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Evaluation of Clan CD C11 peptidase PNT1 and other *Leishmania mexicana* cysteine peptidases as potential drug targets

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Running head: *Leishmania mexicana* cysteine peptidases essential for parasite cell viability

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ABSTRACT

Leishmania mexicana is one of the causative agents of cutaneous leishmaniasis in humans. There is an urgent need to identify new drug targets to combat the disease. Cysteine peptidases play crucial role in pathogenicity and virulence in *Leishmania spp.* and are promising targets for developing new anti-leishmanial drugs. Genetic drug target validation has been performed on a number of cysteine peptidases, but others have yet to be characterized. We targeted 16 *L. mexicana* cysteine peptidases for gene deletion and tagging using CRISPR-Cas9 in order to identify essential genes and ascertain their cellular localization. Our analysis indicates that two clan CA, family C2 calpains (LmCAL27.1, LmCAL31.6) and clan CD, family C11 PNT1 are essential for survival in the promastigote stage. The other peptidases analysed, namely calpains LmCAL4.1, LmCAL25.1, and members of clan CA C51, C78, C85 and clan CP C97 were found to be non-essential. We generated a gene deletion mutant (Δ*pnt1*) which was severely compromised in its cell growth and a conditional gene deletion mutant of *PNT1* (Δ*pnt1*:: $PNT1^{flox}$/Δ *pnt1*::*HYG* [SSU DiCRE]). PNT1 localizes to distinct foci on the flagellum and on the surface of the parasite. The conditional gene deletion of *PNT1* induced blebs and pits on the cell surface and eventual cell death. Over-expression of PNT1, but not an active site mutant $PNT1^{C134A}$, was lethal, suggesting that active PNT1 peptidase is required for parasite survival. Overall, our data suggests that *PNT1* is an essential gene and one of a number of cysteine peptidases that are potential drug targets in *Leishmania*.

Highlights

Localization and essentiality study of 16 uncharacterized peptidases in *L. mexicana.*

Clan CD family C11 PNT1 is essential for survival of *Leishmania mexicana* parasites.

1. Introduction

Human infective *Leishmania* parasites are the causative agents of one of the major tropical diseases, leishmaniasis. Infections may be life changing (cutaneous form) or even fatal (Visceral form of the disease) [1]. Millions of people are at risk of contracting the disease with an estimated 20,000 to 40,000 deaths occurring each year [2]. *L. mexicana*, one of the causative agents of the cutaneous form of the disease, has several life cycle stages that include amastigotes that live in mammalian macrophages and flagellated promastigotes in the sandfly midgut [3]. *Leishmania* parasites have mechanisms by which they evade the host immune system and can survive as well as replicate in the harsh environment of phagolysosomes within host phagocytes [4]. Deciphering the molecular mechanisms that underlie the pathogenicity, virulence and survival mechanisms of these parasites is vital for the design of innovative control measures.

Leishmania peptidases are among the widely studied virulence factors. These enzymes have been implicated in pathogenesis, virulence and cellular remodelling of life cycle stages during differentiation of the Leishmania parasite [5, 6]. They are also potential drug targets [7, 8]. Peptidases can be divided into different sub groups, Clans and Families, based on their catalytic mechanism and primary amino acid sequence in the catalytic domain [9]. There are several families of peptidases identified to date [9], among these *L. mexicana* encodes members of Aspartic (A), Cysteine (C), Metallo- (M), Mixed (P), Serine (S) and Threonine (T) [9].

There have been a number of studies investigating the importance of *Leishmania* cysteine peptidases in the life cycle of the parasite. In the cysteine peptidase family, Clan CA family C1 members cathepsin L-related peptidases; CPA and CPB play an important role in differentiation and survival of *Leishmania* parasite [10]. Clan CA, family C54 member ATG4 was also found to be important for virulence in *L. major* [11]. Whilst Clan CD C14 member metacaspase, was originally thought to have an essential cell cycle role in *L. major* [12], gene deletion mutants [13] indicate a potential role in cell death and in autophagy. In contrast the phenotype of an *L. mexicana* metacaspase null mutant indicates the enzyme has a role as a negative regulator of amastigote proliferation [14]. No detectable phenotype could be observed for *L. major* Clan CF, Family C15 proglutamyl protease I (PPI)-deficient mutants, which retained infectivity to macrophages in vitro and mice. However, over-expression of the active PPI, in *L. major* impaired differentiation [15]. The aforesaid studies clearly highlight the role of cysteine peptidases in the virulence of *Leishmania*, however, a more comprehensive study of the proteolytic capacity of the parasite is required in order to identify and validate more potential drug targets.

In this study, we concentrated our efforts to address the gap in knowledge for 16 *Leishmania* cysteine peptidases in Clan CA, families C2, C51, C78, C85, Clan CD families C11 and C50 and Clan CP, family C97. We used *L. mexicana* as the species of choice for genetic manipulation due to the well-established methods for CRISPR-Cas9 genome engineering [16, 17].

2. Materials and Methods

2.1 Plasmid Vector generation and CRISPR-Cas9 strategies

For U6snRNA strategy the SpCas9 gene was amplified from the plasmid p5RT70DDmyc-FlagCas9 (a kind gift from Professor Markus Meissner, University of Glasgow) using primers containing 5'overlap to pNUS-GFPcN NdeI linearized vector, which allows for episomal c-terminal expression of GFP fused proteins in *Leishmania* sp [18]. The plasmid was linearized using *NdeI* and the 4.2 kb SpCas9 fragment purified and cloned using Gibson assembly (NEB) to generate pNUS-Cas9. In order to express guide RNAs (sgRNA) using a U6snRNA promoter and terminator as described before for *Leishmania* species [17]. The gRNA cassette was synthesized containing PNT1 specific gRNA obtained from EuPaGDT [19], (GCCTTGGCATAGTTCTTGAGC). In order to express both SpCas9 and gRNA from the same plasmid the synthetic expression cassette was cloned in pNUS-Cas9 linearized with *HindIII* using Gibson assembly. The final plasmid (pNUS-Cas9_PNT1.1sgRNA) was used for transient expression. Double strand break (DSB) repair was facilitated by providing cells with a donor DNA, allowing for homologous recombination, replacement of PNT1 alleles and selection of mutants. The donor DNA consists of 1000 bp PNT1 UTR specific regions, amplified from *Leishmania* genomic DNA with primers containing adaptors for cloning using Gateway (Thermo Fisher Scientific) into a hygromycin (HYG) resistance gene flanked by 5'and 3' untranslated regions (UTR) of *Leishmania* dihydrofolate reductase (DHFR). The donor DNA vector was linearized using *PacI*/*PmeI* and gel purified prior to transfection.

