a 60% growth reduction was observed in mildly acidic medium (pH = 5.5). No phenotypic changes were observed under the tested conditions for the *CPN60.3* single allele replacement mutants and for *CPN60.4* double allele replacement mutants.

Specific antibodies for the four different CPN60s were generated by immunising laying hens with the respective recombinant protein. It was shown that CPN60.1 is not expressed to detectable levels, in agreement with previous findings. Furthermore, it was not possible to distinguish between the closely related CPN60.2 and CPN60.3. Nevertheless, the combined CPN60.2/CPN60.3 abundance increase ~2.5-fold during *in vitro* axenic amastigote development,

also in agreement with previous findings. The abundance of CPN60.4 remained stable throughout an *in vitro* life cycle of the parasite.

Co-immune precipitation experiments and luciferase refolding assays were preformed to analyse the interaction with the CPN10 co-chaperonin and the chaperone activity of each of the four CPN60s, using recombinantly expressed and affinity purified proteins. Preliminary data suggest chaperone functionality for CPN60.2, CPN60.3 and CPN60.4. The role of the co-chaperone CPN10 during the folding process remains unclear and needs to be further investigated. The co-immune precipitation experiments showed an interaction of CPN60.2 and CPN60.4 with the co-chaperonin CPN10 while no stable interaction between CPN60.3 and CPN10 could be detected.

The results obtained in this study broaden the understanding of the different CPN60s in and show that CPN60.3 is the major chaperone of *L. donovani*.

2. Zusammenfassung

Die Leishmaniose ist eine durch den protozoischen Parasiten der Gattung *Leishmania* verursachte Krankheit und gehört zu den wichtigsten und am häufigsten vernachlässigten tropischen Krankheiten. Patienten die mit dem Parasiten *Leishmania* infiziert sind, leiden unter ulzerierenden, meist selbst heilenden Hautläsionen, unter Infektionen der Schleimhäute und unter systemischen, viszeralen Infektionen. Letztere sind tödlich wenn sie nicht behandelt werden. Der Parasit ist während seines parasitischen Lebenszyklus, welcher zwischen der Sandmücke und dem Vertebraten statt findet, verschiedenen Stressoren ausgesetzt. Während dieses Lebenszykluses expremiert der Parasit verschiedene Hitze Schock Proteine. Die Hitze Schock Proteine der Größe 60 kDa (Chaperone 60, CPN60) sind in vier verschiedenen Isoformen vorhanden und mindestens eines wird während des Lebenszyklus durchgängig expremiert. Die Hauptfunktion der Chaperone ist die passende Faltung von neu synthetisierten, denaturierten, fehl gefalteten oder nicht gefalteten Proteinen in deren korrekte tertiäre Struktur zu unterstützen. Es ist jedoch nicht bekannt warum der Parasit *Leishmania* vier verschiedene CPN60 Isoformen, CPN60.1, CPN60.2, CPN60.3 und CPN60.4, besitzt.

Das Ziel dieser Arbeit ist es, die Rolle der vier verschiedenen CPN60 Proteine in *Leishmania donovani*, dem Hauptverursacher der viszeralen Leishmaniose, zu untersuchen. Zuerst wurden Doppel Allele Austausch Mutanten der vier CPN60 Proteine mittels homologer Rekombination und CRISPR/Cas Genom Engineering hergestellt. Für CPN60.1, CPN60.2 und CPN60.4 wurden lebende Null Mutanten erhalten, während CPN60.3 ein essentielles Gen in *L. donovani* ist und ausschließlich Einzel Allele Austausch Mutanten hergestellt werden konnten. Um beobachtete Phänotyp Analysen zu verifizieren wurden ektopische Kopien des Zielgens (*engl.* gene of interest, GOI) in die Mutanten transfiziert. Weiterhin wurden, um das GOI über exprimierende Mutanten zu erhalten, episomale Kopien der Gene in den Wild Typ (WT) eingefügt. Alle Mutanten wurden auf phänotypische Änderungen, wie verändertes Wachstum unter verschiedenen Bedingungen, Morphologie, Infektivität oder Virulenz untersucht.

Es wurde beobachtet, dass CPN60.1 eine Rolle in der Virulenz des Parasiten spielt, da das Fehlen von CPN60.1 zu einer Verringerung der relativen Parasitenlast um 50% in murinen Makorphagen führt. Weiterhin konnte gezeigt werden, dass das Fehlen von CPN60.2 zu einem etwas kleineren Zellkörper führt, sowie das Wachstum in mildem sauren Milieu (pH = 5,5) auf 60% gehemmt ist. Es konnten keine phänotypischen Änderungen für die Einzel Allele Austausch

Mutanten von CPN60.3 und für die Null Mutanten von CPN60.4 beobachtet werden.

Für die vier verschiedenen CPN60 wurden spezifische Antikörper hergestellt. Dazu wurden Legehennen mit dem entsprechenden Antigen immunisiert und die Antikörper aus den Eiern gewonnen. Es wurde gezeigt, dass CPN60.1 nicht in nachweisbaren Mengen expremiert wird, was mit früheren Beobachtungen übereinstimmt. Weiterhin wurde gezeigt, dass es nicht möglich ist, zwischen den nah verwandten Proteinen CPN60.2 und CPN60.3 zu unterscheiden. Dennoch war die kombinierte, erhöhte Expression um das 2,5 fache von CPN60.2 und CPN60.3 während der *in vitro* Entwicklung zu axenischen Amastigote zu beobachten, was ebenfalls mit früheren Beobachtungen übereinstimmt. Die Proteinmenge von CPN60.4 blieb in allen *in vitro* Lebensstadien des Parasiten gleich.

Ko-Präzipitations Experimente und Luziferase Rückfaltungs Assays wurden durchgeführt, um die Interaktion mit dem Co-Chaperon CPN10 und um die Chaperon Aktivität der vier CPN60 nachzuweisen. Hierfür wurden rekombinant exprimierte und Affinitäts aufgereinigte Proteine verwendet. Vorläufige Daten zeigen eine Chaperone Aktivität von CPN60.2, CPN60.3 und CPN60.4 Die Rolle des Co-Chaperone CPN10 während des Faltungsprozesses bleibt weiterhin unklar und bedarf weiterer Untersuchungen. Die Ko-Präzipitation zeigte, dass CPN60.2 und CPN60.4 mit CPN10 interagieren, während keine stabile Interaktion zwischen CPN60.3 und CPN10 nachgewiesen werden konnte.

Die in dieser Arbeit erhaltenen Ergebnisse erweitern das Verständnis über die vier verschiedenen CPN60s von *L. donovani* und zeigen, dass CPN60.3 das Hauptchaperone dieses Parasiten ist.

3. Introduction

3.1. Leishmaniasis

Leishmaniasis is a neglected tropical disease, caused by the protozoan parasites of the genus *Leishmania*. According to the WHO, over one billion people are living at risk in endemic areas and 650.000 - 1.090.000 people are newly infected every year (WHO, 2018).

The endemic areas are mainly South-America, North-Africa, and Asia, but also neighbouring countries as shown in Figures 1 and 2. The treatment of the disease has some major limitations, not only due to the availability of the drugs, but also by the regional distribution and costs. As the disease occurs in rural regions, patient care is hindered by the infrastructure. The available drugs, e.g. Amphotericin B, Miltefosin, sodium stibogluconate, Paramomycin, and Pentamidin, are either expensive or have severe side-effects. Furthermore, the numbers of drug resistant infections are increasing and vaccinations are not available yet. This makes it urgent to increase research, develop new drugs, and search for an effective vaccination strategy.

The parasite *Leishmania* is transmitted to the host via the bite of the female sand fly which is the natural vector of *Leishmania*. Different forms of Leishmaniasis, of *Leishmania* species and of sand fly species exist. The first classification was based on the regional distribution, termed as Old World (Europe, Africa and Asia) and New World (South and Central America) Leishmaniasis. To date, at least 32 different *Leishmania* spp. are known in the Old World, 23 in the New World, and one in Australia. Moreover, three species are present in both the Old World and the New World. With regards to the sand flies (Phlebotominae), three genera are known in the Old World (Phlebotomus, Sergentomyia, and Chinius) and three genera in the New World (Lutzomyia, Brumptomyia, and Warileya) (Akhoundi, Kuhls et al. 2016). The different *Leishmania* spp. are transmitted, according to the regional distribution, by the different sand fly genera. The disease itself is further categorised into cutaneous, mucocutaneous, and visceral Leishmaniasis.

Status of endemicity of visceral leishmaniasis worldwide, 2015

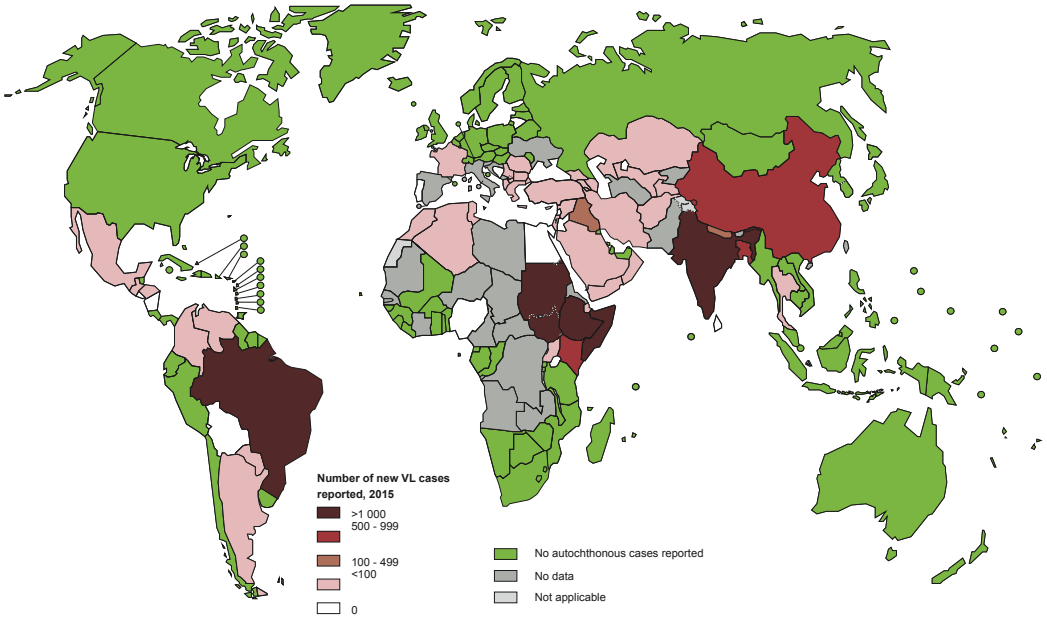


Figure 1: Global distribution of endemic areas of visceral Leishmaniasis worldwide. Non endemic countries are represented in green. Increasing numbers of visceral Leishmaniasis cases are shown from rose to dark red. Countries with no data available are shown in grey (WHO, 2018).

Status of endemicity of cutaneous leishmaniasis worldwide, 2015

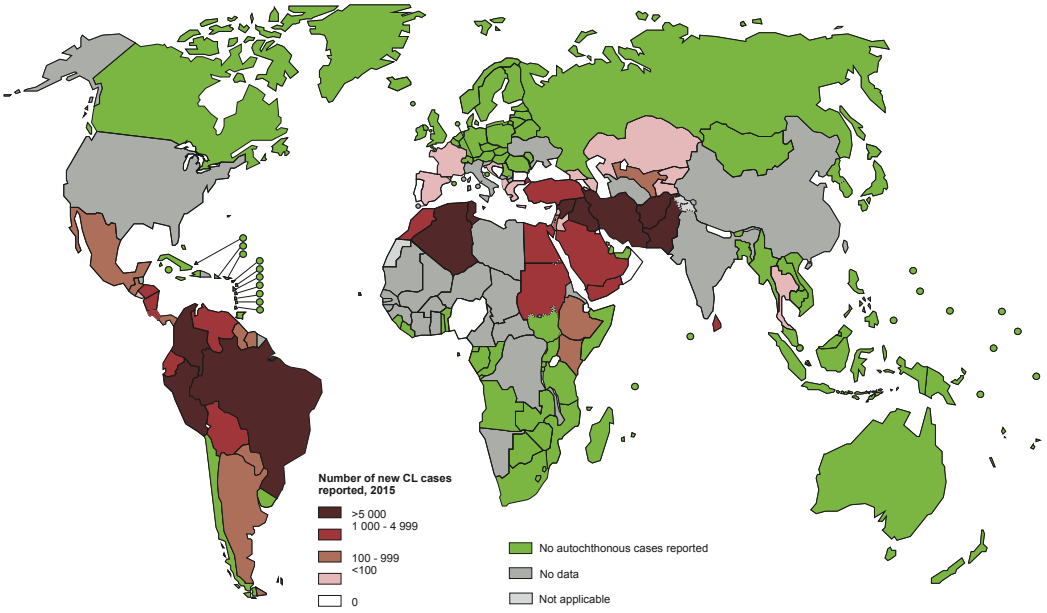


Figure 2: Global distribution of endemic areas of cutaneous Leishmaniasis worldwide. Non endemic countries are represented in green. Increasing numbers of visceral Leishmaniasis cases are shown from rose to dark red. Countries with no data available are shown in grey (WHO, 2018).

The cutaneous Leishmaniasis (CL), also known as the oriental sore, affects the skin, leading to ulcerating skin lesions. These lesions usually occur at the site of infection, which are mainly the head and the extremities. In cases of diffuse

cutaneous Leishmaniasis, lesions occur at multiple sites on the body. Patients do not only suffer from the partial self-healing lesions itself, but also from long-term effects, as scars remain, leading to social stigma and disfigurement. Cutaneous Leishmaniasis is caused by all parasites of the Viannia spp., and of the complexes of *L. mexicana*, *L. tropica*, and *L. major* (Banuls, Hide et al. 2007), while 95% of all infections are caused by the latter two. The diffuse cutaneous Leishmaniasis is mainly caused by *L. aethiopica* and *L. amazonensis*.

The mucocutaneous Leishmaniasis (MCL), also known as *Espundia*, affects the mucous membranes of the mouth, nose, and throat. If untreated, it will lead to destruction of palate, septum and lips. Through secondary infections even lethal outcomes have been reported. The MCL occurs mainly in central parts of South-America and is caused by *L. brazilensis* (90%), *L. panamensis* and *L. guyanensis (Banuls, Hide et al. 2007)*.

Visceral Leishmaniasis (VL), also know as kala-azar, makes up 50.000 - 90.000 of the new cases each year and affects liver, spleen and bone marrow. It is fatal if left untreated. It afflicts chiefly children and is often reported as a co-infection of HIV (WHO, 2018). Over 90% of the cases occur in the following six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan (Alvar, Velez et al. 2012). VL is caused by the parasites of the *L. donovani* complex, i.e. *L. donovani* and *L. infantum*. VL may also lead to Post-Kala-Azar dermal Leishmaniasis (PKDL). PKDL is a relapse of the VL and appears month to years after a VL infection. In these cases, the patient suffers from skin lesions or facial erythema. PKDL is caused by parasites of the species *L. donovani*.

The parasite *Leishmania* is an Eukaryote belonging to the taxon Euglenozoa and the class of the Kinetoplastea. The Kinetoplastea are characterised by a) the presence of a flagellum and b) the presence of a kinetoplast. The kinetoplast is, similar as the cell nucleus, a DNA containing organelle and part of the mitochondrium. The DNA is represented in two different forms, as maxicircles and as minicircles. While the maxicircles code for proteins involved in the maturation of mRNA, the minicircles code for the guide RNA needed for editing the cytochrome oxidase subunit III mRNA (Lukes, Guilbride et al. 2002). Within the class of the Kinetoplastea, the *Leishmania* belong to the order of the Trypanosomatida, the family of the Trypanosomatidae, and the genus Leishmania (Ross 1903). Cupolillo (Cupolillo, Medina-Acosta et al. 2000) proposed to further distinguish between the Euleishmania and the Paraleishmania. The Euleishmania contain the subgenus of *L. donovani* to which the species *L. donovani* belongs

(Figure 3). *L. donovani*, which was used in all experiments, was independently described by C. Donovan and by W. B. Leishman in 1903.

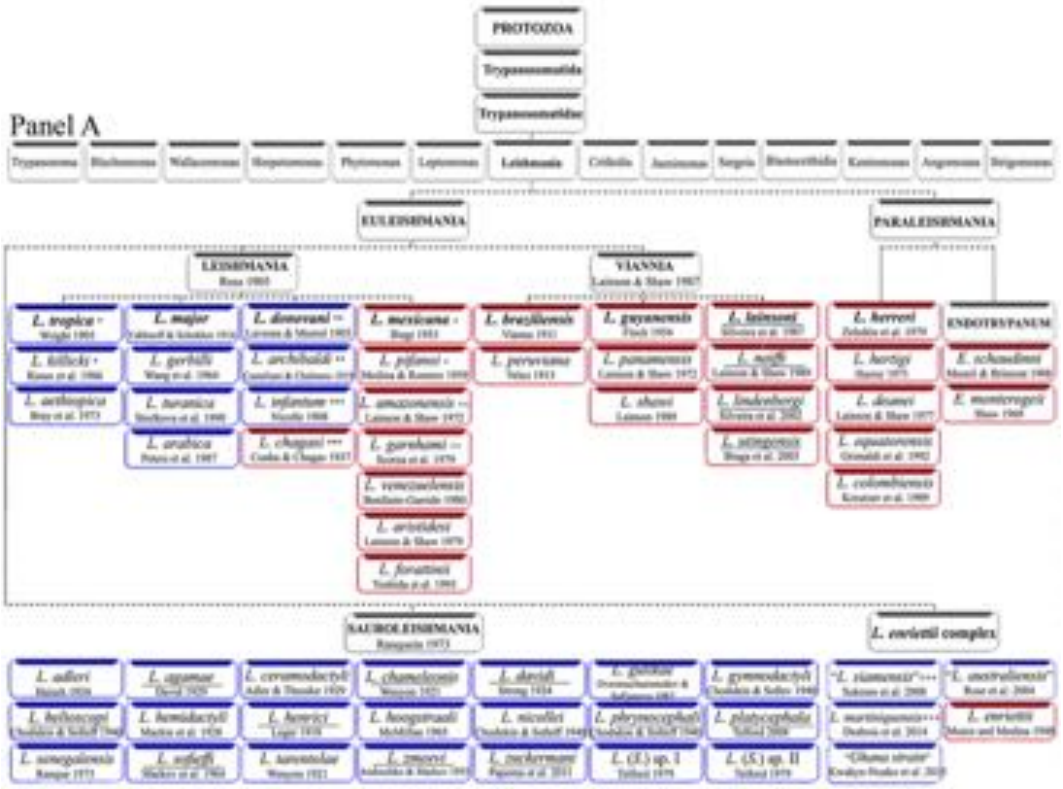


Figure 3: Phylogenetic tree of the *Leishmania* spp. (Akhoundi, Kuhls et al. 2016).

3.2. Life cycle

The parasitic life cycle of the *Leishmania* spp is biphasic and consists of an extracellular and an intracellular stage. It starts when the sand fly bites a vertebrate for blood feeding. During this process, metacyclic promastigotes in the sand fly are regurgitated into the host. The characteristics of the metacyclic promastigotes are an elongated cell body (~8 µm) with a flagellum longer than the cell body (Sunter and Gull 2017). Furthermore, the metacyclic parasites are highly virulent, motile, and cannot proliferate. Inside the host, the parasites attach to and are engulfed by phagocytes. The final host cell is the macrophage. Inside the macrophage the metacyclic promastigotes are enclosed by a phagolysosome and change their morphology into the amastigote form. This morphological change is triggered by the elevated temperature of the host (~37°C) and a low pH of ~5.5 inside the phagolysosome. The characteristics of the amastigotes are a reduced cell body length, an ovoid form and a rudimentary flagellum, rendering the parasite non-motile. Nevertheless, the parasite proliferates in its amastigote form, as long as it is enclosed in the phagolysosome. After the rupture of the macrophage, the amastigotes are set free into the surrounding medium. There,

they can be either taken up by new macrophages repeating the proliferation cycle, or they can be taken up by a blood-feeding sand fly (Figure 4). Inside the sand fly, the amastigotes end up in the midgut, where they convert from the amastigote form to the procyclic promastigote stage. The procyclic promastigote displays a variable cell body length of ~6.5 µm to 11.5 µm, with the flagellum being shorter than the cell body (Sunter and Gull 2017). Furthermore, the procyclic form of *Leishmania* is highly proliferative. As long as the parasite is in its procyclic form it is enclosed by the peritrophic matrix which it escapes by changing into the nectomonad promastigote. This form is characterised by a cell body longer than 12 µm (Sunter and Gull 2017). The nectomonad promastigote attaches via the flagellum to the microvilli of the midgut, but cannot proliferate anymore. Afterwards, it moves back up to the thoracic midgut and changes into the leptomonad form which is quite similar to the metacyclic form. In the leptomonad form the parasite can proliferate again, before it changes to the infective metacyclic form (Figure 5). With another bite of the sand fly and the transfer of the metacyclic promastigotes to the host the life cycle closes.

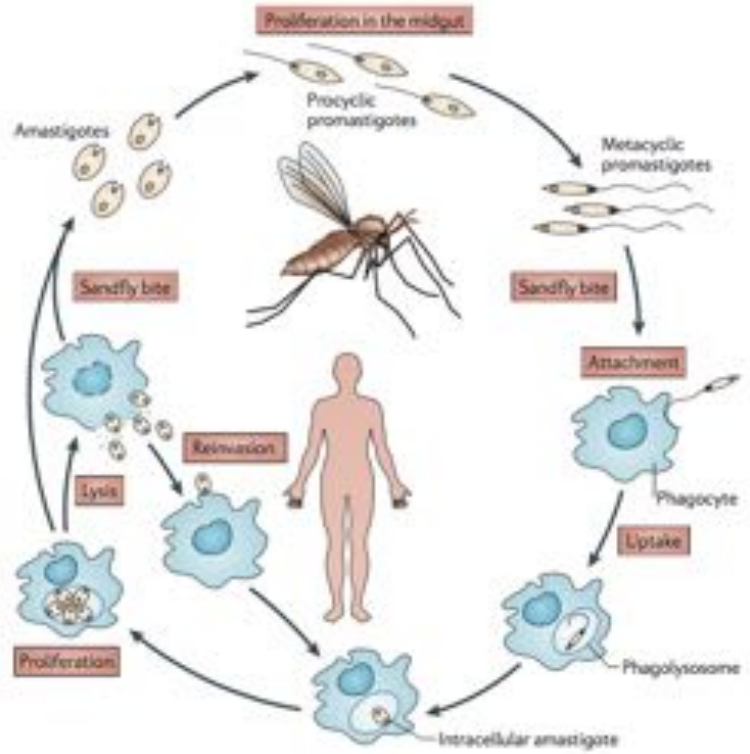


Figure 4:
Life cycle of *Leishmania*. The metacyclic promastigote is transferred to the host during a blood meal of the sand fly. After uptake by a phagocyte the parasite is encapsuled into a phagolysosome. In here it changes into an amstigote and proliferate. After lysis of the phagocyte it can either reinvade other phagocytes or can be taken up by another sand fly. In the sandfly the amastigote changes into the procyclic promastigote and proliferates in the midgut. Next, it moves to the foregut where is changes into the metacyclic promastigote and the life cycle closes (Kyle and Scott, 2011).

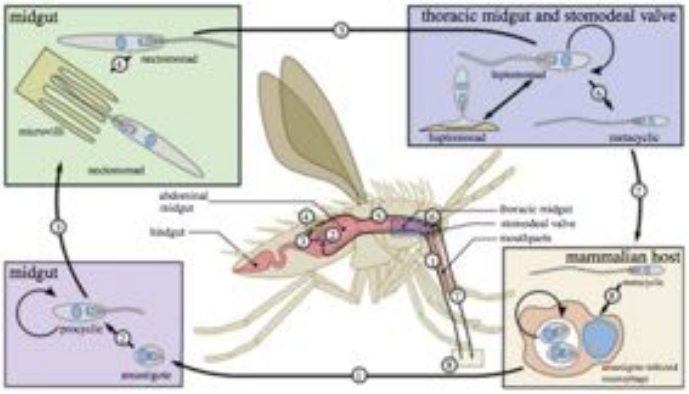


Figure 5: Life cycle of *Leishmania* in the sand fly. After the uptake of the parasite in its amastigote from the host it moves from the mouthpart into the midgut. In here the amastigote changes into its procyclic promastigote form and proliferates. Next, it changes into the nectomonad form an attaches to the membrane of the midgut. The nectomonad promastigote moves the thoracic midgut where changes to its leptomonad form and proliferates. After changing into the metacyclic form the parasite can infect another host (Sunter and Gull 2017).

For research purposes it is often necessary to analyse parasites throughout their complete complex life cycle. As it is quite difficult to perform the whole life cycle under laboratory conditions, it can be mimicked in vitro. *Leishmania* parasites are usually cultivated in their procyclic form in a neutral medium (pH = 7.4) at 25°C. The conditions for the stage conversion from the metacyclic promastigote to the amastigote in vivo are the elevated temperature in the host and the decreased pH value in the phagolysosome (Zilberstein and Shapira 1994). These two physical changes can also be applied to the in vitro culture. By lowering the pH to 5.5 and increasing the temperature to 37°C, axenic amastigotes of *L. donovani* and certain New World leishmaniae can be produced (Bates 1993, Zilberstein and Shapira 1994, Barak, Amin-Spector et al. 2005). However, these axenic amastigotes show very limited proliferation. The re-conversion from amastigotes to promastigotes is also possible by changing the temperature and the pH value back to the promastigote culture conditions.

3.3. The Heat Shock Proteins of *Leishmania*

In 1980 S. Lindquist (Velazquez, DiDomenico et al. 1980) researched the protein expression of *Drosophila* after elevated temperature treatment and discovered that several protein levels were upregulated. The proteins were termed Heat Shock Proteins (HSP). HSPs are now known to be ubiquitous and highly conserved. Their expression is not only induced by the increase of temperature, but also by other stressors. The main known stressors are temperature increase, change in the pH, oxidative stress, heavy metal ions and ethanol. The *Leishmania* spp express a full set of HSPs and increase their synthesis at elevated temperatures, but not under chemical stress (Clos, Brandau et al. 1998).

The set of HSPs expressed by *Leishmania* consists of the HSP100, HSP90, HSP70, CPN60 & CPN10, HSP40, and small HSPs. The structures and functions of the HSP families are not related and have to be seen individually (Netzer and Hartl 1997). The main functions of HSPs are to fold un- or mis-folded proteins, dissolve protein aggregates, or prevent aggregation (Bukau and Horwich 1998).
Significantly, elevated temperature and acidic pH are triggers for the promastigote to amastigote conversion (Bates, 1993, Zilberstein and Shapira, 1992, Barak et al., 2005). Most of the HSPs are present during the promastigote form and are upregulated after a temperature increase. The regulation occurs on the post transcriptional level (Hunter, Cook et al. 1984, Miller 1988, Argaman, Aly et al. 1994, Brandau, Dresel et al. 1995, Schlüter, Wiesgigl et al. 2000) as gene-specific transcription regulation does not exist in *Leishmania* and other Trypanosomatida (Clayton 2002). Even though the mRNA stability and partially the concentration is increased (Coulson and Smith 1990, Argaman, Aly et al. 1994, Hübel, Krobitsch et al. 1997, Quijada, Soto et al. 1997), this cannot account for the increased protein synthesis.

Leishmania parasites encounter heat stress during the transition from the promastigote form in the sand fly to the amastigote form in the host (see chapter 3.2).
Heat shock proteins play diverse roles in *Leishmania*. HSP100 shows elevated protein levels in the axenic amastigote (Hübel, Brandau et al. 1995, Hübel, Krobitsch et al. 1997, Krobitsch, Brandau et al. 1998). Furthermore, null mutants of HSP100 failed to infect macrophages *in vitro* and showed attenuation in the mouse model (Hübel, Krobitsch et al. 1997, Krobitsch and Clos 1999). This indicates an essential role of HSP100 in the amastigote stage. The likely reason for this is the pivotal role of HSP100 in the assembly of immune-modulatory exosomes (Silverman, Clos et al. 2010, Silverman, Clos et al. 2010).
In contras, inhibition of HSP90 in *L. donovani* leads to the differentiation of promastigotes into amastigote-like parasites (Wiesgigl and Clos 2001). Inhibition of HSP90 with geldanamycin (GA) or radicicol (RAD) also leads to cell stress and induced levels of HSP60, HSP70, HSP90 and HSP100 (Wiesgigl and Clos 2001).
Very little is known about HSP70 response to stress in *Leishmania*. HSP70 is encoded in different gene copy numbers depending on the species or even the strain (Wallace, Ball et al. 1992, Folgueira and Requena 2007). It is highly expressed and constitutes up to 2% of the soluble protein load (Brandau, Dresel et al. 1995). The likely co-chaperone of HSP70 is HSP40. About HSP40 very little is known, either.
Four different genes for CPN60 (=HSP60 = GroEL) are known (see chapter 6.1). Earlier work showed that CPN60.2 levels increase during the differentiation into

axenic amastigotes, while no CPN60.1 could be detected (Schlüter, Wiesgigl et al. 2000) (for more detailed information on CPN60 in *Leishmanai* see chapter 3.4.2). The co-chaperone CPN10 which is encoded in two copies also shows elevated protein levels in the axenic amastigote (Zamora-Veyl, Kroemer et al. 2005). Furthermore, it is essential for the parasite. During the infection process of macrophages CPN10 localises in the cytosol of the macrophages and plays an essential role during the infection process (Colineau, Clos et al. 2017).

3.4. CPN60

The Heat Shock Protein 60 kDa (HSP60 / CPN60) belongs to the type I chaperonin family and is highly conserved throughout bacteria and eucaryotes (Cheng, Hartl et al. 1989, Goloubinoff, Christeller et al. 1989, Ostermann, Horwich et al. 1989). Yet it is absent from archaebacteria and eukaryotic cell cytosol. In those a similar but unrelated protein termed t-complex polypeptide 1 (TCP-1, TRiC/CCT) which belongs to the type II chaperonins is expressed (Ellis 1990, Gupta 1990). Its localisation is mainly in the mitochondrium of the organisms. Type I chaperones are defined as molecular folding machines that require a co-chaperone. The co-chaperone is not required for type II chaperones, as they contain an additional closing protein domain (Ditzel, Lowe et al. 1998). The best studied CPN60 is the *E. coli* GroEL. GroEL forms tetradecamers and acts with its heptameric co-chaperone GroES as a chaperone (Martin, Langer et al. 1991, Xu, Horwich et al. 1997).

Crystal structure analyses of GroEL/GroES and of the mammalian HSP60/HSP10 revealed that the main subunit is a homo-heptameric ring of CPN60, with a bullet-like shape. Inside of the bullet there is a wide space into which unfolded peptides can enter. On the poles of the bullet, an opening is located. As shown by Ishida (Ishida, Okamoto et al. 2018) mammalian HSP60 forms mainly single heptamers in the absence of HSP10 and ATP. The well described tetradecameric structure starts to form as soon as heptameric HSP10 binds to the open end of the bullet. The binding of the heptameric HSP10 complex closes the bullet like a lid. Next, the resulting closed bullet binds to an open HSP60 heptamer resulting in the football shaped complex (Figure 6) (after (Nisemblat, Yaniv et al. 2015)).

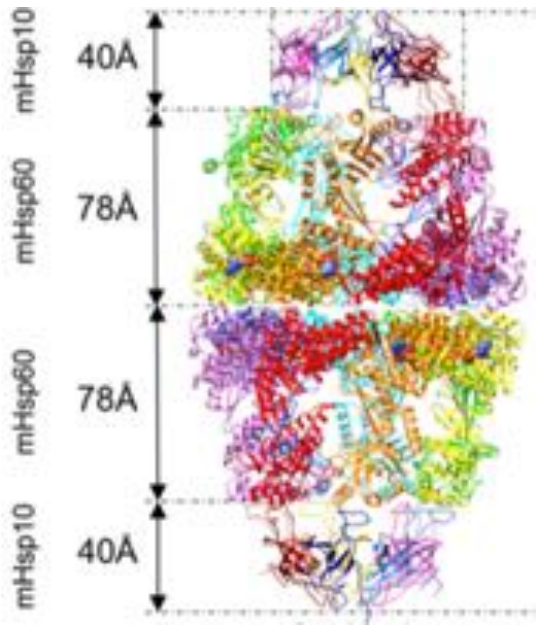


Figure 6: Crystalline protein structure of the human HSP60-HSP10 complex. At the top and the bottom the two heptamer rings of mHSP10 are displayed. The two bullet-like forms of the mHSP60 heptamers are shown in the middle forming the football-like complex (Nisemblat, Yaniv et al. 2015).

As soon as the football shaped complex is formed the folding of proteins into their correct quaternary structure starts. The unfolded peptide enters the open bullet, the heptameric HSP10 ring on the other end dissociates in the presence of ATP and a second heptameric HSP10 ring binds to the hydrophilic end and closes the bullet. At the same time ATP binds in an equimolar ratio to the closed bullet and initiates a structural twist. The twist enlarges the cavity with the encapsulated protein enabling it to start its correct folding. Because of the absence of any other hydrophilic monomers, the protein is protected against hydrophobic interactions and aggregation. The release of the correctly folded protein is initiated by closing and twisting of the second bullet. The folding mechanism is shown in Figure 7.

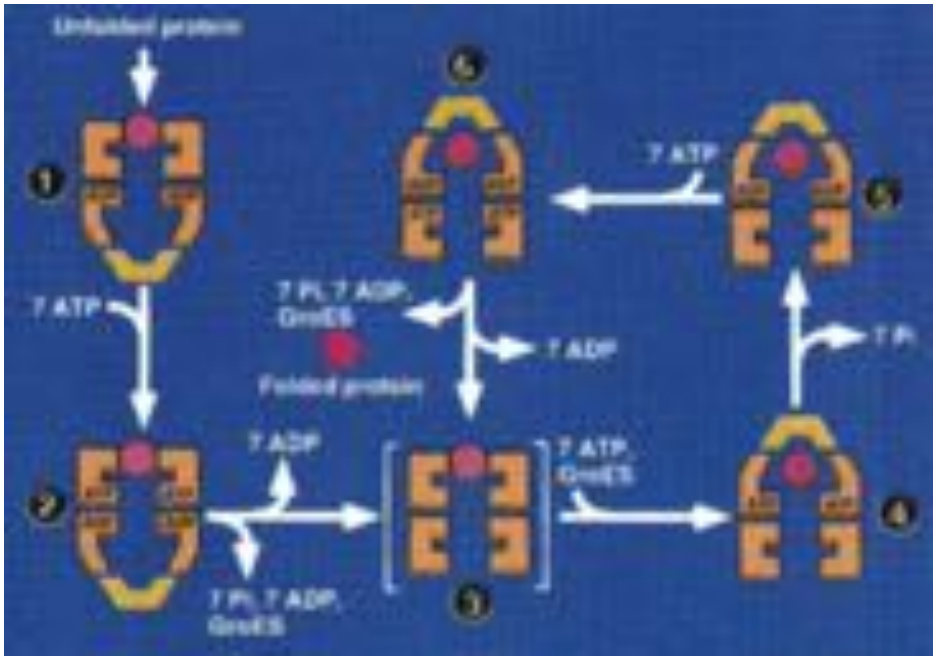


Figure 7: Representative figure of the molecular folding mechanism of the GroEL/GroES complex. An unfolded protein (red) enters the open heptamer of the GroEL (orange) while the second GroEL of the tetradecamer complex is in a closed conformation with GroES (yellow). Due to binding of ATP to open GroEL ring the GroES heptamer dissociates at the other side. By binding of GroES and ATP to the open GroEL which is encapsulating the unfolded protein the cavity is closed. Additional ATP leads to folding of the unfolded protein and the cycle starts over again (Hartl, 1996).

The CPN60/CPN10 complex is specialised on the folding of a limited number of proteins. It was thought that ~12% of all newly synthesised proteins are folded by the chaperonin complex (Hartl 1996, Feldman and Frydman 2000). However, Kerner *et al.* (Kerner, Naylor et al. 2005) showed that in *E. coli* only ~0.4% of all proteins depend on CPN60 as a folding machinery. Nevertheless, 15% of these proteins are essential for the cell. Interestingly, nearly all of these proteins showed a $(\beta\alpha)_8$ TIM-barrel domain. These domains are hydrophobic and exposed during the folding process. Thus, their correct folding without chaperones is impaired leading either to missfolded proteins or to aggregation (Ellis 2005).

Beside the CPN60s role as chaperones, they display other functions as well. Proteins that have more than one function, which is not due to gene fusion or multiple proteolytic fragments, are termed “moonlighting proteins” (Jeffery 2017). The main characteristic of a protein or an enzyme is that they are highly specialised. An enzyme will work only under very narrow physiological conditions. A slight change in the pH or the temperature usually leads to a strong decrease in the activity. This raises the question how proteins, such as the moonlighting proteins can have two or more functions. Physiological changes may alter the original function, but may also lead to conformational changes. A polypeptide with a different 3D structure may also form a different active site. Therefore, it is possible that a protein inside a compartment or inside a cell shows a different

function than in a different compartment or outside the cell. Furthermore, a protein that acts as an antigen does not require a specific active site. Specific signal molecules can contain very small peptides on the surface which are sufficient for recognition by an antibody (Jeffery, Bahnson et al. 2000). In such a case a protein does not even have to change its conformation.

3.4.1. CPN60 as a moonlighting protein

The first speculation about CPN60 being a moonlighting protein was offered in 1993 by Friedland *et al.* (Friedland, Shattock et al. 1993). It was reported that the CPN60.2 of *Mycobacterium tuberculosis* stimulates human monocytes leading to a pro-inflamatory response. This finding was later confirmed for other organisms, such as *Legionella pneumophila*, *E. coli*, and *M. leprae*. Macrophages treated with that particular CPN60 responded via upregulation of pro-inflamatory cytokines (Retzlaff, Yamamoto et al. 1994). Beside the treatment of macrophages with CPN60 dendritic cells also produced TNFα, NO, IFNα, and IL6 (Kol, Bourcier et al. 1999, Vabulas, Ahmad-Nejad et al. 2001, Osterloh, Kalinke et al. 2007). Quintana (Quintana and Cohen 2011) showed that similar effects could also be observed for effector T cells, regulatory T cells, and B cells. Maguire *et al.* (Maguire, Coates et al. 2002)concluded that CPN60 has an intracellular role as a chaperone and an extracellular role as a signal protein, similar to pro-inflammatory cytokines. In contrast to these findings of pro-inflammatory effects, some reports also suggest an anti-inflammatory role for CPN60 (Birk, Gur et al. 1999, Luna, Postol et al. 2007).

Several diseases are also linked to CPN60, mainly because of elevated CPN60 levels in the serum of patients. Reports include cardiovascular diseases (Schett, Metzler et al. 1999, Pockley, Wu et al. 2000, Shamaei-Tousi, Stephens et al. 2006, Nahas, Nahas-Neto et al. 2014), periodontis (Rizzo, Cappello et al. 2012), and autoimmune diseases such as juveline idiopathic arthritis (Wu, Ou et al. 2011). The role of soluble CPN60 in the blood as a marker for inflammatory diseases was suggested by Henderson (Henderson 2010).

Another extracellular role of CPN60 is as a signal molecule between immune cells (Pockley, Wu et al. 2000, Srivastava 2002, Henderson 2010). Henderson (Henderson 2010) proposed that CPN60 actively activate the immune system via a receptor. A first hint was given by the findings that bacterial CPN60 can activate the toll-like receptor 4 (TLR4) pathway in macrophages and dendritic cells (Bulut, Faure et al. 2002). The binding to the TLR4 on the surface of macrophages was proven by Ohashi *et al.* (Ohashi, Burkart et al. 2000). The activation of the TLR4

led to the activation of the intracellular NF-κB pathway resulting in the production of inflammatory cytokines. This is in agreement with the previous findings.
Several reports also indicate a role for CPN60 during apoptosis. Zhu *et al.* (Zhu, Fang et al. 2016) reported a translocation of CPN60 to the membrane and an extracellular release from apoptotic cells. Furthermore, they reported that presenting CPN60 on the cell surface facilitates the uptake of cells via the LOX-1 receptor. Another report by Samali (Samali, Cai et al. 1999) showed that CPN60 binds in a complex with pro-caspase 3 which is an early signal for apoptosis. Here, CPN60 acts as a regulator for pro-caspase 3, as the complex dissociates during apoptosis.

3.4.2. CPN60 in pathogens

The role of CPN60 in pathogens had been analysed in several organisms.
In contrast to the four CPN60s of *L. donovani*, *M. tuberculosis* expresses just just two CPN60s named *Mt*CPN60.1 and *Mt*CPN60.2. It was found that *Mt*CPN60.1 is a non essential gene in *M. tuberculosis* and *M. bovis* while *Mt*CPN60.2 is essential for the survival of *M. tuberculosis* (Hu, Henderson et al. 2008, Wang, Lu et al. 2011). Bacteria lacking *Mt*CPN60.1 failed to induce an inflammatory response in animal models. Hickey *et al.* (Hickey, Ziltener et al. 2010) showed that *Mt*CPN60.2 is located on the surface and facilitates the association with macrophages. Furthermore, it was shown that *Mt*CPN60.2 does not form tetradecamers but dimers and *Mt*CPN60.1 requires post-translational phosphorylations to form higher oligomers (Hu, Henderson et al. 2008, Wang, Lu et al. 2011).
The protozoan parasite *Plasmodium falciparum* expresses only one copy of *Pf*CPN60. The heat shock response for *Pf*CPN60 was assessed by Ashis *et al.* (Das, Syin et al. 1997) and revealed a RNA level increase by 3x - 4x fold while the protein abundance did not change. Its intracellular localisation showed that *Pf*CPN60 accumulates during the ring-, the trophozoite-, and the schizont-stage in the cytoplasm before it is imported into the mitochondrion. In the cytosol chaperones such as the *Pf*HSP90 and the *Pf*HSP70 bind to the precursor *Pf*CPN60 before its translocation (Padma Priya, Grover et al. 2015).
For the parasite *Toxoplasma*, 11 entries are found for CPN60 in the toxodb data base. Analysis of *Tg*CPN60 in *T. gondii* revealed an important role in intracellular survival, differentiation and virulence (Shonhai, Maier et al. 2011). The latter finding was further supported by the finding that *Tg*CPN60 is a prominent antigen in infected rabbits (Ma, Zhang et al. 2009).

The distantly related parasite *Entamoeba histolytica* expresses one copy of *Eh*CPN60 which is located in the mitosome and might have a classical chaperonin activity. As *E. histolytica* has lost its mitochondrion the mitosome is considered an equivalent compartment (Clark and Roger 1995). An interesting finding was that the *Eh*CPN60 contains an amino-terminal 15 amino acid signal sequence which is required for the translocation into the mitosome. Parasites lacking the signal sequence could no translocate *Eh*CPN60 into the mitosome. Furthermore, the translocation could be restored if the signal sequence of *T. cruzi* was integrated (Tovar, Fischer et al. 1999).

One of the *T. cruzi Tc*CPN60s locates in the mitochondrion. Its RNA abundance is elevated 6-fold after a temperature increase from 25°C to 37°C. Contrary it was found that the RNA abundance remains stable when the temperature increases from 25°C to 42°C. The protein synthesis remains stable at different temperatures (Sullivan, Olson et al. 1994). The CPN60 of *T. brucei* shows 3 different isoforms which are located in the mitochondrial matrix, the kinetoplast, and the flagellar pocket (Bringaud, Peyruchaud et al. 1995, Radwanska, Magez et al. 2000, Folgueira and Requena 2007). The expression of the different isoforms depends on the life cycle stage. The *Tb*CPN60.1 is an essential gene which appears to display the classical chaperonin activity while CPN60.2 and CPN60.3 are non essential (Zhang, Cui et al. 2010, Alsford, Turner et al. 2011, Abdeen, Salim et al. 2016).

The individual role of the four distinct CPN60s in *Leishmania* has not been investigated in detail so far. The first indication of CPN60 expression during an infection was reported by Smejkal *et al.* (Smejkal 1988). This finding was strengthened by the finding that sera from infected patients detected recombinant *L*CPN60 (Rey-Ladino 1997). It was also shown in *in vitro* experiments that infection of macrophages with *Leishmania* led to an increase in CPN60 expression by 52% - 100% (Rey-Ladino and Reiner 1993). Also sera from infected dogs showed an immune response to *L*CPN60. The sera were tested against full *Leishmania* lysates and analysed by mass-spectrometry. The results showed that the detected CPN60 was the *L*CPN60.4 (Agallou, Athanasiou et al. 2016). About the intracellular and extracellular role of CPN60 in *Leishmania* is known that *Leishmania* counters TNFα, which is released by macrophages upon a *Leishmania* infection, and H_2O_2 treatment with an increase in CPN60 RNA synthesis (Salotra, Ralhan et al. 1994). Furthermore, it was shown by Silverman (Silverman, Clos et al. 2010) that CPN60.2 and CPN60.3 are part of the exosome cargo of *Leishmania* and dependent on HSP100. About the intracellular role little is known about the intracellular role, but Schlüter (Schlüter, Wiesgigl et al. 2000) showed that CPN60.2 is located in the mitochondrial matrix which argues for a role as a chaperone. Schlüter (Schlüter, Wiesgigl et al. 2000) also showed that

CPN60.2 expression is increased 2.5-fold during the axenic amastigote stage. No results were obtained for CPN60.1 as it was below the level of detection. Nevertheless, CPN60.1 is expressed as it was detected by ribosome profiling analysis (E. Bifeld, unpublished).

3.5. Gene editing techniques

To study a gene of interest (GOI) in *Leishmania* via reverse genetics, homologous recombination (HR) was the method of choice since its development in 1990 (Cruz and Beverley 1990). Recently, newly developed techniques are becoming more prominent, as they offer additional options, but also have some drawbacks in comparison to HR. The two most promising techniques are the Cre-loxP recombination system (**C**ausing **re**combination - **lo**cus of crossing (**x**) over **P**1) and the CRISPR/Cas strategy (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats / **C**RISPR **as**sociated).

3.5.1. DiCre - based inducible gene disruption

The Cre-loxP system was originally discovered by Stanberg in the bacteriophage P1 in 1978. By that time the molecular function and the mechanism of action were not clear and further research was needed. The first usage of the Cre-loxP system in a protozoan parasite (*Toxoplasma gondii*) was described by Andenmatten *et al.* (Andenmatten, Egarter et al. 2013). The mechanism of action is as follows: The GOI is flanked by two loxP sites which consist of 34 bp. Of these 34 bp the first 16 bp and the last 16 bp are reverse complementary to each other while the 8 bp in the centre are characteristic for the lox site (Albert, Dale et al. 1995). One Cre enzyme detects one of the 16 bp of the loxP sites and binds to it. As soon as four Cre enzymes are bound to the four 16 bp of the two loxP sites they form a tetramer resulting in a DNA loop. The DNA loop is the region between the two loxP sites. Depending on the orientation of the loxP sites – orientated either in the same direction or in the opposite direction – the loop will be cleaved out or inverted, respectively (Van Duyne 2001). The big advantage of the Cre-loxP system is its inducibility. To achieve inducibility, the Cre enzyme was divided into two inactive subunits of 59 kDa and 60 kDa. One is fused to the protein FKBP12 and the other to the protein FRB. These two proteins fuse upon Rapamycin induction. This also results in the fusion of the two Cre subunits leading to the active enzyme DiCre (**Di**merised **Cre**) (Jullien, Sampieri et al. 2003). The successful implementation in the *Leishmania* spp. system was first shown by

Duncan *et al.* (Duncan, Myburgh et al. 2016) and by Santos *et al.* (Santos, Silva et al. 2017).

3.5.2. CRISPR/Cas

The first description of the Cas9 enzyme from *Hemophilus influenzae* was by Smith *et al.*, in 1970 (Smith and Wilcox 1970). Scocca (Scocca, Poland et al. 1974) already suggested that the enzyme could be used for successful integration of DNA. The first complete description of the molecular mechanism and the possible application of the CRISPR/Cas system was by Gasiunas *et al.* (Gasiunas, Barrangou et al. 2012) in 2012. The first application in prokaryotes was in 2012 by Jinek (Jinek, Chylinski et al. 2012) and in eukaryotes in 2013 by Cong *et al.* (Cong, Ran et al. 2013).

The CRISPR/Cas system consists of a single guide RNA (sgRNA) and the Cas enzyme which cuts the DNA strand at a precise position. The sgRNA is 20 nucleotides (nt) long and highly specific for the region where the Cas enzyme will cleave. The region where the sgRNA will anneal is further defined by being directly upstream of a protospacer adjacent motif sequence NGG (PAM). The Cas enzyme will cleave the DNA 3 bp upstream of the PAM sequence. The sgRNA originally consists of a crRNA and a tracrRNA. The crRNA contains the specific 20 nt while the tracrRNA guides the Cas enzyme to the PAM sequence. The two RNAs contain a reverse complementary region where they anneal (Figure 8). For gene editing applications, any specific region upstream of a PAM sequence can be chosen for design of a sgRNA. A very suitable online tool (www.leishgedit.net) is provided by Eva Gluenz *et al.* (Beneke, Madden et al. 2017). For this application the two RNAs (crRNA and tracrRNA) are produced as one PCR product which is transcribed *in vivo* into a single sgRNA. The PCR product does not only contain the specific cleavage site and the Cas guiding sequence, but also a T7 RNA polymerase promoter sequence upstream of the specific 20 nt. This sequence facilitates T7 RNA polymerase-dependent transcription of the PCR product *in vivo* into the sgRNA. As the T7 RNA polymerase and Cas enzyme genes do not naturally exist in *Leishmania* spp, they must be expressed either from episomal or for from integrated transgenes. The Cas enzyme most often used is the Cas9 enzyme of *Streptococcus pyogenes*. The PCR product of the sgRNA can be transfected directly into the parasites and the Cas enzyme will cleave at a defined position.

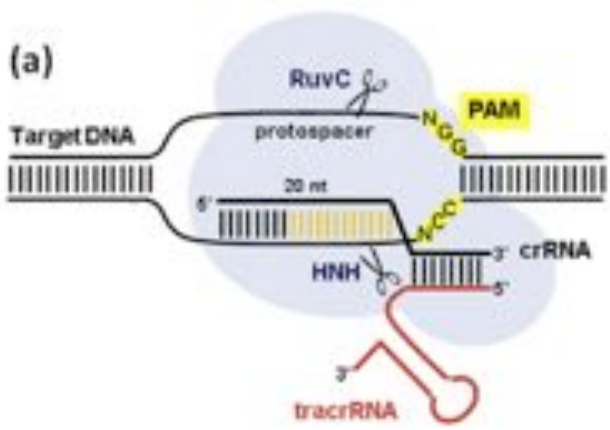


Figure 8: Representative figure of the specific cleavage of the Cas endonuclease. The Cas enzyme (grey), guided by the tracrRNA (red) which is bound to the crRNA (black), binds to the DNA strand. 3 bp upstream the PAM site (yellow) the endonuclease cleaves the DNA (Beneke, Madden et al. 2017).

To further improve the system, a GOI can be cleaved at two positions at the same time and a replacement construct can be inserted. *Leishmania* parasites repair double strand breaks (DSB) either by homology directed repair (HDR) or by microhomolgy-mediated end joining (MMEJ) (Zhang and Matlashewski 2015). Thus, replacement constructs, such as resistance marker genes or tags, can be predesigned in vectors. Short flanking sequences (30 bp) must be added. The replacement or insertion construct can be prepared in a single PCR reaction (Figure 9). The short flanking sequences will lead to MMEJ after cleavage with of the Cas enzyme. The PCR product of the sgRNA and of the replacement constructs can be transfected in one step.

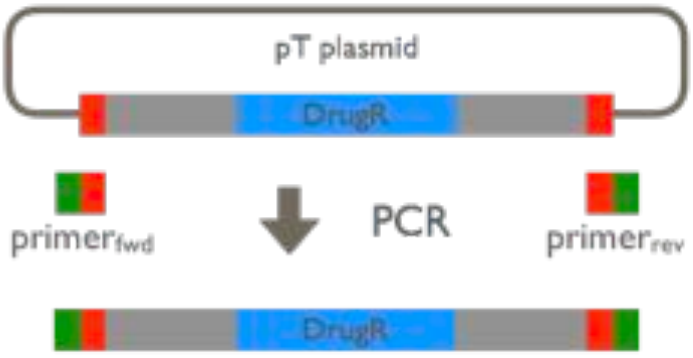


Figure 9: Representative figure of the replacement construct generation by PCR. The pT plasmid contains the drug resistance marker gene (blue) flanked by unspecific NCs (grey). On both sides aligning regions for the specific primers are added (red). The target specific region for MMEJ is displayed in green (Beneke, Madden et al. 2017).

4. Aim of the work

Leishmaniasis is a persistent threat for more than 1 billion people all over the world. As no effective vaccination exists and treatment options are limited, more research is needed. For the development of new drugs or a vaccine a better understanding of the biochemical processes and the biomolecular mechanisms inside the parasite is required. Many heat shock proteins play an essential role during the different life cycle stages of *Leishmania*. To date, very little is known about the four 60 kDa chaperonins (CPN60).

The aim of this thesis is to understand **the role of the four 60 kDa Heat Shock Proteins of *Leishmania donovani*, the reason for the presence of four diverged genes and their individual impact on viability, stress tolerance, and virulence**.

To address these questions, null mutants for each of the four CPN60s must be generated and their phenotypes recorded. The phenotype analysis addresses cell morphology, growth under different conditions and *in vitro* infectivity. Moreover, expression patterns, interaction with the co-chaeronin CPN10, and chaperone activity must be investigated. For the generation of the null mutants different techniques should be used and compared, such as the homologous recombination, the Cre-loxP system and the CRISPR/Cas system.

5. Material & methods

5.1. Material

5.1.1. Parasite strains

Leishmania donovani 1SR (MHOM/SD/00/1SR) is a laboratory strain originally from Sudan. The strain was cloned and is a gift from D. Zilberstein.

5.1.2. Bacterial stains

Name	Usage	Company
DH5α, chemically competent *E.coli*	DNA cloning	New England Bio-Labs, Beverly, USA
BL21, chemically competent *E.coli*	Expression of protein	Sarstedt, Waldbronn, Germany

5.1.3. Animals

Name	Usage	Obtained
Laying hens	isolation of specific IgY after immunisation	Geflügelzucht Zahrte, Wrestedt, Germany
C57BL/6 mice	isolation of monocytes	BNITM, Hamburg, Germany

5.1.4. Primers and vectors

The following Tables show the used primers and vectors. Primers used for PCR were also partly used for qPCR or sequencing.

5.1.4.1. Primer

Name	Sequence
P1 - CPN60.2-5'-NC-EcoRI	GGGGAATTCATTTAAATCACTACGCCTCCTCCTCTAC
P2 - CPN60.2-5'-NC-KpNI	CATGGTACCTTTTGGAGGTCGGGTGTGTC
P3 - CPN60.2-3'-NC-BamHI	GAGGGGATCCCGAAATTGCCCAGAGTTGAG
P4 - CPN60.2-3'-NC-HindIII	GAGGGAAGCTTATTTAAATTATGCGCGTTCCCTCTTCAC
P5 - CPN60.2+3-NdeI	GAGGCATATGCTCCGCTCCGCTGTGTG

Name	Sequence
P6 - CPN60.2+3-KpnI	GAGGGGTACCATGCTCCGCTCCGCTGTGTG
P8 - CPN60.3-BamHI	GAGGGGATCCTAGAAGCCCATGCCGCC
P9 - CPN60.3-5'-NC.KpnI	GAGGGGTACCGATGTTTTCTGTGGGAGGTTG
P10 - CPN60.3-5'-NC-EcoRI	GAGGGAATTCATTTAAATATGGAAACTGCACGTGCAAGG
P11 - CPN60.3-3'-NC-HindIII	GAGGAAGCTTATTTAAATGCTCATTAGGAGCGTGTAGC
P12 - CPN60.3-3'-NC-BamHI	GAGGGGATCCGCACGGTGAGTATACCTTCC
P13 - CPN60.2-BamHI.v2	GAGGGGATCCCTAGTAGCTGCCGAACAGC
P14 - CPN60.1-fwd	CGCCGTACTTTGTGACGAAC
P15 - CPN60.1-rev	GCTGAATGGCGTAGTTCAGC
P16 - CPN60.2-fwd	CCATTGTTGAGGCTCCAAAGG
P17 - CPN60.2-rev	AACAGCTCCTCGTCATCCTC
P18 - CPN60.3-fwd	GCCGCCATTGTTGAGCTG
P19 - CPN60.3-rev	CCGCCCATACCACCCATAC
P20 - CPN60.4-fwd	CAAAAGAGGGGCCAATCAGC
P21 - CPN60.4-rev	CGCTAGTCTTTGTAACTGCGC
P22 - CPN60.1-3'-NC-BamHI	GAGGGGATCCTCGAGGAGGACGGCAAGGAG
P22b/P34 - Cpn60.1-3'NC-BamHI-v2	GGGGATCCCGGCAAGGAGTGCGAGTGAC
P23 - CPN60.1-3'-NC-HindIII	GAGGAAGCTTATTTAAATGTCACCGCTCCTCGCCACCACCG
P23b/P35 - CPN60.1-3'NC-HindIIIv2	ATTTAAATAAGCTTCCTCTTTCTTTCTGTGAGTCTACG
P24 - CPN60.1-5'-NC-EcoRI	GAGGGAATTCATTTAAAGTTGTACTTAGAGTAGATGG
P25 - CPN60.1-5'-NC-KpnI	GAGGGGTACCAGAGTATAGAAGGTGCTGATG
P26 - CPN60.4-3'-NC-BamHI	GAGGGGATCCTGCACGCACTTCGCTGCCGAGGC
P27 - CPN60.4-3'-NC-HindIII	GAGGAAGCTTATTTAAATGCCGCCTCTATGAAGGCGTC
P28 - CPN60.4-5'-NC-EcoRI	GAGGGAATTCATTTAAATACTCATCTCTGTTGAAGATG
P29 - CPN60.4-5'-NC-KpnI	GAGGGGTACCCAACAAAGTGTTGTGCAACTG
P30 - Cpn60.2-5'flank	CGATGGCAAGGCGAAATCTC
P31 - Cpn60.2-3’-flank	CCCACCTGGCCTGCATAACC
P32 - Cpn60.3-5’flank	CATGGCGGCAACACCAATAG
P33 - Cpn60.3-3’flank	GACAAGAAGAGCATTGCCAC
P36 - CPN60.1-NdeI	GAGGCATATGCTCTCCCGTACGGTGCT

Name	Sequence
P37 - CPN60.1-BamHI	GAGGGGATCCTTACAGCCCTTTCATCTCAA
P38 - CPN60.1-KpnI	GAGGGGTACCATGCTCTCCCGTACGGTGCT
P39 - CPN60.4-BamHI	GAGGGGATCCTCACTTGCGGCGCTTGTCGG
P40 - CPN60.4-NdeI	GAGGCATATGTTTTCCTTATCGCGCCG
P41 - CPN60.4-KpnI	GAGGGGTACCATGTTTTCCTTATCGCGCCG
P42 - puro-fwd	GAGGGGATCCACCATGACCGAGTACAAGCC
P43 - puro-rev	GAGGGGATCCTCAGGCACCGGGCTTGCG
P44 - bleo-fwd	GAGGGGATCCACCATGGCCAAGTTGACCAGTG
P45 - bleo-rev	GAGGGGATCCTCAGTCCTGCTCCTCGGCCAC
P46 - loxP-5'rev.nucl	CATAACTTCGTATAATGTATGCTATACGAAGTTATGTAC
P47 - loxP-3'-rev.nucl	GATCGATAACTTCGTATAATGTATGCTATACGAAGTTATG
P49 - loxP-3'-fwd.nucl	GATCCATAACTTCGTATAGCATACATTATACGAAGTTATC
P50 - loxP-5'fwd	ATAACTTCGTATAGCATACATTATACGAAGTTATGGTAC
P55 - LDD-LPG1-BamHI-rev	GAGGGGATCCGCGACTCACTCCAGTGTTG
P56 - LDD-LPG1-BglII-fwd	GAGGAGATCTATGAGTCTTGTGATGTACT
P57 - CPN60.3-3'NC-CSrev	GCACCCCCACGTTAGCCAAG
P58 - CPN60.3-5'NC-CSfwd.nucl	GCTTCGTCGTGCTTTGAAGG
P59 - CPN10.1-NdeI	GAGGCATATGAGAGCGTGCTGCTTGGC
P60 - CPN10.1-BamHI	GAGGGGATCCAACGGATGAAGGGGGGCAAG
P61 - CPN10.2-NdeI	GAGGCATATGCTCCGCTTCACCATCCCCG
P62 - CPN10.2-BamHI	GAGGGGATCCTCAGCTTGACAGCACGCCAA
P63 - 60.1 - 3' sgRNA primer	GAAATTAATACGACTCACTATAGGACGGCAAGCAGTG CGAGTGAGTTTTAGAGCTAGAAATAGC
P64 - 60.1 - 5' sgRNA primer	GAAATTAATACGACTCACTATAGGAGTATAGAAGGTGCT GATGGGTTTTAGAGCTAGAAATAGC
P65 - 60.1 - Downstream reverse primer	CTACAGTCGGCAAAACACATCCCGGCGCCGCCAATT TGAGAGACCTGTGC
P66 - 60.1 - Upstream forward primer	CAAGCAAAAAAAGTTCAAACTGGAACACCCGTATAAT GCAGACCTGCTGC
P67 - 60.2 - 3' sgRNA primer	GAAATTAATACGACTCACTATAGGCGCGCCAAAATCTC AACTCTGTTTTAGAGCTAGAAATAGC
P68 - 60.2 - 5' sgRNA primer	GAAATTAATACGACTCACTATAGGAGACACACCCGAC CTCCAAAGTTTTAGAGCTAGAAATAGC
P69 - 60.2 - Downstream reverse primer	CGACATAGGCGGAAAAAAGAGGCTGTTGGCCCAATTT GAGAGACCTGTGC

Name	Sequence
P70 - 60.2 - Upstream forward primer	CGCGCTAAAGTCACCGAAATCAATCAAGACGTATAATG CAGACCTGCTGC
P71 - 60.3 - 3' sgRNA primer	GAAATTAATACGACTCACTATAGGGGATAGCAGAAGTA GGGTGTGTTTTAGAGCTAGAAATAGC
P72 - 60.3 - 5' sgRNA primer	GAAATTAATACGACTCACTATAGGTTTCTGTGGGAGGT TGGGTGGTTTTAGAGCTAGAAATAGC
P73 - 60.3 - Downstream reverse primer	CGCGGCACACACACACACACACAGAGGCTCCCAATT TGAGAGACCTGTGC
P74 - 60.3 - Upstream forward primer	ACCTCCCCCCTTCTCCATATCCTCTCCCCAGTATAATG CAGACCTGCTGC
P75 - 60.4 - 3' sgRNA primer	GAAATTAATACGACTCACTATAGGAGCAGAGCCGGCA GCTGCCTGTTTTAGAGCTAGAAATAGC
P76 - 60.4 - 5' sgRNA primer	GAAATTAATACGACTCACTATAGGTGTTGTGCAACTGC TATCTGGTTTTAGAGCTAGAAATAGC
P77 - 60.4 - Downstream reverse primer	AAACCCCAACCGCAGCCAACGAACTGATCCCCAATTT GAGAGACCTGTGC
P78 - 60.4 - Upstream forward primer	GCAGTGCCTCTGCGCCTCCTGATTCAGCCAGTATAAT GCAGACCTGCTGC
P79 - G00	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATA ACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA AAAC
P80 - DiCre-3'-rev-verification	CGTGCGGATCGATAACTTCG
P81 - DiCre-5'-fwd-verification	CGGTACATAACTTCGTATAG
P82 - Cpn60.4-5'flank	AGTTCTATGCCCCGACGCTTT
P83 - Cpn60.4-3'flank	AGCTGCAGCTGGAATACATCC
P84 - 60.1 - 3' - Downstream forward primer	CCGCCGATGAAGTTTGAGATGAAAGGGCTGGGTTCT GGTAGTGGTTCCGG
P85 - 60.1 - 3' - Downstream reverse primer	CTACAGTCGGCAAAACACATCCCGGCGCCGCCAATT TGAGAGACCTGTGC
P86 - 60.1 - 5' - Upstream forward primer	CAAGCAAAAAAAGTTCAAACTGGAACACCCGTATAAT GCAGACCTGCTGC
P87 - 60.1 - 5' - Upstream reverse primer	GACACAGCGAAGCACCGTACGGGAGAGCATACTACC CGATCCTGATCCAG
P88 - 60.2 - 3' - Downstream forward primer	GAGGATGACGAGGAGCTGTTCGGCAGCTACGGTTCT GGTAGTGGTTCCGG
P89 - 60.2 - 3' - Downstream reverse primer	CGACATAGGCGGAAAAAAGAGGCTGTTGGCCCAATTT GAGAGACCTGTGC
P90 - 60.2 - 5' - Upstream forward primer	CGCGCTAAAGTCACCGAAATCAATCAAGACGTATAATG CAGACCTGCTGC
P91 - 60.2 - 5' - Upstream reverse primer	GCCGGCAAGACACACAGCGGAGCGGAGCATACTAC CCGATCCTGATCCAG

Name	Sequence
P92 - 60.3 - 3' - Downstream forward primer	GGTATGGGTGGTATGGGCGGCATGGGCTTCGGTTCTGGTAGTGGTTCCGG
P93 - 60.3 - 3' - Downstream reverse primer	CGCGGCACACACACACACACACAGAGGCTCCCAATTTGAGAGACCTGTGC
P94 - 60.3 - 5' - Upstream forward primer	ACCTCCCCCCTTCTCCATATCCTCTCCCCAGTATAATGCAGACCTGCTGC
P95 - 60.3 - 5' - Upstream reverse primer	GCCGGCAAGACACACAGCGGAGCGGAGCATACTACCCGATCCTGATCCAG
P96 - 60.4 - 3' - Downstream forward primer	TACCGCCCGACGCCCGACAAGCGCCGCAAGGGTTCTGGTAGTGGTTCCGG
P97 - 60.4 - 3' - Downstream reverse primer	AAACCCCAACCGCAGCCAACGAACTGATCCCCAATTTGAGAGACCTGTGC
P98 - 60.4 - 5' - Upstream forward primer	GCAGTGCCTCTGCGCCTCCTGATTCAGCCAGTATAATGCAGACCTGCTGC
P99 - 60.4 - 5' - Upstream reverse primer	GCTCGCCAAGCGGCGCGATAAGGAAAACATACTACCCGATCCTGATCCAG
P100 - 60.1+5'-ext - KpnI	GAGGGGTACCATGCCGATTGCGGCAATCAAGCA
P101 - CPN60.1-3'-NdeI	GAGGCATATGTTACAGCCCTTTCATCTCAA
P102 - CPN60.1-3’-NdeI-v.2	GAGGCATATGTAACAGCCCTTTCATCTCAA
RT-Actin-F1	TGGCACCATACCTTCTACAACGAG
RT-Actin-R2	CGTCATCTTCTCACGGTTCTGC

5.1.4.2. Sequencing primer

Name	Sequence
M13-20R	GGAAACAGCTATGACCATG
M13-21F	TGTAAAACGACGGCCAGT
M13-24F	CCAGGGTTTTCCCAGTCACG
M13-24R	CGGATAACAATTTCACACAGG
pIR-p-fwd	CTGCGTTGTTGCCTGTGATG
pIR-P-fwd2	GGCTCTGCGTTTCACTTGC
pIR-p-rev	GCGAACTGGTCGTAGAAATC
pJC45 fwd	GGATAACAATTCCCCTCTAG
pJC45-rev	CTAGTTATTGCTCAGCGGTG

5.1.4.3. RT-qPCR primer

Name	Sequence
Leish_AC-F2	CAGAACCGTGAGAAGATG
Leish_AC-R	ACAGCCTGAATACCAATG
Leish_AC-Probe	FAM-CCTGGCTGGCCGGGACCTGAC-BHQ1
Mouse-Acb-F	CTGGAGAAGAGCTATGAG
Mouse-Acb-R	CTTACCCAAGAAGGAAGGCTG
Mouse-Acb-Probe	Cy5-CATCACTATTGGCAACGAGCGG-BHQ3

5.1.4.4. Vectors

Name	Usage	Provider
pUC19	Cloning vector	Invitrogen
pCL2N	*Leishmania* expression vector	D. Zander-Dinse, BNI
pCLN-3HA	*Leishmania* expression vector	D. Zander-Dinse, BNI
pCL2N-C-mCherry	*Leishmania* expression vector	D. Zander-Dinse, BNI
pJC45	*E. coli* protein expression vector with 10xHis tag	Clos, Brandau, BNI
pCL2N-C-mCherry::CPN60.1-old-start	*Leishmania* expression plasmid mCherry::CPN60.1-old-start	this thesis
pCL2N-C-mCherry::CPN60.1-ext.-start	*Leishmania* expression plasmid mCherry::CPN60.1-ext.-start	this thesis
pJC45-CPN60.1	*E. coli* protein expression vector with $His_{(10)}$::CPN60.1	this thesis
pJC45-CPN60.2	*E. coli* protein expression vector with $His_{(10)}$::CPN60.2	this thesis
pJC45-CPN60.3	*E. coli* protein expression vector with $His_{(10)}$::CPN60.3	this thesis
pJC45-CPN60.4	*E. coli* protein expression vector with $His_{(10)}$::CPN60.4	this thesis
pJC45-CPN10	*E. coli* protein expression vector with $His_{(10)}$::CPN10	this thesis
pCLN-3HA::CPN60.1	*Leishmania* expression plasmid 3HA::CPN60.1	this thesis
pCLN-3HA::CPN60.2	*Leishmania* expression plasmid 3HA::CPN60.2	this thesis
pCLN-3HA::CPN60.3	*Leishmania* expression plasmid 3HA::CPN60.3	this thesis

Name	Usage	Provider
pCLN-3HA::CPN60.4	*Leishmania* expression plasmid 3HA::CPN60.4	this thesis
pCL2N-CPN60.1	*Leishmania* expression plasmid CPN60.1	this thesis
pCL2N-CPN60.2	*Leishmania* expression plasmid CPN60.2	this thesis
pCL2N-CPN60.3	*Leishmania* expression plasmid CPN60.3	this thesis
pCL2N-CPN60.4	*Leishmania* expression plasmid CPN60.4	this thesis
pUC19-CPN60.1-5'bleo3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.1-5'puro3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.2-5'bleo3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.2-5'puro3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.3-5'bleo3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.3-5'puro3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.4-5'bleo3'NC	null mutant cloning plasmid	this thesis
pUC19-CPN60.4-5'puro3'NC	null mutant cloning plasmid	this thesis
pGL2313	*Leishmania* expression plasmid DiCre	J. Mottram, University of York
pUC19-CPN60.3-5'loxP-CPN60.3-Ldd-LPG1-bleo-loxP3'NC	conditional null mutant cloning plasmid	this thesis
pUC19-CPN60.3-5'loxP-CPN60.3-Ldd-LPG1-puro-loxP3'NC	conditional null mutant cloning plasmid	this thesis
pT007_Cas9_T7_Tub	*Leishmania* expression plasmid Cas9 and T7 RNA Polymerase	E. Gluenz, University of Oxford
pTpuro_v1	replacement construct plasmid	E. Gluenz, University of Oxford
pTbleo_v1	replacement construct plasmid	E. Gluenz, University of Oxford
pPLOT-mCH-Phelo	mCh-tagging replacement construct plasmid	E. Gluenz, University of Oxford
pPLOT-mNG-Puro	mCh-tagging replacement construct plasmid	E. Gluenz, University of Oxford

5.1.5. Antibodies

5.1.5.1. Antibodies for Western blot

Name	Origin	Dilution	Provider
anti-CPN60.1 IgY	Laying hens	1:2000	this thesis
anti-CPN60.2 IgY	Laying hens	1:1000	this thesis
anti-CPN60.3 IgY	Laying hens	1:3000	this thesis
anti-CPN60.4 IgY	Laying hens	1:2000	this thesis
anti-CPN10 IgY	Laying hens	1:500	AG Clos, BNITM
anti-CPN70 IgY	Laying hens	1:500	AG Clos, BNITM
anti-IgY (chicken)-AP	rabbit	1:5000	Dianova, Hamburg

5.1.5.2. Antibodies and dyes for IFA

Name	Origin	Dilution	Provider
anti-tubulin IgG	mouse	1:4000	Sigma Aldrich, München
anti-mouse Alexa Fluor® 594 IgG	Goat	1:250	Invitrogen
DAPI		1:50	Sigma Aldrich, München

5.1.6. Enzymes and size standards

Name	Provider
Gene Ruler 1kb DNA Ladder	Thermo Fisher Scientific, Waltham, USA
iProof High-Fidelity PCR Kit	Bio-Rad, München, Germany
KAPA PROBE FAST qPCR Master Mix Kit	VWR, Darmstadt, Germany
PageRuler unstained Protein Ladder	Thermo Fisher Scientific, Waltham, USA
ProSieve QuadColor Protein Marker	Biozym, Hessisch Oldendorf, Germany
Restriction enzymes, diverse	New England Biolabs, Beverly, USA
RNase A	Sigma-Aldrich, München, Germany
T4 DNA Ligase	New England Biolabs, Beverly, USA
Taq DNA Polymerase Kit	Thermo Fisher Scientific, Waltham, USA
Trypsin	Promega, München, Germany

Name	Provider
Quick-Load® 100 bp DNA Ladder	New England Biolabs, Beverly, USA

5.1.7. Commercial buffers required for cloning

Name	Provider
10x T4-DNA-Ligase buffer	New England Biolabs, Beverly, USA
NEB CutSmart buffer	New England Biolabs, Beverly, USA
NEB 3.1 buffer	New England Biolabs, Beverly, USA

5.1.8. Kits

Name	Provider
Agencourt AMPure XP Kit	Beckman Coulter, Fullerton, USA
DyNAmo cDNA Synthese Kit	Thermo Fisher Scientific, Waltham, USA
GeneClean® II Kit	MPO Biomedicals, Santa Ana, USA
High sensitivity DNA analysis kit	Agilent Technologies, Santa Clara, USA
InviTrap Spin Cell RNA Mini Kit	Stratec, Birekenfeld, Germany
ISOLATE II Genomic DNA Kit	Bioline, Luckenwalde, Germany
MiSeq reagent kit v3	Illumina, San Diego, USA
Nextera XT index kit	Illumina, San Diego, USA
Nextera XT library kit	Illumina, San Diego, USA
NucleoSpin Gel and PCR Clean up Kit	Machery-Nagel, Düren, Germany
QuantiTect® Reverse Transcription Kit	Qiagen, Venlo, Netherlands

5.1.9. Medium

Name	Provider	Usage
Dulbecco´s Modified Eagle Medium (DMEM)	Thermo Fisher Scientific, Waltham, USA	cultivation of macrophages
Medium 199 with Earle´s salts	Sigma-Aldrich, München, Germany	cultivation of *Leishmania*
Minimum Essential Medium (MEM)	Sigma-Aldrich, München, Germany	cultivation of LADMAC cells
RPMI-1640 (Roswell Park Memorial Institute)	Sigma-Aldrich, München, Germany	cultivation of LADMAC cells

5.1.10. Cell culture medium

Name	Composition
Cycle Grow LB - agar plates	2% LB 1.5% LB-Agar
Cycle Grow LB - liquid medium	2% LB
DMEM^{+}, complemented	DMEM + Glut Gibco 10% heat inactivated FCS 5% horse serum 1 x Pen/Strep 10%-30% LADMAC supernatant
Freezing medium	30% complemented M199^{+} 50% heat inactivated FCS 20% DMSO
M199^{+}, complemented, pH = 7.4	1 x M199 20% heat inactivated FCS 2 mM L-Glutamin 100 U Penicilin 0.1 mg/mL Streptomycin 40 mM HEPES (pH = 7.4) 15,3 µM Hemin 100 mM Adenine 5 µM 6-Biopterin
M199^{+}, complemented, pH = 5.5	1 x M199 20% heat inactivated FCS 2 mM L-Glutamin 100 U Penicilin 0.1 mg/mL Streptomycin 40 mM HEPES (pH = 5.5) 15,3 µM Hemin 100 mM Adenine 5 µM 6-Biopterin

5.1.11. Chemicals

Name	Provider
Aceton	Carl Roth, Karlsruhe, Germany
Acrylamide/Bis-acryalmide 40% (37.5:1)	Carl Roth, Karlsruhe, Germany
Acrylamide/Bis-acryalmide 40% (19:1)	Carl Roth, Karlsruhe, Germany
Ethanol	Carl Roth, Karlsruhe, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Methanol	Carl Roth, Karlsruhe, Germany
Phenol	Carl Roth, Karlsruhe, Germany
Trichloromethane	Carl Roth, Karlsruhe, Germany

Name	Provider
TEMED	Carl Roth, Karlsruhe, Germany

5.1.12. Buffer and solutions

The chemicals were, if not stated different, purchased from Sigma-Aldrich (München, Germany) and Carl Roth (Karlsruhe, Germany). The solutions were prepared in ddH_2O.