A T7RNAPol strategy was used to generate both endogenously tagged genes and null mutants. Primers to generate donor DNA and sgRNA were designed using LeishGEdit website and protocol therein [16] was followed. Briefly, plasmids containing resistance markers for blasticidin (*BSD*), puromycin (*PUR*) or neomycin (*NEO*), flanked by Leishmania specific UTR regions to control expression, were used as PCR-templates. For in-fusion endogenous tagging a version of the plasmid also containing mNeonGreen fluorescent protein open reading frame (ORF) allowed for N- or C-terminus modification. Donor DNAs contain suitable region of the plasmid for either deletion or tagging of the gene flanked by 30 nt sequence for homologous recombination at the appropriate locus. Expression of sgRNA in vivo is driven by a T7 RNA Polymerase (T7RNAPol) promoter added to primers containing the sgRNA and a portion of SpCas9 scaffold for annealing to a universal reverse primer containing the remaining scaffold for ribonucleoprotein complex assembly. For deletion mutants double stranded breaks were introduced at the 5´ and 3´ UTRs of the gene, and recombination

of both alleles facilitated by transfecting with donor DNAs containing 2 distinct selection markers. PCR-synthesized DNAs (1 sgRNA + 1 donor for fluorescent mutants; 2 sgRNA + 2 donors for deletion mutants) were pooled prior to transfection into a transgenic *L. mexicana* cell line expressing T7RNAPol and SpCas9 [16]. All relevant primers are listed in Table S1.

The floxed PNT1 construct was generated as described earlier **[20]**. Briefly, a DiCre expressing cell line was generated in *L. mexicana*. The first allele of *PNT1* was replaced by a LoxP flanked version of the gene with a C-terminal GFP tag and confirmed by PCR and flow cytometry after propidium iodide staining. The second copy of *PNT1* was then deleted by homologous recombination and was replaced with a HYG resistance cassette (donor DNA vector described above). It is important to note that replacing the first allele with the antibiotic cassette followed by floxing the second allele was problematic and led to cell cycle defects. The complementation plasmids were generated by cloning *PNT1*, $PNT1^{C134A}$ in pGL631 (pNUS vector) for episomal expression under the selection of G418 resistance. $PNT1^{C134A}$ was generated by site directed mutagenesis on the *PNT1* pNUS plasmid.

2.2 *Leishmania* cell culture and transfection

The promastigote form of *Leishmania mexicana mexicana* (MNYC/BZ/62/M379) was cultured at 25°C in HOMEM which was supplemented with 1% penicillin/streptomycin and 10% heat inactivated foetal calf serum. Plasmids and homologous recombination DNA fragments were transfected using Human T-Cell kit (Lonza) as described elsewhere [14]. CRISPR-Cas9 guides and donors (Table S1) were transfected after mixing with Cytomix buffer (66.67 mM Na_2HPO_4, 23.33 mM NaH_2PO_4, 5 mM KCl, 50 mM HEPES pH 7.3) combined to 150µM $CaCl_2$, and the electroporation process performed using Lonza Nucleofector system, program X-001. The cells were incubated at 25ºC overnight and were plated onto 96 well plates after dilution and in presence of antibiotics. Transgenic lines were maintained in the following antibiotics and respective concentrations: blasticidin (10 µg mL^{-1}), G418 (50 µg mL^{-1}), puromycin (50 µg mL^{-1}), hygromycin (50 µg mL^{-1}), and nourseothricin (75 µg mL^{-1}).

2.3 Rapamycin induced conditional gene deletion

The DiCre mediated gene deletion was performed using 200nM rapamycin. Cells were grown at a starting cell density of 1 x 10^5 cells mL^{-1}. Cells were grown for 72 h in the presence of selection antibiotics. Thereafter, the cells were split to an initial concentration 1 x 10^5 cells mL^{-1} with another addition of 200nM rapamycin. The growth curves presented represent cells after the second round of rapamycin treatment.

2.4 Western blot analysis

1 x 10^7 cells lysed in NuPAGE sample buffer (supplemented with β-mercaptoethanol) were loaded on to a 4-12% NuPAGE Bis Tris Gel and run at 150V in MOPS buffer. The gels were transferred onto a

PVDF membrane using the wet transfer system XCell II Blot Module of Invitrogen at a constant voltage of 30V for 90 min. The blot was blocked with 5% Skimmed Milk (Sigma) for 1 h. Primary antibodies were added at the following concentrations: rabbit polyclonal PNT1 antibody- 1:500 overnight at 4°C, sheep polyclonal OPB antibody-1:20,000 1 h at room temperature. After 3 washes in 1X TBST, goat anti rabbit HRP (Promega) and donkey anti-sheep HRP (Santa Cruz) were added at 1;5000 dilution for 1 h. The blots were washed with 1X TBST and overlayed with Clarity Max substrate (BioRad). The blots were developed in a myECL Imager (Thermo Fisher Scientific).