Name	Composition
7.5 M ammonium acetate	7.5 M ammonium acetate in ddH_20
AP-buffer	100 mM Tris-HCl (pH = 9.5), 100 mM NaCl, 10 mM $MgCl_2$
APS	10 % in ddH_2O
B5 buffer	20 mM Tris-HCl (pH = 8.0), 0.5 M KCl, 5 mM imidazole
B100 buffer	20 mM Tris-HCl (pH = 8.0), 0.5 M KCl, 100 mM imidazole
B1000 buffer	20 mM Tris-HCl (pH = 8.0), 0.5 M KCl, 1 M imidazole
BCIP (5-Bromo-4-chloro-3-indolyl-phosphate)	100 mg BCIP, 2 mL DMF
Blocking solution (IFA)	2 % w/v BSA in PBS, 0.1 % Triton X-100
Blocking solution (Immunoblot)	5 % w/v milk powder in TBS, 0.1 % Tween 20
buffer 1	20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole
buffer 2	20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole, 8 M urea
Coomassie brilliant blue staining	1 g/L Coomassie brilliant blue R-250, 40 % Ethanol, 10 % acetic acid
Coomassie brilliant blue destaining solution	40 % Ethanol, 10 % acetic acid
6 × DNA-loading buffer	90 % formamide, 10 mM EDTA (pH = 8.0), 0.05 % bromophenol blue, 0.05 % Xylenxyanol
Ethidium bromide	50 mg/mL in ddH_2O
electroporation buffer	21 mM HEPES (pH = 7.5), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose
KP buffer + 7% PEG 6000	KP buffer + 7% w/v PEG 6000
KP buffer + 24% PEG 6000	KP buffer + 24% w/v PEG 6000

Name	Composition
2 × Laemmli buffer	100 mM Tris-HCl (pH = 6.8), 100 mM DTT, 4 % SDS, 0.01 % bromophenol blue, 20 % glycerine
lysis buffer (Immuno präzipitation)	10 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 0.5 mM EDTA (pH = 8.0). 2 % Triton X-100, 1 mM PMSF, 0.5 mM 1,10-Phenanthroline
MES-Buffer	0.25 M saccharose, 20 mM MOPS, 3 mM EDTA
Mowiol	25 % glycerine, 0.1 M Tris-HCl (pH = 8.5), 10 % Mowiol 4-88
NBT	250 mg NBT, 3.5 mL DMF, 1.5 mL ddH_2O
1 × PBS	0.137 M NaCl, 10.14 mM Na_2HPO_4, 2.68 mM KCl, 1.76 mM K_2HPO_4 (pH 7.4)
permeabilisation solution	50 mM NH_4Cl, PBS (pH = 7.4), 0.1 % Triton X-100
200 mM 1,10-Phenanthroline	200 mM 1,10 Phenanthroline in MeOH
Plasmid preparation buffer 1	50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA (pH = 8.0)
Plasmid preparation buffer 2	0.2 N Natriumhydroxid, 1 % SDS (pH = 14)
Plasmid preparation buffer 3	3 M potassium acetate, 2 M acetic acid (pH = 5.5)
200 mM PMSF	200 mM Phenylmethane sulfonyl fluoride in MeOH
Potassium phosphate buffer (KP buffer)	71.2 mM K_2HPO_4, 28.3 mM KH_2PO_4, 100 mM NaCl
Refolding buffer (Luciferase Assay)	25 mM HEPES/KOH (pH = 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 3 mM ATP
1 x Roditi buffer	10.5 mL 3 x Roditi buffer, 3 mL 1.5 mM $CaCl_2$, 18 mL ddH_2O
3 x Roditi buffer	15 mM KCl, 70 mM NaH_2PO_4, 200 mM Na_2HPO_4, 150 mM HEPES (pH = 7.4)
Saccharose solution	20 mM Tris-HCl, 1 M Saccharose (pH = 7.4)
1 × SDS buffer	25 mM Tris, 250 mM glycine, 0.1 % SDS
10 % separating gel	375 mM Tris-HCl (pH = 8.8), 10 % acrylamide-bisacrylamide (37.5:1, 40 %), 0.1 % SDS, 0.1 % APS, 0.1 % TEMED
5 % stacking gel	125 mM Tris-HCl (pH = 6.8), 5 % acrylamide-bisacrylamide (37.5:1, 40 %), 0.1 % SDS, 0.1 % APS, 0.1 % TEMED
1 × TAE buffer	40 mM Tris-Acetate, 1 mM EDTA (pH = 8.0)
10 × TBE buffer	890 mM Tris, 890 mM boric acid, 20 mM EDTA (pH = 8.2)

Name	Composition
1 × TBS	150 mM NaCl, 10 mM Tris-HCl (pH = 7.2)
TE buffer	10 mM Tris-HCl, 1 mM EDTA (pH = 8.0)
TE-RNase-buffer	10 µg/mL RNaseA in TE buffer
Transfer buffer	48 mM Tris, 39 mM glycine, 0.04 % SDS, 20 % Methanol
Transfer buffer w/o MeOH	48 mM Tris, 39 mM glycine, 0.04 % SDS
Tris buffer with Tween 20	0.02% Tween 20 in TBS
1 M Tris-HCl, pH = 6.8	1 M Tris, pH = 6.8
1.5 M Tris-HCl, pH = 8.0	1.5 M Tris, pH = 8.0
Trypsin-EDTA solution	0.5 mg/mL Trypsin, 0.2 mg/mL EDTA in PBS
wash buffer (co-immune precipitation)	1 x TBS, 0.05% Tween20, 1 mM PMSF, 0.5 mM 1,10-Phenanthrolin
washing solution	PBS (pH = 7.4), 0.1 % Triton X-100

5.1.13. Antibiotics

Antibiotic	$c_{(0)}$	$c_{(final)}$
Ampicillin	10 mg/mL in ddH_2O	100 µg/mL
Blasticidin	5 mg/mL in PBS	5 µg/mL
Bleocin	2,5 mg/mL	5 µg/mL
G418	50 mg/mL	50 µg/mL
Hygromycin	50 mg/mL	50 µg/mL
Kanamycin	10 mg/mL in ddH_2O	10 µg/mL
L-Glutamine Pen/Strep (Penicillin/Streptomycin)	200 mM L-glutamine, 10.000 U Penicillin, 10 mg/mL Streptomycin	2 mM L-glutamine, 100 U Penicillin, 0,1 mg/mL Streptomycin
Nurseothricin/clonNAT	150 mg/mL in ddH_2O	150 µg/mL
Pen/Strep (Penicillin/ Streptomycin)	10.000 U Penicillin, 10 mg/mL Streptomycin	100 U Penicillin, 0,1 mg/mL Streptomycin
Puromycin	2,5 mg/mL in M199	25 µg/mL
Rapamycin	100 µM	

5.1.14. Equipment

Name	Provider
Biomate 3 Spectrophotometer	Thermo Fisher Scientific, Waltham, USA
Biometra UV Band Eluator	Biometra, Göttingen, Germany
Branson Sonifier 250	G. Heinemann, Schwäbisch Gmünd, Germany
CASY® Cellcounter & Analyzer	Roche, Mannheim, Germany
cell culture plate 12-well	TPP, Trasadingen, Schweiz
Cell culture flask T25 cm^2	Sarstedt, Waldbronn, Germany
Cell culture flask T75 cm^2	Sarstedt, Waldbronn, Germany
Cell culture flask T175 cm^2	Sarstedt, Waldbronn, Germany
Cellophane® foil (24x24 cm)	Carl Roth, Karlsruhe, Germany
cell scraper M	TPP, Trasadingen, Schweiz
Electrophoresis Power Supply-EPS301	Amersham, Buckinghamshire, UK
Electroporation cuvettes (0,4 cm)	Bio-Rad, München, Germany
Electroporation cuvettes (0,2 cm)	Bio-Rad, München, Germany
Eppendorf Centrifuge 5415 D	Eppendorf, Hamburg, Germany
Eppendorf Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Eppendorf Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Eppendorf Mastercycler gradient	Eppendorf, Hamburg, Germany
Evos FL Auto Cell Imaging System	Thermo Fisher Scientific, Waltham, USA
Evos XL Cell Imaging System	Thermo Fisher Scientific, Waltham, USA
Fluted filter (⌀ 185 mm)	Schleicher & Schüll, Dassel, Germany
Gene Pulser	Bio-Rad, München, Germany
His Bind Resin	Novagen, Madison, USA
Incubator WTC Binder	Binder, Tuttlingen, Germany
InnovaTM 4400 incubator shaker	New Brunswick Scientific, New Jersey, USA
J2-21 Centrifuge	Beckman Coulter, Fullerton, USA
J2-HS Centrifuge	Beckman Coulter, Fullerton, USA
Microfuge Tube	Beckman Coulter, Fullerton, USA
Neubauer counting chamber (0,1 mm depth)	Assistent, Sondheim, Germany
New Brunswick Galaxy 170S	Eppendorf, Hamburg, Germany
Optima TL Ultracentrifuge	Beckman Coulter, Fullerton, USA
Optima XE-90	Beckman Coulter, Fullerton, USA

Name	Provider
PerfectBlue gel system	Peqlab, Erlangen, Germany
pH-Meter Micropocessor pH 211	Hanna Instruments, Kehl am Rhein, Germany
Quick-seal tubes	Beckman Coulter, Fullerton, USA
Rotor-Gene 6000	Corbett, Sydney, Australien
Ribolyser Beads	Thermo Fisher Scientific, Waltham, USA
Safety cabinet MSC-Advantage	Thermo Fisher Scientific, Waltham, USA
Sonovex Super	Bandelin, Berlin, Germany
Spectra/Por dialysis tubing (MWCO 6-8 kD)	Spectrum, Rancho Dominguez, USA
Taumel roll shaker CAT RM5	neoLab, Heidelberg, Germany
Trans-Blot SD	Bio-Rad, München, Germany
TrayCell	Hellma, Müllheim, Germany
Ultra-Clear centrifuge tubes	Beckman Coulter, Fullerton, USA
Vortexer VF2	IKA-Werke GmbH & Co. KG, Staufen, Germany
3 MM Whatman-Papier	Schleicher & Schüll, Dassel, Germany

5.1.15. Software & Data bases

5.1.15.1. Data base

Data base	Usage
TriTryp	Gene data bank for Trypanosomatidae
RCSB Protein Data bank	Protein data bank
PubMed - NCBI	Literature Research

5.1.15.2. Software

Software	Usage
Adobe® Photoshop® CS3 Extended, Vers. 10.0.1	raster graphics editor for analysis of images
EndNote X7.8	Paper library
GraphPad Prism5, Vers. 5.0a	Statistical Software incl. graphical design
ImageJ, Vers. 1.42q	Image processing program
MacVector, Inc., Vers. 16.0.8 (33)	sequence analysis and in silicon cloning
SnapGene Viewer, Vers. 4.1.3	sequence analysis and in silicon cloning

5.2. Methods

5.2.1. Cultivation of *Leishmania donovani* promastigotes

L. donovani promastigotes were cultured in 10 mL M199^{+} at 25°C and pH = 7.4. Parasites were grown until early stationary phase. To keep them in logarithmic to early stationary phase the parasites were diluted at this point. If needed *L. donovani* parasites were grown to mid or late stationary phase.

5.2.2. Cryopreservation of Leishmania

L. donovani promastigotes were grown to late logarithmic phase (~ 8*10^{6} cells / mL). Cell concentration was determined using either CASY® Cell counter or Beckman Multisizer 3 Coulter Counter. 1*10^{8} cells were sedimented for 10 min at 1000 x g and 4°C. Supernatant was discarded and cells resuspended in 0,5 mL M199^{+}. 0,5 mL Leishmania freezing medium was added and cells were transferred into a 2 mL cryopreservation tube. The tube was placed into a styrofoam box and cooled down slowly to -70°C for 24hrs. Afterwards the tube was transferred to the liquid nitrogen cryogenic tank.
Needed samples were thawn rapid at 37°C and the cells transferred to a cell culture flask containing 9 mL M199^{+} supplemented with the required antibiotics.

5.2.3. Transfection of *Leishmania*

L. donovani promastigotes were grown to late logarithmic phase (~ 8*10^{6} cells / mL). 4*10^{7} cells were needed per transfection. The required amount of cells was sedimented at 1000 x g, 4°C for 10 min, the supernatant discarded and the cells washed twice with 10 mL cold PBS. Afterwards the cells were washed with 10 mL cold electroporation buffer and resuspended in 400 µL cold electroporation buffer per 4*10^{7} cells. 2 µg of linearised DNA or 50 µg of plasmid DNA were placed into a pre-cooled electroporation cuvette. 400 µL of cell suspension was added and carefully mixed by tipping against the cuvette. For electroporation the cuvette was placed in a Bio-Rad Gene Pulser apparatus and electroporation was carried out by three pulses of 1.5 kV, 200 Ω, 25 µFD and a time-constant of 0.9 to 1.5. Next the cuvette was placed on ice for 10 min. Afterwards the cell suspension was transferred into a cell culture flask containing 10 mL M199^{+} and 100 µL PenStrep. After 24hrs the required antibiotics (5.1.13) were added and after 48hrs the cell culture diluted 1:10. Positive selection was finished as soon as the control group without the DNA (MOCK) was dead.

5.2.4. Transfection of Leishmania by CRISPR/Cas9 transfection

To target the CPN60 genes, the Cas9-expressing strain was transfected with the appropriate PCR constructs coding for gene-specific sgRNAs and with the GOI-specific replacement constructs. For this, specific primers were designed as follows:
For the sgRNA the 5'-primer consists on its 5'-end of a 24 nt (nucleotide) promoter region for the T7-RNA-polymerase, followed downstream by 20 nt specific to the target region. Last 20 nt are added as a start for the Cas9 backbone, being homologous to the 3'-primer. The 3'-primer (G00) is a standardised primer. It consists on its 5'-end of the 20 nt being homologous to the 5'-primer, followed by 61 nt creating the guiding loop for the Cas9 enzyme. The required primer dimers were amplified by PCR, resulting in a 125 bp fragment.
Before *L. donovani* parasites could be transfected the sgRNA and the replacement constructs had to be prepared. The following Tables 1, 2, 3, and 4 show the reaction mixture and the cycling programs used. Primers were always frozen at -80°C before Master Mix was added. Samples were thawn at RT, but not mixed before PCR.

Table 1: PCR reaction mix required for sgRNA generation.

Reagent	Volume [µL]	final concentration
G00 primer	0.4	2 µM
dNTP	4	0.2 mM
Primer	10	2 µM
High fidelity polymerase	0.2	1 Unit
10x reaction buffer	2	1x
ddH_2O	7	

Table 2: PCR program required for sgRNA generation.

Step	Temperature	Time
1	98°C	30 sec
2	98°C	10 sec
3	60°C	30 sec
4	72°C	15 sec

Step	Temperature	Time
	35x step 2 - 4	
5	72°C	10 min
6	4°C	hold

Table 3: PCR reaction mix required for replacement construct generation.

Reagent	Volume [µL]	final concentration
template (pPLOT or pT)	0.5	30 ng
dNTP	0.8	0.2 mM
Primer $_{fwd\ \&\ rev}$	8	2 µM (each)
High fidelity polymerase	0.4	1 Unit
DMSO	1.2	3 %
$MgCl_2$	3	1.5 mM
10x reaction buffer	4	1x
ddH_2O	22.1	

Table 4: PCR program required for replacement construct generation.

Step	Temperature	Time
1	94°C	5 min
2	94°C	30 sec
3	65°C	30 sec
4	72°C	2:15 min
	40x step 2 - 4	
5	72°C	7 min
6	4°C	hold

After the PCR 1 µL of the sample was loaded onto a gel for analysis, while the remaining sample was pooled as required and frozen at -80°C. The second sample, either the sgRNA or the replacement construct, was processed the same way and pooled with the already frozen sample. The samples were were shortly before usage heat sterilised at 95°C for 5 min.

L. donovani promastigotes were grown to late logarithmic phase (~ $8*10^6$ cells / mL). $1*10^7$ cells were needed per transfection. The required amount of cells was sedimented at 1000 x g, RT for 10 min, the supernatant discarded and the cells washed once with 10 mL 1x Roditi buffer. Next the parasites were resuspended in

200 µL 1x Roditi buffer per $1*10^7$ cells. The pooled PCR samples were placed into a 1.5 mL tube and 200 µL cell suspension were added and mixed. 250 µL of the suspension were transferred into a pre cooled electroporation cuvette (0.2 cm). For electroporation the cuvette was placed in a Amaxa Nucleofactor IIb apparatus and electroporation was carried out using the program X-001. Next the cell suspension was transferred into a cell culture flask containing 5 mL $M199^+$ and 100 µL PenStrep. After 24hrs the required the antibiotics (5.1.13) were added. Positive selection was finished as soon as the control group without the DNA (MOCK) was dead.

5.2.5. Limiting dilution analysis

To obtain single clones of transfected *L. donovani* parasites, the parasites were diluted via limiting dilution. Therefore, $1*10^7$ cells were resuspended in 1 mL $M199^+$. 0.5 mL of the cell suspension was transferred to a 15 mL tube containing 4,5 mL $M199^+$, resulting in a cell concentration of $1*10^6$ cells/mL. This dilution procedure was repeated until a concentration of $1*10^2$ cells/mL was reached. 2.5 mL of the suspension was transferred to a new 15 mL tube containing 2.5 mL $M199^+$ to obtain a final concentration of 50 parasites/mL. Afterwards 2.5 mL of the final dilution was added to 47 mL $M199^+$ containing 0.5 mL PenStrep and the required antibiotics. The suspension was split into two 96-well plates with 200 µL/ well. The plates were sealed with parafoil and incubated for 2 weeks at 25°C. Analysis of the wells revealed that in 30% - 60% of the wells parasites were growing. Of each plate 5 single clones were picked and transferred into a cell culture flask containing 10 mL $M199^+$ and the required antibiotics.

5.2.6. In vitro differentiation

To analyse the protozoan lifecycle of *L. donovani* in vitro a stage conversion was performed. Therefore, $3*10^8$ logarithmic phase promastigotes were cultured in 10 mL $M199^+$, pH = 7.4 without antibiotics at 25°C. After 24hrs the flask was transferred to 37°C and kept there for another 24hrs. Next the parasites were sedimented at 1000 x g, 10 min, RT and resuspended in acidic $M199^+$ (pH = 5.5). The cell suspension was transferred to a ventilated cell culture flask and kept at 37°C with 5% CO_2. After 48hrs the parasites had converted into axenic amastigotes and the cell suspension had to be diluted 1:5. 72hrs thereafter the cells were sedimented at 1000 x g, 10 min, RT and resuspended in 10 mL $M199^+$, pH7.4 and kept at 25°C to convert the amasitgotes back into promastigotes. Differentiation back to promastigotes is completed within 48hrs to 72hrs.

5.2.7. In vitro proliferation studies

To study proliferation of *L. donovani* under different conditions and stressors in vitro several proliferation studies can be performed. Regardless of the treatment with different stressors these studies can be differentiated into three different proliferation assays. First are the ones being carried out at 25°C at pH = 7.4 (I), second at 37°C and pH = 7.4 (II) and third the ones at 25°C and pH = 5.5 (III).

(I) Proliferation assays at 25°C and pH = 7.4 were performed as following: $1*10^6$ *L. donovani* promastigotes were grown in 10 mL M199$^+$, pH = 7.4. to obtain an initial concentration of $1*10^5$ cells/mL. Cell concentration was determined using CASY® Cell Counter or Beckman Multisizer 3 Coulter Counter every 24hrs for 4 to 5 days. Obtained values were normalised against WT parasites for each day to make comparison of several independent experiments possible.

(II) Proliferation assays at 37°C and pH = 7.4 were performed as following: $5*10^6$ *L. donovani* promastigotes were grown in 10 mL M199$^+$, pH = 7.4. to obtain an initial concentration of $5*10^5$ cells/mL. Cell concentration was determined using CASY® Cell Counter or Beckman Multisizer 3 Coulter Counter every 24hrs for 4 to 5 days. Obtained values were normalised against WT parasites for each day to make comparison of several independent experiments possible.

(III) Proliferation assays at 25°C and pH = 5.5 were performed as following: $1*10^6$ *L. donovani* promastigotes were grown in 10 mL M199$^+$, pH = 7.4. to obtain an initial concentration of $1*10^5$ cells/mL. Cell concentration was determined using CASY® Cell Counter or Beckman Multisizer 3 Coulter Counter every 24hrs for 4 to 5 days. Obtained values were normalised against WT parasites for each day to make comparison of several independent experiments possible.

5.2.8. Isolation and cultivation of bone marrow derived macrophages

Monocytes were isolated from female C57Bl/6 mice. Therefore, the femur and the tibia were removed and disinfected with 70% isopropanol. The bone marrow was flushed out using prewarmed DMEM$^+$ medium (37°C). Remaining bone splitters were removed and the cells sedimented at 400 x g, 4°C for 10 min. The cells were

resuspended in 40 mL DMEM+ and were transferred into a T175 ventilated cell culture flask. Incubation was done at 37°C, 5% CO_2 for 9 days. Whereas every 3 days 50% of the DMEM+ medium was replaced with new DMEM+ medium.

5.2.9. Ex vivo infection studies

Analysis of infectivity and virulence of genetically modified *L. donovani* strains was performed in ex vivo infection assays and analysed using Taqman qPCR (Bifeld, Tejera Nevado et al. 2016). Therefore, $2*10^5$ murine macrophages were seeded in 12 well culture plates. Only 8 well were used, while the edges were filled with 2 mL PBS. 72 hrs thereafter the macrophages were washed twice with 2mL pre-warmed PBS. Afterwards $2*10^6$ *L. donovani* parasites per well were added in 500 mL DMEM (infection ratio 10:1) and incubated for 4hrs at 37°C, 5% CO_2. The cells were then washed twice with 2 mL pre-warmed PBS and pre-warmed DMEM was added according to the following incubation time. Cells were either incubated for 30min or 48hrs and 500 µL or 2 mL DMEM were added respectively. To isolate gDNA the supernatant was transferred to a 2 mL tube and sedimented at 1000 x g, 4°C for 10 min. In the meantime adherent macrophages were lysed using 200 µL Lysis Buffer GL from Isolate II Genomic DNA KIT from BIOLINE. After centrifugation the supernatant was discarded and the lysed macrophage suspension was added to the pellet. All following steps were carried out according to manufactures protocol. Taqman qPCR was carried out as described by (Bifeld, Tejera Nevado et al. 2016) (see 5.2.24).

5.2.10. Fixation of Leishmania for microscopy

Microscopic analysis was carried out by using $1*10^6$ to $1*10^7$ parasites. After centrifugation at 1000 x g, 4°C, 8 min, parasites were resuspended in 200 µL PBS. 20 µL of the suspension were then smeared onto a object slide and let try. Afterwards the object slide was incubated with cold MeOH (-20°C) for 1 min. Thereafter the object slide was ready for further staining or light microscopical analysis.

5.2.11. Polymerase chain reaction (PCR)

For the Polymerase chain reaction two different Taq polymerases were used. The iProof High-Fidelity PCR Kit was mainly used for the production of DNA-constructs being used for transfection or protein synthesis. The Taq DNA Polymerase Kit was mainly used for analytical purposes. The exact configuration

for each PCR had to be determined before and was done by a gradient-PCR. Following two general pipetting schema and temperature schema are shown.

Table 5: PCR reaction mix for iProof High-Fidelity PCR Kit.

Reagent	Volume
iProof GL-buffer	13 µL
DMSO	3 µL
ddH_2O	6.5 µL
$Primer_{fwd}$ (10µM)	1 µL
$Primer_{rev}$ (10µM)	1 µL
DNA (~10-100ng/µL)	1 µL

Table 6: PCR program for iProof High-Fidelity PCR Kit.

Step	Temperature	Time
1	98°C	3 min
2	98°C	30 sec
3	55°C	30 sec
4	72°C	20 sec
	29x step 2 - 4	
5	72°C	5 min
6	4°C	hold

Table 7: PCR reaction mix for Taq DNA Polymerase Kit.

Reagent	Volume
ddH_2O	19.5 µL
10x buffer -$MgCl_2$	2.5 µL
$MgCl_2$ (50mM)	0.75 µL
dNTP-Mix (10µM)	0.5 µL
$Primer_{fwd}$ (10µM)	0.5 µL
$Primer_{rev}$ (10µM)	0.5 µL
DNA	0.5 µL
Taq DNA Polymerase	0.25 µL

Table 8: PCR program for Taq DNA Polymerase Kit.

Step	Temperature	Time
1	95°C	5 min
2	95°C	1 min
3	60°C	30 sec
4	72°C	90 sec
	25x step 2 - 4	
5	72°C	2 min
6	4°C	hold

5.2.12. Agarose gel electrophoresis

Analysis on DNA was done by agarose gel electrophoresis. Therefore, a 1,5% to 2% agarose gel in TAE-buffer was made. The gel was run at 80V to 120V for 30 min up to 2hrs.

5.2.13. Extraction of DNA fragments from an agarose gel & DNA purification from a PCR

Purification of DNA from agarose gels was carried out by using the “Nucleo Spin Gel & PCR cleanup” kit from Machery-Nagel. Samples were cut out from an agarose gel under UV-light and weight was estimated. Double the amount of NTI-buffer was added and samples heated up to 70°C for 15 min. PCR-products were resuspended in the double amount of NTI-buffer, but no heating was required. Samples were loaded onto a spin column and sedimented at 11000 x g, for 30 sec. After washing twice with 700 µL NT3 buffer the columns were sedimented at 11000 x g for 1 min to dry them from remaining traces. 10 µL to 15 µL of preheated (70°C) elution buffer were loaded onto the column, incubated for 5 min and sedimented at 11000 x g for 1 min. The elution step was repeated to increase the amount of DNA. Eluted DNA was stored at either 4°C or -20°C.

5.2.14. Enzymatic digest of DNA

The enzymatic digest was used for two different purposes. Either to fully digest DNA for further usage or a partial digest for analytical examination. The following Table shows a pipetting schemata for analytical usage.

Table 9: Reaction mix for enzymatic digest.

Reagent	Volume
ddH2O	16.5 µL
Restriction buffer	2 µL
DNA	1.5 µL
Enzyme I	0.25 µL
Enzyme II	0.25 µL

5.2.15. Ligation of DNA fragments

To ligate DNA fragments the DNA concentration had to be determined. This was achieved by running an agarose gel, followed by optical analysis. If the concentration of the vector was equal to the concentration of the insert 0.5 µL of the vector and 2 µL of the insert were used. Otherwise the amount was changed to obtain the same ratio. Following a pipets schema os shown:

Table 10: Reaction mix for ligation of DNA fragments.

Reagent	Volume
T4 DNA Ligase	**1 µL**
T4 DNA Ligase buffer	1 µL
vector	x µL
insert	y µL
ddH_2O	8 µL - x - y
total volume:	**10 µL**

5.2.16. Transformation of *E. coli*

Two different E.coli strains were used depending on the experiment. Subcloning Efficiency™ Dh5α™ Competent Cells were used for amplifying DNA, while Competent BL21 (DE3)[pABlaclQ] cells were used for protein expression. Cells (50 µL aliquot) were than on ice and 5 µL of ligation mix (1 µg of plasmid for protein expression) were added. After 30 min incubation on ice cells were heated up to 42 °C for 20 sec (30 sec for BL21 (DE3)[pABlaclQ] cells) and kept on ice for

2 min. 950 µL (500 µL for BL21 (DE3)[pABlaclQ] cells) pre-warmed LB-medium were added and incubated at 37°C, 350 rpm for 1h. Samples were sedimented at 605 g, 5 min and 850 µL (500 µL for BL21 (DE3)[pABlaclQ] cells) of the supernatant were removed, cells resuspended in the remaining volume and plated onto pre-warmed agar plates containing the required antibiotics. Incubation was done over night at 37°C and plates stored for up to 48 hrs at 4°C.

5.2.17. Isolation of genomic DNA from Leishmania

Genomic DNA from Leishmania parasites was isolated using "ISOLATE II Genomic DNA Kit" from BIOLINE. $5*10^7$ to $2*10^8$ cells were sedimented at 1000 x g, 8 min, 4°C and washed with 10 mL PBS and 1 mL PBS. The pellet was resuspended in to 200 µL Lysis buffer GL and 200 µL Lysis buffer G3 augmented with 25 µL Proteinase K solution were added. The samples were carefully mixed and heated up to 70°C for 15 min at 400 rpm. After brief vortexing 210 µL 96% EtOH were added, the sample was vortexed vigorously and transferred to an ISOLATE II Genomic DNA spin column. Samples were sedimented at 11000 x g, 1 min, washed with 500 µL Wash buffer GW1 and with 600 µL Wash buffer GW2. The columns were dried at 11000 x g for 1 min and 50 µL preheated (70°C) Elution buffer G were added, incubated for 5 min at RT and sedimented at 11000 x g, 1 min. The elution step was repeated to increase the total amount of gDNA. Samples were stored at 4°C.

5.2.18. Isolation of plasmid DNA by alkaline lysis

Transformed single clones of *E.Coli* were grown in 2 mL LB-medium containing 20 µL Ampicilin over night at 37°C. Samples were transferred to a 2 mL reaction tube and sedimented at 16000 x g, 2 min, 4°C. The supernatant was discarded, the pellets resuspend in 100 µL Solution 1 and 200 µL Solution 2 added. Samples were inverted 5x, incubated at RT for 3 min, 150 µL cold (4°C) Solution 3 were added and inverted 5x. Afterwards the samples were sedimented at 16000 x g, 10 min, 4°C and the supernatant transferred to a 1.5 mL reaction tube. 1 mL 96% EtOH were added, incubated at RT for 5 min and sedimented at 16000 x g, 10 min, RT. The supernatant was discarded, the pellets were washed with 500 µL 70% EtOH and let dried at RT. Afterwards the pellets were resuspended in 40 µL TE-RNAse-buffer, incubated at 37°C for 40 min and stored for further usage at 4°C.

5.2.19. Isolation of highly purified plasmid DNA

Transformed single clones of *E.Coli* were grown in 200 mL LB-medium containing 2 mL Ampicilin over night at 37°C. Samples were transferred to a 400 mL tube and sedimented at 2820 x g, 20 min, 4°C. The supernatant was discarded, the pellets resuspend in 5 mL Solution 1, transferred to a 50 mL reaction tube and 10 mL Solution 2 added. Samples were incubated on the roll shaker at RT for 5 min, 7.5 mL cold (4°C) Solution 3 were added, inverted 5x and incubated at 4°C for 10 min. Afterwards the samples were sedimented at 10600 x g, 10 min, 4°C and the supernatant filtrated into a 50 mL reaction tube. 0.7%/vol. of isopropanol were added, mixed and incubated at RT, 20 min. Afterwards the samples were sedimented at 2700 x g, 20 min, RT, the supernatant discarded and the pellets washed with 10 mL 70% EtOH. The dried pellets were resuspended in 4 mL TE-buffer and incubated on the roll shaker until the pellets were completely dissolved. 4.9 g CsCl and 100 µL Ethidiumbromide were added, the samples vortexed vigorously and transferred to a 5.2 mL QuickSeal tube. Tubes were closed and sedimented for 8h, 70000 x rpm, 25°C. The DNA band was extracted by using a syringe, the volume was determined and the solution was washed twice with 1 volume of NH_4-acetate saturated isopropanol. 0.1 volume (of original volume) 7.5 M NH_4-acetate, 2 volumes (new volume) ddH_2O and 2.5 volumes (new volume) 96% EtOH were added, mixed and incubated 20min at RT. Afterwards the samples were sedimented at 2700 x g, 30 min, RT, the supernatant discarded, the pellets dried and resuspended in 100 µL to 500 µL TE-buffer at 4°C, overnight. DNA concentration was determined and samples set to 1000 µg/mL with TE-buffer and stored at 4°C.

5.2.20. DNA sequencing

Sequence analysis of DNA samples was carried out by LGC Genomics. Therefore, 1 µg of DNA, 1 µL primer (10 µM) and 12 µL ddH_2O were added to a 1.5 mL reaction tube and send to LGC genomics. For analysis MacVector, Inc., version 16.0.8 (33) was used.

5.2.21. Next generation sequencing

Whole genome analysis of the genetic modified parasites was performed by using Next Generation Sequencing (NGS). The NGS technique used was sequencing by synthesis from Illumina. Therefore, gDNA was isolated as described in 5.2.17 and diluted to a final concentration of 0.5 ng/µL. For an exact measurement the Qubit

(3.0)-system from Invitrogen was used. Next 1 ng was used for the creation of the library. Therefore, the Nextera XT library kit and the Nextera XT index kit were used according to the manufacturer's protocol. The obtained library was purified using the Agencourt AMPure XP Kit according to the manufacturer's protocol. Quality control of the obtained DNA was performed using a Bioanalyzer (Agilent) with the high sensitivity DNA analysis kit (Agilent). The library was diluted to 4 nM and denatured in the presence of 0.2 N NaOH at a concentration of 20 pM. The solution was diluted further to 10 pM using chilled hybridisation buffer HT1 and a reference library (PhyX) is added. The obtained library was heat denatured (95°C, 2 min) and incubated on ice for 5 min. Next the reagent cartridge was loaded with 10 pmol and sequencing performed using the MiSeq System. The obtained sequences were the analysed by aligning to a reference genome using the MacVector Bowtie 2.0 algorithm.

5.2.22. Isolation of RNA from Leishmania and transcription into cDNA

RNA isolation from Leishmania parasites was carried out by using the "InviTrap® Spin Cell RNA Mini" Kit. $2*10^7$ - $5*10^7$ cells were sedimented at 1000 x g, 8 min, 4°C, washed with 10 mL cold PBS and 1 mL cold PBS. Supernatant was discarded and pellets resuspended in remaining PBS. 350 µL Lysis solution R augmented with DTT were added and solution was transferred to a DNA-binding spin filter, incubated for 1 minute RT and sedimented at 13400 x g, 2min. The spin filter was discarded, 250 µL 96% EtOH added and the solution loaded onto a RNA-RTA spin filter. After 1 min, RT incubation the samples were sedimented at 9300 x g, 30 sec, the flow through discarded and the filter washed with 600µL Wash buffer R1, followed by 700 µL Wash buffer R2. The columns were dried at 13400 x g, 5 min at RT and the RNA eluted by adding 40 µL Elution buffer R, followed 2 min incubation at RT and centrifugation at 9300 x g for 1 min. Samples were immediately transferred on ice. The RNA concentration was determined and of each sample duplicates transcribed in cDNA. Therefore, 0.8 µg RNA were augmented with 2 µL gDNA wipe out buffer and x µL H_2O, to obtain a total volume of 14 µL. The samples were heated up to 42°C for 4 min and cooled down to 4°C. Afterwards 5 µL of RT buffer augmented with RT primer mix and 1 µL Reverse Transcriptase were added heated up to 42°C for 30 min, followed by 95°C for 3 min and cooled down to 4°C. Samples were immediately stored at -20°C (for max. 48 hrs) until further usage. Remaining cDNA was precipitated for

longer storage Therefore, 400 µL 70% EtOH and 4 µL 4.5M Na-acetate were added, incubated for 20 min at -20°C and sedimented at 16000g, 0°C, 30 min. The supernatant was discarded and the samples stored at -20°C.

5.2.23. Semi-quantitative real-time RT-PCR (qRT-PCR)

RNA analysis of gene edited Leishmania parasites was carried out by performing qRT-PCR using "DyNAmo Flash SYBR Green qPCR Kit" from Biozym. As reference gene Actin was used. The pipetting schema is shown in the following Table.
19 µL of the prepared mix and 1 µL of cDNA / H_2O were mixed and the qRT-PCR run at Rotor Gene 6000.

Table 11: Reaction mix for analysis of Actin via qRT-PCR.

Actin	
SYBR Green MasterMix	10 µL
L.don. Actin B2 primer (5 µM)	2 µL
L.don. Actin F1 primer (5 µM)	2 µL
H_2O	5 µL

Table 12: Reaction mix for analysis of the GOI via qRT-PCR.

Gene of interest	
SYBR Green MasterMix	10 µL
GOI forward primer (10 µM)	1 µL
GOI reverse primer (10 µM)	1 µL
H_2O	7 µL

5.2.24. Semi-quantitative real-time TaqMan-PCR (qPCR)

The duplex semi-quantitative real-time TaqMan-PCR was used to analyse the relative parasite load of infected macrophages. Therefore, gDNA isolated from infected macrophages was used instead of isolated RNA. The genes analysed were Actin for both the macrophages and the *Leishmania* parasites, as described by (Bifeld, Tejera Nevado et al. 2016).
The qPCR was carried out by using the KAPA PROBE FAST qPCR Master Mix (2x) / SensiFAST™ Probe No-ROX Kit. 18 µL of the prepared mix and 2 µL of the isolated gDNA / H_2O were mixed and the qPCR run at the Rotor Gene 6000.

Table 13: Reaction mix for qPCR.

Mastermix	Volume [µL]
Mastermix (Bioline)	10
Leish-AcF2 (900nM)	0.3
Leish-AcR (900nM)	0.3
Leish-AcProbe (200nM)	0.5
mouse-ACß_F (900nM)	0.3
mouse-ACß_R2 (900nM)	0,3
mouse-ACß (200nM)	0.5
MgCl2 (25mM)	1.2
H2O	4.6
gDNA	2

5.2.25. Denaturing cell lysis

$1*10^7$ *L. donovani* cells were sedimented at 1000 x g, 8 min, 4°C. The supernatant was discarded and the cells washed with 10 mL cold PBS. The pellet was transferred into a 1.5 mL tube and washed with 1 mL cold PBS. The cells were resuspended in 1 vol. PBS. 0.9 vol. of 2x Laemmli and 0.1 vol. DTT were mixed and added to the cell suspension. The sample was mixed, sonicated at 100% output for 10 min and heated up to 95°C for 10 min. Samples were stored at -20°C.

5.2.26. SDS-Polyacrylamid-Gelelectrophoresis (SDS-PAGE)

Protein lysates (see 5.2.25) were loaded onto a discontinuous SDS-PAGE. SDS gels were prepared at different concentrations regarding the needed purpose. Standard gels were prepared with a 10% separating gel and a 5% stacking gel using 39.5:1 acrylamide. Some gels were prepared using 19:1 acrylamide. The following Table shows the required volumes for a separating and a 5% stacking gel.

Table 14: Separating gel for SDS-PAGE.

Separating Gel	Volume [% v/v]	Final Concentration
ddH_2O	36 - 53.5	
1M Tris/HCl (pH = 8.8)	25	375 mM
40% Acrylamide	20 - 37.5	8 % - 15 %
20% SDS	0.5	0.1 %
10% APS	1	0.1 %
TEMED	0.085	0.1 %

Table 15: Stacking gel for SDS-PAGE.

Stacking Gel	Volume [% v/v]	Final Concentration
ddH_2O	74.5	
1M Tris/HCl (pH = 8.8)	12.5	125 mM
40% Acrylamide	11.4	5 %
20% SDS	0.5	0.1 %
10% APS	1	0.1 %
TEMED	0.1	0.1 %

The separating gel was poured between two glass plates of the electrophoresis chamber and topped with isopropanol to obtain an even surface. After polymerisation of the separating gel the isopropanol was removed and the stacking gel and the comb was added. Depending of its further usage two different markers were used. For further Coomassie staining an unstained marker was used. While a pre-stained marker was used for Immunoblot analysis. The gel was run at 15 V/cm for 1.5 hrs. Following it was subjected to either Coomassie staining or Western Blotting.

5.2.27. Non-denaturing cell lysis

$2*10^7$ *L. donovani* cells were sedimented at 1000 x g, 8 min, 4°C. The supernatant was discarded and the cells washed with 10 mL cold PBS. The pellet was transferred into a 1.5 mL tube and washed with 1 mL cold PBS. The cells were resuspended in 40 µL extraction buffer (15% glycerol, 0.5 mM 1,10-phenanthroline, 10 mM Tris-HCl (pH = 8.0), 70 mM KCl). The sample was frozen three times in liquid nitrogen and thawed three times at 37°C for 3 min each. Next

a small amount of ceramic beads (Rybolyser beads) was added and vortexed for 30 sec. After centrifugation at 16000 x g, 4°C for 10 min the supernatant was transferred into a new 1.5 mL tube and 4 volumes loading buffer were added. The samples were immediately loaded onto the native PAGE.

5.2.28. Native PAGE

To separate proteins under native conditions a native PAGE had to be made. Native gels were prepared by creating a gradient gel. The following Tables 16, and 17 show the two solutions, with the maximum and minimum amount of acrylamide, which had been used.

Table 16: Solution A for Native PAGE.

Solution A	Volume [% v/v]	Final Concentration
ddH_2O	83.33	
5 x TBE	11.11	
50% Glycerin	5.55	2.77 %
40% Acrylamide (19 : 1)	11.11	4.44 %
10% APS	0.44	0.04 %
TEMED	0.044	0.04 %

Table 17: Solution B for Native PAGE.

Solution B	Volume [% v/v]	Final Concentration
ddH_2O	16.07	
5 x TBE	10.71	
50% Glycerin	32.14	16,07 %
40% Acrylamide (37,5 : 1)	48.21	19,3 %
Bromophenol blue	0.18	
10% APS	0.43	0.04 %
TEMED	0.043	0.04 %

The gradient gel was created by pumping continuously Solution A to Solution B, while Solution B was continuously poured between two glass plates. After polymerisation the gel was equilibrated in blot buffer at 200 V, at 4°C for 1 hr.

After loading the samples the gel was run at 200 V at 4°C for 24 hrs. Next the gel was transferred into Transfer Buffer and heated up to 60°C for 1 hr. Afterwards the gel was blotted as described in 5.2.30.

5.2.29. Coomassie-Brillant-Blue staining

SDS-gels obtained in 5.2.26 could be stained using Coomassie Brilliant Blue R-250. Therefore, gels were covered with a solution of 40% EtOH, 10% acetic acid and 1 g/L Coomassie Brilliant Blue R-250 for 1.5 hrs at RT or over night at 4°C. Next gels were destained using destaining solution for 30min - 2hrs at RT. Obtained gels were washed twice with H_2O. For further analysis gels were scanned and dried between cellophane for long time storage.

5.2.30. Western Blot

To transfer protein from a SDS-PAGE onto a polyvinylidene difluoride membrane semi-dry Western blotting was used. Therefore, the membrane was activated by covering it with cold methanol for 30 seconds. Afterwards the membrane was washed twice with ddH_2O and placed in ddH_2O until usage. Two pieces of Whatman paper of the same size as the membrane were placed in blot transfer buffer. One piece of the Whatman paper was placed on the cathode of the blotting chamber. Next the gel placed on top of it and covered with the polyvinylidene difluoride membrane. Last the second Whatman paper was placed on top and the blotting chamber was closed with the anode. The transfer was performed by applying a current of $1mA/cm^2$ of membrane for 1 h to 1.5 hrs regarding the thickness of the gel. The blotted polyvinylidene difluoride membrane could either be stored dry or directly subjected to Immunoblot analysis.

5.2.31. Immunoblot

To visualise protein bands on polyvinylidene difluoride membranes antibody staining was performed. Unspecific binding had to be avoided by blocking the membrane using blocking solution. Therefore the membrane was covered with blocking solution for 1h at RT or over night at 4°C. Next it was probed with the primary antibody in blocking solution. The concentration used for the specific antibodies is shown in 5.1.13. Afterwards the membrane was washed three time with wash solution, 5 min each. The secondary antibody used was conjugated to AP fro detection. After incubation with the secondary antibody in blocking

solution for 1 h at RT the membrane was washed twice with washing solution. Afterwards the membrane was washed with developing buffer. The membrane was developed using BCIP and NBT in developing solution with a ratio of BCIP 1:2 NBT. Development was carried out at RT until the desired bands appeared. Afterwards the membrane was washed three times with water and scanned after drying.

5.2.32. Recombinant protein expression and purification from E.Coli

5.2.32.1. Protein expression

BL21 (DE3)[pABlaclQ] cells were transfected with the desired plasmid as described in 5.2.16. Two single clones were picked and added to one flask containing 100 mL pre-warmed LB-Medium, 1 mL Ampicilin (100µg/mL), 100 µL Kanamycin and 1 mL sterile filtrated 10% Glucose. Cells were incubated at 37°C, shaking until an OD_{600} = 0.1 was reached. Next protein expression was induced by adding isopropyl-β-D-thiogalactopyranosid (IPTG). Required volume was calculated by (remaining cell suspension volume)/2500. Before induction 1 mL of the suspension was taken as aliquot for later analysis and stored at -70°C (=S1). Incubation was done for 2h at 37°C, shaking. OD_{600} was measured and a second 1 mL aliquot was taken for later analysis and stored at -70°C (=S2). Cell suspension was transferred to a 400 mL centrifuge bottle and sedimented at 5000 rpm, 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 mL cold PBS. Afterwards the suspension was transferred to a 50 mL tube and sedimented again at 5000 rpm, 20 min at 4°C. The supernatant was discarded and the pellet could be stored at -70°C for further processing. Next the pellet was resuspended in 20 mL buffer 1 (20 mM Tris, 50 mM NaCl, 5 mM Imidazolee, pH = 7.9). The sample was ultrasonificated (6 x 20 sec with 10 sec break, on ice). Afterwards the suspension was sedimented at 10330 x g for 30 min at 4°C and the supernatant was transferred into a new 50 mL tube. A 250 µL aliquot was taken (=S3) and both the sample and the aliquot stored at -70°C. The remaining pellet was resuspended in 10 mL buffer 2, incubated fro 60 min on the roller-shaker at 4°C and sedimented at 10330 x g, 30 min at 4°C. Next the supernatant was transferred into a new 50 mL tube and a 250 µL aliquot was taken (=S4). Both, the sample and the aliquot, were stored at -70°C.