2.5 Confocal microscopy

Staining was carried out with 1 x 10^6 cells from log phase cultures. The cells were washed with 1X PBS and fixed with of 4% paraformaldehyde (PFA) for 10 min. The wells were washed twice with 1X PBS and excess PFA was quenched with 100 mM glycine for 10 min. The cells were then permeabilized using 0.1% Triton X-100 for 10 min. Cells were blocked in 1% BSA for 1 h. The PNT1 antibody (1:200) diluted in 1% BSA was added to the cells and incubated for 1 h at room temperature. The cells were washed thrice with PBS. Anti-rabbit secondary antibody Alexa Fluor 594 (Molecular probes) was used at 1:500 dilution for 1 h. Cells were washed thrice in PBS, overlaid with a drop of SlowFade Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole - DAPI (Invitrogen). The final cell pellet was resuspended in 50 µL PBS and stored at 4°C until ready to image. Stained cells were spread on a slide and overlaid with coverslip. For DAPI staining of rapamycin induced cells the antibody incubation step was omitted and cells mounted with anti fading mounting medium with DAPI (Invitrogen) on slides coated with Poly-L-lysine. For live cell imaging cells were stained with Hoescht 33342 and immobilized in CyGel as previously described [21]. All images were obtained on a Zeiss LSM 880 and processed using Zeiss Zen.

2.6 SEM and TEM

For TEM *Leishmania* parasites were fixed in 8% formaldehyde, 5% glutaraldehyde in 100mM Sodium phosphate buffer pH 7.3 mixed 50:50 with culture medium and incubated for 10 min at room temperature. The cells were centrifuged at 1000g for 10 min and resuspended in 4% formaldehyde 2.5% glutaraldehyde in 100mM phosphate for 30 min. After fixation, the cells were washed with 100mM phosphate 2 x 10 min and then treated with 1% OsO4 on ice 45 min followed by 100mM phosphate 2 x 10 min and the following series of treatments as follows: 1% tannic acid in 100mM phosphate 10 min, 100mM phosphate 2 x 10 min, 25% ethanol 15 min, 30% ethanol + 2%UA in dark 1hr, 50% ethanol 15 min, 50% acetone 15 min, 70% acetone 15 min, 90% acetone 15 min, 100% acetone 2 x 15 min, 25% Spurr (R) : 75% acetone 20 min, 50% Spurr (R) : 50% acetone 20 min, 75% Spurr (R) : 25% acetone 30 min, 100% Spurr (R) 3 changes 30 min each, and polymerised at 70°C overnight. The samples were spun on micro centrifuge as required.

For SEM, the *Leishmania* parasites Primary fixation of double strength fixative (8% formaldehyde and 5% glutaraldehyde) in 0.1M phosphate buffer pH 7.3 mixed 50:50 with culture medium for 10 min followed by fixation in 4% formaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer for 30 min. The cells were washed twice in 0.1M phosphate buffer for 10 min each. Post-fixation of 1% osmium tetroxide for 45 min on ice. Washed twice in 0.1M phosphate buffer for 10 min each. The samples were fixed in 1% tannic acid in 0.1M phosphate buffer for 20 min. Washed twice in 0.1M phosphate buffer for 10 min each. The samples were dehydrated in graded series of ethanol (25-100%), 15 mins each change. 100% ethanol with hexamethyldisilazane (HMDS), 2 changes and left to dry in desiccator overnight. Samples were affixed to SEM stubs and sputter coated with 5nm of gold/palladium on Polaron SC7640 sputter coater then imaged using Jeol JSM 6490LV scanning electron microscope operating at 8kV accelerating voltage.

3. Results

3.1 CRISPR-Cas9 gene deletion identifies three essential peptidases.

The automated algorithms of MEROPS database [9] identified 177 peptidases in the *L. mexicana* genome, 61 being annotated as cysteine peptidases or non-peptidase homologues. The gene models and annotation for each of the identified proteins was confirmed in TriTrypDB [22]. 17 C2 non-peptidase homologues were confirmed to lack a predicted functional peptidase domain and were not included in the current study. The cysteine peptidases are distributed in clans and families based on sequence, structure and active site residues (Fig. 1A). The *L. mexicana* cysteine peptidase clans identified in MEROPS were Clan CA, CD, CF, CP and PC. Several proteins have only recently been identified as having cysteine peptidase activity [23], including Clan CA family C78 [24], family C85 [25], and PPPDE (Permuted Papain fold Peptidases of dsRNA viruses and Eukaryotes) Clan CP family C97 [26] (Fig 1A). The details of the proteins of interest are shown in Table 1. A detailed domain analysis of those cysteine peptidases was also performed, which gives more insight into the roles of these peptidases (Fig. 1B). The domain information was obtained from the SMART domain analysis server [27, 28]. In addition to the peptidase domains that were specific to each family, the other identifiable domains were transmembrane, coiled coil domain, WD40 domain, zinc finger domain and Pfam DUF domains. Among all the peptidases in this study, we conducted a detailed analysis of the Clan CD C11 peptidase PNT1 [29], which is present in the *L. mexicana* genome, but not annotated in MEROPS.

To test whether cysteine peptidases of interest (Fig. 1A, Table 1) are essential for parasite survival we used CRISPR-Cas9 to attempt to generate null mutants [16]. We first tested a U6 snRNA promoter-based CRISPR-Cas9 system for *L. mexicana* promastigotes on *PNT1* and on the 5 calpains we examined in this study (CAL4.1, CAL25.1, CAL27.1, CAL31.6 and CAL33.1). We were unable to

isolate transfectants for any of the calpains using this system. Among the 6 gene editing attempts, we only managed to generate gene targeted mutants for *PNT1*. The PCR analysis using primers outwith the cassette (schematic shown in Fig.2A) confirmed correct integration of the *HYG* resistance cassette and deletion of *PNT1* in two out of six clones (Cl3 and Cl4, Fig. 2B). The promastigotes of Cl3 and Cl4 were severely compromised in their cell growth, unable to maintain a long-term viable culture, and died (Fig. 2C). Mutants that retained at least one copy of *PNT1* had the same growth rate as the parental CRISPR-Cas9 line (Fig. 2C). This indicates that PNT1 is essential for promastigote viability. As the U6SnRNA system was apparently only effective against a limited number of targets and we could not predict which ones would work, we used an alternative CRISPR-Cas9 system where single-guide RNA (sgRNA) delivery uses PCR-generated DNA templates transcribed *in vivo* by a transgenic cell line expressing T7 RNA polymerase and SpCas9 [16]. This approach allows knock-in of fluorescent tags and generation of gene deletion mutants.

Initially, the 16 cysteine peptidases were either N or C-terminally tagged with mNeonGreen (Fig. 3A-M), with fluorescent signal being detected for 13 of the lines. Localisation was classified to distinct cellular structures according to *Leishmania* cellular landmarks [30], Table 1. The calpains were localised to the flagellum (CAL4.1), the flagellar pocket (CAL27.1), small cytoplasmic organelles (CAL33.1) and to a lysosome-like compartment within the endomembrane system (CAL25.1 and CAL31.6). The two members of C51 and the OTU-like cysteine peptidase were found in small cytoplasmic organelles, whilst PPPDE1 and PPPDE3 Clan CP peptidases had a cytosolic expression and very low signal. PPPDE4 and PPPDE5 are localised at the endomembrane system and at a distinct punctum at the posterior tip of the cell, respectively. Finally, separase localised to the nucleus, sometimes exhibiting localised points in the nucleus in cells containing 1 nucleus and 1 kinetoplast (1N1K). In cells where mitosis was complete a low and disperse signal at the nucleus was detected. PNT1 was tagged at the C-terminus, however, there was no detectable mNeonGreen signal in the cells. The C- or N-terminus tagging were apparently lethal for LmxM.09.1300 as no mutants were isolated, whilst no mNeonGreen signal was detected for LmxM.33.4000.

Gene deletion mutants were successfully generated for 13 of the 16 cysteine peptidases, as validated by PCR; correct integration of the antibiotic marker for each gene was confirmed and the endogenous target gene was absent (Fig. 4, Table 1). Null mutants were created for 3 of the calpains (CAL4.1, CAL25.1 and CAL33.1), both D-alanyl-glycyl endopeptidase-like proteins (LmxM.32.2830, LmxM.32.2850), Clan CA C78 (LmxM.33.4000), OTU-like peptidase (LmxM.36.6020), the 5 PPPDE family proteins (LmxM.09.1310, LmxM.09.1300, LmxM.24.0650, LmxM.31.1330, LmxM.32.2260) and separase (LmxM.20.1680). The CRISPR-Cas9 knockout experiments were performed by replacing both alleles of the target genes with antibiotic resistance genes, *BSD* and *NEO*, or BSD and *PUR*. The absence of a PCR product for the CDS, cells resistant to both antibiotics and correct integration of drug resistance markers is strongly indicative of a knockout. Null mutants

could not be generated for LmCAL27.1, LmCAL31.6 and *PNT1*, a weak evidence of essentiality, as discussed previously [31] since we cannot completely eliminate the possibility of technical failure However, we can confirm that at least one of the sgRNAs used to insert the DSB into 5'- and 3'-UTR of the gene of interest is functional, since the same sequence was used to facilitate the endogenous labeling of the genes. After failure to recover transfectants or generation of mutants retaining a WT copy, transfections of the gene were repeated at least 3 times.

3.2 PNT1 is essential for the viability of *L. mexicana.*

PNT1 is a Clan CD (C11) cysteine peptidase that was recently shown to be essential for replication of the kinetoplast in *T. brucei* [29]. The subcellular localization of PNT1 was ascertained in *L. mexicana* by labelling with an antibody raised against an insoluble recombinant protein containing the C11 domain. PNT1 localizes to the cell body and in the flagellum in distinct intense foci (Fig. 5A,B). PNT1 staining was more intense in cells that were in the process of cell division (Fig 5C,D). Western blotting supported this cell cycle-dependent expression, as levels of PNT1 decreased as the cells approached stationary growth phase (Fig. 5E)

To gain further insights into the function of PNT1 in *L. mexicana* we used the DiCre system to carry out an inducible deletion of *PNT1* [20]. The Cre monomers were expressed constitutively from the ribosomal locus (SSU::DiCre). The first allele of *PNT1* was floxed and the second copy replaced using *HYG* selectable marker (Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::*HYG* [SSU DiCRE]). We found that the order in which gene replacements were carried out influenced the system efficiency, with single marker cell lines resulting in aneuploidy and therefore were not suitable for further analysis. To avoid this problem we first integrated the floxed version of *PNT1* and subsequently we deleted the second *PNT1* allele using *HYG* cassette.

Two Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] clones (number 4 and 9) were tested for excision of the floxed *PNT1* after addition of rapamycin, with an expected 3.6 kb DNA PCR fragment being evident prior to treatment and the presence of 0.68 kb DNA fragment showing excision after rapamycin addition (Fig. 6A). Reduction in PNT1 levels in the induced cell line was confirmed by western blot with polyclonal PNT1 antibody (Fig. 6B). Clone 9 was found to have a greater depletion of PNT1 than clone 4, possibly because the genetic manipulation led to clone 4 being slightly compromised in its growth in the absence of rapamycin (Fig. 6C). To assess further the proliferation competence of these parasites after induction the cells were cultured for a period of 72 h in the presence of 200nM rapamycin and then diluted to 1 x 10^5 cells mL^{-1} in the presence of the same concentration of rapamycin, before counting over a five-day period. Both clones showed a loss of viability in comparison with the DiCre parental line (Fig. 6C). Interestingly, the induced PNT1 floxed cell line recovered growth after 72 h, possibly due to the presence in the population of cells that had not induced the loss of *PNT1*. A clonogenic assay was therefore performed in order to test this. The

Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] induced cell line was cloned in 96 well plates 72 h after rapamycin addition. The clones growing on the plates were checked for excision of the floxed *PNT1* by PCR. It was observed that all the isolated clones retained the floxed *PNT1* allele (Fig. 6D), suggesting that parasites non-responsive to DiCre induced *PNT1* gene deletion are present in small numbers and these eventually grow out 72 h after the second addition of rapamycin.