To analyse which fraction contained the expressed protein a SDS-PAGE was made as described in 5.2.26. Therefore, the samples S1, S2, S3 and S4 were loaded onto the gel. To obtain equal loading the samples had to be prepared as

following. S1 and S2 were sedimented at 11300 x g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 1 mL cold PBS. The samples were sedimented as previous and the supernatant discarded. S1 was resuspended in 25 µL PBS and 25 µL 2x Laemmli (+10% DTT) were added. S2 had to be diluted according the following formula:

$Volume_{1xLaemmli,\ S2} = (OD_{600,\ S2} \times Volume_{1xLaemmli,\ S1}) / OD_{600,\ S1}$

Whereas 1x Laemmli is 50%/vol. PBS, 45%/vol. 2x Laemmli and 5%/vol. DTT. 250 µL 2x Laemmli were added to S3 and S4. All samples were subjected to ultrasonification for 10 min, 100% output, followed by 10 min, 95°C, shaking. After loading 25 µL of each sample onto the gel, it was run as described previously. Next the gel was stained with Coomassie as described in 5.2.29.

5.2.32.2. Protein purification

All following steps were carried out in the 4°C room. To purify the expressed recombinant protein the Coomassie stained gel was analysed and the required fraction (either S3 oder S4) was thawed on ice. Meanwhile 10 mL His-beads were transferred to a column and the storage buffer via flow through discarded. Running dry of the column must be avoided. Next the column was equilibrated with 10 mL $NiSO_4$ solution for 30 min. The flow through was discarded and the column washed with 10 mL B5 buffer at a flow through rate of 0.5 mL/min. To refold the thawed protein, it was dropwise added to 200 mL cold B5 buffer under constant stirring. Afterwards the protein solution was added slowly to the column and a flow through rate of 1mL/min was adjusted. The last 10 mL of the flow through were collected (= sample DF). The column was washed with 10 mL B5 buffer (= sample W1), followed by 5 mL B100 buffer (=sample W2). The protein was eluted by adding 10 mL B1000 buffer. The flow through was fractionated into 10 x 1.5 tubes with 1mL each (=samples E1 - E10). 5 mL B1000 buffer were added and the flow through collected (= sample W3). The column was cleaned and stored with 10 mL B5 buffer + 0.002% NaN_3. For analysis of the eluted fractions a SDS-PAGE was made and the fractions DF, W1, W2, E1 - E10 and W3 where loaded and stained afterwards with Coomassie.

5.2.32.3.Protein dialysis

To remove imidazole from purified protein the samples had to be dialysed. Therefore, the desired fractions (see 5.2.32.2) were pooled. A dialysis tubing was heated up in ddH_2O supplemented with a small amount of sodium carbonate to 100°C. Next the tubing was closed on one side and the protein fractions were

filled into at 4°C. Afterwards the second end was closed and the tubing transferred into 500 mL cold PBS. The tubing was stirred carefully for 60 min. The tube was placed in fresh 500 mL cold PBS and stirred again for 60 min at 4°C. The dialysed protein was transferred to 1.5 mL tubes, the concentration determined at 215 nm and the samples stored at -70°C.

5.2.33. Native recombinant protein expression and purification from *E. coli*

5.2.33.1. Native recombinant protein expression

BL21 (DE3)[pABlaclQ] cells were transfected with the desired plasmid as described in 5.2.16. A single clone was picked and added to one flask containing 100 mL pre-warmed LB-Medium, 1 mL Ampicilin (100µg/mL), 100 µL Kanamycin and 1 mL sterile filtrated 10% Glucose. Cells were incubated at 37°C, shaking until an OD_{600} = 0.1 was reached. Next protein expression was induced by adding isopropyl-β-D-thiogalactopyranosid (IPTG). Required volume was calculated by (remaining cell suspension volume)/2500. Before induction 1 mL of the suspension was taken as aliquot for later analysis and stored at -70°C (=S1). Incubation was done for 25 min at 37°C, shaking. OD_{600} was measured and a second 1 mL aliquot was taken for later analysis and stored at -70°C (=S2). Cell suspension was cooled down to 4°C rapidly, transferred to a 400 mL centrifuge bottle and sedimented at 5000 rpm, 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 mL cold PBS. Afterwards the suspension was transferred to a 50 mL tube and sedimented again at 5000 rpm, 20 min at 4°C. The pellet was resuspended in 2 mL buffer 1+ (20 mM Tris, 100 mM KCl, 5 mM Imidazolee, 1mM EDTA, 1mM 1,10-Phenanthroline, 10% Glycerol, pH = 7.9). The sample was ultrasonificated (3 x 20 sec with 20 sec break, on ice). Afterwards the suspension was sedimented at 10330 x g for 30 min at 4°C and the supernatant was transferred to a 1.5 mL tube. A 25 µL aliquot was taken (=S3) and both the sample (frozen in liquid N_2) and the aliquot stored at -70°C.

5.2.33.2. Native protein purification

All following steps were carried out in the 4°C room. The sample was thawn on ice. Meanwhile 2 mL His-beads were transferred to a 15 mL tube and the storage buffer discarded. Next the beads were equilibrated with 2 mL $NiSO_4$ solution on the roller shaker for 30 min. After 20 min incubation without movement the beads separated from the solution and the sample was sedimented at 200 x g, 4°C, for

2min. The supernatant was discarded and the beads washed with 10 mL B5 buffer (30 min, roll shaker). The protein solution was added and incubated for 1 hr. After settlement and centrifugation the supernatant was discarded. The beads were washed with 10 mL B5 buffer, followed by 3 mL B100 buffer. The protein was eluted by adding 2 mL B1000 buffer, supplemented with 2 mM EDTA and 10% Glycerol. The sample was immediately subjected to protein dialysis (see 5.2.32.3). Native recombinant proteins have to be frozen in liquid N_2 to avoid denaturing.

5.2.34. Immunisation of laying hens

To obtain a specific IgY antibody against a desired protein laying hens were immunised with the required antigen. The purified recombinant proteins (see 5.2.32.3) were used as the desired antigen.
Therefore, 500 µg of recombinant protein was mixed with 1 volume of TiterMax® Gold Adjuvant using a 3-way valve. The mixture was kept on ice until the hen was immunised. After 10-14 days eggs were collected and IgY was isolated to analyse immediate immune response. Hens were immunised a second time with the amount of protein to booster the IgY production. After 10-14 days eggs were collected and IgY was isolated as described in 5.2.35.

5.2.35. Isolation of IgY

To isolate the IgY from eggs from immunised hens the egg yolk was separated from the egg white. The egg yolk was washed with ddH_2O, the vitelline membrane removed and the egg yolk transferred into a 50 mL tube. The volume was measured and 1 volume of potassium phosphate buffer (PPB) added. The sample was briefly mixed on the role shaker and the volume determined. 1 volume of PPB supplemented with 7% PEG 6000 was added and the sample mixed at 4°C on the roll shaker fro 30 min. Next the sample was sedimented at 16000 x g, 4°C, for 10 min. The supernatant was filtered through a gauze swab and a filter paper. 10% (g/mL) PEG 6000 was added and incubated on the roll shaker at RT until the PEG 6000 was completely dissolved. Next the sample was sedimented at 16000 x g, 4°C, for 10 min. The supernatant was discarded and the pellet resuspended in 10 mL potassium phosphate buffer. 1 volume of potassium phosphate buffer supplemented with 24% PEG 6000 was added and the sample sedimented at 16000 x g, 4°C for 10 min. The supernatant was discarded and the pellet resuspended in 5 mL potassium phosphate buffer. The sample was sedimented at

16000 x g, 4°C for 30 min. The supernatant was carefully aliquoted into 1.5 mL tubes and supplemented with 0,02% NaN_3.

5.2.36. Indirect Immunofluorescence staining

L. donovani parasites were sedimented at 2500 x g, 8 min 4°C and washed twice with 1 mL cold PBS. Supernatant was discarded and the pellet resuspended in 200 µL cold PBS. 20 µL of the suspension were smeared onto a glass microscope slide and left air dried. The microscope slides were subjected to ice cold methanol for cell fixation. After drying the parasites were circled with a PapPen. As soon as the PapPen was dried the cells were washed 3 times with washing solution for 5 min each washing step. Next permeabilisation solution was added for 20 min and washed afterwards 3 times with washing solution for 5 min each washing step. Blocking solution was added for 30 min, followed by the primary antibody in blocking solution for 1hr. The required concentrations of the primary antibody are shown in Table 5.1.5.2. The slides were washed 3 times with washing solution for 5 min each washing step. Afterwards the secondary antibody was added in blocking solution. The required concentrations of the secondary antibody are shown in Table 5.1.5.2. If needed DAPI was added at the same time. The slides were washed 3 times with washing solution for 5 min each washing step. One drop of MOWIOL was added per slide and the slides were closed with a cover slide. The stained slides were stored at 4°C and analysed by using a Life Technologies EVOS FL Auto Cell Imaging System.

5.2.37. Luciferase Renaturation Assay

The aim of an *in vitro* luciferase renaturation assay is to test refolding activity of suspected chaperones. The experimental setup links the activity indirectly to a bioluminescence signal. In a first step the native enzyme luciferase is denatured either by an elevated temperature or by chemical denaturing. Next, the denatured enzyme is added to the protein of interest (POI). The reaction is carried out in a physiological buffer at a well defined temperature. In the case of CPN60 the reaction buffer needs to be at a neutral pH value and containing ATP which is required for the twisting of the protein. After incubation of the denatured Luciferase together with the POI the Luciferase activity is accessed. For this, luciferin, originally obtained from the firefly, is added to the reaction mix. The active Luciferase catalyses the oxidation of the luciferin resulting in a dioxetane. From this CO_2 decays leading to an excited ketone. By changing to an energetically lower level the ketone releases energy which can be detected as a

bioluminescent signal. The detected light intensity correlates with the amount of folded Luciferase.

Active Luciferase (50 µM) was diluted 1:10 in dilution buffer (Promega®) to a final concentration of 5 µM. The Luciferase was heat inactivated by heating up to 42°C for 15 min. The concentration of the recombinant proteins was determined and the proteins set to a final concentration of ~20 nM in refolding buffer. The refolding reaction was carried out in 0.2 mL PCR-tubes. The tubes were blocked by adding dilution buffer (containing BSA) and incubation for 15 min. The dilution buffer was removed and the refolding mix prepared as shown in Table 18. Preparation was carried out on ice.

Table 18: Reaction mix for luciferase repatriation assay.

Reagent	Final concentration
CPN60	2 nM
CPN10	2 nM
inactivated Luciferase	40 nM
refolding buffer	final Volume 125 µL

The reaction was started by adding the inactivated Luciferase and incubated at 25°C for the required time. 40 µL of the reaction mix was added to 40 µL Steady Glo Reagent (Promega ®) in a black Nunc™ 96-well plate. The mixture was incubated for 10 min at RT, followed by Luminescence measurement.

5.2.38. Co-immune precipitation

The co-immune precipitation was carried out using α-GFP-beads. Ribosomal expressed CPN10 was tagged to GFP and episomal expressed CPN60 tagged to a 3-HA tag. $1*10^8$ parasites were sedimented at 1000 x g, 4°C for 8 min and washed twice with 10 mL cold PBS. The supernatant was discarded and the pellet resuspended in 300 µL 0.5 mM DSP. After incubation at RT for 30 min 300 µL of 25 mM Tris-HCl (pH = 7.5) were added and incubated at RT for 15 min. The suspension was sedimented at 1000 x g, 4°C for 8 min and the pellet resuspended in 300 µL Lysis buffer. The mixture was incubated on ice for 60 min, while the sample was mixed every 10 min. Next, the sample was sedimented at 16000 x g, 4°C for 10 min and the supernatant transferred into a pre-cooled 1.5 mL tube. Wash buffer was added to a total volume of 450 µL. 50 µL were removed and as a lysate prepared as sample 1 (=S1).

50 µL of the α-GFP-beads were transferred into a 1.5 mL tube and 175 µL wash buffer were added. The sample was mixed gently and the beads separated via a magnet. Next, the beads were washed with 1 mL wash buffer.
The lysate sample was added to the equilibrated beads and incubated for 2 hrs, RT, end-over-end mixing. The magnets were separated using a magnet and 50 µL of the supernatant were collected (=S2). The remaining supernatant was discarded. The beads were washed with 300 mL wash buffer, followed by 300 µL pure ddH_2O. 100 µL pre-heated 1 x Laemmli (95°C) were added and gently mixed. The sample was incubated at 95°C for 10 min. Next, the beads were separated and the supernatant collected. 10 µL DTT were added and the sample heated up to 95°C for 10 min (=S3). The samples were subjected to immunoblotting.

6. Results

6.1. Identification of Leishmania 60 kDa Chaperonines

Given that *Leishmania donovani* possess four different putative CPN60s (Table 19), the phylogenetic relationships were examined, to analyse possibly separate lineages of CPN60. Therefore, a ClustalW amino acid sequence comparison and alignment of the *Ld*CPN60 paralogues was performed with the genes from three other *Leishmania* species, *L. major*, *L. braziliensis*, and *L. mexicana*, but also from *Trypanosoma brucei* and *T. cruzi*. The latter are closely related pathogens causing Human African Trypanosomiasis (HAT, a.k.a. sleeping sickness) and Chagas Disease, respectively. The *Escherichia coli* GroEL sequence was used as root for the dendrogram (Figure 10).

Table 19: List of putative CPN60 genes in *L. donovani*.

Name	Gene ID
CPN60.1	LdBPK_321940
CPN60.2	LdBPK_362130
CPN60.3	LdBPK_362140
CPN60.4	LdBPK_302830

The dendrogram (Figure 10) reveals that CPN60.1 ((Schlüter, Wiesgigl et al. 2000); LdBPK_321940) forms a distinct, single copy gene family in all the leishmaniae, but also in the trypanosomes. CPN60.2 ((Schlüter, Wiesgigl et al. 2000); LdBPK_362130) and CPN60.3 (LdBPK_263140) form two distinct lineages in the four *Leishmania* species, although they are neighbouring genes on chromosome 36 or 35. In the Trypanosomes, the neighbouring genes corresponding to CPN60.2 and CPN60.3 are more closely related and group according to the two species, indicating that the two lines of CPN60 may have evolved from a single gene by duplication in an ancestral species of *Trypanosoma* spp. and formed separate lines in the leishmaniae. CPN60.4 (LdBPK_302830) is also a conserved and distinct lineage in *Leishmania* spp with representatives in *Trypanosoma* spp., but there are two additional paralogues of CPN60.4 in *T. cruzi*.

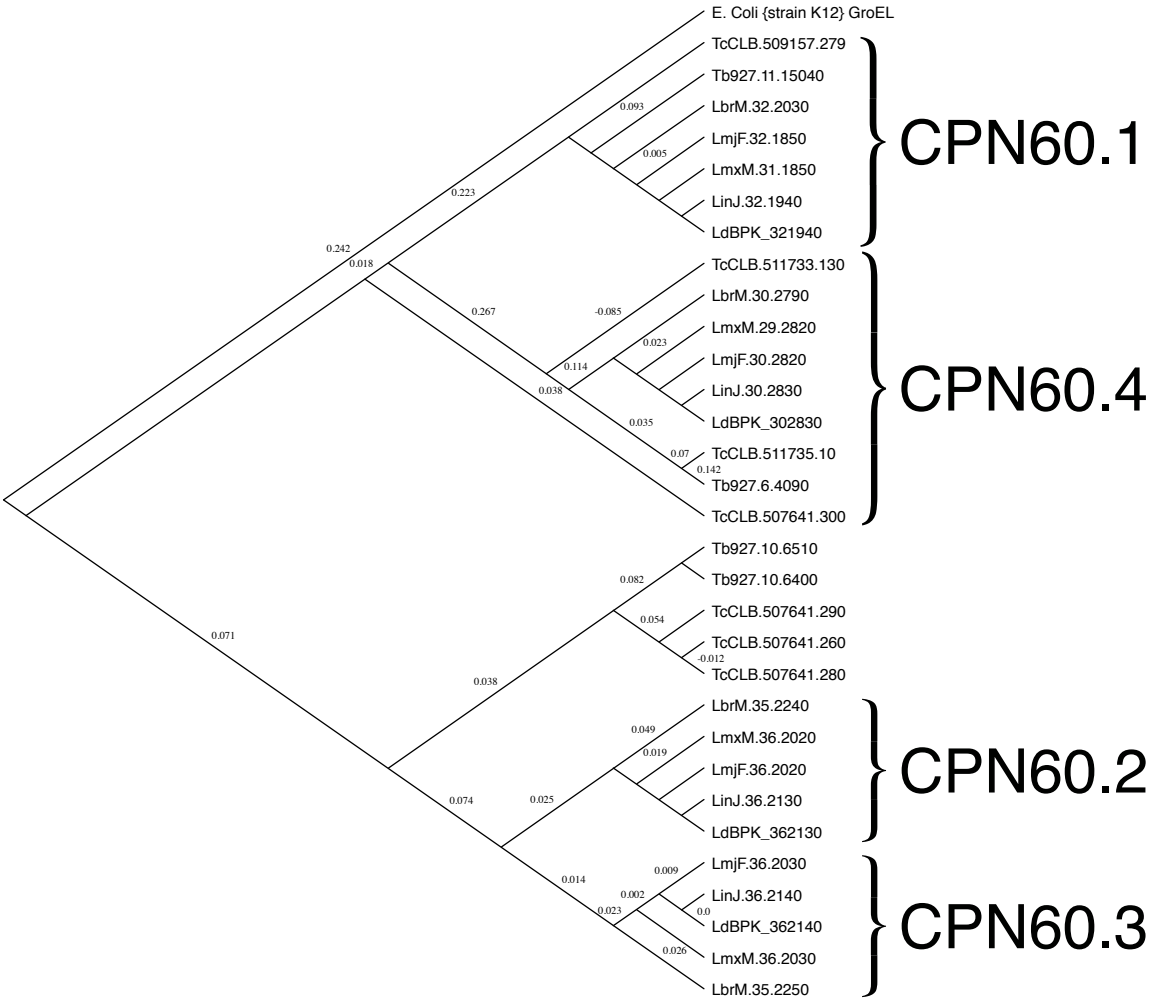


Figure 10: Phylogenetic tree of the different CPN60s expressed by *L. donovani*, *L. infantum*, *L. major*, *L. mexicana*, *L. braziliensis*, *T. cruzi, T. brucei*. The *E. Coli* GroEL was used as root.

Table 20: Pairwise alignment of different CPN60s displayed in the phylogenetic tree.

	E. Coli {strain K12} GroEL	TcCLB.509157.279	Tb927.11.15040	LbrM.32.2030	LmjF.32.1850	LmxM.31.1850	LinJ.32.1940	LdBPK_321940	TcCLB.511733.130	LbrM.30.2790	LmxM.29.2820
E. Coli {strain K12} GroEL	100.0	18.2	42.4	43.4	43.2	43.2	43.2	43.2	11.3	34.5	35.3
TcCLB.509157.279	24.6	100.0	32.2	31.3	30.9	31.1	31.1	31.1	34.6	18.9	19.5
Tb927.11.15040	60.9	34.0	100.0	84.7	84.5	84.9	84.7	84.7	11.8	38.4	38.6
LbrM.32.2030	61.2	33.1	93.9	100.0	97.8	97.3	97.5	97.5	12.4	38.4	39.2
LmjF.32.1850	60.7	33.1	93.8	99.2	100.0	98.8	99.0	99.0	12.1	38.0	38.7
LmxM.31.1850	60.7	33.1	94.1	99.0	99.5	100.0	99.2	99.2	12.3	38.0	38.9
LinJ.32.1940	60.7	33.1	94.3	99.0	99.5	100.0	100.0	100.0	12.3	38.2	38.9

	E. Coli {strain K12} GroEL	TcCLB.509157.279	Tb927.11.15040	LbrM.32.2030	LmjF.32.1850	LmxM.31.1850	LinJ.32.1940	LdBPK_321940	TcCLB.511733.130	LbrM.30.2790	LmxM.29.2820
LdBPK_321940	60.7	33.1	94.3	99.0	99.5	100.0	100.0	100.0	12.3	38.2	38.9
TcCLB.511733.130	15.9	41.3	14.6	15.0	14.8	15.1	15.0	15.0	100.0	18.8	19.4
LbrM.30.2790	56.0	25.1	58.1	58.6	58.3	58.5	58.5	58.5	20.3	100.0	87.2
LmxM.29.2820	56.9	25.4	58.2	59.1	58.5	58.6	58.6	58.6	20.9	93.0	100.0
LmjF.30.2820	56.2	25.2	57.2	58.1	57.5	57.6	57.6	57.6	20.9	93.7	97.6
LinJ.30.2830	56.4	25.4	57.7	58.6	58.0	58.1	58.1	58.1	20.9	94.3	98.0
LdBPK_302830	56.4	25.4	57.7	58.6	58.0	58.1	58.1	58.1	20.9	94.3	98.0
TcCLB.511735.10	22.8	0.0	22.5	22.8	22.7	22.7	22.7	22.7	0.0	34.6	34.5
Tb927.6.4090	56.5	25.4	57.6	57.3	57.5	57.6	57.5	57.5	21.4	83.8	85.4
TcCLB.507641.300	30.6	0.0	29.9	29.0	29.2	29.2	29.2	29.2	0.0	27.8	28.0
Tb927.10.6510	70.5	26.9	69.1	68.3	68.1	68.3	68.3	68.3	16.6	62.9	63.6
Tb927.10.6400	70.5	26.9	69.1	68.3	68.1	68.3	68.3	68.3	16.6	62.9	63.6
TcCLB.507641.290	69.6	27.4	69.1	69.0	68.8	69.0	69.0	69.0	16.7	62.7	63.1
TcCLB.507641.260	44.6	27.3	43.0	43.6	43.4	43.6	43.6	43.6	16.7	41.3	41.8
TcCLB.507641.280	63.9	27.4	62.9	62.8	62.8	62.9	62.9	62.9	16.7	60.2	60.2
LbrM.35.2240	66.2	26.4	69.1	69.0	68.8	69.0	69.0	69.0	15.8	62.9	62.9
LmxM.36.2020	66.5	26.2	69.2	69.5	69.3	69.5	69.5	69.5	16.0	63.3	63.3
LmjF.36.2020	66.0	26.4	69.2	69.1	68.6	68.8	68.8	68.8	16.0	62.8	62.9
LinJ.36.2130	66.4	26.2	68.7	69.1	68.6	68.8	68.8	68.8	16.0	62.8	63.3
LdBPK_362130	66.4	26.2	68.7	69.1	68.6	68.8	68.8	68.8	16.0	62.8	63.3
LmjF.36.2030	68.3	26.5	69.2	69.0	68.8	69.0	69.0	69.0	15.7	63.0	63.2
LinJ.36.2140	68.4	26.5	69.2	69.0	68.8	69.0	69.0	69.0	15.5	63.0	63.2
LdBPK_362140	68.4	26.5	69.2	69.0	68.8	69.0	69.0	69.0	15.5	63.0	63.2
LmxM.36.2030	68.3	26.4	69.2	69.0	68.8	69.0	69.0	69.0	15.6	62.5	62.7
LbrM.35.2250	68.8	26.7	69.6	69.3	69.1	69.3	69.3	69.3	15.9	63.6	63.7

	LmjF.30.2820	LinJ.30.2830	LdBPK_302830	TcCLB.511735.10	Tb927.6.4090	TcCLB.507641.300	Tb927.10.6510	Tb927.10.6400	TcCLB.507641.290	TcCLB.507641.260	TcCLB.507641.280
E. Coli {strain K12} GroEL	34.8	35.3	35.3	14.8	36.7	22.0	51.4	51.4	48.8	27.4	44.3
TcCLB.509157.279	19.9	20.0	20.0	0.0	19.9	0.0	20.6	20.6	20.7	20.6	20.7
Tb927.11.15040	38.9	39.3	39.3	16.5	39.3	22.9	51.1	51.1	51.1	27.3	46.7
LbrM.32.2030	39.2	39.7	39.7	16.8	39.4	22.0	51.3	51.3	51.7	28.7	47.3

	LmjF.30.2820	LinJ.30.2830	LdBPK_302830	TcCLB.511735.10	Tb927.6.4090	TcCLB.507641.300	Tb927.10.6510	Tb927.10.6400	TcCLB.507641.290	TcCLB.507641.260	TcCLB.507641.280
LmjF.32.1850	38.9	39.4	39.4	16.3	39.4	22.1	51.2	51.2	51.7	28.5	47.3
LmxM.31.1850	39.0	39.5	39.5	16.3	39.4	22.0	51.0	51.0	51.5	28.5	47.1
LinJ.32.1940	39.0	39.5	39.5	16.3	39.4	22.1	51.2	51.2	51.7	28.5	47.3
LdBPK_321940	39.0	39.5	39.5	16.3	39.4	22.1	51.2	51.2	51.7	28.5	47.3
TcCLB.511733.130	19.6	19.6	19.6	0.0	19.8	0.0	13.3	13.3	12.5	12.4	12.4
LbrM.30.2790	87.8	88.5	88.5	28.9	69.2	19.8	44.2	44.2	42.8	24.3	42.0
LmxM.29.2820	95.4	96.3	96.3	29.1	71.7	19.4	44.9	44.9	43.0	24.6	41.2
LmjF.30.2820	100.0	98.3	98.3	29.1	72.5	19.4	44.5	44.5	42.6	24.3	41.0
LinJ.30.2830	99.3	100.0	100.0	29.1	71.9	19.4	44.9	44.9	43.2	24.8	41.5
LdBPK_302830	99.3	100.0	100.0	29.1	71.9	19.4	44.9	44.9	43.2	24.8	41.5
TcCLB.511735.10	34.9	35.1	35.1	100.0	31.4	40.5	19.7	19.7	18.9	3.0	18.9
Tb927.6.4090	85.8	86.0	86.0	36.2	100.0	19.5	44.9	44.9	43.5	25.5	41.7
TcCLB.507641.300	27.8	27.8	27.8	54.6	27.4	100.0	40.0	40.0	45.9	2.5	44.6
Tb927.10.6510	63.1	63.1	63.1	26.3	63.1	43.4	100.0	100.0	86.7	42.8	77.4
Tb927.10.6400	63.1	63.1	63.1	26.3	63.1	43.4	100.0	100.0	86.7	42.8	77.4
TcCLB.507641.290	62.5	62.7	62.7	25.5	63.2	45.9	93.4	93.4	100.0	48.5	88.6
TcCLB.507641.260	41.1	41.3	41.3	7.4	41.3	8.9	52.9	52.9	56.1	100.0	52.0
TcCLB.507641.280	60.1	60.2	60.2	25.5	59.5	44.8	85.1	85.1	90.2	59.3	100.0
LbrM.35.2240	62.1	62.2	62.2	26.1	61.9	38.7	87.1	87.1	87.7	51.8	78.7
LmxM.36.2020	62.6	62.8	62.8	26.2	63.1	37.7	85.2	85.2	86.6	52.2	78.3
LmjF.36.2020	62.2	62.4	62.4	26.6	61.9	37.7	84.0	84.0	84.9	50.1	76.6
LinJ.36.2130	62.6	62.8	62.8	27.1	62.6	37.3	84.9	84.9	85.2	51.3	76.9
LdBPK_362130	62.6	62.8	62.8	27.1	62.6	37.3	84.9	84.9	85.2	51.3	76.9
LmjF.36.2030	62.3	62.5	62.5	25.7	62.5	41.1	89.0	89.0	89.9	51.9	81.2
LinJ.36.2140	62.3	62.5	62.5	26.0	62.5	41.3	89.7	89.7	90.6	52.7	81.9
LdBPK_362140	62.3	62.5	62.5	26.0	62.5	41.3	89.7	89.7	90.6	52.7	81.9
LmxM.36.2030	61.8	62.0	62.0	25.7	62.0	40.7	88.9	88.9	89.8	52.5	81.1
LbrM.35.2250	63.0	63.2	63.2	25.8	62.9	41.5	90.4	90.4	91.1	53.3	82.6

	LbrM.35.2240	LmxM.36.2020	LmjF.36.2020	LinJ.36.2130	LdBPK_362130	LmjF.36.2030	LinJ.36.2140	LdBPK_362140	LmxM.36.2030	LbrM.35.2250
E. Coli {strain K12} GroEL	46.3	46.5	46.1	46.8	46.8	48.8	49.1	49.1	48.9	49.1

	LbrM.35.2240	LmxM.36.2020	LmjF.36.2020	LinJ.36.2130	LdBPK_362130	LmjF.36.2030	LinJ.36.2140	LdBPK_362140	LmxM.36.2030	LbrM.35.2250
TcCLB.509157.279	19.1	19.4	19.2	19.2	19.2	19.7	19.7	19.7	19.6	19.5
Tb927.11.15040	50.9	50.4	50.4	49.7	49.7	49.7	49.9	49.7	49.9	50.3
LbrM.32.2030	51.8	52.0	51.5	51.5	51.5	51.5	51.5	51.5	51.5	52.0
LmjF.32.1850	52.0	52.2	51.5	51.5	51.5	51.8	52.0	51.8	51.7	52.2
LmxM.31.1850	51.8	52.0	51.3	51.3	51.3	51.7	51.8	51.7	51.5	52.0
LinJ.32.1940	52.0	52.2	51.5	51.5	51.5	51.8	52.0	51.8	51.7	52.2
LdBPK_321940	52.0	52.2	51.5	51.5	51.5	51.8	52.0	51.8	51.7	52.2
TcCLB.511733.130	12.1	12.1	12.1	12.1	12.1	12.0	12.0	12.0	12.0	12.2
LbrM.30.2790	43.0	43.9	43.5	43.4	43.4	42.1	42.1	42.1	42.2	42.6
LmxM.29.2820	43.0	44.4	44.4	44.2	44.2	42.6	42.8	42.8	42.6	42.6
LmjF.30.2820	43.2	44.2	44.2	43.7	43.7	42.4	42.6	42.6	42.4	42.6
LinJ.30.2830	43.2	44.6	44.6	44.1	44.1	42.4	42.6	42.6	42.4	42.6
LdBPK_302830	43.2	44.6	44.6	44.1	44.1	42.4	42.6	42.6	42.4	42.6
TcCLB.511735.10	18.7	18.8	18.8	18.8	18.8	18.6	18.8	18.8	18.7	18.4
Tb927.6.4090	42.1	43.4	42.7	42.7	42.7	42.3	42.4	42.4	42.2	42.1
TcCLB.507641.300	33.1	31.3	31.7	31.2	31.2	36.3	36.9	36.7	36.0	36.2
Tb927.10.6510	74.6	72.2	71.5	71.1	71.1	77.8	78.5	78.5	77.6	79.3
Tb927.10.6400	74.6	72.2	71.5	71.1	71.1	77.8	78.5	78.5	77.6	79.3
TcCLB.507641.290	76.1	74.3	73.1	72.7	72.7	79.8	80.7	80.5	79.5	80.3
TcCLB.507641.260	39.2	39.4	37.8	38.0	38.0	40.2	40.6	40.6	40.4	40.9
TcCLB.507641.280	67.3	66.5	65.3	65.0	65.0	71.5	72.3	72.2	71.3	72.0
LbrM.35.2240	100.0	86.1	87.3	86.1	86.1	86.9	87.5	87.5	87.2	91.0
LmxM.36.2020	93.3	100.0	94.9	94.4	94.4	85.2	85.4	85.5	85.9	82.7
LmjF.36.2020	93.6	96.1	100.0	95.2	95.2	84.8	83.8	84.0	83.3	81.1
LinJ.36.2130	93.5	96.6	97.2	100.0	100.0	82.7	83.4	83.4	82.4	80.4
LdBPK_362130	93.5	96.6	97.2	100.0	100.0	82.7	83.4	83.4	82.4	80.4
LmjF.36.2030	91.4	90.7	90.7	89.6	89.6	100.0	98.6	98.8	97.7	94.1
LinJ.36.2140	92.1	91.4	89.9	90.1	90.1	98.9	100.0	99.8	97.9	94.7
LdBPK_362140	92.1	91.4	89.9	90.1	90.1	98.9	100.0	100.0	98.1	94.7
LmxM.36.2030	91.4	91.4	89.1	89.5	89.5	97.7	98.4	98.4	100.0	94.0
LbrM.35.2250	93.3	90.3	88.9	89.1	89.1	97.3	98.0	98.0	96.8	100.0

The pairwise analysis of the dendrogram, shown in table 20, shows that the four CPN60s of *L. donovani* have the highest degree of similarity with the respective CPN60s of *L. infantum* (7/8 with 100%). Also, the synteny of these 4 CPN60s is conserved in both species.
With regard to CPN60.1, the table confirms the dendrogram. All analysed *Leishmania* CPN60.1 sequences are conserved with ~99% identity and are highly conserved in *T. brucei* (85%). Only *T. cuzi* shows a lower degree of conservation. Nevertheless, CPN60.1 is the most highly conserved CPN60 throughout the Trypanosomatida.
CPN60.2 and CPN60.3 show sequence identities between 81% and 100% within the different *Leishmania* species. The *T. brucei* CPN60.2 and CPN60.3 are duplicates, while the *T. cruzi* CPN60.2 and CPN60.3 are less similar. Nevertheless, both proteins align most likely with each other, indicating that CPN60.2 and CPN60.3 may be the result of a relatively recent gene duplication in *Trypanosoma* spp. and diverged in the *Leishmania* genus. The pairwise analysis of CPN60.4 shows, similar to CPN60.2 and CPN60.3, a high conservation throughout all *Leishmania* spp., while the CPN60.4 of the *Trypanosoma* spp. is less similar to *Leishmania*.
These findings raise the question whether the different CPN60 paralogues have redundant or distinct functions in *Leishmania*.

6.2. Gene replacement via homologous recombination

To investigate the role of the 4 different CPN60s in *L. donovani,* the individual genes were targeted for gene replacement by using homologous recombination, as described by (Cruz and Beverley 1990). As the *Leishmania* genome is mainly diploid two different replacement constructs are needed. The two replacement constructs differ in the resistance marker genes that are used. One construct has a puromycin resistance marker gene, the other one has a bleomycin resistance marker gene. In a first step, one allele of the gene of interest (GOI) is replaced by an antibiotic resistance marker. The replacement is driven by the pressure of the cognate antibiotic. The resulting parasites are termed single-allele replacement mutants ($^{+/-}$). Subsequently, the procedure is repeated using a construct with a second resistance marker gene and the second allele of the GOI is replaced under dual antibiotic pressure.
For each CPN60-coding gene two individual replacement constructs had to be designed. First a ~1000 bp fragment of the 5'-NC (Non Coding region) and a ~1000 bp fragment of the 3'-NC immediately upstream and downstream, respectively, of the individual CPN60 genes were identified, using the genome

sequence information from the TriTryp database. The chosen regions are shown in Table 21. The 5'-NC regions of CPN60.1 and CPN60.2 were reduced to 692 bp and 542 bp, respectively. This was done to avoid overlaps with neighbouring coding sequences. Specific primers, introducing the required restriction sites, were designed to amplify the NCs. Subsequently, the 5'-NC and the 3'-NC were consecutively ligated into a pUC19 plasmid. Next, the resistance marker genes, puroAC or bleoR, were inserted between the NCs to generate the final targeting vector (Figure 11). Using the restriction endonuclease *Swa*I, the construct was linearised, purified and transfected into *L. donovani*. *Leishmania* parasites positive for integration were selected by the addition of the respective antibiotic.

Table 21: Table of gene positions of the 5'-NC and 3'-NC for homologous recombination.

Gene ID	Gene position	5'-NC	3'-NC
LdBPK_321940 (CPN60.1)	724390 - 726174 (1784 bp)	723698 - 724390 (692 bp)	726174 - 727157 (983 bp)
LdBPK_362130 (CPN60.2)	804147 - 805847 (1700 bp)	803608 - 804150 (542 bp)	805859 - 806845 (986 bp)
LdBPK_362140 (CPN60.3)	808282 - 809970 (1688 bp)	807246 - 808276 (1030 bp)	809970 - 810981 (1011 bp)
LdBPK_302830 (CPN60.4)	1048890 - 1050506 (1616 bp)	1047874 - 1048884 (1010 bp)	1050506 - 1051440 (934 bp)

Single-allele replacement of the CPN60.1 coding sequence was successful using either one of both replacement constructs. Viable parasites were obtained, being resistant to either bleomycin or puromycin. This result shows that both replacement constructs are functional. Targeting the second allele for the generation of double replacement mutants did not result in viable parasites. The replacement was tried starting from each of the both obtained single-allele replacement mutants for at least three individual trials. These data suggest, that the CPN60.1 gene is essential for *L. donovani*.

Targeting the CPN60.2 gene, candidate single-allele and double-allele replacement mutants could be obtained, suggesting that CPN60.2 is a non-essential gene in *L. donovani*.

The first allele of the CPN60.3 gene was successfully targeted using either one of both replacement constructs, resulting in viable, putative single-allele replacement parasites. Second allele replacements were attempted in at least 6 electroporation reactions, using both combinations of replacement constructs. Generation of viable parasites was not possible. These data suggest that the CPN60.3 gene is essential for *L. donovani* growth and/or viability.

Viable single-allele and double-allele candidate replacement mutants of the CPN60.4 gene were obtained with both replacement constructs. This result suggests that CPN60.4 is a non-essential gene.

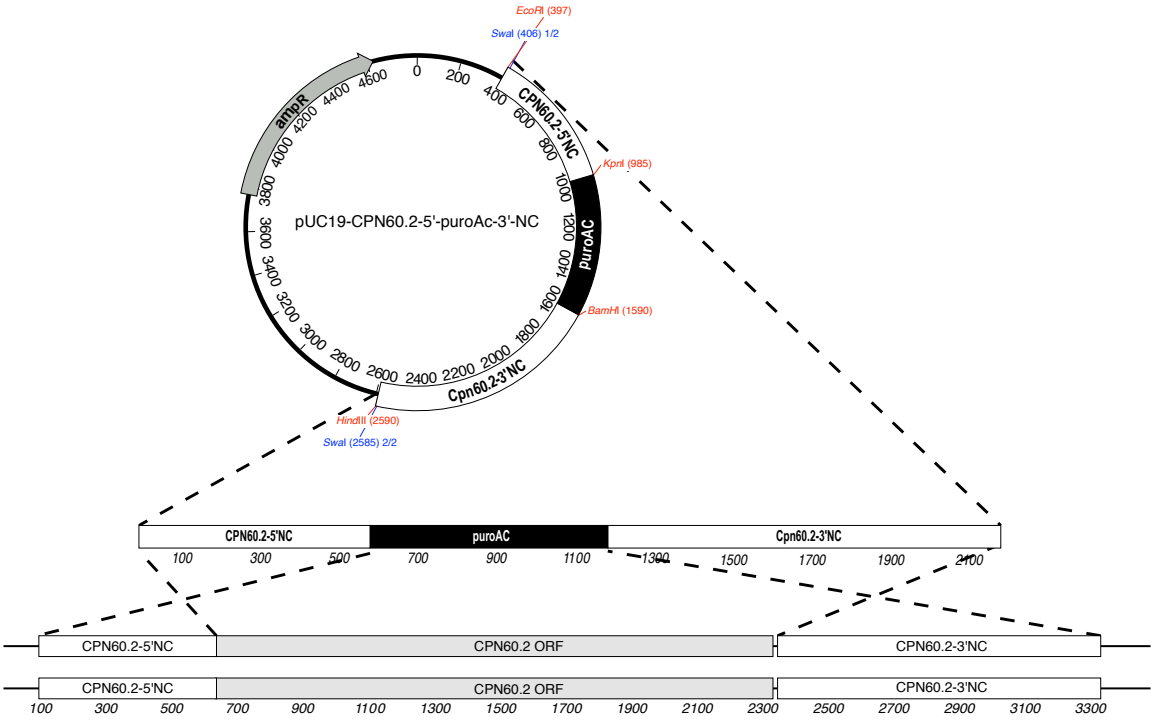


Figure 11: Representative Figure of the pUC19-60.2-5'-puroAc-3'-NC vector and the integration of the linearised construct into the gene locus via homologous recombination.

As described above, it was not possible to obtain viable double-resistant mutants targeting the CPN60.1 or the CPN60.3 genes. These results raise the question, whether these genes are essential. Also, over expression of a gene of interest is often used to analyse if an observed phenotype can be reversed. To facilitate this, ectopic copies of the respective GOIs were introduced in single-allele replacement mutants. Before this background of ectopic GOI expression, the second allele was then targeted for replacement, ideally resulting in double-allele replacement mutants with add-back ($^{-/-/+}$). Therefore, each individual coding sequence was amplified and ligated into the pCL2N vector, allowing for G418 selection. The circular plasmid DNA was transfected into the single-allele replacement mutants ($^{+/-/+}$) followed by second allele replacement.

The viable, putative CPN60.2$^{-/-}$ and CPN60.4$^{-/-}$ mutants were transfected directly with their cognate episomal add-back constructs.