The only structure that is available for C11 cysteine peptidases is from the gut bacterium *Parabacteroides merdae* [32]. Efforts to purify soluble PNT1 protein for either *T. brucei* or *L. mexicana* in *E.coli* or baculovirus expression system were unsuccessful. We were, therefore, unable to validate the cysteine peptidase activity of *L. mexicana* PNT1 using biochemical approaches, so an alternative approach was tried. Transfection of *L. mexicana* with an episome to constitutively overexpress GFP tagged PNT1 appeared to be lethal for the parasites, as no viable clones were isolated. In contrast, transfection of a GFP tagged PNT1 cysteine active site mutant (PNT1^{C134A}) was successful and viable parasites isolated. Taken together our observations indicate that PNT1 is an essential gene, and that active PNT1 is required for cell viability.

3.3 Depletion of PNT1 retains kinetoplast but leads to formation of blebs and pits on the cell surface

In order to analyse the possible role of PNT1 in kinetoplast segregation, as observed in *T. brucei* [29], Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] cell line was induced with rapamycin and stained with DAPI. These cells were confirmed to have lost PNT1 after induction with rapamycin (Fig 7A). All cells had nuclear and kinetoplast configurations consistent with normal cell cycle progression, with no loss of kinetoplast observed (Fig. 7B). Scanning electron microscopy analysis of Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] parasites treated for 48 h (second round of induction) with rapamycin revealed mutants had blebs and pits on the cell surface (Fig. 7C). Cells with normal morphology were also observed in the Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE cell line, possibly due to the presence of non-induced cells. Transmission electron microscopy analysis of the induced Δpnt1::PNT1 flox /Δpnt1::HYG [SSU DiCRE] cell line confirmed the presence of intact kinetoplast and bulges in the flagellar pocket area, resembling the protuberances observed in SEM images (Fig. 7D). Taken together our data suggests that PNT1 is essential in *Leishmania* and that loss of PNT1 leads to cell death with concurrent formation of blebs and pits on the surface of the parasite.

4. Discussion

In the 30 years since the first successful genetic manipulation of *Leishmania* was published [33] only about 200 genes have been targeted for gene deletion [34]. Peptidases, and cysteine peptidases in particular, comprise one of the largest groups of genes that have been investigated in this way and studies have shown that they play a very important role in parasite survival and virulence [5, 6, 8].

Despite this, many more *Leishmania* cysteine peptidases remain to be studied as potential drug targets [23] so we used a variety of bioinformatics tools to interrogate the *L. mexicana* genome and provide an up to date list of encoded cysteine peptidases. This list (Fig. 1) includes several families of genes that have only recently been recognised to be cysteine peptidases in *Leishmania spp*., including Clan CA family C78, family C85 and PPPDE Clan CP family C97. In mouse, two Clan CA C78 peptidases UfSP1 and UfSP2 act on a ubiquitin like protein, the ubiquitin fold modifier 1(Ufm1) for targeting proteins for degradation and other cellular processes akin to the classical ubiquitin molecule [24]. The OTU (Ovarian tumor) family Clan CA C85 DUB's are involved in a range a cellular processes. Members of this family have been implicated in modulating inflammatory signalling cascades [35], immune regulation [36] and inhibition of DNA damage dependent ubiquitination [37]. PPPDE family peptidases have been implicated in important role in deubiqutination of proteins involved in crucial processes such as cell cycle regulation [26] It will be very important to understand the processes that these peptidases and how these work in coordination with the known DUB's in *Leishmania spp*.

Genetic manipulations in *Leishmania spp*. at scale has been challenging due to the requirement for creating repair cassettes with long homology arms, the need for sequential rounds of transfection to delete two alleles and the presence of supernumerary chromosomes [38] . The recent development of a variety of CRISPR-Cas9 approaches in *Leishmania* [16, 17] has enabled fast and robust methods for investigating the function of these peptidases at scale. We used two different systems, here named after the promoter used to express the sgRNA, both using SpCas9 to produce the double stranded break and facilitate the generation of null mutants in *L. mexicana* promastigotes. The U6snRNA system was adapted from the original report by Sollelis and colleagues [17], using the minimal sequences required to promote U6snRNA transcription, comprising the $tRNA^{Gln}$ and BOX A and B flanked by 5' UTR of U6snRNA. The terminator comprises a 120 bp 3'UTR from the U6snRNA locus [39]. For our strategy we decided to express both SpCas9 and the sgRNA ectopically and transiently, in order to obtain mutants with fewer genetic modifications and retaining virulence. Both the plasmid and the donor DNA containing homology regions for recombination in the UTRs of the gene to be deleted was transfected together. Our second strategy was reproduced from Beneke and colleagues [16] and depends on a cell line expressing SpCas9 and T7RNAPol to drive expression of the sgRNA and transfection of two linear DNA fragments produced using PCR: a ˜130 bp fragment (containing a T7 promoter, the sgRNA and the SpCas9 scaffold) and a ˜30nt fragment corresponding to the 5' upstream and 3' downstream to the corresponding sgRNA. Furthermore, in order to increase the chances of deleting both alleles of the gene of interest, we transfected cells with 2 sgRNAs (targeting the 5' and the 3' ends of the target genes) and donor DNAs having NEO/BSD or BSD/PUR resistance cassettes for selection.

Our study reveals that in our hands the CRISPR-Cas9 approach using the T7RNAPol system worked better than the U6snRNA system in *L. mexicana.* Initially, the clan CD, family C11 PNT1 and the 5

calpains (*CAL4.1, CAL25.1, CAL27.1, CAL31.6 and CAL33.1*) were used to test both systems. Although we could generate null mutant clones for PNT1 using the U6snRNA, none of the other 5 calpain genes tested resulted in transfectants, either heterozygotes or null mutants. On the other hand, using the T7RNAPol we were able to generate null mutants for 3 out of the 5 calpains (*CAL27.1, CAL31.6* and *CAL33.1*) but not for *PNT1*. This comparison between systems indicated for us that the T7RNAPol appears to be more reliable than the U6snRNA system. On the other hand, the U6snRNA system has advantages in that it allows for transient expression of Cas9 and the sgRNA and therefore can be used in wild type strains and several species of *Leishmania*. The expression of the sgRNA using the U6snRNA promoter, while transient, still allows for the maintenance of the circular vector for an extended period, as well as SpCas9 and sgRNA expression. Therefore, once the first allele is replaced the second one can still be targeted by sgRNAs and deleted using the donor DNA already integrated as a homology template for recombination. Whilst we were able to generate *PNT1* null mutant clones using the U6snRNA system, these mutants were not viable and died after several passages in culture. Our inability to generate *PNT1* null mutants using the T7RNAPol system might be explained by the T7RNAPol cell line having a slight loss of fitness in comparison to wild type parasites. Taken together, we believe both systems have advantages and disadvantages that must be taken in consideration when choosing which system to use.