Transfection of the episomal copy of CPN60.1 into the single-allele replacement mutant of CPN60.1 was successful. However, targeting the the second allele did not result in viable, triple-resistant parasites. The experiments were repeated

three times. Therefore, it was not possible to determine whether the CPN60.1 gene is essential.
The integration of an CPN60.2 episomal add-back into the CPN60.2 null mutant strain resulted in viable, triple-resistant parasites.
The CPN60.3 single-allele replacement mutants were successfully transfected with an episomal CPN60.3 transgene. The subsequent generation of double allele replacement mutants in the presence of the CPN60.3 add-back resulted in viable, triple-resistant mutants. This result suggests that CPN60.3 is an essential gene in *L. donovani*.
The integration of the CPN60.4 episomal add-back into the CPN60.4 null mutants resulted in viable, triple-resistant parasites.
The episomal constructs were also used to generate over expressing strains for the GOIs ($^{+/+/+}$) to observe whether over expression causes a phenotype. Therefore, the episomal add-back constructs were transfected into the *L. donovani* WT. Individual transfection with each of the episomal add-back constructs was successful.
The obtained mutant strains for the four different CPN60s are summarised in Table 22.

Table 22: List of gene edited *L. donovani* by homologous recombination.

Gene	+/-	-/-	-/-/+	+/+/+
CPN60.1$_{(HR)}$	✔	✖		✔
CPN60.2$_{(HR)}$	✔	✔	✔	✔
CPN60.3$_{(HR)}$	✔	✖	✔	✔
CPN60.4$_{(HR)}$	✔	✔	✔	✔

6.3. CRISPR/Cas9

6.3.1. T7-RNA-polymerase and Cas9 expressing *Leishmania donovani*

As shown in 6.2, it was not possible to obtain double resistant mutants targeting CPN60.1.
Furthermore, it was not possible to obtain double gene replacement mutants in the presence of an episomal add-back. Therefore, another strategy was attempted, namely the CRISPR/Cas9 gene editing that was recently established for *Leishmania* spp.

In brief, *L. donovani* must be transfected with a plasmid bearing the genes for T7-RNA-polymerase Cas9 nuclease. Next, the parasites are transfected with PCR-products containing the T7 RNA polymerase promoter and single guide RNA sequence, and with two replacement constructs containing the puroAc or the blasticidin resistance marker genes (Figure 13). The PCR products coding for sgRNAs are then transcribed by the T7-RNA-polymerase to yield sgRNAs, which guided the Cas9 to cleave the target DNA locus. The two replacement constructs are then integrated at the cleavage site via microhomology-mediated end joining (MMEJ), and the gene of interest is excised (Zhang and Matlashewski 2015).
The successful episomal expression of Cas9 in *L. donovani* was verified via the expression of the FLAG-tag joined to the enzyme. The tag was detected by Western blot using a specific anti-FLAG-tag antibody (Figure 12).

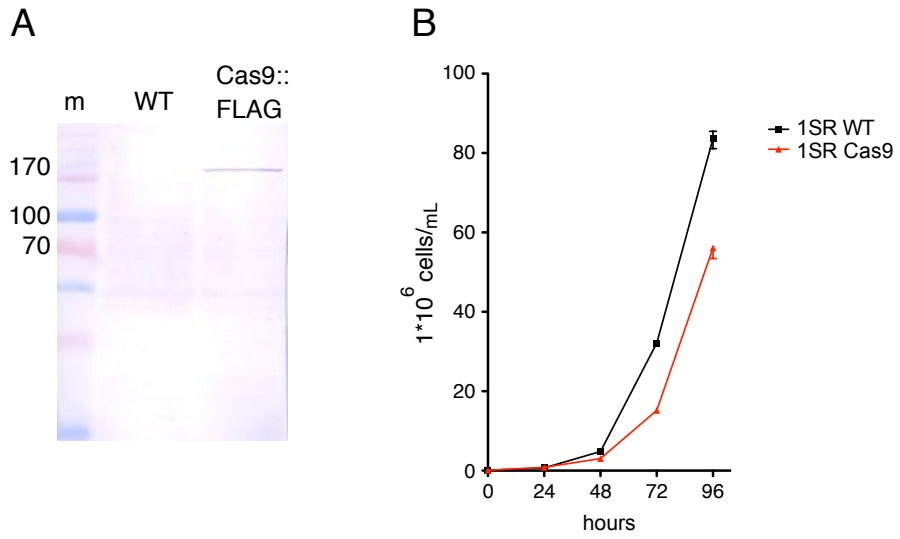


Figure 12: **A**. Western blot of lysates from *L. donovani* WT and Cas9::FLAG expressing parasites. 1st antibody: anti-FLAG. **B**. Growth curve of *L. donovani* WT and *L. donovani* Cas9 over 96 hrs in M199+ (pH = 7.4) at 25°C.

Figure 12 A shows that a band in the Western Blot was visible at a size of ~180 kDa., which verifies the successful expression of the Cas9 enzyme. No band was visible in the negative control (WT).

Expressing an ectopic protein such as the Cas9 enzyme in *L. donovani* may influence the parasites' viability. Therefore, the effect of the Cas9 expression on the growth rate of the parasites was assessed.
Figure 12 B shows that Cas9 expression delayed the growth of the parasites by 24 hrs compared to the wild type *L. donovani*. This delayed growth was compensated by the parasites after 3 month (data not shown). Apart from this, no other differences in growth or in the morphology were observed.

6.3.2. Generation of sgRNAs and replacement constructs for CRISPR/Cas9

Design of the sgRNA PCR templates is described in (5.2.4). For the replacement constructs, pre-designed constructs are used (Beneke, Madden et al. 2017). These are encoding either a puromycin or a blasticidin resistance marker gene, flanked by unrelated NCs. The specific 30 nt required for MMEJ were added by PCR using specifically designed primers at both ends of the replacement construct. A schematic representation of the replacement constructs and the resulting PCR products of replacement constructs and sgRNAs are shown in 13.

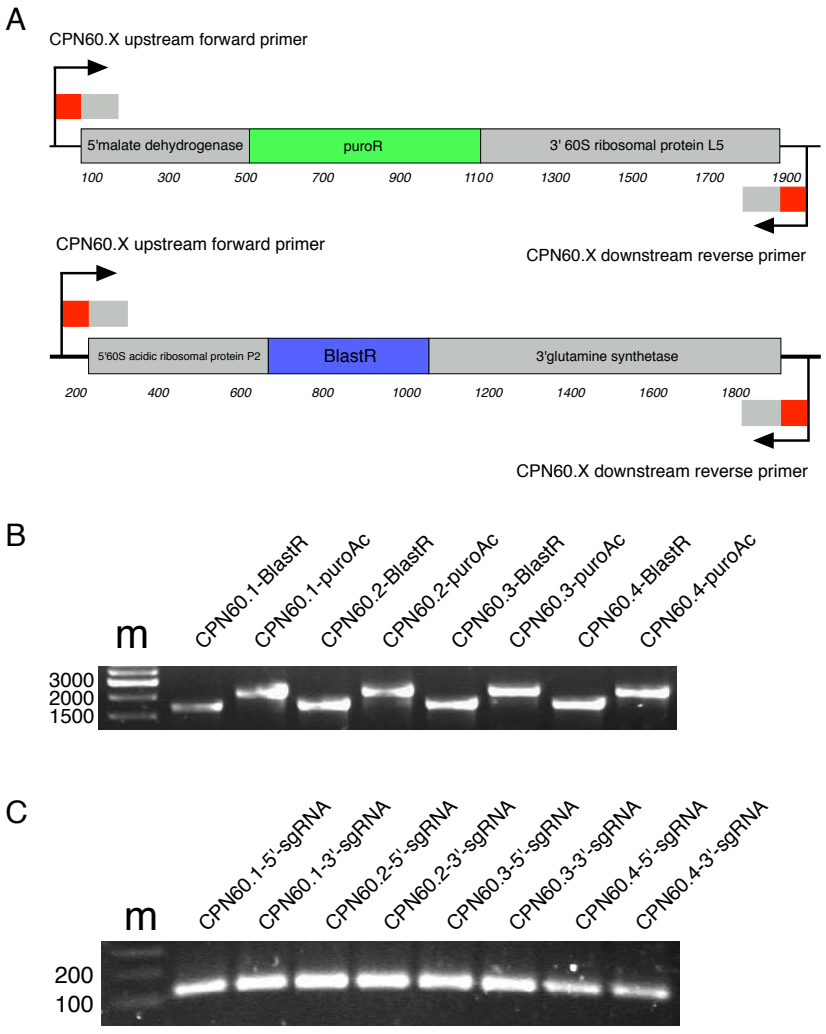


Figure 13: **A**. Representative figure of the replacement constructs before PCR for insertion via CRISPR/Cas and the required primers. **B**. PCR products of each replacement construct for the CPN60 genes. **C**. PCR products of the respective sgRNAs required for each gene.

As Figure 13 shows, PCR amplifications of the replacement construct and the sgRNAs were successful.
To target the CPN60 genes, the Cas9 enzyme-expressing *L. donovani* were transfected with mixtures of GOI-specific replacement constructs and sgRNA amplificates. Transfection was performed as described in 5.2.4. The resulting parasites were treated with puromycin and blasticidin for positive selection. The Table 23 shows the outcome.

Table 23: List of gene edited *L. donovani* by CRISPR/Cas.

Gene	-/-
CPN60.1	✔
CPN60.2	✖
CPN60.3	✖
CPN60.4	✔

Viable double resistant mutants were obtained for the CPN60.1 gene. This is in contrast to the result from the homologous recombination (6.2), where it was not possible to obtain viable double resistant mutants.
However, it was not possible to obtain viable double resistant mutants for the CPN60.2 gene, also in contrast to the results from homologous recombination (6.2).
Targeting the CPN60.3 gene did not result in viable double resistant mutants, confirming the homologous recombination results and suggesting that CPN60.3 is an essential gene in *L. donovani*.
Viable double resistant mutants were also obtained for *CPN60.4*, as also shown by homologous recombination. This suggests that CPN60.4 is a non-essential gene in *L. donovani*.

6.4. Verification of gene-edited mutants

Verification of gene-edited parasites was performed on three levels. Successful and correct integration of replacement constructs was verified on the DNA level using PCR analysis. Putative null mutants and *over expressing* mutants were also analysed on the mRNA level using semi-quantitative *real time* RT-PCR. The putative *L. donovani* CPN60.4$^{-/-}$ mutant was also verified on the protein level.

6.5. Verification at the DNA level

6.5.1. CPN60.1

The CPN60.1 single-allele replacement mutants (6.2) were verified by detecting the bleoR resistance marker gene. For the PCR a bleoR-5'-fwd and a bleoR-3'-rev primer were used and should result in PCR product with a size of 386 bp. As a negative control *L. donovani* WT and as a positive control the *L. donovani* CPN60.2$^{-/-}$ mutant was used.

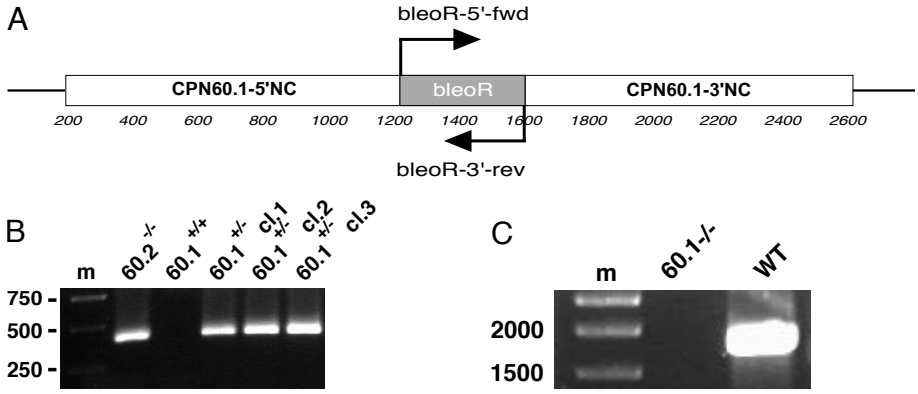


Figure 14: **A**. Representative figure of the CPN60.1 gene locus and the two bleoR binding primers. **B**. PCR products obtained by using the bleoR specific primers and genomic DNA from *L. donovani* CPN60.2$^{-/-}$, *L. donovani* WT, or three different clones of *L. donovani* CPN60.1$^{+/-}$. The presence of the bleoR gene results in a 386 bp construct. **C**. PCR products obtained by using CPN60.1 specific primers and gDNA from *L. donovani* CPN60.1$^{-/-}$ and *L. donovani* WT. The presence of the CPN60.1 results in a 1785 bp construct.

Figure 14 B shows a PCR product of the predicted size of the bleoR gene for all three clones of *L. donovani* CPN60.1$^{+/-}$. A product of the same size was absent in *L. donovani* WT, but present in *L. donovani* CPN60.2$^{-/-}$. This result shows that the bleoR gene was integrated into the genomic DNA.

To verify the absence of the CPN60.1 gene in the putative *L. donovani* CPN60.1$^{-/-}$ $_{(CRISPR)}$ mutants, a PCR using the CPN60.1-5'-fwd primer and the CPN60.1-3'-rev primer was performed. The presence of CPN60.1 results in a 1785 bp construct. Figure 14 C shows a ~1800 bp product for the *L. donovani* WT gDNA, which was absent for the *L. donovani* CPN60.1$^{-/-}$ gDNA, showing that the CPN60.1 gene was successfully removed and that a viable null mutant was obtained.

6.5.2. CPN60.2

To verify the absence of CPN60.2 in the putative *L. donovani* CPN60.2$^{-/-}$ mutants and the correct insertion of the two replacement constructs 6 different PCRs were performed. First the absence of CPN60.2 was verified by performing a CPN60.2-PCR. By using a 60.2-5'-fwd- and a 60.2-3'-rev-primer a 1701 bp product was generated for *L. donovani* WT (60.2$^{+/+}$) gDNA (Figure 15).

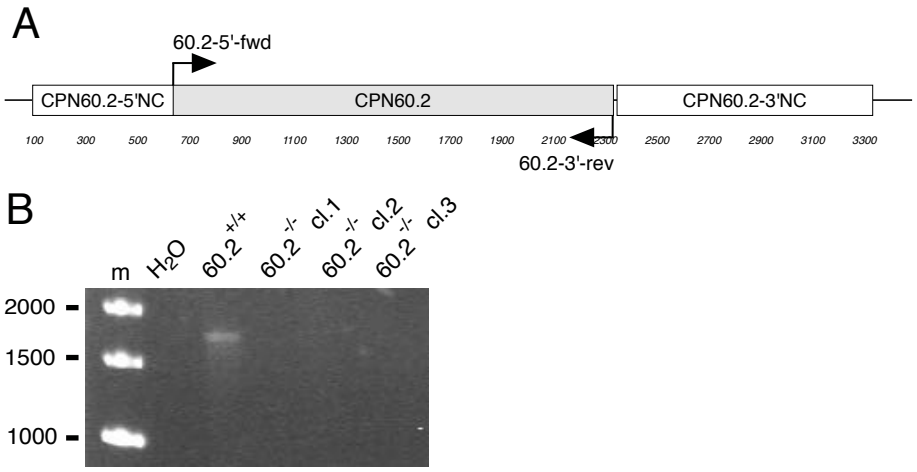


Figure 15: **A**. Representative figure of the CPN60.2 gene locus and the two CPN60.2 binding primers. **B**. PCR products obtained by using the CPN60.2 specific primers and genomic DNA from *L. donovani* WT, or three different clones of *L. donovani* CPN60.2$^{-/-}$. The presence of the CPN60.2 gene results in a 1701 bp construct.

All three clones of *L. donovani* CPN60.2$^{-/-}$ were negative, confirming that removal of CPN60.2 by homologous recombination was successful.

By using the CPN60.2-5'-flank and the CPN60.2-3'-flank primers, a shorter product, from 3346 bp to 2250 bp, should be obtained due to the replacement of *CPN60.2* with the resistance marker genes. The product is reduced (Figure 15 B).

To verify the correct insertion of the resistance marker genes, four PCRs were performed. Two PCRs were preformed for the allele containing the puroAC resistance marker gene and two for the allele containing the bleoR resistance marker gene. Using the CPN60.2-5'-flank and the puroAc-5'-rev primers a PCR product of 680 bp size is expected. A 1029 bp PCR product is expected when using the puroAC-3'-fwd and the CPN60.2-3'-flank primer. The CPN60.2-5'-flank and the bleoR-5'-rev primers create an expected PCR product of 681 bp, while the bleoR-3'-fwd and the CPN60.2-3'-flank primers should give rise to a PCR product of 1048 bp.

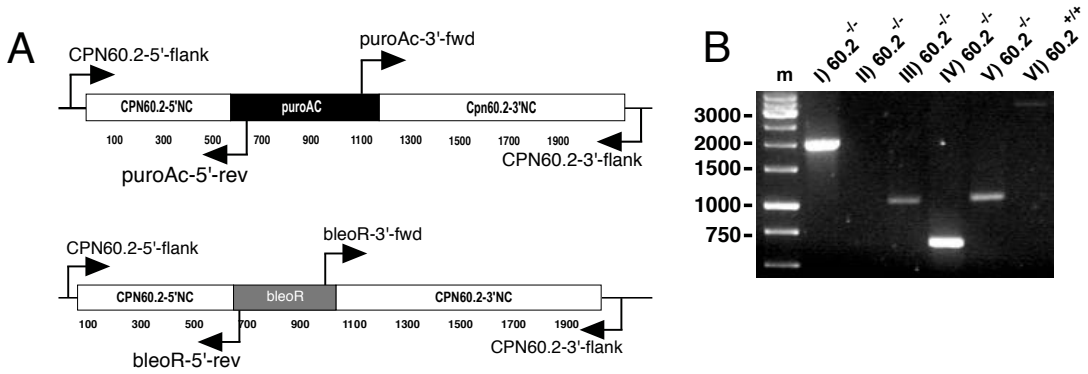


Figure 16: **A**. Representative figure of the CPN60.2 gene locus containing either the puroAC or the bleoR resistance marker gene and the four specific primers required for verification. **B**. PCR products obtained by using the CPN60.2 specific primers and genomic DNA from *L. donovani* WT (**VI)**), or one clone of *L. donovani* CPN60.2$^{-/-}$ (**I)** - **V)**). **I)** CPN60.2-5'-flank + CPN60.2-3'-flank **II)** CPN60.2-5'-flank + puroAc-5'-rev **III)** puroAc-3'-fwd + CPN60.2-3'-flank **IV)** CPN60.2-5'-flank + bleoR-5'-rev **V)** bleoR-3'-fwd + CPN60.2-3'-flank **VI)** CPN60.2-5'-flank + CPN60.2-3'-flank.

The gDNA used to generate the PCR products displayed in Figure 16 B, lanes I) to V) was from the putative *L. donovani* CPN60.2$^{-/-}$, the gDNA used for the PCR product displayed in VI) was from *L donovani* WT. The PCR product generated when using the two flanking primers (CPN60.2-5'-flank and CPN60.2-3'-flank) can be seen on the gel (Figure 16 B, lane I)) at an indicated size of 2100 bp. In comparison the lane VI) from Figure 16 B shows the PCR product generated when using the CPN60.2-5'-flank and the CPN60.2-3'-flank primer with gDNA from *L. donovani* WT, resulting in a band migrating at a size of ~3500 bp. This indicates that the CPN60.2 coding gene was successfully removed and replaced by the resistance marker genes.

The lanes II) and III) from Figure 16 B show the PCR products generated when using either the CPN60.2-5'-flank and the puroAc-5'-rev primers or the puroAc-3'-fwd and the CPN60.2-3'-flank primers. The bands migrate at a size of 700 bp and of 1000 bp, respectively and are in accordance to the predicted sizes. This shows that the puroAc resistance marker gene was correctly integrated.

The lanes IV) and V) from Figure 16 B show the PCR products generated when using either the CPN60.2-5'-flank and the bleoR-5'-rev primers or the bleoR-3'-fwd and the CPN60.2-3'-flank primers. The bands migrate at a size of 650 bp and of 1100 bp, respectively and are in accordance to the predicted sizes. This shows that the bleoR resistance marker gene was correctly integrated.

6.5.3. CPN60.2 - Whole genome sequencing (WGS)

To analyse if removal of CPN60.2 led to compensatory effects on the genomic level whole genome sequencing (WGS) was performed. By using WGS the

chromosome number, gene copy number variations, INDELs (INsertion or DELetion) and SNPs (single nucleotide polymorphism) were analysed. As reference strain the original *L. donovani* 1SRWT strain, before removal of CPN60.2, was used. Figure 17 shows the region coding for CPN60.2 and CPN60.3 on chromosome 36.

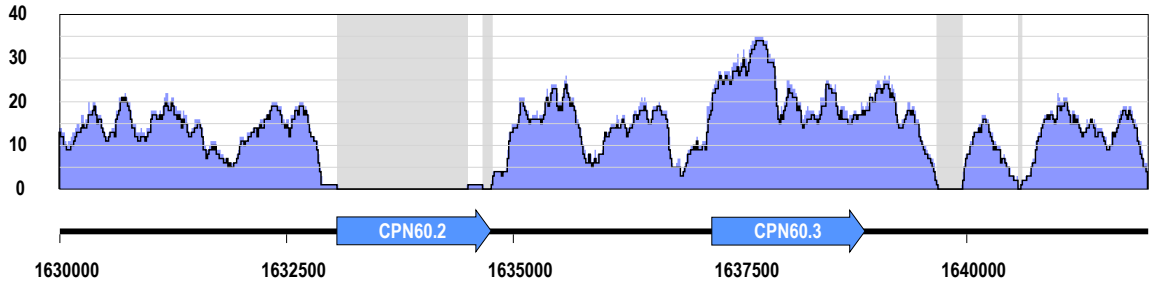


Figure 17: Representative figure of the NGS analysis of the CPN60.2 and CPN60.3 gene locus. The CPN60.2 coding sequence is absent (grey bars).

As Figure 17 shows CPN60.2 was successfully removed, while the neighbouring gene CPN60.3 was not affected. Furthermore, no chromosome ploidy variations were detected, neither of the chromosome copy number level, nor of parts of the chromosomes (data not shown). INDELs and SNPs can be caused by various factors. Beside the naturally occurring mutations, which were excluded by comparing the *L. donovani* CPN60.2$^{-/-}$ to the *L. donovani* WT, other mutations arise from the selective pressure of the antibiotics, the presence of the resistance marker gene, its protein or the absence of *CPN60.2*. By comparing the NGS data of *L. donovani* CPN60.2$^{-/-}$ to other genetically modified *L. donovani*, such as *L. donovani* HSP20$^{-/-}$ or *L. donovani* HSP23$^{-/-,}$ INDELs and SNPs caused by the presence of selection maker genes were excluded. The following Table 24 shows the INDEL and SNP analysis specifically caused by the absence of CPN60.2.

Table 24: List of SNPs and frame shifts in *L. donovani* CPN60.2$^{-/-}$ specifically caused by double allele replacement of CPN60.2.

Chromosome	Gene ID	Protein	SNP position	Change DNA	Change amino acid
Ld03	LdBK_030370	hypothetical protein	125593	G -> C	Ala339Pro
Ld10	LdBK_100860	hypothetical protein	382159	CG -> CTG	Ala212fs
Ld10	LdBK_100860	hypothetical protein	382162	C -> T	Gly211Ser
Ld30	LdBK_301370	SNF2 family N-terminal domain/ Helicase conserved	453042	C -> T	Pro884Leu

Chromosome	Gene ID	Protein	SNP position	Change DNA	Change amino acid
Ld30	LdBK_303340	hypothetical protein	1215119	C -> A	Pro148Thr
Ld34	LdBK_341500	N-terminal region of Chorein, a TM vesicle-mediated sorter/Protein function unknown	648484	C -> T	Glu451Lys
Ld36	LdBK_363090	phosphatidylinositol 3- and 4-kinase, putative	1220559	A -> G	Val958Ala

Table 24 shows seven SNPs that were detected exclusively for the CPN60.2$^{-/-}$ mutant. Of the three known proteins, none is linked to any known CPN60 interaction or pathway. The LdBPK_301370 (a SNF2 family N-terminal domain/ Helicase conserved) shows a point mutation from C to T at position 453042 leading to change from a proline at position 884 to a leucine. The LdBPK_301500 (a N-terminal region of Chorein, a TM vesicle-mediated sorter/ Protein function unknown) shows a point mutation from C to T at position 648484 leading to change from a glutamic acid at position 451 to a lysine. The LdBPK_301370 (a phosphatidylinositol 3- and 4-kinase, putative) shows a point mutation from A to G at position 1220559 leading to change from a valine at position 958 to a alanine. Of the three hypothetical proteins LdBPK_030370 and Ld_BPK303340 show one point mutation leading to an amino acid change from alanine to proline at position 339 and from proline to threonine at position 148, respectively. A BLASTn search against the non-redundant (nr) database was performed for the three hypothetical proteins. Of these three proteins the hypothetical protein LdBPK_100860 is the only protein that shows two genomic changes. Both changes, one mutation and one frame shift are directly side by side. Structural analysis of LdBPK_100860 showed similarities to the Type I hyperactive antifreeze protein from *Pseudopleuronectes americanus*. The function of this protein is to encapsulate water and prevent it from freezing. No hits were found for the remaining two hypothetical proteins. These data show that replacement of CPN60.2 coding gene and the loss of the CPN60.2 protein result in 7 specific mutations which might complement the loss of the gene.

6.5.4. CPN60.3

The putative *L. donovani* CPN60.3$^{+/-}$ mutants were verified by the correct insertion of the puroAc gene. Therefore, three different PCRs were performed. First, the two flanking primers CPN60.3-5'-flank and CPN60.3-3'-flank were used. The resulting products should show the allele containing the CPN60.3 coding gene

(3800 bp), as well as the genetically modified allele containing the puroAC gene (2716 bp). The aim of the second and the third PCR was to verify the correct insertion of the puroAc gene. Therefore, the CPN60.3-5'-flank primer and the puroAc-5'-rev primer were used, resulting in a 1086 bp product. At the 3'-end the CPN60.3-3'-flank and the puroAC-3'-fwd primers were used, resulting in a 1079 bp product.

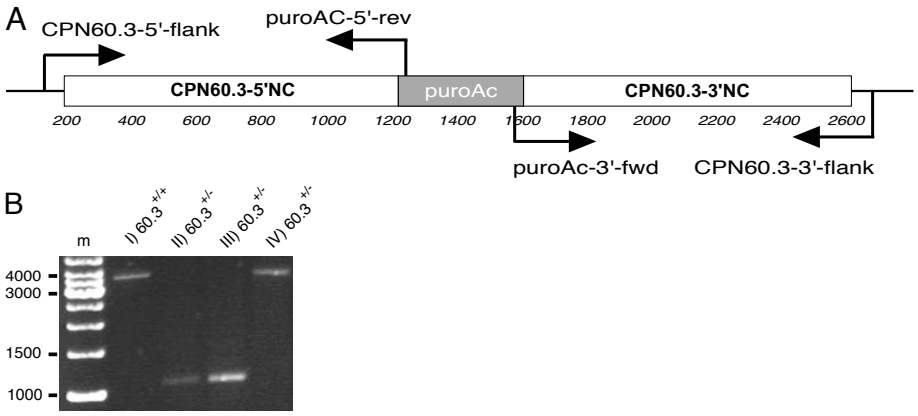


Figure 18: **A**. Representative figure of the CPN60.3 gene locus containing the puroAC resistance marker gene and the four specific primers required for verification. **B**. PCR products obtained by using the CPN60.3 specific primers and genomic DNA from *L. donovani* WT, or one clone of *L. donovani* CPN60.3$^{+/-}$ (**II)** - **IV)**). **I)** gDNA from *L. donovani* WT + CPN60.3-5'-flank + CPN60.3-3'-flank. **II)** CPN60.3-5'-flank + puroAc-5'-rev **III)** CPN60.3-3'-flank + puroAc-3'-fwd **IV)** CPN60.3-5'-flank + CPN60.3-3'-flank.

Figure 18 shows the PCR products visualised on an agarose gel. The PCR products generated when using gDNA from *L. donovani* WT or *L. donovani* CPN60.3$^{+/-}$ together with the CPN60.3-5'-flank and CPN60.3-3'-flank primers are displayed in the second (60.3$^{+/+}$ I)) and the last (60.3$^{+/-}$ IV)) lane respectively. The products run at the expected size of 3800 bp for the *L. donovani* WT (60.3$^{+/+}$) and also for the *L. don*CPN60.3$^{+/-}$ mutant while no band is visible at a size of ~2700 bp. The latter result might be due to the PCR program favouring the larger fragment instead of the smaller. Nevertheless, the calculated product sizes for the correct insertion of the puroAc gene of 1086 bp and 1079 bp are visible at line 60.3$^{+/-}$ II) and 60.3$^{+/-}$ III). Therefore, the results show that the puroAc resistance marker gene was integrated successfully, while at least one copy of CPN60.3 is still present.

6.5.5. CPN60.4

To verify the absence of the CPN60.4 gene in the putative *L. donovani* CPN60.4$^{-/-}$ and the *L. donovani* CPN60.4$^{-/-}$ $_{(CISPR)}$ mutants, a PCR targeting the CPN60.4 was performed. Amplification with CPN60.4-5'-fwd and a CPN60.4-3'-rev primers yields a 1617 bp product in the wild type that should be absent in null mutants.

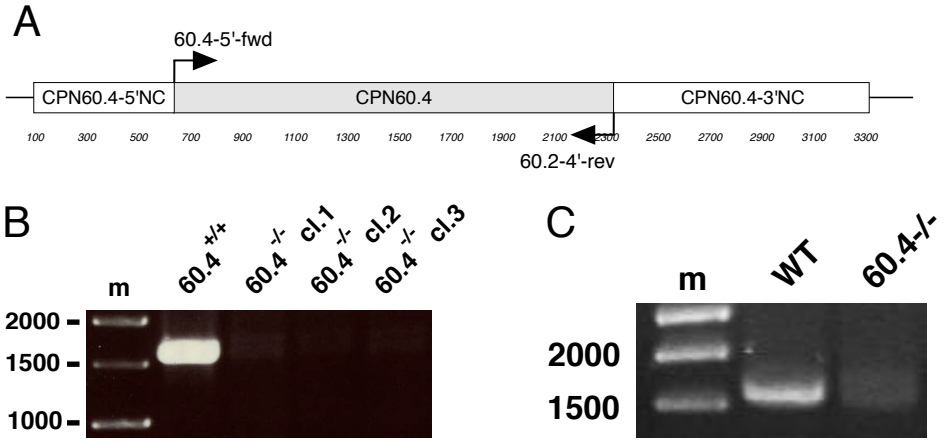


Figure 19: **A**. Representative figure of the CPN60.4 gene locus and the two specific primers required for verification. **B**. PCR products obtained by using the CPN60.4 specific primers and genomic DNA from *L. donovani* WT, or three different clones of *L. donovani* CPN60.4$_{(HR)}^{-/-}$. **C**. PCR products obtained by using the CPN60.4 specific primers and genomic DNA from *L. donovani* WT, or gDNA from *L. donovani* CPN60.4$_{(CRISPR)}^{-/-}$.

Figure 19 shows the presence of a 1617 bp product for the *L. donovani* WT (60.4$^{+/+}$), while no bands of that size were visible for the three clones of *L. donovani* CPN60.4$^{-/-}$ and for *L. donovani* CPN60.4$^{-/-}$ $_{(CISPR)}$. This shows that CPN60.4 was removed successfully by using either homologous recombination or CRISPR/Cas9 technology. It follows from this that CPN60.4 is a non-essential gene, as viable null mutants could be obtained.

6.6. Verification at the RNA level

To ascertain the absence of gene-specific RNAs in the putative single-allele or double-allele gene replacement mutants, a semi-quantitative *real time* RT-PCR was performed. The genetically modified parasites were compared to the *L. donovani* WT parasites. To perform the semi-quantitative *real time* RT-PCR, specific primer pairs for each CPN60 gene were designed. The primers target a short region within the gene of interest. For normalisation, *Leishmania* actin RNA was also quantified to account for variations in RNA isolation. Figure 20 shows the relative GOI RNA levels for the putative mutants.

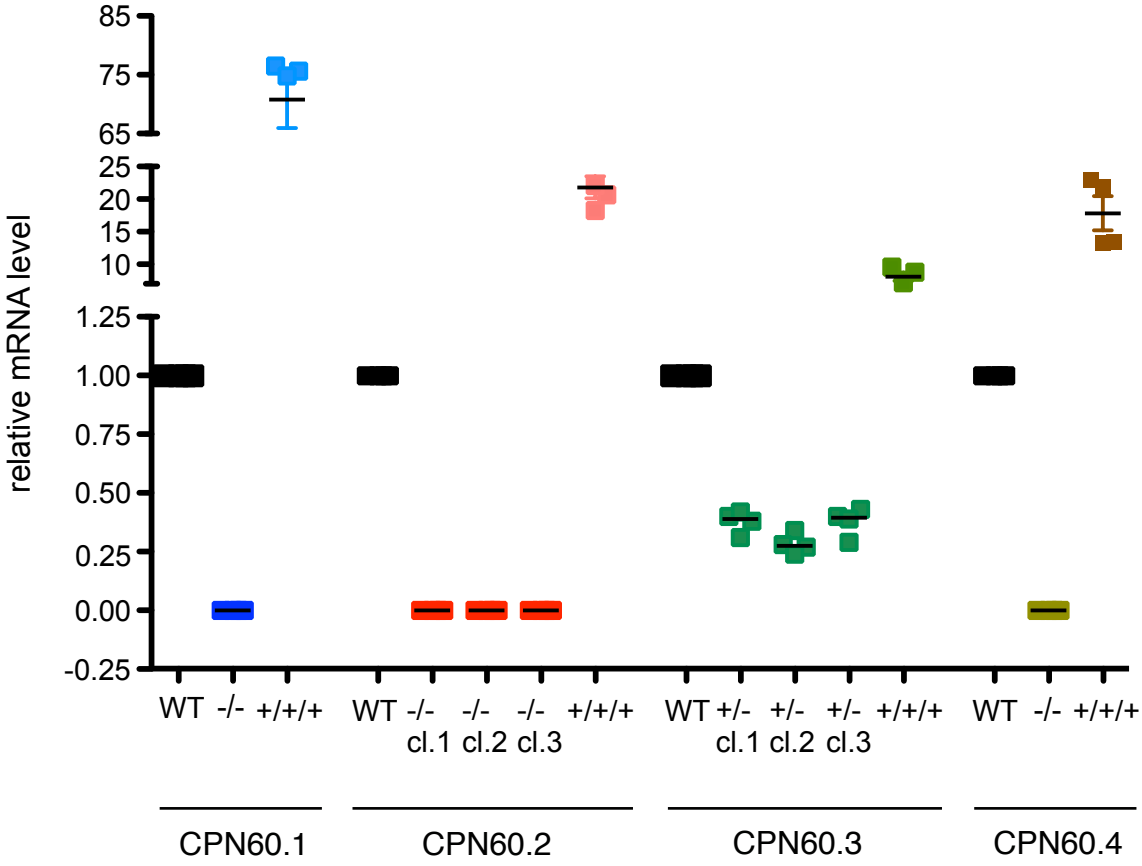


Figure 20: RNA level of single and double allele replacement mutants and gene add-back mutants. The RNA was isolated and transcribed into cDNA. RT-PCR was performed using gene specific primers. Experiments were performed in duplicates. From left to right: CPN60.1 (blue), CPN60.2 (red), CPN60.3 (green), and CPN60.4 (brown). The wild type (WT) is displayed in black.

The CPN60.1$^{-/-}$ mutant shows a relative mRNA level for CPN60.1 of 0, meaning no mRNA was detectable. The *over expressing* strain (CPN60.1$^{+/+/+}$) had a relative mRNA level of 70.5-fold over wild type level.

The mRNA levels of CPN60.2 were analysed for three different clones of CPN60.2$^{-/-}$ and for CPN60.2$^{+/+/+}$. For each clone of CPN60.2$^{-/-}$ no mRNA was detected, while the mRNA level of CPN60.2$^{+/+/+}$ was 22.1-fold over wild type level.

The mRNA level of CPN60.3 was analysed for CPN60.3$^{+/-}$ and CPN60.3$^{+/+/+}$. Clone 1 of CPN60.3$^{+/-}$ had a mRNA level of 0.38 of wild type level, while clone 2 and clone 3 had mRNA levels of 0.27- and 0.39-fold, respectively.

The mRNA level of CPN60.4 in CPN60.4$^{-/-}$ was 0 and in CPN60.4$^{+/+/+}$ 17.52 -fold of wild type level. The data indeed verify the null mutants of CPN60.1$^{-/-}$, CPN60.2$^{-/-}$ and CPN60.4$^{-/-}$ showing no mRNA of the respective GOI, while the single-allele replacement for CPN60.3$^{+/-}$ led to a reduction of the corresponding RNA to less than 50%. Episomal expression of a GOI led to strongly increased mRNA levels, with differences between the different CPN60.X add-back mutants.

6.7. Generation of CPN60 antibodies

Specific antibodies were needed to verify the *L. donovani* CPN60.X null mutants, as well as to study the different CPN60s. Generation of antibodies was performed by immunising hens with the respective recombinant antigen, followed by isolation of the IgY from egg yolk. The antigens were produced by recombinant expression in *E.coli* and purification by liquid metal chelate chromatography. In a first step, the coding sequences were amplified from *L. donovani* gDNA and fused into the *E. coli* expression vector pJC45, to yield a $(His)_{10}$-tagged fusion gene. The resulting plasmid was then used to transform *E. coli* strain (DE3)[pAPlacI^{Q}], for T7 RNA polymerase-directed over expression. The His-tagged protein was purified by Ni^{2+} metal chelate chromatography, followed by dialysis as described (Clos and Brandau 1994). The antigen was then used to immunise laying hens and raise antigen-specific antibodies. The isolated IgY was tested against whole cell lysate from *L. donovani* parasites grown at 25°C and 37°C, as well as against the recombinant protein.

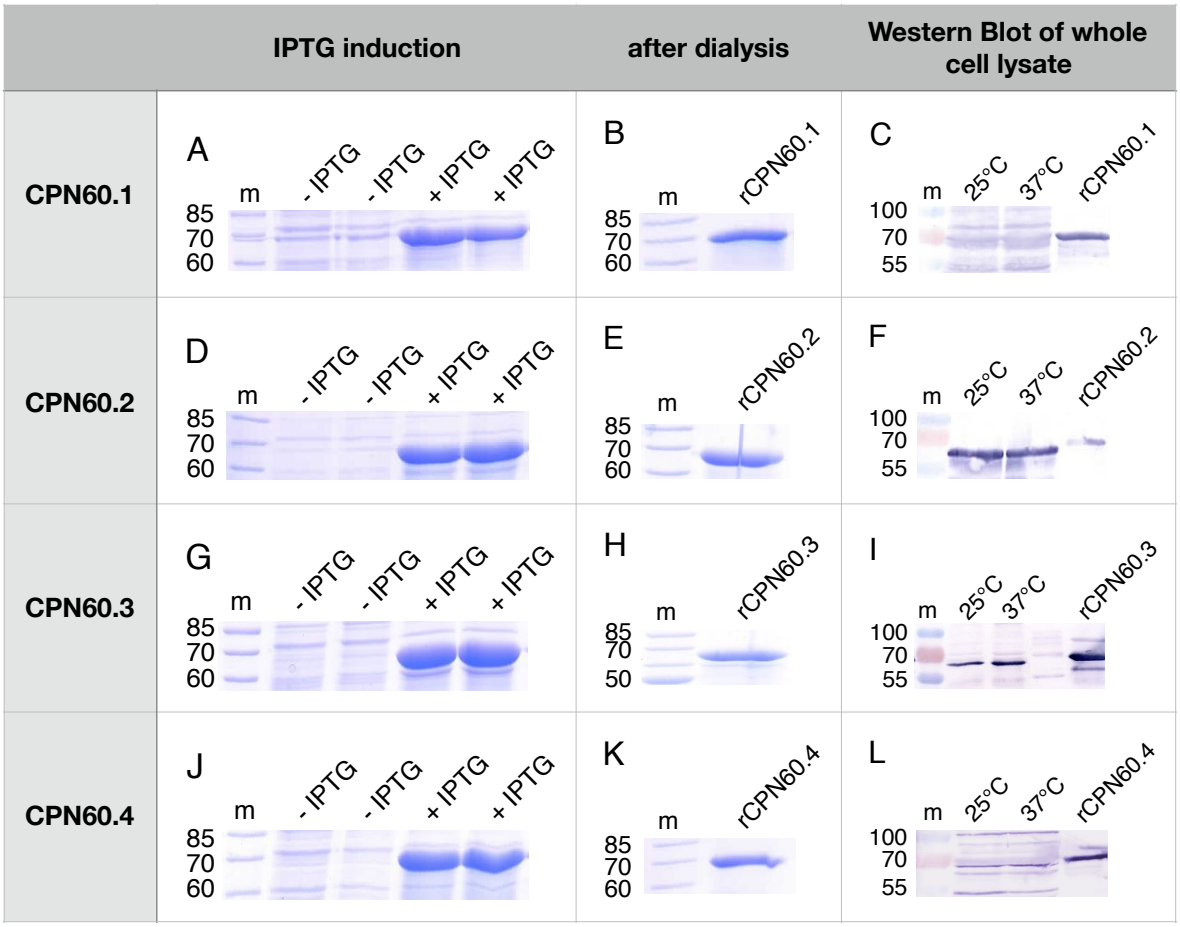


Figure 21: Production and verification of anti-CPN60 antibodies. On the left bar (**IPTG induction**) protein expression in *E. coli* is shown for all four CPN60s upon IPTG induction. The SDS-gel was stained with Coomassie-blue. The middle bar (**after dialysis**) displays the the purified proteins after dialysis run on a SDS-PAGE and stained with Coomassie-blue. The right bar (**Western Blot**

of whole cell lysate) displays the respective immuno-blots. On the left lane (25°C) $1*10^7$ parasites grown at 25°C were loaded, in the middle lane (37°C) $1*10^7$ parasites grown at 37°C were loaded, and on the right lane the recombinant protein was loaded.

Figure 21 shows that the protein expression of the 4 different CPN60s was successfully induced by IPTG.
Purified recombinant rCPN60.1 migrates at a size of ~70 kDa. The anti-CPN60.1 antibody (CPN60.1-Ab) detects a distinct band with a size of ~70 kDa for the rCPN60.1, but no bands are visible in lysates from *L. donovani* WT grown at 25°C or 37°C. This is in keeping with earlier findings (Schlüter, Wiesgigl et al. 2000).
The rCPN60.2 migrates at a size of ~69 kDa. Well defined bands are visible in the immune blot corresponding to a size of 67 to 68 kDa, while the rCPN60.2 band is visible at 69 kDa, due to its $(His)_{10}$-tag.
The rCPN60.3 antigen migrates at a size of 68 kDA. in the Coomassie-blue-stained gel and in the Western blot, while bands corresponding to 67 kDa are detected for the *Leishmania* lysates.
The rCPN60.4 migrates at a size of ~70 kDa in the Western Blot and in the Coomassie blue-stained gel, while bands corresponding to 69 kDa are detected in the lysates.

6.8. Analysis of CPN60 antibodies

As revealed by the phylogenetic analysis (Figure 10), the four different CPN60s of *L. donovani* are highly conserved. It was therefore necessary to analyse the specificity of the CPN60 antibodies. In a first test, the specific antibody was blotted against all four recombinant CPN60 proteins. For the second analysis, the antibody was tested against lysates of four CPN60 over expressing strains*:* CPN60.1$^{+/+/+}$, *L. donovani* CPN60.2$^{+/+/+}$, *L. donovani* CPN60.3$^{+/+/+}$ and *L. donovani* CPN60.4$^{+/+/+}$.

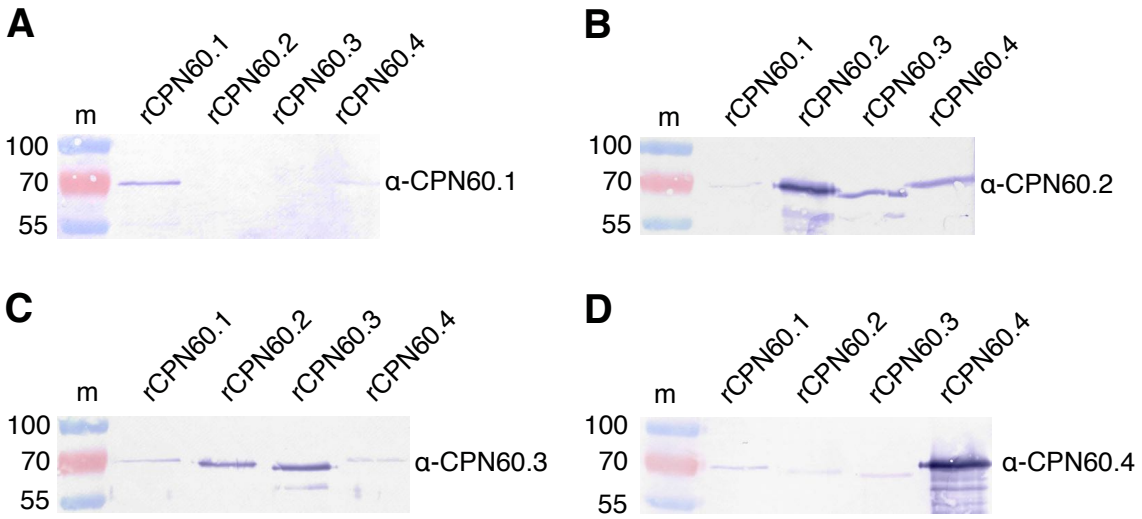


Figure 22: Western blots of recombinant *Ld*CPN60.1, *Ld*CPN60.2, *Ld*CPN60.3, and *Ld*CPN60.4 with different anti-sera from immunised hens. **A**. anti-CPN60.1 **B**. anti-CPN60.2 **C**. anti-CPN60.3 **D**. anti-CPN60.4

Figure 22 shows that the CPN60.1 antibody recognises rCPN60.1, but not rCPN60.2 and *r*CPN60.3, while *r*CPN60.4 cross-recognition is very weak. This result shows that the CPN60.1 antibody is highly specific to recombinant CPN60.1.

The CPN60.2 antibody in contrast recognises all 4 *r*CPN60.X, even though *r*CPN60.1 recognition is weak compared with the other three orthologues. Therefore, the CPN60.2 antibody cross-reacts with all recombinant CPN60s of *L. donovani*.

The CPN60.3 antibody mainly recognises *r*CPN60.2 and *r*CPN60.3 due to their high degree of sequence similarity. Cross-reactivity to*r*CPN60.1 and *r*CPN60.4 is weak.

The CPN60.4 antibody is highly specific for *r*CPN60.4 with a low background recognition of *r*CPN60.1, *r*CPN60.2 and *r*CPN60.3. Therefore, the CPN60.4 antibody can be used to detect recombinant CPN60.4.

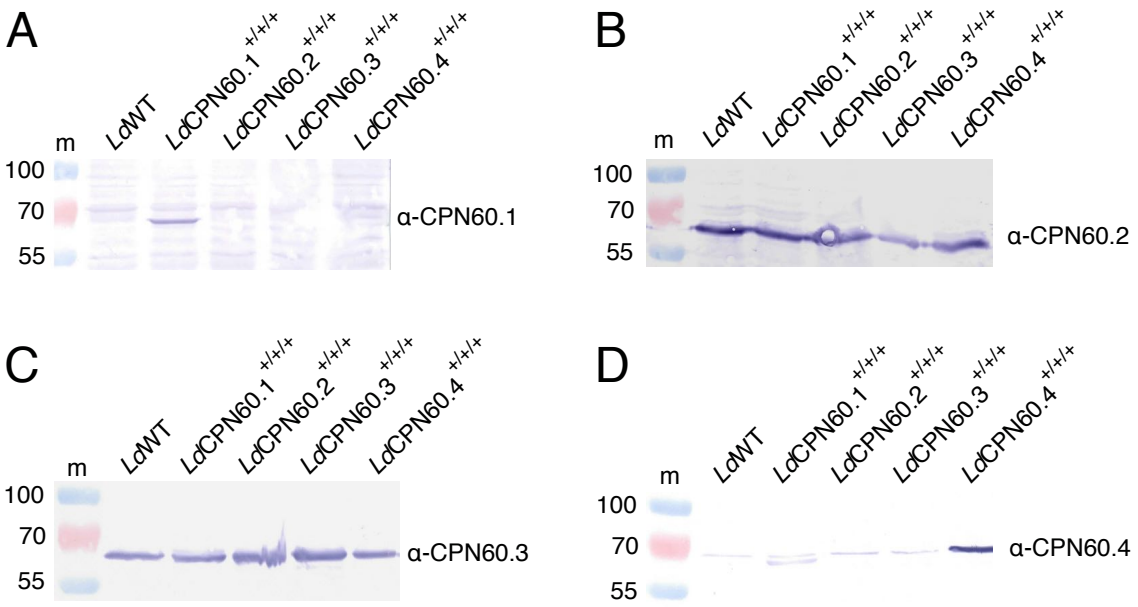


Figure 23: Western blots showing the lysates (1*10^7 cells / lane) of *L. donovani* wild type, *L. donovani* CPN60.1$^{+/+/+}$, *L. donovani* CPN60.2$^{+/+/+}$, *L. donovani* CPN60.3$^{+/+/+}$, and *L. donovani* CPN60.4$^{+/+/+}$ with different anti-sera from immunised hens. **A**. anti-CPN60.1 **B**. anti-CPN60.2 **C**. anti-CPN60.3 **D**. anti-CPN60.4

Anti-CPN60.1 can detect its antigen when over expressed in *L.* donovani (Figure 23 A), but does not cross-react with other over expressed CPN60 antigens. It can therefore be considered as specific. Both anti-CPN60.2 and anti-CPN60.3 cross-react with all four CPN60 proteins in the wild type and in the over expressing strains, precluding any antigen-specific analysis. The anti-CPN60.4 antibody detects its antigen in all five samples, with strongly enhanced intensity in the CPN60.4 over expressing strain, and with a weak cross-reactivity against over expressed CPN60.1. It can also be considered as antigen-specific.

6.9. Verification at the protein level

The results of 6.8 show, that it is not possible to verify *L. donovani* CPN60.1$^{-/-}$ null mutants on the protein level, as the anti-CPN60.1 antibody is not able to detect CPN60.1 in normal *L. donovani* WT lysate.

Due to a lack of specificity of the anti-CPN60.2 and anti-CPN60.3 antibodies (6.8), it is not possible to distinguish between the CPN60 proteins, making it impossible to verify the null mutants at the protein level.

Only the anti-CPN60.4 antibody could be used for mutant verification. Lysates of three CPN60.4$^{-/-}$ clones and of *L. donovani* WT were prepared and analysed by SDS-PAGE and Western blot with anti-CPN60.4 antibody.

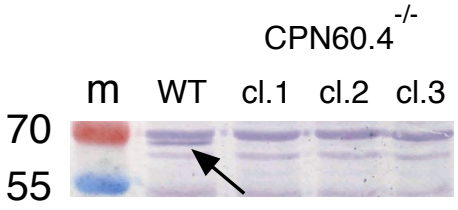


Figure 24: Western blot of a *L. donovani* wild type lysate and lysates from three *L. donovani* CPN60.4$^{-/-}$ clones using specific anti-CPN60.4 serum (1*10^{7} cells / lane).

A specific band for CPN60.4 is visible in the *L. donovani* WT lysate (Figure 24, arrow), which is absent in all three clones of *L. donovani* CPN60.4$^{-/-}$. This verifies the CPN60.4$^{-/-}$ mutants as null mutants on the protein level.

6.10. Phenotypic analysis

To identify the possible role of the CPN60s of *Leishmania* the phenotypes after gene replacement were characterised. By identifying phenotypic changes, it is possible to deduce the role of the protein. Therefore, the obtained *L. donovani* CPN60 mutants were analysed for phenotypical changes. The morphology, the cell growth under different conditions, the infectivity and the protein expression were characterised.

6.10.1. Morphology

To analyse morphological changes in the genetically modified parasites, an anti-tubulin staining was performed. First, the *L. donovani* WT promastigote parasites were characterised during their life cycle from the logarithmic (day 2) to the stationary phase (day 5) (Figure 25 A). From these data, day 4 was chosen to determine the the cell body size (Figure 25 B) and the length of the flagellum (Figure 26).

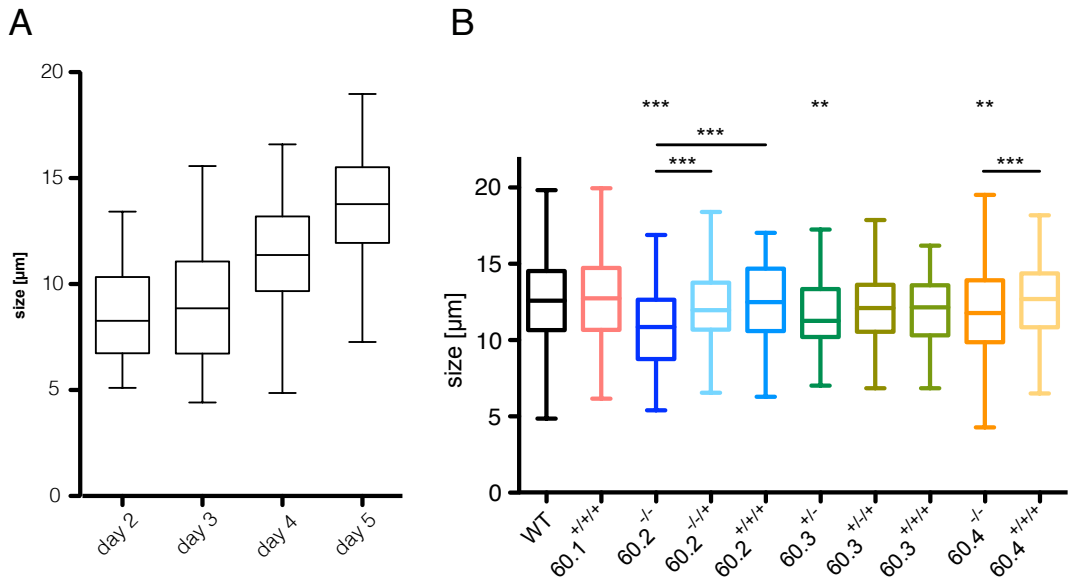


Figure 25: Cell body length of the promastigote form of *Leishmania*. **A**. *L. donovani* WT was grown for 5 days and the cell body length was monitored each day. The size of at least 200 parasites was measured and is displayed as a Whiskers (Tukey) graph. **B**. The cell body length of the *L. donovani* WT and the different CPN60 mutants were analysed at day 4 and are displayed as Whiskers (Tukey) graph (n ≥ 200).

The cell body length of *L. donovani* WT increases from day 2 from an average of 8.16 µm in the logarithmic phase to an average of 12.47 µm in the stationary phase (day 4) (Figure 25 A). This finding is in accordance with previous results (Sunter and Gull 2017).

As Figure 25 B shows, over expression of CPN60.1 does not have an effect on the cell body length.

The removal of CPN60.2 leads to a slightly, but significantly reduced cell body length (10.45 µm). This effect is compensated in the presence of the episomal add back. Over expression of CPN60.2 on the other hand does not reverse the effect. This results indicates that the removal of CPN60.2 in *Leishmania* induces cell stress, resulting in a reduced cell body length,.

Similarly, the *L. donovani* CPN60.3$^{+/-}$ mutants lacking one copy of CPN60.3 show a slightly decreased cell body length of 11.36 µM, possibly by causing a cell stress. This effect was not significantly compensated by the presence of an add-back.

CPN60.4$^{-/-}$ mutants shows a significantly decreased cell body length of 11.6 µm. By over expressing CPN60.4 the effect was compensated and an average cell body length of 12.52 µm was measured. As a smaller cell body length is an indicator for cell stress, these results indicate that the lack of CPN60.4 chaperone causes stress in the parasites.

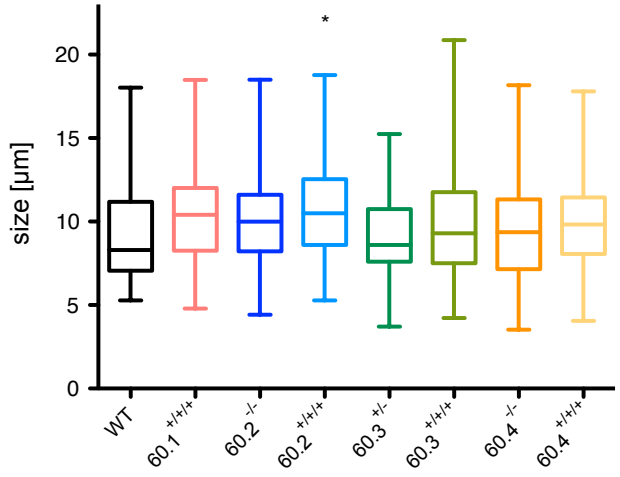


Figure 26: Flagellar length of the promastigote form of *Leishmania*. The flagellar length of the *L. donovani* WT and the different CPN60 mutants were measured at day 4 and are displayed as Whiskers (Tukey) graph (n ≥ 200).

An average flagella length of of 8.14 µm for the *L. donovani* WT on day 4 is recorded. The total average length of *L. donovani* WT on day 4 is 20.08 µm, hence, 60% of the size of the parasite consists of the cell body and 40% of the flagellum. This ratio and the size are characteristic for procyclic to nectomonad promastigotes (Sunter and Gull 2017).

Except for *L. donovani* CPN60.2$^{+/+/+}$ no significant changes in the flagella length were observed. *L. donovani* CPN60.2$^{+/+/+}$ shows a slightly but significant increased flagellar length. However, this finding did not affect the ratio between the cell body length and the flagella length.

Nevertheless, the ratio between cell body and flagella length changed in the *L. donovani* CPN60.2$^{-/-}$ mutants to 50:50, while it remained stable in the other mutants. This results indicates either that the CPN60.2 null mutants are delayed in the procyclic phase or changed directly to leptomonad promastigotes.

Apart from that, parasite morphology remained unaltered by the lack of CPN60s.

6.10.2. In vitro proliferation

Growth characteristics of the *L. donovani* CPN60 mutants were analysed by *in vitro* proliferation assays under various conditions. The axenic promastigote form of *L. donovani* grows at 25°C in M199^{+} at a pH of 7.4. First, the growth of the promastigote *L. donovani* WT was monitored over 4 days, starting with a cell concentration of 1×10^{5}/mL. The Figure 27 shows the growth curve of *L. donovani* WT over 4 days and the proliferation of the mutant parasites relative to the WT at day 4.

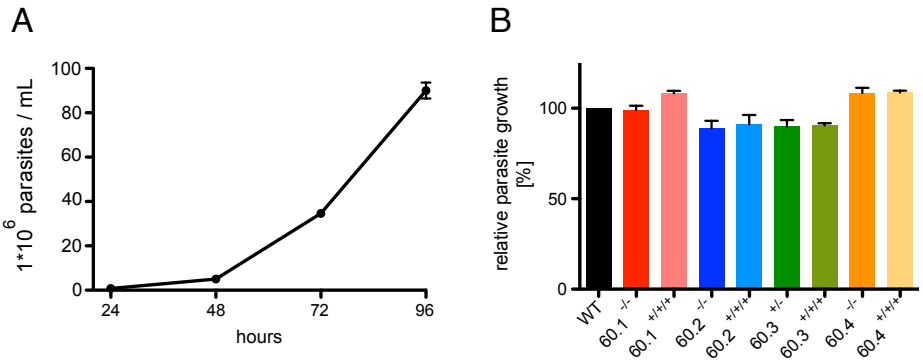


Figure 27: *L. donovani* promastigote mutants were seeded at $1*10^5$ cells mL^{-1} and grown in $M119^+$, pH = 7.4 at 25°C for 4 days. Growth kinetic of *L. donovani* WT was determined each day (**A**). Relative growth of mutants compared to the WT is displayed in (**B**). (n ≥ 3, median, student's T-test).

Figure 27 A shows the logarithmic proliferation of *L. donovani* WT over 4 days to a final concentration of ~$1×10^8$ cells/mL. Beyond that, the concentration of the cells does not increase any further (data not shown), indicating that the stationary phase is reached. The relative parasite growth of all mutants at day 4 does not show any significant differences compared to the WT. This result indicates that CPN60.1, CPN60.2, or CPN60.4 have no essential role in the promastigote proliferation while the role of CPN60.3 in this stage is essential. The *L. donovani* $CPN60.3^{+/-}$ mutant does not show any differences in proliferation compared to the WT.

As CPN60 is a chaperone and expected to play a role in the stress tolerance of the parasite, the different *L. donovani* mutants were grown under challenge with different stressors. First the pH was lowered to 5.5, which reflects the acidic pH in the parasitophorous vacuole of a macrophage.

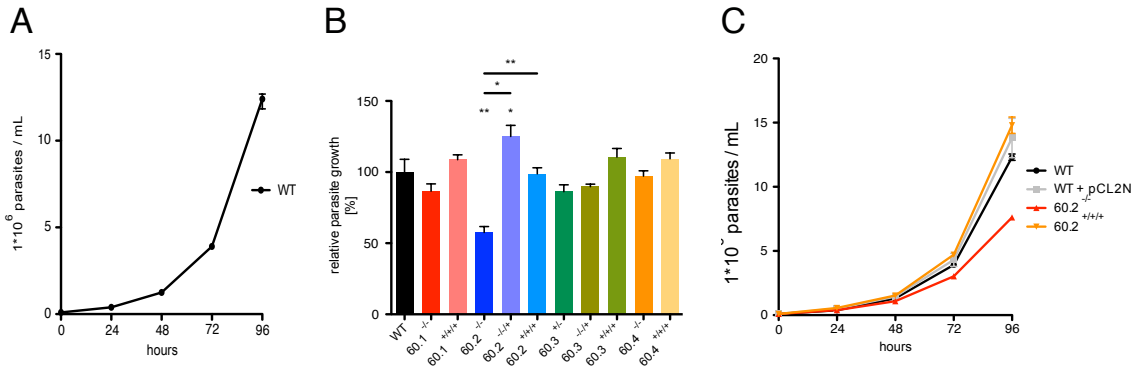


Figure 28: *L. donovani* promastigote mutants were seeded at 1*10^5 cells mL^{-1} and grown in M119$^+$, pH = 5.5 at 25°C for 4 days. Growth kinetic of *L. donovani* WT was determined each day (**A**). Relative growth of mutants compared to the WT is displayed in (**B**). Growth kinetic of *L. donovani* WT, *L. donovani* pCL2N, *L. donovani* CPN60.2$^{-/-}$, and *L. donovani* CPN60.2$^{+/+/+}$ was determined each day (**C**). (n ≥ 3, median, student's T-test).

As Figure 28 A shows, the *L. donovani* WT parasites grow logarithmic for 4 days to a final concentration of ~1.2*10^7 parasites/mL.

Parasites lacking CPN60.1 (*L. donovani* CPN60.1$^{-/-}$) or over expressing it (*L. donovani* CPN60.1$^{+/+/+}$) show no significant difference after 4 days (Figure 28 B).

L. donovani CPN60.2$^{-/-}$ parasites show only 57.5% growth compared with wild type. This effect is complemented by expression of an episomal add-back (*L. donovani* CPN60.2$^{-/-/+}$). Over expression of CPN60.2 does not lead to faster growth at acidic pH. This results indicates a protective effect of CPN60.2 in acidic environments.

To further analyse the growth deficiency of *L. donovani* CPN60.2$^{-/-}$ the growth experiments were repeated and the cell concentration recorded daily for 4 days. Figure 28 C confirms the growth defect of *L. donovani* CPN60.2$^{-/-}$ in acidic milieu starting at 48 hours. The effect became more pronounced after 72 hours, reaching the 57.5% difference after 96 hours.

L. donovani CPN60.3$^{+/-}$ and *L. donovani* CPN60.3$^{+/+/+}$ do no show any significant changes on their cell concentration after 4 days indicating that CPN60.3 does not play a pronounced role under acidic conditions (Figure 28 B).

The proliferation of *L. donovani* CPN60.4$^{-/-}$ and *L. donovani* CPN60.4$^{+/+/+}$ is comparable to the growth of the wild type and indicates that CPN60.4 is non essential at acidic milieu (Figure 28 B).

Next, I tested the sensitivity of the mutants against temperature stress. The cultivation temperature was increased from 25°C to 37°C. This reflects the temperature increase during transmission from the vector to the host. Again, *L. donovani* WT proliferation was monitored for 4 days with daily counting. At the

4-day time point, the mutants' cell density was determined relative to the wild type.

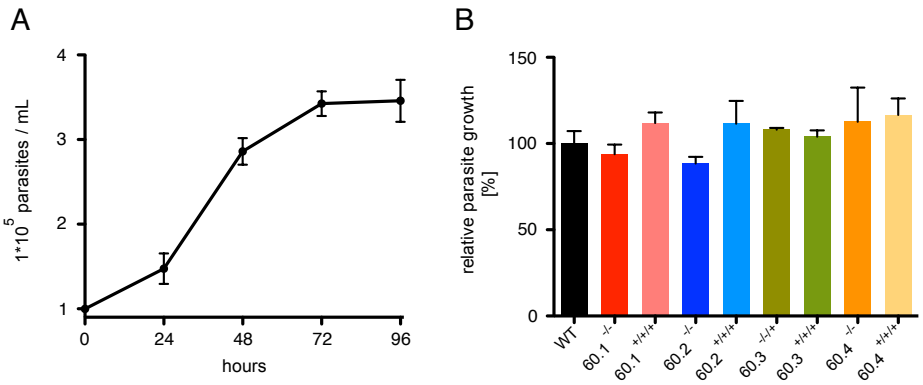


Figure 29: *L. donovani* promastigote mutants were seeded at $5*10^5$ cells mL^{-1} and grown in $M119^+$, pH = 7.4 at 37°C for 4 days. Growth kinetic of *L. donovani* WT was determined each day (**A**). Relative growth of mutants compared to the WT is displayed in (**B**). (n ≥ 3, median, student's T-test).

Figure 29 A shows that *L. donovani* WT parasites show a difference growth behaviour under heat stress, as compared to growth at 25°C or in acidic medium. After logarithmic growth for 48 hrs the maximum cell density of ~$3.4*10^5$ parasites/mL is reached after 72 hours.
When comparing the *L. donovani* CPN60 mutants with the *L. donovani* WT no significant changes in the relative proliferation at 37°C are visible (Figure 29 B). The loss or reduction of any CPN60 protein alone does not impact on the temperature tolerance.

Another well known stressor to cells is ethanol, which mimics protein folding stress. We tested the impact of ethanol on wild type and mutants. *L. donovani* WT and the *L. donovani* CPN60 mutants were grown at 25°C in $M199^+$ (pH = 7.4) supplemented with 2% EtOH and the cell concentration was recorded after 4 days.

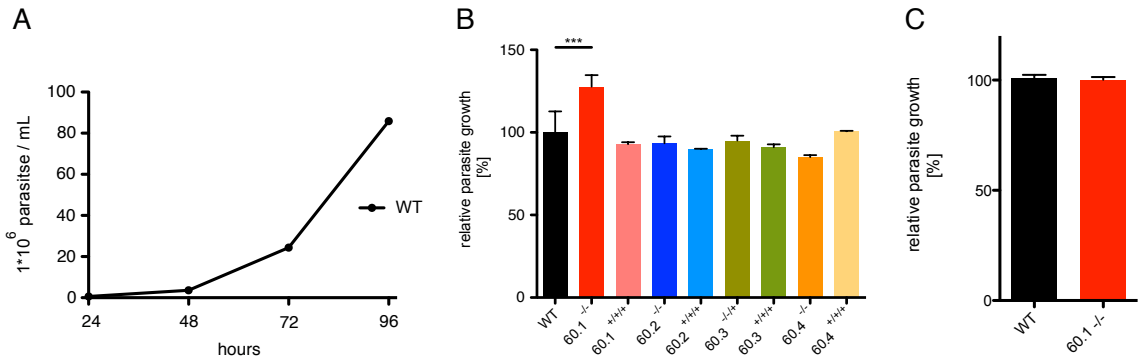


Figure 30: *L. donovani* promastigote mutants were seeded at $1*10^5$ cells mL^{-1} and grown in $M119^+$ supplemented with 2% ethanol, pH = 7.4 at 25°C for 4 days. Growth kinetic of *L. donovani* WT was determined each day (**A**). Relative growth of mutants compared to the WT is displayed in (**B**). Relative growth of *L. donovani* CPN60.1$^{-/-}$ compared to the WT after two month of culture is displayed in (**C**). (n ≥ 3, median, student's T-test).

Figure 30 A shows *L. donovani* WT parasites show a similar logarithmic growth under EtOH stress as compared to non stressed cells.
L. donovani parasites lacking CPN60.1 (*L. donovani* CPN60.1$^{-/-}$) show an increased relative parasite growth of 127.7%, as compared to the *L. donovani* WT (Figure 30 B). After two month the *L. donovani* CPN60.1$^{-/-}$ lost the phenotype of increased growth in the presence of 2% EtOH (Figure 30 C).
Mutants either lacking, or over expressing CPN60.2, CPN60.3 or CPN60.4 did not show any significant changes.

Beside ethanol other well known stressors, such as As^{III}, Sb^{III} or Miltefosine were tested. Miltefosine is a a major oral drug for the treatment of leishmaniasis. It induces apoptosis and disturbs the lipid-dependent cell singling pathway (Dorlo, Balasegaram et al. 2012). Antimony was used because another major drug, sodium stibogluconate contains Sb^V which is converted into Sb^{III}. The mechanism is not clear yet but the anti parasitic effect is mediated via the macrophage (Dissertation Tejera, 2016). As^{III} was chosen as it is chemically similar to Sb^{III} and a correlation was reported (Perry, Wyllie et al. 2013). For the analysis only the strains of *L. donovani* WT, *L. donovani* CPN60.2$^{-/-}$, *L. donovani* CPN60.2$^{+/+/+}$, *L. donovani* CPN60.3$^{+/-}$ and *L. donovani* CPN60.3$^{+/+/+}$ were chosen. The work was performed with the help of Paloma Tejera Nevado. The Figure 31 shows the relative growth depending on the drug concentration.

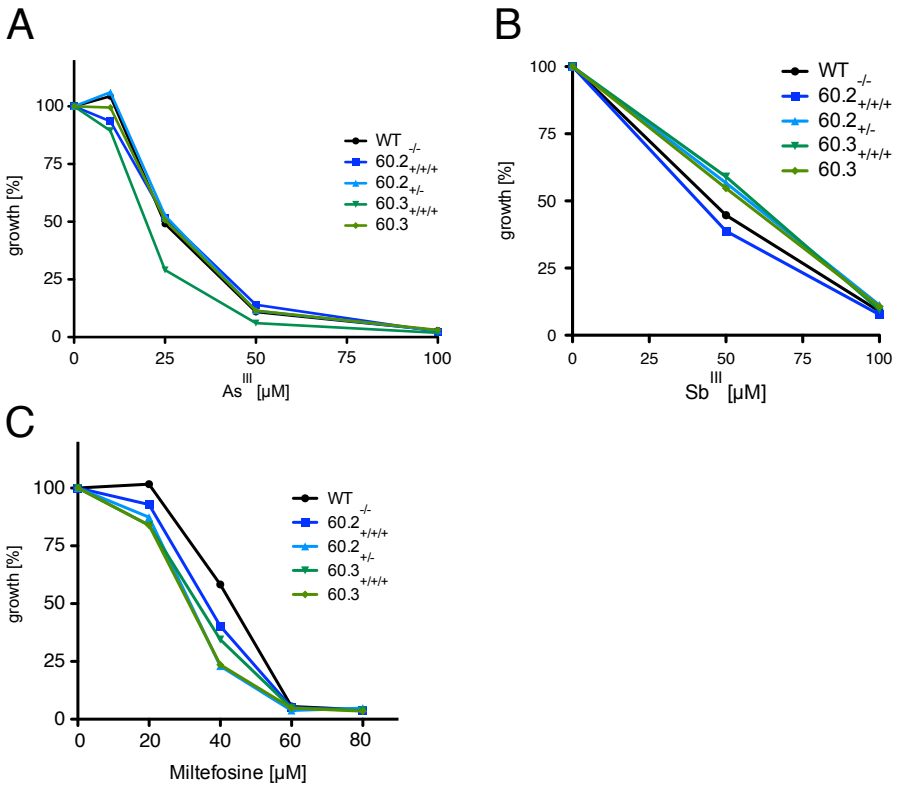


Figure 31: *L. donovani* promastigote mutants were seeded at $1*10^5$ cells mL^{-1} and grown in $M119^+$ supplemented with different drugs at various concentrations, pH = 7.4 at 25°C for 4 days. The relative growth was determined in comparison to the *L. donovani* WT and in the presence of As^{III} (**A**), Sb^{III} (**B**), and Miltefosine (**C**). (n ≥ 2, median, student's T-test).

Figure 31 shows the following IC_{50} (inhibitoric concentration) values for *L. donovani* WT. The IC_{50} value for As^{III} is 25 µM, for Sb^{III} 50 µM and for Miltefosine 40 µM. No significant changes in the relative parasite growth rate under the challenges with the three compounds could be observed for any of the mutants.

6.10.3. Ex vivo infection

To analyse the infectivity and the virulence of the mutants, in vitro infection experiments were performed. For this, murine bone marrow-derived macrophages were infected at a parasite/host cell ratio of 10 : 1. After 4 h, unbound parasites were removed and incubation was continued for up to 72 h. At 3 time points, the relative parasite load was determined by Taqman qPCR. First, the relative parasite load of *L. donovani* WT after 4 hrs, 48 hrs and 72 hrs was determined and is displayed in Figure 32.

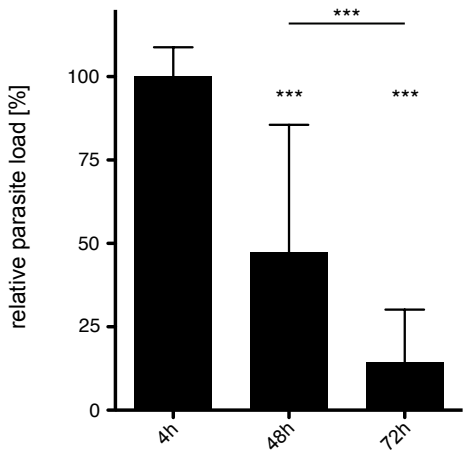


Figure 32: Relative parasite load of *L. donovani* WT in murine macrophages. Bone marrow derived macrophages were infected with *L. donovani* WT in a ratio of 1:10 for 4h and the relative parasite load was assessed after 4h, 48h and 72h. (n ≥ 4, median, student's T-test).

The parasite load drops to ~50% of the initial parasite load after 48 hrs, and to ~15% after 72 h. This result shows that the chosen infection model mimics the infection and the clearance of the parasites by macrophages.

We then analysed the initial infection rate after 4hrs and the relative parasite load after 48 hrs for the wild type and the mutants. The Figure 33 shows the initial infection rate after 4 hrs.

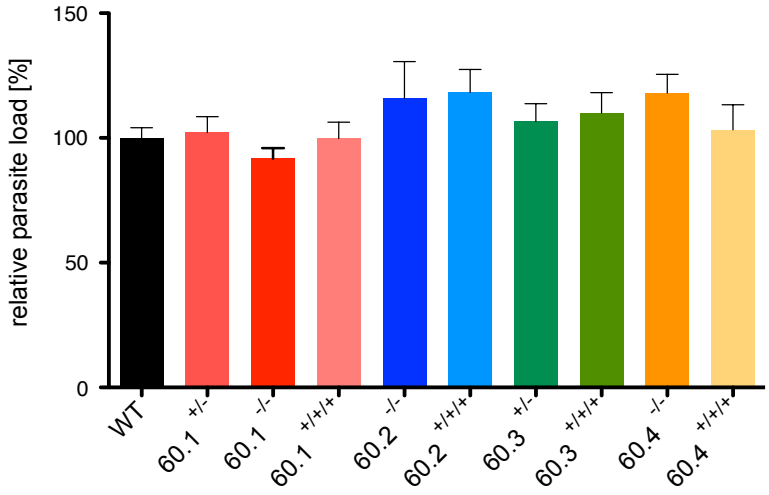


Figure 33: Relative parasite load of *L. donovani* mutants 4h p.i. of murine macrophages. (n ≥ 4, median, student's T-test).

As Figure 33 shows, no significant changes in the initial infection rate of the *L. donovani* CPN60 mutants compared to the *L. donovani* WT are observed. Obviously, CPN60 does not play a role during the process of infection.

Next, the fate of the mutants was analysed after 48 hrs.

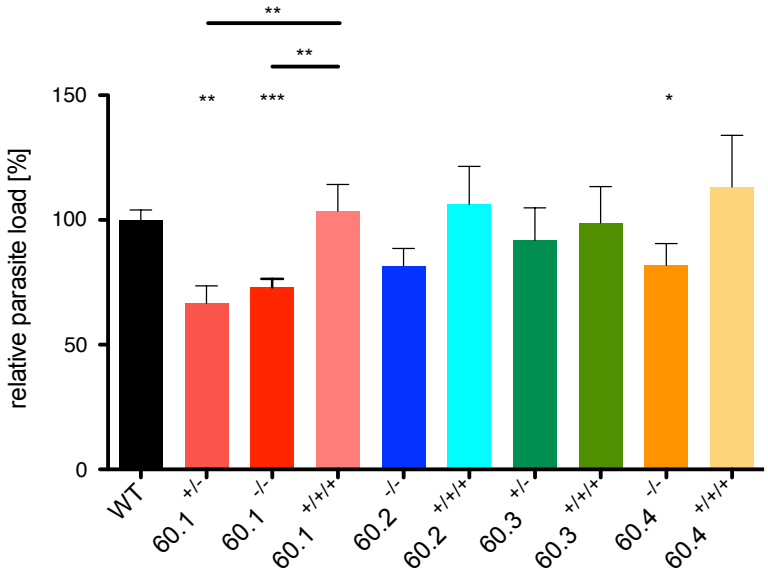


Figure 34: Relative parasite load of *L. donovani* mutants 48h p.i. of murine macrophages. (n ≥ 4, median, student's T-test).

After 48h, *L. donovani* CPN60.1$^{+/-}$ show a significantly lower parasite load relative to the wild type (66.6%). Similarly, the relative parasite load of *L. donovani* CPN60.1$^{-/-}$ is also reduced to 73% compared to the wild type. Over expression of CPN60.1 does not lead to an increased relative parasite load. The lack of CPN60.1 reduces parasite viability in the infected macrophages.
For *L. donovani* CPN60.2$^{-/-}$ and for *L. donovani* CPN60.3$^{+/-}$ no significant changes were observed, in spite of the observation that CPN60.2 protects against acidification (see 6.10.2).
L. donovani CPN60.4$^{-/-}$ show a slightly reduced relative parasite load of 81.8%, indicating a minor role of CPN60.4 for parasite survival in the infected macrophages.

6.10.4. In vitro protein expression

6.10.4.1. Promastigote protein expression

As described by Samali (Samali, Cai et al. 1999), CPN60 plays a role in several processes including programmed cell death. To study the role of CPN60 in *L. donovani* at different life stages (logarithmic, stationary, late stationary/ apoptotic phase), *L. donovani* WT parasites were grown for 14 days and the CPN60 expression was analysed by immunoblotting. Signal intensity was measured and standardised against total protein loading. The Figure 35 shows expression of CPN60.1, CPN60.4 and HSP70, as well as combined expression of CPN60.2 and CPN60.3, as it was not possible to distinguish between those two chaperonins (see 6.8).