Promastigote null mutants were generated for 13/16 of the cysteine peptidases that were targeted for deletion. For three of the cysteine peptidases null mutants were not isolated. These were provisionally ascribed an essential status based on the inability to generate null mutants, either because no transfectants were recovered, as observed for *PNT1* and *CAL27.1*, or because an extra copy of the gene was retained after double selection as observed to *CAL31.6,* a gene found on supernumerary chromosome 30. Some of the non-essential peptidases in this list, from Clan CA C78, C85 and Clan CP C97 are predicted to be involved in the ubiquitination pathway. It could be that these peptidases are redundant given that there are other deubiquitinases in the *Leishmania* parasite. The roles of these peptidases also remain to be investigated in the amastigote form of the parasite. The calpains investigated in this study have the catalytic residues that suggest that these should be active in the parasite. Of note, depletion of the *T. brucei* homolog of *lmCAL27.1*, which is annotated as ClpGM6 and localizes to the flagellar pocket, leads to shortening of the flagellar attachment zone (FAZ) and a transition from a trypomastigote to epimastigote form [40]. A similar deregulation of differentiation could be a reason why null mutants for *CAL27.1* could not be isolated in *Leishmania*. The D-alanyl-glycyl endopeptidase has important role in bacterial proliferation [41] however, their role in *Leishmania* life cycle is yet to be elucidated. Both the D-alanyl-glycyl endopeptidase were found to be non-essential for the survival of the parasite, so these enzymes might have redundant roles. The finding that Clan CD C50 member, separase, is non-essential in the promastigote stage of the parasite is unusual. Separase undertakes the proteolytic cleavage of cohesin molecules at the end of anaphase

[42-44] which allows cell cycle to proceed. Chromosome segregation cannot proceed until the cohesion links are broken by separase. The absolute requirement for cleavage of the cohesion molecules by separase and cell cycle progression suggests that the most likely explanation for the survival of *Leishmania mexicana* parasites after deletion of separase is the presence of another peptidase with a compensatory role. It could be hypothesised that the most likely candidate is Clan CD (C14) metacaspase, which has been implicated in chromosome segregation in *L. major* [12].

DiCre mediated inducible knockout of the single copy *PNT1* showed that PNT1 is essential for the viability of *L. mexicana.* CRISPR-Cas9 mediated knockout of PNT1 was lethal. IFA data also shows that PNT1 staining is more intense in cells that are dividing and that levels of PNT1 decreased as the cells approached stationary phase, suggesting that PNT1 has a role in the cell division of *L. mexicana*. Interestingly, PNT1 did not localize to the kinetoplast and that kinetoplasts were not lost after depletion of PNT1 in the DiCre line. In addition, blebs and pits were also formed on the surface of the parasites This phenotype is different from RNAi of PNT1 in bloodstream form *T. brucei* where PNT1 is exclusively required for the maintenance of the kinetoplast in the parasite. Additionally, it can also be inferred that an active PNT1 is required for the parasite survival. Altogether, our data suggests that PNT1 has a cell cycle related function in *L. mexicana*, which is distinct from maintenance of the kinetoplast found in *T. brucei*. Elucidating the structure of *L. mexicana* PNT1 will help in drug development efforts.

Figure Legends

Fig. 1: Domain analysis of *L. mexicana* cysteine peptidases. (A) Distribution in different clans and families using the MEROPS classification system [9]. (B) Domain analysis of the 16 cysteine peptidases analysed in this study. Various domains are depicted at the side of the image. Analysis was done using the SMART domain server.

Fig. 2: *PNT1* deletion mediated by CRISPR-Cas9 U6snRNA system. (A) Diagram of the pNUS-CAS9_PNT1.1sgRNA plasmid and donor DNA containing *PNT1* specific 5' and 3'UTR for recombination. Diagnostic PCR primers were designed to amplify the integrated *HYG* cassette, with primers binding upstream of the open reading frame (PCR1), and *PNT1* full-length (PCR2). (B) Diagnostic PCRs of clones obtained after transfection and selection with HYG confirming integration of the resistance cassette and presence or absence of *PNT1* (C) Proliferation of *PNT1* clones 1-6 compared to WT parasites, cells counted every 24h until 120h.

Fig. 3: CRISPR-Cas9 mediated tagging of 16 cysteine peptidases. Intracellular localization of mNeonGreen tagged cysteine peptidases using live cell imaging of *L. mexicana* (A-M). DNA was stained with Hoescht 33342. Scale bar: 2 μm.

Fig. 4: Confirmation of T7Cas9 mediated gene knockout using PCR. Primers were designed to amplify internal regions of the CDS (approximately 400 bp) and the integrated resistance cassette, with forward primer binding upstream the integration region and reverse binding to the 5'of resistance marker, either *BSD* (blasticidin), *NEO* (geneticin) or *PUR* (puromycin). The genomic DNA from the clones was used for the PCR. The lanes on the gels show the presence or absence of the CDS of the gene of interest and the integration PCRs showing the integration of *BSD*/*PUR* or *BSD*/*G418* resistance cassettes. The arrow adjacent to LmCALP4.1 shows integration of the G418 resistance cassette.

Fig. 5: Subcellular localization of PNT1. Immunofluorescence staining for PNT1 in interphase (A-B) and dividing (C-D) cells. DNA was stained with Hoescht 33342 (shown in blue) and PNT1 was stained with PNT1 Ab followed by secondary mouse TRITC antibody (Molecular probes)-. Scale bar: 2 μm (C) *L. mexicana* cell lysates were analysed by Western blot on different days of growth with a PNT1 antibody. OPB was used as a loading control. 2d, 3d, 4d and 5d refers to cell extract after 2, 3, 4 and 5 days of growth respectively.