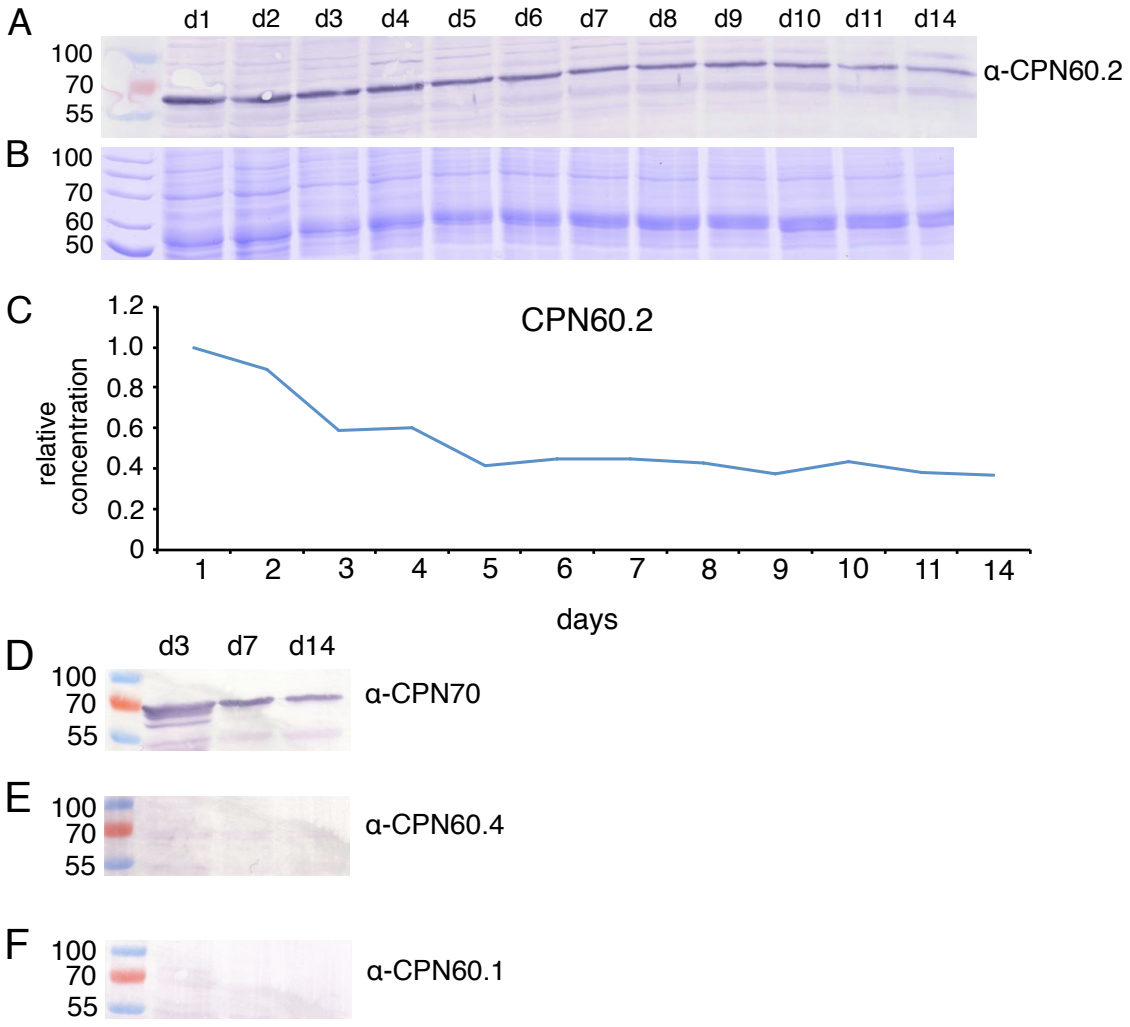


Figure 35: Relative protein abundance during promastigote proliferation. *L. donovani* WT were grown for 14 days in m199^{+}, pH = 7.4, at 25°C. Lysates were prepared each day. The immuno-blot was probed using the CPN60.2 antibody (**A**), normalised against the whole protein lysate (**B**), and the relative concentration in comparison to day 1 displayed (**C**). The immune-blots using CPN70 (**D**), CPN60.4 (**E**), and CPN60.1(**F**) are displayed below.

Figure 35 shows that abundance of CPN60.2 and/or CPN60.3 constantly decreases over the first 5 days. By day 5, which represents early stationary phase, the abundance is reduced by >50%, after which it remains stable until day 14.
Similar results were obtained for HSP70 (C), indicating that reduction of cell proliferation leads to reduced chaperone levels.
Unfortunately, the detection of CPN60.1 and CPN60.4 in the same samples failed for reasons unknown, despite repeated attempts.

6.10.4.2. In vitro stage conversion

To mimic the *Leishmania* life cycle, an in vitro stage conversion experiment was performed. In brief, promastigote parasites were grown in neutral M199^{+} (pH = 7.4) and at 25°C. After a heat shock of 37°C for 24 hrs the parasites were grown in acidic M119^{+} (pH = 5.5) at 37°C for 4 days to reach the axenic amastigote stage. The axenic amastigote-like stages were transferred back to neutral milieu and 25°C for 4 day to revert to the promastigote stage. At each step cell lysates were prepared and analysed by Western blot using anti-CPN60.2, for combined quantification of CPN60.2 and CPN60.3, and anti-CPN60.4 antibodies.

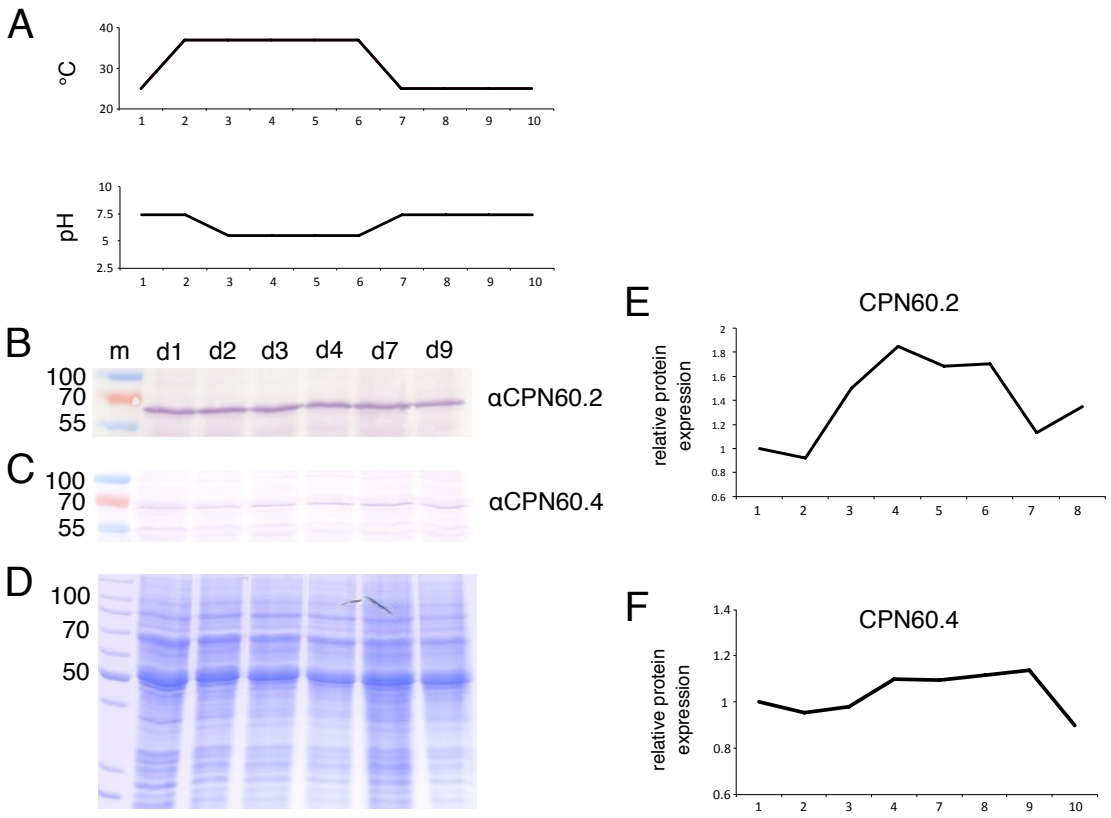


Figure 36: Protein expression during stage conversion of *L. donovani*. The temperature was changed from 25°C to 37°C while the pH was lowered from 7.4 to 5.5 at the displayed time points (**A**). Lysates were prepared each day and $1*10^7$ cells / lane loaded onto a SDS-PAGE. The Western Blots using anti-CPN60.2 antibody (**B**) and anti-CPN60.4 antibody (**C**) are displayed and were quantified by normalising against the whole protein lysate (**D**). Relative protein expression over time is shown for CPN60.2 in (**E**) and for CPN60.4 in (**F**).

Figure 36 A shows the temperature and pH change required for the stage conversion.Figure 36 B and C show the specific bands for CPN60.2 and CPN60.4 in the Western Blot while Figure 36 D shows the total protein amount loaded into the SDS gel. The relative levels of CPN60.2 and CPN60.3 are displayed in Figure 36 E and F. The relative protein expression of CPN60.2 is increased by ~1.8-fold in amastigote like parasites compared to promastigotes. This is in accordance to previous findings (Schlüter, Wiesgigl et al. 2000). Relative CPN60.4 levels remain constant throughout the in vitro life cycle.

6.10.5. Morphology during stage conversion

To analyse whether any of the CPN60s plays an essential role during stage conversion, an *in vitro* stage conversion experiment was performed using the CPN60 mutants. *L. donovani* WT was used as a control. *L. donovani* CPN60.2$^{-/-}$ and *L. donovani* CPN60.4$^{-/-}$ were used as representative null mutant strains. Anti-

tubulin and DAPI staining was performed at different time points during stage conversion.

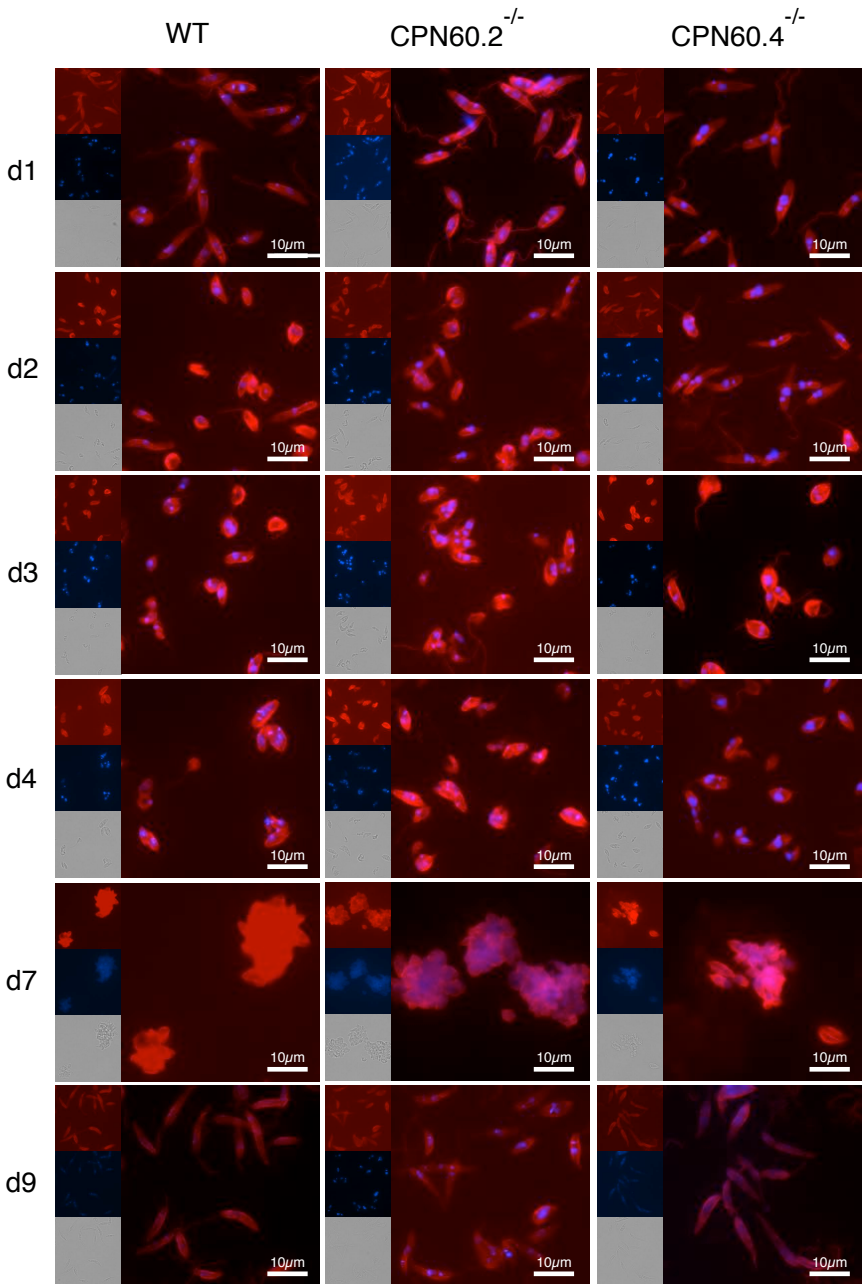


Figure 37: Immune fluorescence pictures of the CPN60 mutants during stage conversion. Promastigotes (25°C, pH = 7.4, day 1) of *L. donovani* WT, CPN60.2$^{-/-}$, and CPN60.4$^{-/-}$ were subjected to an elevated temperature (37°C, day 2). The cells were then transferred to acidic medium (pH = 5.5, day 3). After day 7 the parasites were set back to the promastigote culture conditions. Fixated cells were permeabilised, stained with anti-tubulin antibody, followed by anti-mouse IgG Alexa Fluor 594 (red) and DAPI (nucleus & kinetoplast / blue) and analysed using an EVOS microscope.

As Figure 37 shows, *L. donovani* WT parasites undergo a size reduction after heat stress and acidification, forming amastigote like parasites that form rosetta-like cell aggregates after 7 days. Transfer into neutral M199^{+} (pH = 7.4) at 25°C leads

to re-conversion into elongated promastigote forms (day 9). *L. donovani* CPN60.2$^{-/-}$ and *L. donovani* CPN60.4$^{-/-}$ undergo the same morphological changes during the *in vitro* stage conversion. I obtained identical results for mutants of *L. donovani* CPN60.1$^{-/-}$ and *L. donovani* CPN60.3$^{+/-}$, as well as for the CPN60 over expressing strains (data not shown). I conclude that none of the CPN60s play an essential role in the morphological conversions during the stage conversion process.

6.11. DiCre - based inducible gene disruption

Analysis of essential genes, such as CPN60.3, is not feasible with homologous recombination of CRISPR/cas technology since no viable null mutants can be raised. To overcome this limitation, the rapid loss of the gene of interest after induction was attempted. For this I used the DiCre approach. First, the gene of interest is flanked by two loxP sites (floxed), while the genes for two Cre recombinase subunits are expressed ectopically as fusion proteins with FKBP12 and FRB, respectively. After induction with rapamycin the two subunits are fused via FKBP12 and FRB, and a functional Cre enzyme is obtained. This cleaves the coding sequence between the two loxP sites, leading to the loss of the gene of interest.
To this end, two replacement constructs were designed containing the gene of interest in array with a LDD-LPG1-sequence and either a puroAC or a bleoR resistance marker gene for positive selection. The constructs were flanked on each site with a loxP site. For correct integration ~1000bps of the 5'-and 3'-NCs of CPN60.3 were added to the 5'- and the 3'-ends. After integration via homologous recombination, DiCre enzyme activity was induced with rapamycin. The loss of gene of interest was assessed by PCR.

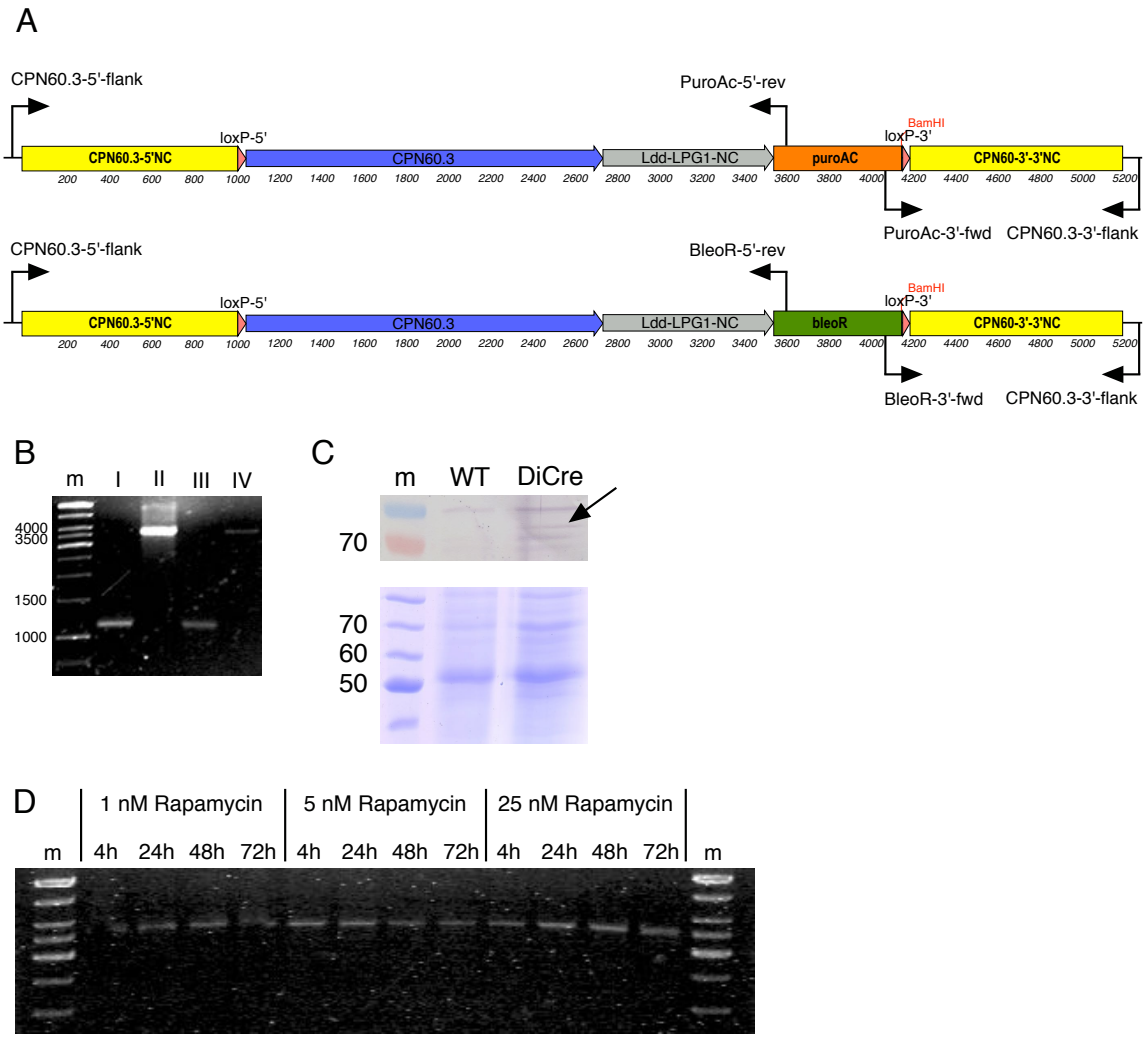
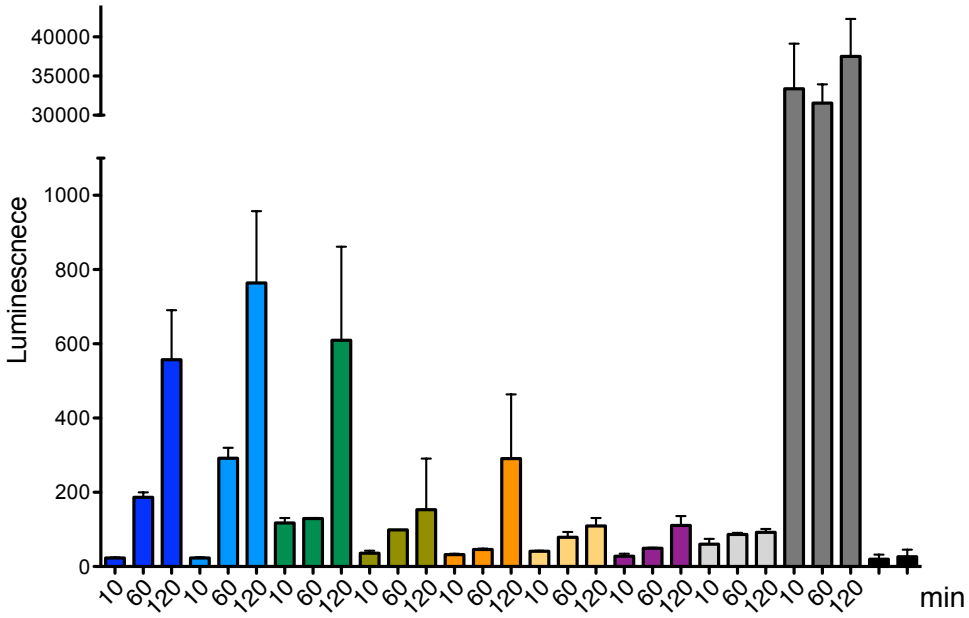


Figure 38: Representative Figure of the floxP-CPN60.3 replacement construct and the primer positions required for verification (**A**). PCR products for successful integration of the replacement construct. CPN60.3-5'-flank + PuroAC-5'-rev (line I), CPN60.3-3'-flank + PuroAC-3'-fwd (line II), CPN60.3-5'-flank + BleoR-5'-rev (line III), CPN60.3-3'-flank + BleoR-3'-fwd (line IV) (**B**). Western Blot using anti-FKBP antibody (1*10^7 cells/lane) (**C**). PCR products (CPN60.3-5'-flank + PuroAC-5'-rev) after Rapamycin induction at different concentration and time points (**D**).

After synthesis of the pre-designed insertion constructs, they were verified by sequencing (data not shown). In a first step, one allele of the gene of interest was replaced via homologous recombination (*L. donovani* CPN60.3$^{floxP+/+}$). In a second step the second gene of interest on the second allele was replaced (*L. donovani* CPN60.3$^{floxP+/floxP+}$). Correct insertion of the single-allele replacement constructs were analysed by two PCRs. First, a CPN60.3-5'-flank and either a puroAC-5'-rev or a bleoR-5'-rev primer were used (~ 3600 bp). Second, a CPN60.3-3'-flank and either a puroAC-3'-fwd or a bleoR-3'-fwd primer were used (~ 1100 bp).
Successful integration is shown in Figure 38 B. The successful expression of the Cre enzyme is shown in Figure 38 C. To show functionality of the DiCre system the first PCR was repeated after Rapamycin induction. Figure 38 D shows that induction of the DiCre enzyme does not lead to excision of the flanked CPN60.3.

6.12. Chaperone activity Assay

To test the chaperoning function of the four *Leishmania* CPN60s, a luciferase assay was established. For this, active luciferase was heat-inactivated at 42°C for 15 min. The denatured luciferase was added to soluble, recombinant CPN60s with or without soluble, recombinant CPN10 in refolding buffer. After incubating the samples at 25°C, the Luciferase activity was assessed using the Steady-Glo® Luciferase Assay System. Figure 39 shows the luciferin luminescence depending on the chaperone combinations and incubation time.



CPN60.2	X	X									
CPN60.3			X	X							
CPN60.4					X	X					
CPN10	X		X		X		X				
denatured Luciferase	X	X	X	X	X	X	X	X			
native Luciferase									X		
Steady Glo	X	X	X	X	X	X	X	X	X	X	
refolding buffer	X	X	X	X	X	X	X	X	X	X	X

Figure 39: Luciferase refolding assay. Luminescence measured after digest of luciferin (Ingredient of the Steady Glo). Luciferin was digested by refolded Luciferase. Luicferase was heat denatured at 42°C for 15 min and refolded in the presence of different chaperones at 25°C for 10 min, 60 min and 120 min.

Figure 39 shows that luciferase is refolded by CPN60.2 with and without the presence of CPN10. CPN60.2 has little refolding activity within 10 min, but luciferase activity is detectable after 60 min. Activity increases further after 120 min. By contrast, CPN60.3-mediated refolding follows a similar kinetic, but requires CPN10. Similar results are obtained for CPN60.4. Without CPN10 CPN60.4 is not able to fold luciferase while it takes 120 min in the presence of CPN10 until active luciferase is obtained. CPN10 alone does not show any folding activity.

6.13. Pull Down Interaction

To analyse which of the CPN60s are the interaction partners of CPN10, a pull down experiment was performed. For this, CPN10 was tagged with GFP and expressed from an integrated gene copy in the ribosomal RNA locus. As it is not possible to distinguish between some of the CPN60s (see 6.8), each CPN60 was tagged with a 3xHA tag and individually expressed from episomes. Using magnetic anti-GFP-beads, tagged CPN10 was pulled down together with bound proteins and subjected to SDS-PAGE and Western blot. The resulting immuno blots show two bands for each CPN60, one from the endogenously coded CPN60 and a 3-HA-tagged CPN60.X. Western blots for input proteins, unbound and bound fractions for each tagged CPN60 are shown in the Figure 40.

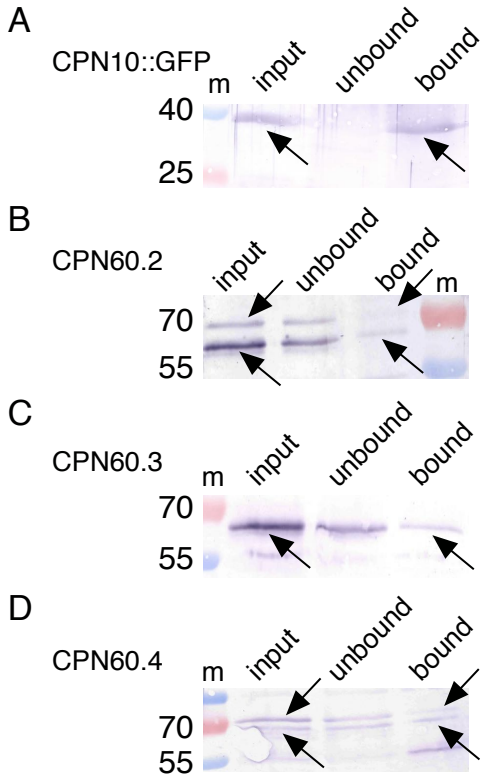


Figure 40: Western Blot of co-immuno precipitated CPN10::GFP with different CPN60.X::3HA. $1*10^8$ parasites were linked using DSP and co-immuno precipitated using anti-GFP beads. Input, unbound and bound fractions are loaded onto a SDS-PAGE and are displayed. Antibodies used: anti-CPN10, arrow indicates CPN10::GFP (**A**), anti-CPN60.2, arrows indicate CPN60.2 (bottom) and CPN60.2::3HA (top) (**B**), anti-CPN60.3, arrow indicates CPN60.3 (bottom) (**C**), and anti-CPN60.4, arrows indicate CPN60.4 (bottom) and CPN60.4::3HA (top) (**D**).

Figure 40 A shows the identification of the CPN10::GFP fusion protein in the input and bound fractions, confirming a complete pull-down of the fusion protein.
The anti-CPN60.2 antibody detects two bands in the input fraction, the native CPN60.2 and the 3-HA tagged CPN60.2 (B). Both bands are also visible in the unbound, and bound fractions, establishing CPN60.2 as an interaction partner of CPN10.
The anti-CPN60.3 antibody is not able to detect any CPN60.3::3-HA, as shown in the input fraction (C) . Therefore, it is not possible to deduce whether CPN60.3 interacts with CPN10.
Two bands are visible in the input fraction using the anti-CPN60.4 antibody. Proteins of the same size are also detected in the unbound and bound fractions, indicating that CPN60.4 too is an interaction partner of CPN10 (D).
Due to its low concentration and unreliable detection, CPN60.1 was omitted from this experiment.
At least two CPN60 chaperones, CPN60.2 and CPN60.4 are co-precipitated with CPN10::GFP, indicating a stable binding between either CPN60 species and the CPN10 co-chaperonin. Expression of 3-HA-tagged CPN60.3 could not be detected. Possible reasons for this will be discussed.

7. Discussion

Molecular chaperones are defined as proteins that assist in the folding of newly synthesised, unfolded or mis-folded proteins, or helping in the dissolution of protein aggregates. By definition, chaperones are not covalently linked to the client proteins and are not part of the final active protein complex, but act as folding catalysators (Ellis 2005, Pockley and Henderson 2018). The most thoroughly investigated protein of the CPN60/CPN10 family is the GroEL/GroES complex from *E.coli*, of which detailed insights into the molecular folding mechanism are available (Hartl 1996, Kim, Hipp et al. 2013). Orthologous proteins from other organisms are usually termed 60 kD chaperones (CPN60s) and are highly conserved throughout the phyla (Maguire, Coates et al. 2002). CPN60 genes were also found in the protozoan parasite *Leishmania donovani*, a unicellular, early branching eukaryote. Most organisms carry multiple CPN60 genes (Karlin and Brocchieri 2000), and *L.donovani* possesses four isoforms. At this point, it remains unclear why the parasite maintains 4 different CPN60s. In this context, the aim of this work was to understand the roles of the four putative 60 kDa Heat Shock Proteins in *L. donovani.*

To analyse the lineage of the *Leishmania CPN60* genes*,* a phylogenetic analysis within the Trypanosomatidae was performed. The analysis showed that CPN60.1 has the highest degree of conservation at almost 99% within the *Leishmaniae* and also to the closely related *T. brucei*, but not in *T. cruzi* (~31%). CPN60.4 too is a highly conserved protein throughout the Trypanosomatidae with similar identities as CPN60.1 between the *Leishmaniae*, also excluding *T. cruzi*. Especially the identities of CPN60.1 and CPN60.4 between *L. donovani* and *L. infantum* are close to 100%, with only ~5 silent mutations on the genome level. It is very likely that the two parasites had branched off quite late and are highly related which is in accordance to previous reports (Schonian, Kuhls et al. 2011). With regard to CPN60.1 and CPN60.4 it is obvious that both proteins are encoded by separate gene lineages, as they are located on different chromosomes with a relative low sequence identity.
Since CPN60.1 and CPN60.4 are individual proteins whose gene loci are on different chromosomes, it may be assumed, that their genes did not arise from gene duplication, but from lateral gene transfer. Due to their high conservation within the *Trypanosoma* and *Leishmania* genera they seem to play essential but individual roles in both parasitic genera.
In contrast, the neighbouring genes encoding CPN60.2 and CPN60.3 show a high sequence similarity. This finding leads to two questions. First, did CPN60.2 and

CPN60.3 arise from a recent gene duplication event? Second, can CPN60.2 and CPN60.3 compensate functionally for each other? The first question cannot be answered exactly. Nevertheless, both proteins show 83% similarity in *L. donovani*. From this I assume, that they may have arisen from gene duplication, but have evolved since then. Spontaneous gene duplication might give the parasite an advantage, as by mutating one copy without affecting the original protein the mutated protein can give the parasite an additional functionality. *L. donovani* does not show any transcriptional, but only post-transcriptional regulation and therefore, a gene duplication does not directly affect the protein concentration which might have a negative effect on the parasite (Hunter, Cook et al. 1984, Miller 1988, Argaman, Aly et al. 1994, Brandau, Dresel et al. 1995, Schlüter, Wiesgigl et al. 2000).

Another possibility is that *Leishmania* may also have obtained one or more additional CPN60s by lateral gene transfer (Vikeved, Backlund et al. 2016), as also shown for several other organisms (Karlin and Brocchieri 2000). This could also explain the high number of different, but still related CPN60s in the Trypanosomatidae family, as incorporation seems to have occurred before the genera branched off. Furthermore, maintenance of different CPN60s might give an advantage, especially with regard to the parasite's protozoan life cycle. In addition, *Leishmania* parasites, even though characterised by genome plasticity (Cruz, Titus et al. 1993, Sterkers, Lachaud et al. 2012, Laffitte, Leprohon et al. 2016), do not seem to respond to stress with rapid point mutations, but more likely with allele multiplication (Dumetz, Imamura et al. 2017). Point mutations seem to appear before the parasite encounters cell stress. The parasites harbouring a mutation might have an advantage and are more likely selected for. From this it seems possible, that *L. donovani* obtained two CPN60s through lateral gene transfer, with a high probability of gene duplication for CPN60.2 and CPN60.3.

The second question is whether the different isoformes of CPN60 can compensate for each other. The precondition for this is that they execute the same function under the same condition. Contrary, if the four CPN60s execute the same function, but at different conditions, such as temperature or pH, this indicates otherwise. It is also possible that they compensate partly for each other and have an additional unique function. The latter is supported by the fact that CPN60s from other organisms are known as "moonlighting" proteins and display additional functions beside their chaperone activity (Friedland, Shattock et al. 1993, Jeffery 2017).

To tackle the question of compensation, the role of CPN60 from other organisms is compared. *Rhizobium leguminosarum* expresses three different CPN60 isoformes and is up to my knowledge the only organism for which a complete

functional analysis had been carried out. With regard to the publications I will term these CPN60s *Rl*CPN60.1, *Rl*CPN60.2, and *Rl*CPN60.3, but I want to clarify that there is no direct link to *Ld*CPN60.1, *Ld*CPN60.2, and *Ld*CPN60.3. The three *Rl*CPN60s were expressed recombinantly and analysed via Circular Dichroism (CD) spectroscopy. The data reveal similar results for all three CPN60s (George, Kelly et al. 2004). Nevertheless, the functional analysis showed that *Rl*CPN60.1 is essential while *Rl*CPN60.2 and *Rl*CPN60.3 are non-essential. The lack of *Rl*CPN60.1 can be compensated by over expression of *Rl*CPN60.3. Additionally *Rl*CPN60.1 and *Rl*CPN60.2 are constantly expressed and show increased protein abundance after HS while *Rl*CPN60.3 shows protein abundance only under anaerobic conditions. (Rodriguez-Quinones, Maguire et al. 2005, Gould, Burgar et al. 2007). A amino acid sequence analysis between *R. leguminosarum* CPN60s and *L. donovani* CPN60s revealed 30% to 50% identity (data not shown). Additionally, a functional analysis of the three different CPN60s of *Mycobacterium smegmatis* showed that *Ms*CPN60.2 is essential and shows high protein abundance while *Ms*CPN60.1 and *Ms*CPN60.3 are non-essential and show less to no abundance, respectively. After a HS the protein levels of *Ms*CPN60.2 and *Ms*CPN60.1 increase while no *Ms*CPN60.3 levels could be detected (Rao and Lund 2010). From these data different roles of the *Ld*CPN60s seem to be quite likely. To answer this question more precisely double allele replacement mutants were required. Targeting the CPN60 orthologue from *E. coli* named GroEL resulted in non viable cells as GroEL is essential (Fayet, Ziegelhoffer et al. 1989). Further studies on double allele replacement of CPN60 were not conducted.

For specific targeting of genes in *Leishmania*, reverse genetics via homologous recombination (HR), first described by Cruz (Cruz and Beverley 1990), is still the most widely used method. Targeting the 4 CPN60s of *L. donovani* using HR to generate null mutants was successful for CPN60.2 and CPN60.4 , showing that these genes are non-essential genes. Yet, it was not possible to obtain CPN60.1 and CPN60.3 null mutants. Double gene replacement in the presence of an episomal add-back was successful for CPN60.3, proving its essentiality. Essentiality of CPN60.1 could not be verified, as no double gene replacement mutants in the presence of an episomal add-back were obtained. By using CRISPR/Cas null mutants were successfully generated for CPN60.1 and CPN60.4, but no null mutants were generated for CPN60.2 and CPN60.3. The following sections will discuss each CPN60 individually.

7.1. CPN60.1

The role of CPN60.1 in *Leishmania donovani* was unclear so far. The synthesis of the protein was verified by ribosome profiling analysis (E. Bifeld, unpublished), but could not be confirmed by Western blot analysis using a specific antibody (Schluter, Wiesgigl et al. 2000, this thesis). This finding is interesting as it is comparable to the non detectable protein levels of *Ms*CPN60.3 and *Rl*CPN60.3 (Rodriguez-Quinones, Maguire et al. 2005, Rao and Lund 2010). Furthermore, CPN60.1 is neither part of the exosomal load nor has it been shown to be recognised by sera from infected hosts. It seems that parasites and bacteria which express more than one isoform of CPN60 also maintain one CPN60 isoform expressed to non detectable levels.

For a better understanding, double allele replacement mutants of CPN60.1 were required. The generation of viable *L. donovani* CPN60.1$^{-/-}$ mutants was successful using the CRISPR/Cas method. The resulting *L. donovani* CPN60.1$^{-/-}$ parasites were in the promastigote form, proving that CPN60.1 is a non essential gene *in vitro*. Further experiments revealed that performing a full stage conversion from promastigotes to axenic amastigote-like forms and back to promastigotes was possible. From this I conclude that CPN60.1 is also neither essential in the axenic amastigote form, nor for any of both transitions. To probe CPN60.1s potential role during proliferation growth experiments under different conditions were performed. The conditions, chosen to mimic the different life cycle stages, were promastigote growth at 25°C and pH = 7.4, at acidic pH = 5.5, at elevated temperature at 37°C, and under 2% ethanol stress. Neither the *L. donovani* CPN60.1$^{-/-}$ mutants nor the CPN60.1 over expressing mutants showed a changed growth. Hence, CPN60.1 does not play a role during proliferation. CPN60.1 is also not involved in any morphogenic processes of parasite development as the immune fluorescence microscopy showed.

An interesting result was obtained when murine macrophages were infected with *L. donovani* CPN60.1$^{-/-}$ mutants or with single allele replacement mutants. The initial infection rate did not change in comparison to the WT parasites, but the parasite load after 48 hrs post infection dropped to 50%. The results were similar for the *L. donovani* CPN60.1$^{-/-}$ mutant and the single allele replacement mutant. This indicates a role for CPN60.1 in the survival of *L. donovani* within the macrophage. Yet it remains unclear whether this is due to a failure to convert to the amastigote stage or a weakened stress tolerance of the parasite, in particular in the defence against harmful molecules released by the macrophage. Furthermore, it remains unclear whether CPN60.1 might be released into the phagolysosome or the host cell cytoplasm, the latter via exosomes similar to CPN60.2 and CPN60.3 (Silverman, Clos et al. 2010), or whether it plays an

exclusively intracellular role. Nevertheless, the role of CPN60.1 for the virulence of the parasite is comparable to the one from *Mt*CPN60.1. *Mt*CPN60.1 is as *Ld*CPN60.1 a non essential gene, and *M. tuberculosis* lacking *Mt*CPN60.1 fails to induce an inflammatory response in animal models (Hickey, Ziltener et al. 2010). The macrophage model used in this thesis also lacks essential immune-modulatory effects, such as the t-cell and b-cell induced response and therefore, phenotype specific changes might be missed. This has also taken into account for the remaining three CPN60s. Contrary, it was shown by Bifeld *et al.* that exosomes isolated from *Leishmania major* stimulate murine macrophages (Bifeld 2014). To assess the role of CPN60.1 in the virulence of the parasite further research is needed.

Second, the finding that both CPN60.1$^{-/-}$ mutants and single allele replacement mutants showed the same result in the *in vitro* macrophage infection studies raises the question of CPN60.1 protein concentration inside the parasite. Since CPN60.1 abundance was not detectable on the Western Blot a very low concentration of CPN60.1 was expected. This would be in accordance with the previous finding that CPN60.1 is expressed to non detectable levels (Schlüter, Wiesgigl et al. 2000). If the low protein concentration is then further decreased by single allele replacement it might fall below a functional threshold and is similar to a null mutant. This is supported by the findings of the homologous recombination, as it was not possible to generate double allele replacement mutants, even though both replacement constructs are functional. This indicates that the resistance marker genes were not translated to a sufficient amount. Yet, it also could be possible that CPN60.1 undergoes several post-translational modifications that makes it undetectable by antibodies raised against recombinantly expressed protein. In contrast to that the specific antibodies were able to detect CPN60.1 expressed by an episomal copy. However, post-translational modifications could be limited to a certain amount while remaining CPN60.1 remains without modifications and is therefore detectable. To overcome this limitation and to answer the previous questions one endogenous allele of CPN60.1 could be tagged by CRISPR/Cas editing. A small tag such as a 3xHA tag could be integrated for a better analysis of the expression by Western Blot. A fluorescent tag could also be added for localisation experiments, especially during the life cycle and in *in vitro* infection experiments. Adding a tag to a protein however, could impair its original function or lead to a wrong localisation.

To further understand the role of CPN60.1 possible interaction partners need to be identified, which could be achieved by using the endogenous tagged proteins and performing co-immune precipitation experiments. Attempts to verify the interaction with the co-chaperone CPN10 were not successful so far (see results 6.13). Another possibility beside the co-immune precipitation is the BioID-

approach (Khosh-Naucke, Becker et al. 2017). This approach may also allow the identification of client proteins for folding. Further research should also focus on the intracellular role of CPN60.1 as a chaperone. As no recombinant native protein could be expressed, it is necessary to fill this gap in order to be able to analyse the chaperonin activity of CPN60.1 via the Luciferase Assay. To gain more information about CPN60.1 in the family of the Trypanosomatidae CPN60.1 from other phylogenetically related species should be transfected into the *L. donovani* CPN60.1$^{-/-}$ mutant and its phenotype analysed. This will also give important information about CPN60.1 as a virulence factor. Due to its high conservation throughout the Trypanosomatidae it is quite likely that the role of CPN60.1 is conserved within the different species.

7.2. CPN60.2

The CPN60.2$^{-/-}$ mutants were generated via homologous recombination (HR). The viability of the confirmed null mutants shows that CPN60.2 is a non-essential gene in the promastigote. It was also shown by stage conversion experiments that CPN60.2 is non essential for the axenic amastigote or for the process of *in vitro* stage conversion. This finding is interesting as previous work showed that CPN60.2 abundance is increased 2.5-fold during *in vitro* axenic amastigote differentiation (Schlüter, Wiesgigl et al. 2000). A possible essential role during the amastigote-like form was expected, but not confirmed since *L. donovani* CPN60.2$^{-/-}$ parasites did not show any differences compared to the WT. However, it is unclear whether CPN60.2, CPN60.3 or the abundance of both increases in axenic amastigotes, as the available antibodies cannot distinguish between the two isoforms.

For further characterisation of CPN60.2 growth kinetics were performed under different conditions. Growth analysis of the *L. donovani* CPN60.2$^{-/-}$ mutants and CPN60.2 over-expressing mutants showed that *L. donovani* CPN60.2$^{-/-}$ parasites have a reduced proliferation rate (~ 60%) under mildly acidic conditions (pH = 5.5). The growth defect of the *L. donovani* CPN60.2$^{-/-}$ mutant could be complemented by the presence of an episomal copy of CPN60.2. As the acidic conditions reflect one aspect of the phagolysosome this result strongly indicates a role of CPN60.2 either in the amastigote stage or in the stress tolerance of the promastigote. In addition, the CPN60.2$^{-/-}$ mutant showed a slightly shorter cell body under normal *in vitro* promastigote conditions compared with the WT. Furthermore, the cell body to flagellum ratio changed from 60% to 50%. This effect was also reversed by ectopic expression of CPN60.2. A shorter cell body is usually a major indicator for cell stress in *Leishmania* (Wiesgigl and Clos 2001). One may speculate that CPN60.2 plays a role in the stress response, especially

during the conversion from the promastigote to the amastigote. Yet, no differences of morphology and proliferation were observed during the stage conversion compared to the WT. Additionally, no differences to the WT were found when macrophages were infected *in vitro* with the CPN60.2$^{-/-}$mutants. This argues against an important role of CPN60.2 during infection. Contrary to this finding it was shown by Silverman *et al.* (Silverman, Clos et al. 2010) that CPN60.2 is part of the protein load of immune-modulatory exosomes. It is possible that the loss of CPN60.2 is compensated by the closely related CPN60.3. The analysis of the RNA level of CPN60.3 showed that it remained stable after double allele replacement of CPN60.2. An analysis at the protein level was not possible and it remains unclear whether the *L. donovani* CPN60.2$^{-/-}$ parasites overcome the lack of CPN60.2 with an increased abundance of CPN60.3. Other possible compensatory changes in the CPN60.2$^{-/-}$ mutants were analysed on the genome level by whole genome sequencing and comparison to the *L. donovani* wild type. A total of 7 SNPs in 6 coding sequences were identified. The most interesting candidate is the gene LdBK_100860, which encodes a hypothetical protein. Two mutations are present in this protein, one SNP and directly beside a frameshift of 1bp. By performing a BLASTn search, structural similarities were found to the Type I hyperactive antifreeze protein from *Pseudopleuronectes americanus*. So far no Cold Shock Proteins of *Leishmania* have been identified, but the Type I hyperactive antifreeze protein might indicate a possible link to them. Furthermore, the gene LdBK_100860 might encode an antagonistic protein to the *Ld*CPN60.2.

To investigate the possible chaperonin activity of CPN60.2, a luciferase refolding assay was performed. CPN60.2 has a chaperonine function independent of CPN10, which is the putative co-chaperone. It also showed that the correct folding requires around 1h. The concentration of correctly refolded luciferase increased over time. Nevertheless, the co-immune precipitation experiments confirmed that CPN60.2 binds to CPN10. The results confirm a classical chaperonin role of CPN60.2 , but raise the question question why CPN60.2 can act without CPN10. Impurities of *E. coli* GroES cannot be excluded during the native expression of the proteins, but are quite unlikely as the protein was purified by its His-tag and no protein contaminations were observed after SDS-PAGE. This issue can be further analysed by using a tryptophan fluorescence assay for the analysis of impurities as a major characteristic is the absence of tryptophan in chaperones (Todd and Lorimer 1998). Possible hypotheses for the CPN10 independent folding are that CPN60.2 can chaperone unfolded proteins either via a second folding mechanism, or that the bullet form of CPN60.2 is already sufficient for correct folding. For further research a lactate dehydrogenase (LDH) assay beside the luciferase refolding assay can be used. It was shown that this

assay is suitable for chaperones and does not require the presence of the co-chaperone CPN10 (Badcoe, Smith et al. 1991). Additionally, it can provide detailed information about the ATP dependence (George, Kelly et al. 2004). Nevertheless, the folding assays have to be performed with a few variations in terms of CPN60.2 conformation to answer that question correctly.
Future research should also focus on the role of CPN60.2 in the amastigote stage and during the conversion into the amastigote and the role of CPN60.2 in the exosomes must be clarified. The first question may be answered by endogenous fluorescent tagging of CPN60.2 and intracellular localisation. CPN60.2 from other trypanosomatid parasites could be ectopically expressed in the *L. donovani* CPN60.2$^{-/-}$ mutant and the generation of CPN60.3 double allele replacement mutants tried. This would give further insight into possible compensatory mechanisms. Additionally, the possible antagonist of the CPN60.2, LdBK_100860, can be over expressed to test the impact on the *Leishmania* phenotype.

7.3. CPN60.3

Several attempts to replace or delete the *L. donovani CPN60.3* alleles using HR and two attempts using CRISPR/Cas failed to produce viable *Leishmania* promastigotes. Generation of single allele replacement mutants via HR was possible using both gene replacement constructs, verifying their correct function. To further study the role of the essential CPN60.3, the inducible loxP-system was tried, but this was not successful. These results show that CPN60.3 is an essential gene in *Leishmania* whose loss cannot be complemented by the other CPN60 paralogues, as it is discussed for CPN60.2. As none of the other 3 CPN60s is essential, CPN60.3 appears to be the crucial CPN60 chaperone for *Leishmania* viability. The other 3 chaperonins may have a limited or a completely different function. It might also be that CPN60.3 requires under specific conditions other CPN60s to form more stable or more active heteromeric chaperone-complexes. This might depend on the pH-value and the temperature and could have a minor impact on the growth.
To analyse the intracellular role of CPN60.3 a luciferase refolding assay and co-immune precipitation experiments were carried out. CPN60.3 requires CPN10 to refold denatured luciferase. In contrast to CPN60.2, CPN60.3 is not able to fold luciferase without CPN10. CPN60.3 shows slower folding kinetics than CPN60.2. Active luciferase was first detectable after 120 min. From this I hypothesise that either CPN60.2 might function as an unspecific chaperone, which acts as a fast response to stress while CPN60.3 is the main CPN60 chaperone that is quite specific for defined client proteins or vice versa CPN60.3 is unspecific and compensate for the specific CPN60.2 while CPN60.2 can not cover CPN60.3.

Nevertheless, it has to be taken into account that these luciferase refolding data are preliminary and that the experiments have to be repeated to verify their results. Interestingly, no stable interaction between CPN60.3 and CPN10 was verified in the co-immune precipitation experiments. This might be due to the peptide tags impairing binding. To further analyse the interaction, interaction experiments followed by mass-spectrometry (MS) analysis could be performed as described by Kerner, *et al.* (Kerner, Naylor et al. 2005).

Using the single allele gene replacement mutant CPN60.3$^{+/-}$, no impact of CPN60.3 on the infectivity or the virulence of *L. donovani* could be observed, even though CPN60.3 was found to be part of the exosomal load (Silverman, Clos et al. 2010). Also, no impact on the parasite's morphology or on its proliferation was observed for CPN60.3$^{-/+}$ *in vitro*. The experiments were carried out with single allele replacement mutants and CPN60.3 over-expressing mutants and cannot be compared with the true null mutants obtained for the other CPN60 genes. Other organisms expressing more than one CPN60 isoform also contain one CPN60 which cannot or just partly be complemented by the remaining CPN60s. The CPN60.2 from *M. tuberculosis* is also essential while the *Mt*CPN60.1 cannot complement. The *R. leguminosarum* CPN60.1 is the essential chaperone, but can be complemented by 4-fold over-expression of CPN60.3.

As CPN60.3 is the essential CPN60 chaperone of *Leishmania* further research is required. Experimental setups should focus on establishing a functional loxP-system for phenotypical analysis. Furthermore, the complete chaperone function should be analysed via the Luciferase Assay under different conditions, such as the temperature or the pH-value also including different concentrations of the other three CPN60s. Also endogenous fluorescent tagging might confirm its localisation and could be used in the axenic amastigote like form as well as during infection experiments. Domain swapping into the different CPN60s could give additional information about the structure.

7.4. CPN60.4

CPN60.4 is a non essential gene in *L. donovani*. CPN60.4$^{-/-}$ mutants were obtained by using HR and CRISPR/Cas. The role of CPN60.4 in *Leishmania* had not been investigated before. Only one publication reported that sera from infected dogs were positive for CPN60.4 (Agallou, Athanasiou et al. 2016). CPN60.4 is also not known as part of the exosomal protein load as described by Silverman *et al.* (Silverman, Clos et al. 2010). CPN60.4 displays in contrast to the other three CPN60s one major change in its amino acid sequence. It contains one tryptophan at position 105 which is completely atypical for chaperones.

The phenotype analysis of the CPN60.4$^{-/-}$ mutants showed a small and weakly significant reduction of the cell body length, which did neither alter the flagellum length nor the cell body to flagellum ratio significantly. Over expression of CPN60.4 did not affect cell body length. No other phenotypical changes were observed and no data indicate that CPN60.4 is involved either in the infectivity or the virulence of *L. donovani*.

Analysis of the protein abundance during the stage conversion *in vitro* showed that the CPN60.4 abundance remained stable. Unfortunately, the specific CPN60.4 antibody lost its specificity quite rapidly and results could not be confirmed at a later time point.

The chaperone activity of CPN60.4 was assessed by the luciferase refolding assay and co-immune precipitation experiments. The latter showed a stable interaction between CPN60.4 and CPN10 strongly indicating a chaperonin function. In contrast, no chaperone activity could be verified in a first luciferase refolding assay. At this point, the role of CPN60.4 remains unclear, but it can be speculated that CPN60.4, like the other non essential chaperones, may have a chaperone function at specific points in the parasitic life cycle.

7.5. Comparison HR vs CRISPR/Cas

In the course of this thesis, three methods for gene editing were used: homologous recombination, the CRISPR/Cas system, and the loxP-system. Of these three, the first two were successfully applied while no positive results were obtained for the loxP-system. Even so, the results obtained with HR and CRISPR/cas were different. Using HR, viable double allele replacement mutants for CPN60.2 and CPN60.4 were obtained, but not for CPN60.1 and CPN60.3. Using CRISPR/Cas, resulted in viable double allele replacement mutants for CPN60.1, but not for CPN60.2. Comparable results were obtained for CPN60.3 and CPN60.4.

When using HR, the time required until viable double allele replacement mutants are obtained is at least 45 days while viable null mutants mutants can be obtained using CRISPR/Cas within 10 days. This rapid process of CRISPR/Cas gives the parasite less time to compensate the gene editing with off-target gene rearrangements, possibly exacerbating the phenotype of a mutation. Double allele replacement mutants generated via HR might not show any phenotype as the loss of the gene is complemented by off-target gene rearrangements. During the selection of the parasites, those with genetic adaptions may have a fitness advantage. In the context of CPN60.2 such compensatory effects have to be investigated, as HR-mediated replacement allowed the generation of *L. donovani* CPN60.2$^{-/-}$ mutants while CRISPR/Cas did not. The results of a whole genome

sequencing revealed no obvious differences when compared to the WT. The hypothesis that the loss of a gene might be compensated by amplification of another gene (Dumetz, Imamura et al. 2017) was not confirmed in this study.

CRISPR/Cas is more suitable when targeting genes with a low expression. By targeting CPN60.1 via HR it was shown that both insertion constructs are suitable, but it was not possible to generate double allele replacement mutants. The primary idea that CPN60.1 is an essential gene was refuted when *L. donovani* CPN60.1$^{-/-}$ mutants were readily obtained using CRISPR/Cas. It is conceivable that the NC regions flanking CPN60.1 gene sides may play a role, as was shown for the *L. major* virulence factor P46 (Bifeld 2014). The inserted replacement constructs of CRISPR/Cas are pre-designed with non-specific NCs that facilitate the expression of the resistance marker genes.

Another difference of the CRISPR/Cas system is that the parasites that are engineered are not WT parasites. The parental parasites have to express the Cas9 endonuclease and the T7-RNA-polymerase, necessitating selection with two more selection antibiotics. As shown in 6.3.1, this has an effect on the proliferation. This growth deficiency is lost within a few *in vitro* passages. The necessity to express two more proteins can be overcome by transfecting these two proteins as recombinant proteins directly into the parasite (Soares Medeiros, South et al. 2017). This would also reduce the side effects of the additional protein synthesis, while allowing gene editing.

A big advantage of the CRISPR/Cas system in comparison to HR is that this system can be used as a high-throughput system. As soon as the Cas9 and T7-RNA-polymerase expressing parasites are generated, only two PCR reactions are required per GOI, at least allowing in theory the large scale mutational analysis of entire gene families in short time.

Bibliography

1. Abdeen, S., N. Salim, N. Mammadova, C. M. Summers, K. Goldsmith-Pestana, D. McMahon-Pratt, P. G. Schultz, A. L. Horwich, E. Chapman and S. M. Johnson (2016). "Targeting the HSP60/10 chaperonin systems of Trypanosoma brucei as a strategy for treating African sleeping sickness." Bioorg Med Chem Lett **26**(21): 5247-5253.
2. Agallou, M., E. Athanasiou, M. Samiotaki, G. Panayotou and E. Karagouni (2016). "Identification of Immunoreactive Leishmania infantum Protein Antigens to Asymptomatic Dog Sera through Combined Immunoproteomics and Bioinformatics Analysis." PLoS One **11**(2): e0149894.
3. Akhoundi, M., K. Kuhls, A. Cannet, J. Votypka, P. Marty, P. Delaunay and D. Sereno (2016). "A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies." PLoS Negl Trop Dis **10**(3): e0004349.
4. Albert, H., E. C. Dale, E. Lee and D. W. Ow (1995). "Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome." Plant J **7**(4): 649-659.
5. Alsford, S., D. J. Turner, S. O. Obado, A. Sanchez-Flores, L. Glover, M. Berriman, C. Hertz-Fowler and D. Horn (2011). "High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome." Genome Res **21**(6): 915-924.
6. Alvar, J., I. D. Velez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin and M. den Boer (2012). "Leishmaniasis worldwide and global estimates of its incidence." PloS One **7**(5): e35671.
7. Andenmatten, N., S. Egarter, A. J. Jackson, N. Jullien, J. P. Herman and M. Meissner (2013). "Conditional genome engineering in Toxoplasma gondii uncovers alternative invasion mechanisms." Nat Methods **10**(2): 125-127.
8. Argaman, M., R. Aly and M. Shapira (1994). "Expression of heat shock protein 83 in Leishmania is regulated post- transcriptionally." Mol Biochem Parasitol **64**(1): 95-110.
9. Badcoe, I. G., C. J. Smith, S. Wood, D. J. Halsall, J. J. Holbrook, P. Lund and A. R. Clarke (1991). "Binding of a chaperonin to the folding intermediates of lactate dehydrogenase." Biochemistry **30**(38): 9195-9200.
10. Banuls, A. L., M. Hide and F. Prugnolle (2007). "Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans." Adv Parasitol **64**: 1-109.
11. Barak, E., S. Amin-Spector, E. Gerliak, S. Goyard, N. Holland and D. Zilberstein (2005). "Differentiation of Leishmania donovani in host-free system: analysis of signal perception and response." Mol Biochem Parasitol **141**(1): 99-108.
12. Bates, P. A. (1993). "Axenic amastigote culture of*Leishmania*amastigotes." Parasitol. Today **9**: 143-146.
13. Beneke, T., R. Madden, L. Makin, J. Valli, J. Sunter and E. Gluenz (2017). "A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids." R Soc Open Sci **4**(5): 170095.
14. Bifeld, E. (2014). Der Virulenzfaktor P46 und seine Bedeutung für Wirtsspezifität und Infektiosität des Parasiten Leishmania major [Bray et al., 1973]. Doctor rer. nat., University of Hamburg.

15. Bifeld, E., P. Tejera Nevado, J. Bartsch, J. Eick and J. Clos (2016). "A versatile qPCR assay to quantify trypanosomatidic infections of host cells and tissues." Med Microbiol Immunol **205**(5): 449-458.
16. Birk, O. S., S. L. Gur, D. Elias, R. Margalit, F. Mor, P. Carmi, J. Bockova, D. M. Altmann and I. R. Cohen (1999). "The 60-kDa heat shock protein modulates allograft rejection." Proc Natl Acad Sci U S A **96**(9): 5159-5163.
17. Brandau, S., A. Dresel and J. Clos (1995). "High constitutive levels of heat-shock proteins in human-pathogenic parasites of the genus Leishmania." Biochem J **310**(Pt 1): 225-232.
18. Bringaud, F., S. Peyruchaud, D. Baltz, C. Giroud, L. Simpson and T. Baltz (1995). "Molecular characterization of the mitochondrial heat shock protein 60 gene from Trypanosoma brucei." Mol Biochem Parasitol **74**(1): 119-123.
19. Bukau, B. and A. L. Horwich (1998). "The Hsp70 and Hsp60 chaperone machines." Cell **92**(3): 351-366.
20. Bulut, Y., E. Faure, L. Thomas, H. Karahashi, K. S. Michelsen, O. Equils, S. G. Morrison, R. P. Morrison and M. Arditi (2002). "Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway." J Immunol **168**(3): 1435-1440.
21. Cheng, M. Y., F. U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg and A. L. Horwich (1989). "Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria." Nature **337**(6208): 620-625.
22. Clark, C. G. and A. J. Roger (1995). "Direct evidence for secondary loss of mitochondria in Entamoeba histolytica." Proc Natl Acad Sci U S A **92**(14): 6518-6521.
23. Clayton, C. E. (2002). "Life without transcriptional control? From fly to man and back again." Embo J **21**(8): 1881-1888.
24. Clos, J. and S. Brandau (1994). "pJC20 and pJC40— two high-copy-number vectors for T7 RNA polymerase-dependent expression of recombinant genes in*Escherichia coli*." Prot. Expression Purif. **5**: 133-137.
25. Clos, J., S. Brandau and C. and Hoyer (1998). "Chemical stress does not induce heat shock protein synthesis in*Leishmania donovani*." Protist **149**(2): 167-172.
26. Colineau, L., J. Clos, K. M. Moon, L. J. Foster and N. E. Reiner (2017). "Leishmania donovani chaperonin 10 regulates parasite internalization and intracellular survival in human macrophages." Med Microbiol Immunol **206**(3): 235-257.
27. Cong, L., F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini and F. Zhang (2013). "Multiplex genome engineering using CRISPR/Cas systems." Science **339**(6121): 819-823.
28. Coulson, R. M. and D. F. Smith (1990). "Isolation of genes showing increased or unique expression in the infective promastigotes of Leishmania major." Mol Biochem Parasitol **40**(1): 63-75.
29. Cruz, A. and S. M. Beverley (1990). "Gene replacement in parasitic protozoa." Nature **348**: 171-173.
30. Cruz, A. K., R. Titus and S. M. Beverley (1993). "Plasticity in chromosome number and testing of essential genes in Leishmania by targeting." Proc Natl Acad Sci U S A **90**(4): 1599-1603.

31. Cupolillo, E., E. Medina-Acosta, H. Noyes, H. Momen and G. Grimaldi, Jr. (2000). "A revised classification for Leishmania and Endotrypanum." Parasitol Today **16**(4): 142-144.
32. Das, A., C. Syin, H. Fujioka, H. Zheng, N. Goldman, M. Aikawa and N. Kumar (1997). "Molecular characterization and ultrastructural localization of Plasmodium falciparum Hsp 60." Mol Biochem Parasitol **88**(1-2): 95-104.
33. Ditzel, L., J. Lowe, D. Stock, K. O. Stetter, H. Huber, R. Huber and S. Steinbacher (1998). "Crystal structure of the thermosome, the archaeal chaperonin and homolog of CCT." Cell **93**(1): 125-138.
34. Dorlo, T. P., M. Balasegaram, J. H. Beijnen and P. J. de Vries (2012). "Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis." J Antimicrob Chemother **67**(11): 2576-2597.
35. Dumetz, F., H. Imamura, M. Sanders, V. Seblova, J. Myskova, P. Pescher, M. Vanaerschot, C. J. Meehan, B. Cuypers, G. De Muylder, G. F. Spath, G. Bussotti, J. R. Vermeesch, M. Berriman, J. A. Cotton, P. Volf, J. C. Dujardin and M. A. Domagalska (2017). "Modulation of Aneuploidy in Leishmania donovani during Adaptation to Different In Vitro and In Vivo Environments and Its Impact on Gene Expression." MBio **8**(3).
36. Duncan, S. M., E. Myburgh, C. Philipon, E. Brown, M. Meissner, J. Brewer and J. C. Mottram (2016). "Conditional gene deletion with DiCre demonstrates an essential role for CRK3 in Leishmania mexicana cell cycle regulation." Mol Microbiol **100**(6): 931-944.
37. Ellis, R. J. (1990). "The molecular chaperone concept." Semin Cell Biol **1**(1): 1-9.
38. Ellis, R. J. (2005). "Chaperomics: in vivo GroEL function defined." Curr Biol **15**(17): R661-663.
39. Fayet, O., T. Ziegelhoffer and C. Georgopoulos (1989). "The groES and groEL heat shock gene products of Escherichia coli are essential for bacterial growth at all temperatures." J Bacteriol **171**(3): 1379-1385.
40. Feldman, D. E. and J. Frydman (2000). "Protein folding in vivo: the importance of molecular chaperones." Curr Opin Struct Biol **10**(1): 26-33.
41. Folgueira, C. and J. M. Requena (2007). "A postgenomic view of the heat shock proteins in kinetoplastids." FEMS microbiology reviews **31**(4): 359-377.
42. Friedland, J. S., R. Shattock, D. G. Remick and G. E. Griffin (1993). "Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells." Clin Exp Immunol **91**(1): 58-62.
43. Gasiunas, G., R. Barrangou, P. Horvath and V. Siksnys (2012). "Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria." Proc Natl Acad Sci U S A **109**(39): E2579-2586.
44. George, R., S. M. Kelly, N. C. Price, A. Erbse, M. Fisher and P. A. Lund (2004). "Three GroEL homologues from Rhizobium leguminosarum have distinct in vitro properties." Biochem Biophys Res Commun **324**(2): 822-828.
45. Goloubinoff, P., J. T. Christeller, A. A. Gatenby and G. H. Lorimer (1989). "Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfoleded state depends on two chaperonin proteins and Mg-ATP." Nature **342**(6252): 884-889.

46. Gould, P. S., H. R. Burgar and P. A. Lund (2007). "Homologous cpn60 genes in Rhizobium leguminosarum are not functionally equivalent." Cell Stress Chaperones **12**(2): 123-131.
47. Gupta, R. S. (1990). "Sequence and structural homology between a mouse T-complex protein TCP-1 and the 'chaperonin' family of bacterial (GroEL, 60-65 kDa heat shock antigen) and eukaryotic proteins." Biochem Int **20**(4): 833-841.
48. Hartl, F. U. (1996). "Molecular chaperones in cellular protein folding." Nature **381**(6583): 571-579.
49. Henderson, B. (2010). "Integrating the cell stress response: a new view of molecular chaperones as immunological and physiological homeostatic regulators." Cell Biochem Funct **28**(1): 1-14.
50. Hickey, T. B., H. J. Ziltener, D. P. Speert and R. W. Stokes (2010). "Mycobacterium tuberculosis employs Cpn60.2 as an adhesin that binds CD43 on the macrophage surface." Cell Microbiol **12**(11): 1634-1647.
51. Hu, Y., B. Henderson, P. A. Lund, P. Tormay, M. T. Ahmed, S. S. Gurcha, G. S. Besra and A. R. Coates (2008). "A Mycobacterium tuberculosis mutant lacking the groEL homologue cpn60.1 is viable but fails to induce an inflammatory response in animal models of infection." Infect Immun **76**(4): 1535-1546.
52. Hübel, A., S. Brandau, A. Dresel and J. Clos (1995). "A member of the ClpB family of stress proteins is expressed during heat shock in Leishmania spp." Mol Biochem Parasitol **70**(1-2): 107-118.
53. Hübel, A., S. Krobitsch, A. Horauf and J. Clos (1997). "Leishmania major Hsp100 is required chiefly in the mammalian stage of the parasite." Mol Cell Biol **17**(10): 5987-5995.
54. Hunter, K. W., C. L. Cook and E. G. Hayunga (1984). "Leishmanial differentiation in vitro: induction of heat shock proteins." Biochem Biophys Res Commun **125**(2): 755-760.
55. Ishida, R., T. Okamoto, F. Motojima, H. Kubota, H. Takahashi, M. Tanabe, T. Oka, A. Kitamura, M. Kinjo, M. Yoshida, M. Otaka, E. Grave and H. Itoh (2018). "Physicochemical Properties of the Mammalian Molecular Chaperone HSP60." Int J Mol Sci **19**(2).
56. Jeffery, C. J. (2017). "Moonlighting proteins - nature's Swiss army knives." Sci Prog **100**(4): 363-373.
57. Jeffery, C. J., B. J. Bahnson, W. Chien, D. Ringe and G. A. Petsko (2000). "Crystal structure of rabbit phosphoglucose isomerase, a glycolytic enzyme that moonlights as neuroleukin, autocrine motility factor, and differentiation mediator." Biochemistry **39**(5): 955-964.
58. Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier (2012). "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Science **337**(6096): 816-821.
59. Jullien, N., F. Sampieri, A. Enjalbert and J. P. Herman (2003). "Regulation of Cre recombinase by ligand-induced complementation of inactive fragments." Nucleic Acids Res **31**(21): e131.
60. Karlin, S. and L. Brocchieri (2000). "Heat shock protein 60 sequence comparisons: duplications, lateral transfer, and mitochondrial evolution." Proc Natl Acad Sci U S A **97**(21): 11348-11353.

61. Kerner, M. J., D. J. Naylor, Y. Ishihama, T. Maier, H. C. Chang, A. P. Stines, C. Georgopoulos, D. Frishman, M. Hayer-Hartl, M. Mann and F. U. Hartl (2005). "Proteome-wide analysis of chaperonin-dependent protein folding in Escherichia coli." Cell **122**(2): 209-220.
62. Khosh-Naucke, M., J. Becker, P. Mesen-Ramirez, P. Kiani, J. Birnbaum, U. Frohlke, E. Jonscher, H. Schluter and T. Spielmann (2017). "Identification of novel parasitophorous vacuole proteins in P. falciparum parasites using BioID." Int J Med Microbiol.
63. Kim, Y. E., M. S. Hipp, A. Bracher, M. Hayer-Hartl and F. U. Hartl (2013). "Molecular chaperone functions in protein folding and proteostasis." Annual review of biochemistry **82**: 323-355.
64. Kol, A., T. Bourcier, A. H. Lichtman and P. Libby (1999). "Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages." J Clin Invest **103**(4): 571-577.
65. Krobitsch, S., S. Brandau, C. Hoyer, C. Schmetz, A. Hübel and J. Clos (1998). "*Leishmania donovani*heat shock protein 100: characterization and function in amastigote stage differentiation." J. Biol. Chem. **273**: 6488-6494.
66. Krobitsch, S. and J. Clos (1999). "A novel role for 100 kD heat shock proteins in the parasite*Leishmania donovani*." Cell Stress Chaperones **4**(3): 191-198.
67. Laffitte, M. N., P. Leprohon, B. Papadopoulou and M. Ouellette (2016). "Plasticity of the Leishmania genome leading to gene copy number variations and drug resistance." F1000Res **5**: 2350.
68. Lukes, J., D. L. Guilbride, J. Votypka, A. Zikova, R. Benne and P. T. Englund (2002). "Kinetoplast DNA network: evolution of an improbable structure." Eukaryot Cell **1**(4): 495-502.
69. Luna, E., E. Postol, C. Caldas, L. A. Benvenuti, J. M. J. Rodrigues, K. Lima, J. Kalil and V. Coelho (2007). "Treatment with encapsulated Hsp60 peptide (p277) prolongs skin graft survival in a murine model of minor antigen disparity." Scand J Immunol **66**(1): 62-70.
70. Ma, G. Y., J. Z. Zhang, G. R. Yin, J. H. Zhang, X. L. Meng and F. Zhao (2009). "Toxoplasma gondii: proteomic analysis of antigenicity of soluble tachyzoite antigen." Exp Parasitol **122**(1): 41-46.
71. Maguire, M., A. R. Coates and B. Henderson (2002). "Chaperonin 60 unfolds its secrets of cellular communication." Cell Stress Chaperones **7**(4): 317-329.
72. Martin, J., T. Langer, R. Boteva, A. Schramel, A. L. Horwich and F. U. Hartl (1991). "Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate." Nature **352**(6330): 36-42.
73. Miller, J. (1988). "Effects of temperature elevation on mRNA and protein synthesis in Leishmania mexicana amazonensis." Mol. Biochem. Parasitol. **30**: 175-184.
74. Nahas, E. A., J. Nahas-Neto, C. L. Orsatti, A. P. Tardivo, G. Uemura, M. T. Peracoli and S. S. Witkin (2014). "The 60- and 70-kDa heat-shock proteins and their correlation with cardiovascular risk factors in postmenopausal women with metabolic syndrome." Cell Stress Chaperones **19**(4): 559-568.
75. Netzer, W. J. and F. U. Hartl (1997). "Recombination of protein domains facilitated by co-translational folding in eukaryotes." Nature **388**(6640): 343-349.

76. Nisemblat, S., O. Yaniv, A. Parnas, F. Frolow and A. Azem (2015). "Crystal structure of the human mitochondrial chaperonin symmetrical football complex." Proc Natl Acad Sci U S A **112**(19): 6044-6049.
77. Ohashi, K., V. Burkart, S. Flohe and H. Kolb (2000). "Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-Like Receptor-4 Complex." The Journal of Immunology **164**(2): 558-561.
78. Osterloh, A., U. Kalinke, S. Weiss, B. Fleischer and M. Breloer (2007). "Synergistic and differential modulation of immune responses by Hsp60 and lipopolysaccharide." J Biol Chem **282**(7): 4669-4680.
79. Ostermann, J., A. L. Horwich, W. Neupert and F. U. Hartl (1989). "Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis." Nature **341**(6238): 125-130.
80. Padma Priya, P., M. Grover, U. S. Tatu and V. Natarajan (2015). "Characterization of Precursor PfHsp60 in Plasmodium falciparum Cytosol during Its Asexual Development in Human Erythrocytes." PLoS One **10**(8): e0136401.
81. Perry, M. R., S. Wyllie, A. Raab, J. Feldmann and A. H. Fairlamb (2013). "Chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs in a mouse model of visceral leishmaniasis." Proceedings of the National Academy of Sciences of the United States of America **110**(49): 19932-19937.
82. Pockley, A. G. and B. Henderson (2018). "Extracellular cell stress (heat shock) proteins-immune responses and disease: an overview." Philos Trans R Soc Lond B Biol Sci **373**(1738).
83. Pockley, A. G., R. Wu, C. Lemne, R. Kiessling, U. de Faire and J. Frostegard (2000). "Circulating heat shock protein 60 is associated with early cardiovascular disease." Hypertension **36**(2): 303-307.
84. Quijada, L., M. Soto, C. Alonso and J. M. Requena (1997). "Analysis of post-transcriptional regulation operating on transcription products of the tandemly linked Leishmania infantum hsp70 genes." J Biol Chem **272**(7): 4493-4499.
85. Quintana, F. J. and I. R. Cohen (2011). "The HSP60 immune system network." Trends Immunol **32**(2): 89-95.
86. Radwanska, M., S. Magez, N. Dumont, A. Pays, D. Nolan and E. Pays (2000). "Antibodies raised against the flagellar pocket fraction of Trypanosoma brucei preferentially recognize HSP60 in cDNA expression library." Parasite Immunol **22**(12): 639-650.
87. Rao, T. and P. A. Lund (2010). "Differential expression of the multiple chaperonins of Mycobacterium smegmatis." FEMS Microbiol Lett **310**(1): 24-31.
88. Retzlaff, C., Y. Yamamoto, P. S. Hoffman, H. Friedman and T. W. Klein (1994). "Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures." Infect Immun **62**(12): 5689-5693.
89. Rey-Ladino, J. A., Joshi, P. B., Singh, B., Gupta, R., and Reiner, N. E. (1997). "Leishmania major: molecular cloning, sequencing, and expression of the heat shock protein 60 gene reveals unique carboxy terminal peptide sequences." Exp Parasitol **85**(3): 249-263.
90. Rey-Ladino, J. A. and N. E. Reiner (1993). "Expression of 65- and 67-kilodalton heat-regulated proteins and a 70- kilodalton heat shock cognate protein of Leishmania donovani in macrophages." Infect Immun **61**(8): 3265-3272.

91. Rizzo, M., F. Cappello, R. Marfil, L. Nibali, A. Marino Gammazza, F. Rappa, G. Bonaventura, P. Galindo-Moreno, F. O'Valle, G. Zummo, E. Conway de Macario, A. J. Macario and F. Mesa (2012). "Heat-shock protein 60 kDa and atherogenic dyslipidemia in patients with untreated mild periodontitis: a pilot study." Cell Stress Chaperones **17**(3): 399-407.
92. Rodriguez-Quinones, F., M. Maguire, E. J. Wallington, P. S. Gould, V. Yerko, J. A. Downie and P. A. Lund (2005). "Two of the three groEL homologues in Rhizobium leguminosarum are dispensable for normal growth." Arch Microbiol **183**(4): 253-265.
93. Ross, R. (1903). "Note on the Bodies Recently Described by Leishman and Donovan." Br Med J **2**(2237): 1261-1262.
94. Salotra, P., R. Ralhan and R. Bhatnagar (1994). "Differential expression of stress proteins in virulent and attenuated promastigotes of Leishmania donovani." Biochem Mol Biol Int **33**(4): 691-697.
95. Samali, A., J. Cai, B. Zhivotovsky, D. P. Jones and S. Orrenius (1999). "Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells." EMBO J **18**(8): 2040-2048.
96. Santos, R., G. L. A. Silva, E. V. Santos, S. M. Duncan, J. C. Mottram, J. D. Damasceno and L. R. O. Tosi (2017). "A DiCre recombinase-based system for inducible expression in Leishmania major." Mol Biochem Parasitol **216**: 45-48.
97. Schett, G., B. Metzler, R. Kleindienst, A. Amberger, H. Recheis, Q. Xu and G. Wick (1999). "Myocardial injury leads to a release of heat shock protein (hsp) 60 and a suppression of the anti-hsp65 immune response." Cardiovasc Res **42**(3): 685-695.
98. Schluter, A., M. Wiesgigl, C. Hoyer, S. Fleischer, L. Klaholz, C. Schmetz and J. Clos (2000). "Expression and subcellular localization of cpn60 protein family members in Leishmania donovani." Biochim Biophys Acta **1491**(1-3): 65-74.
99. Schlüter, A., M. Wiesgigl, C. Hoyer, S. Fleischer, L. Klaholz, C. Schmetz and J. Clos (2000). "Expression and Subcellular Localization of Cpn60 Protein Family Members in*Leishmania donovani*." Biochim. Biophys. Acta **1491**: 65-74.
100. Schonian, G., K. Kuhls and I. L. Mauricio (2011). "Molecular approaches for a better understanding of the epidemiology and population genetics of Leishmania." Parasitology **138**(4): 405-425.
101. Scocca, J. J., R. L. Poland and K. C. Zoon (1974). "Specificity in deoxyribonucleic acid uptake by transformable Haemophilus influenzae." J Bacteriol **118**(2): 369-373.
102. Shamaei-Tousi, A., J. W. Stephens, R. Bin, J. A. Cooper, A. Steptoe, A. R. Coates, B. Henderson and S. E. Humphries (2006). "Association between plasma levels of heat shock protein 60 and cardiovascular disease in patients with diabetes mellitus." Eur Heart J **27**(13): 1565-1570.
103. Shonhai, A., A. G. Maier, J. M. Przyborski and G. L. Blatch (2011). "Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis." Protein Pept Lett **18**(2): 143-157.
104. Silverman, J. M., J. Clos, C. C. de'Oliveira, O. Shirvani, Y. Fang, C. Wang, L. J. Foster and N. E. Reiner (2010). "An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages." J Cell Sci **123**(Pt 6): 842-852.
105. Silverman, J. M., J. Clos, E. Horakova, A. Y. Wang, M. Wiesgigl, I. Kelly, M. A. Lynn, W. R. McMaster, L. J. Foster, M. K. Levings and N. E. Reiner (2010). "Leishmania

exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells." J Immunol **185**(9): 5011-5022.

106.Smejkal, R. M., Wolff, R., and Olenick, J.G. (1988). "*Leishmania braziliensis panamensis*: increased infectivity resulting from heat shock." Exp. Parasitol. **65**: 1-9.

107.Smith, H. O. and K. W. Wilcox (1970). "A restriction enzyme from Hemophilus influenzae. I. Purification and general properties." J Mol Biol **51**(2): 379-391.

108.Soares Medeiros, L. C., L. South, D. Peng, J. M. Bustamante, W. Wang, M. Bunkofske, N. Perumal, F. Sanchez-Valdez and R. L. Tarleton (2017). "Rapid, Selection-Free, High-Efficiency Genome Editing in Protozoan Parasites Using CRISPR-Cas9 Ribonucleoproteins." MBio **8**(6).

109.Srivastava, P. (2002). "Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses." Annual review of immunology **20**: 395-425.

110.Sterkers, Y., L. Lachaud, N. Bourgeois, L. Crobu, P. Bastien and M. Pages (2012). "Novel insights into genome plasticity in Eukaryotes: mosaic aneuploidy in Leishmania." Mol Microbiol **86**(1): 15-23.

111.Sullivan, M. A., C. L. Olson, A. G. Winquist and D. M. Engman (1994). "Expression and localization of Trypanosoma cruzi hsp60." Mol Biochem Parasitol **68**(2): 197-208.

112.Sunter, J. and K. Gull (2017). "Shape, form, function and Leishmania pathogenicity: from textbook descriptions to biological understanding." Open Biol **7**(9).

113.Todd, M. J. and G. H. Lorimer (1998). "Criteria for assessing the purity and quality of GroEL." Methods Enzymol **290**: 135-141.

114.Tovar, J., A. Fischer and C. G. Clark (1999). "The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite Entamoeba histolytica." Mol Microbiol **32**(5): 1013-1021.

115.Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Hacker and H. Wagner (2001). "Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells." J Biol Chem **276**(33): 31332-31339.

116.Van Duyne, G. D. (2001). "A structural view of cre-loxp site-specific recombination." Annu Rev Biophys Biomol Struct **30**: 87-104.

117.Velazquez, J. M., B. J. DiDomenico and S. Lindquist (1980). "Intracellular localization of heat shock proteins in Drosophila." Cell **20**(3): 679-689.

118.Vikeved, E., A. Backlund and C. Alsmark (2016). "The Dynamics of Lateral Gene Transfer in Genus Leishmania - A Route for Adaptation and Species Diversification." PLoS Negl Trop Dis **10**(1): e0004326.

119.Wallace, G. R., A. E. Ball, J. MacFarlane, S. H. el Safi, M. A. Miles and J. M. Kelly (1992). "Mapping of a visceral leishmaniasis-specific immunodominant B-cell epitope of Leishmania donovani Hsp70." Infect Immun **60**(7): 2688-2693.

120.Wang, X. M., C. Lu, K. Soetaert, C. S'Heeren, P. Peirs, M. A. Laneelle, P. Lefevre, P. Bifani, J. Content, M. Daffe, K. Huygen, J. De Bruyn and R. Wattiez (2011). "Biochemical and immunological characterization of a cpn60.1 knockout mutant of Mycobacterium bovis BCG." Microbiology **157**(Pt 4): 1205-1219.

121.Wiesgigl, M. and J. Clos (2001). "Heat Shock Protein 90 Homeostasis Controls Stage Differentiation in*Leishmania donovani*." Mol Biol Cell **12**(11): 3307-3316.

122.Wiesgigl, M. and J. Clos (2001). "*Leishmania*and the Leishmaniases: the heat shock protein 90 of*Leishmania donovani*." Med. Microbiol. Immunol. **190**: 27-31.

123.Wu, C. T., L. S. Ou, K. W. Yeh, W. I. Lee and J. L. Huang (2011). "Serum heat shock protein 60 can predict remission of flare-up in juvenile idiopathic arthritis." Clin Rheumatol **30**(7): 959-965.

124.Xu, Z., A. L. Horwich and P. B. Sigler (1997). "The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex." Nature **388**(6644): 741-750.

125.Zamora-Veyl, F. B., M. Kroemer, D. Zander and J. Clos (2005). "Stage-specific expression of the mitochondrial co-chaperonin of Leishmania donovani, CPN10." Kinetoplastid Biol Dis **4**(1): 3.

126.Zhang, W. W. and G. Matlashewski (2015). "CRISPR-Cas9-Mediated Genome Editing in*Leishmania donovani*." MBio **6**(4): e00861.

127.Zhang, X., J. Cui, D. Nilsson, K. Gunasekera, A. Chanfon, X. Song, H. Wang, Y. Xu and T. Ochsenreiter (2010). "The Trypanosoma brucei MitoCarta and its regulation and splicing pattern during development." Nucleic Acids Res **38**(21): 7378-7387.

128.Zhu, H., X. Fang, D. Zhang, W. Wu, M. Shao, L. Wang and J. Gu (2016). "Membrane-bound heat shock proteins facilitate the uptake of dying cells and cross-presentation of cellular antigen." Apoptosis **21**(1): 96-109.

129.Zilberstein, D. and M. Shapira (1994). "The role of pH and temperature in the development of Leishmania parasites." Annu. Rev. Microbiol. **48**: 449-470.