Fig. 6: DiCre mediated inducible knockout of *PNT1* (A) Left; Schematic representation of the genotype of Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] before (upper) and after (lower) excision with rapamycin. The position of primers and the expected PCR fragment size used to confirm DiCre mediated *PNT1* excision are highlighted. Right: Clone 4 and 9 were used for the analysis. Gel image shows PCR fragments before and after rapamycin (Rap) induction. (B) Western blot analysis of clones 4 and 9 before and after rapamycin induction. EF1-α was used as a loading control. (C) Growth curves of PNT1 clones 4 and 9 before and after induction with rapamycin. The graph shows the effect of the second round of rapamycin induction after an initial 72 hr induction. The graph was generated by plotting average values from 2 independent experiments (D) Clones isolated after rapamycin induction in *PNT1* floxed clone 9.

Fig. 7: (A) PCR confirmation of the excision of *PNT1* floxed clones 4 and 9 after rapamycin treatment (B) DAPI staining of Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] clone 9 before and after rapamycin treatment. Scale bar: 5 μm. (C) and (D) SEM and TEM of Δ*pnt1*::*PNT1*^{flox}/Δ *pnt1*::HYG [SSU DiCRE] clone 9 before and after rapamycin treatment. N and K stand for Nucleus and Kinetoplast respectively.

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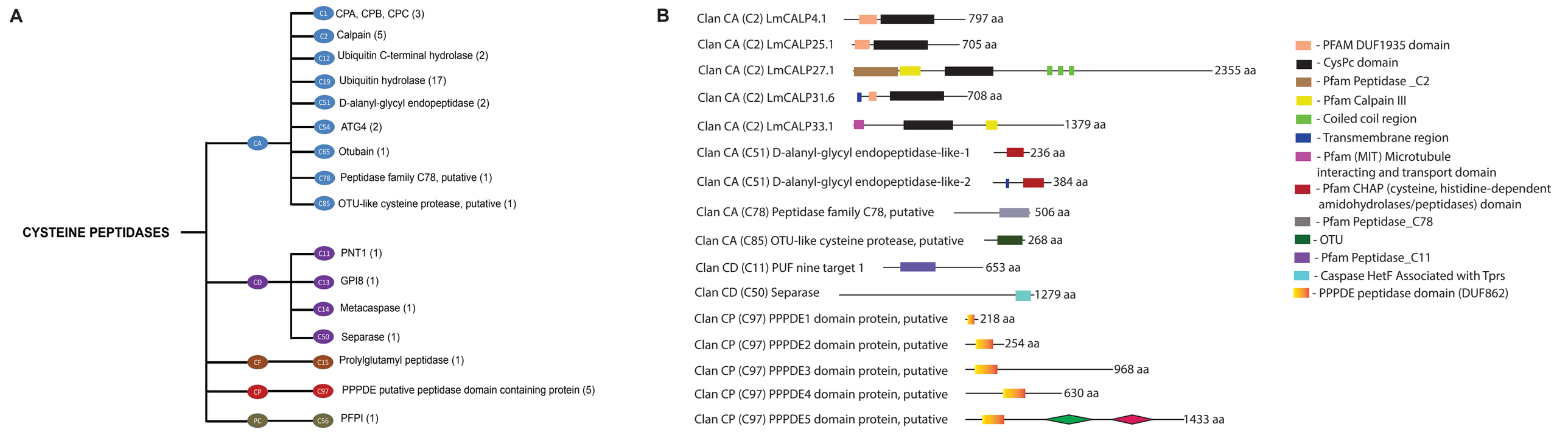
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C2
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C12
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C19
Ubiquitin hydrolase (17)
C51
D-alanyl-glycyl endopeptidase (2)
C54
ATG4 (2)
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Otubain (1)
C78
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C85
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C11
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GPI8 (1)
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Metacaspase (1)
C50
Separase (1)
CF
C15
Prolylglutamyl peptidase (1)
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C56
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1433 aa
- PFAM DUF1935 domain
- CysPc domain
- Pfam Peptidase _C2
- Pfam Calpain III
- Coiled coil region
- Transmembrane region
- Pfam (MIT) Microtubule interacting and transport domain
- Pfam CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain
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- Pfam Peptidase_C11
- Caspase HetF Associated with Tprs
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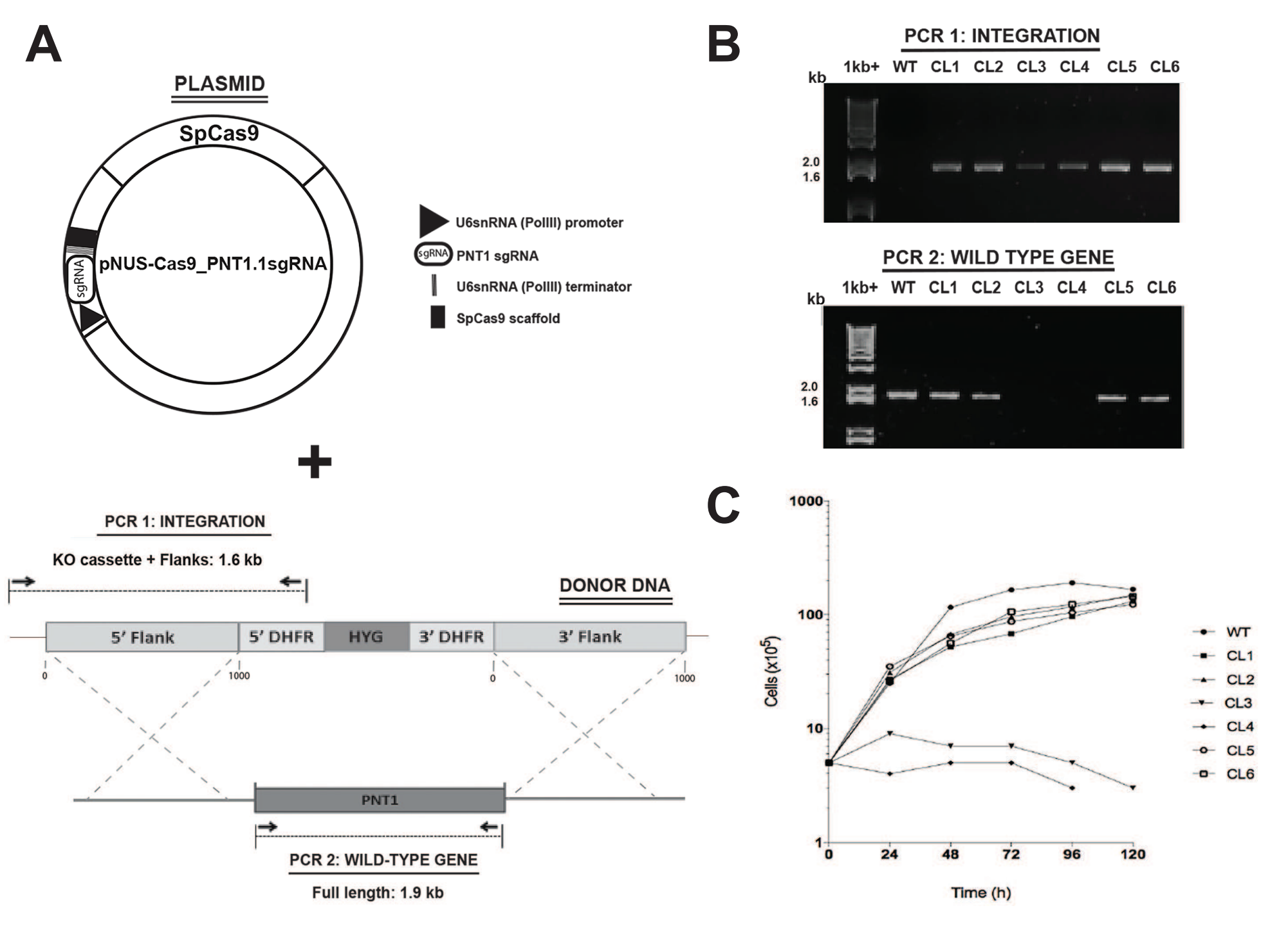
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sgRNA
PNT1 sgRNA
U6snRNA (PolIII) terminator
SpCas9 scaffold
+
PCR 1: INTEGRATION
KO cassette + Flanks: 1.6 kb
DONOR DNA
5' Flank
5' DHFR
HYG
3' DHFR
3' Flank
0
1000
0
1000
PNT1
PCR 2: WILD-TYPE GENE
Full length: 1.9 kb
B
PCR 1: INTEGRATION
1kb+ WT CL1 CL2 CL3 CL4 CL5 CL6
kb
2.0
1.6
PCR 2: WILD TYPE GENE
1kb+ WT CL1 CL2 CL3 CL4 CL5 CL6
kb
2.0
1.6
C
1000
100
10
1
Cells (x10^5)
0
24
48
72
96
120
Time (h)
WT
CL1
CL2
CL3
CL4
CL5
CL6

Clan C51
LmxM.32.2850

Clan C51
LmxM.32.2830

CAL33.1
LmxM.32.2010

CAL31.6
LmxM.30.0460

CAL27.1
LmxM.27.0490

CAL25.1
LmxM.25.1480

CAL4.1
LmxM.04.0450

G F E D C B A

DIC

Hoescht 33342

mNeonGreen

Merge

Separase (1N1K)
LmxM.20.1680

PPPDE5
LmxM.32.2260

PPPDE4
LmxM.31.1330

PPPDE3
LmxM.24.0650

PPPDE1
LmxM.09.1310

OTU-like
LmxM.36.6020

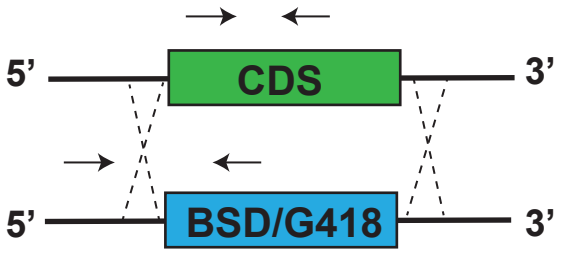
M L K J I H

DIC

Hoescht 33342

mNeonGreen

Merge



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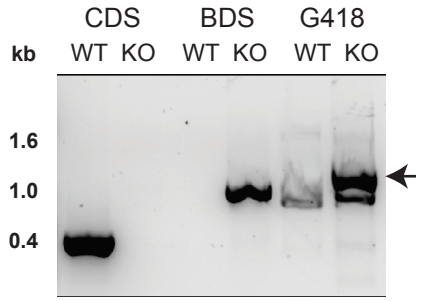

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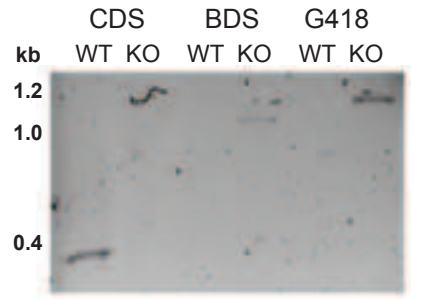
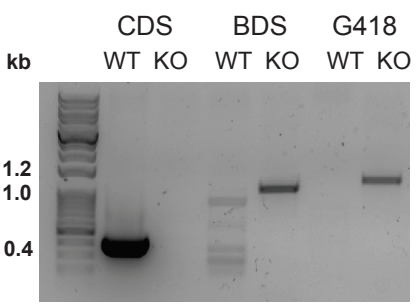
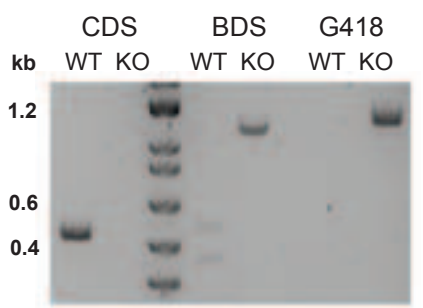


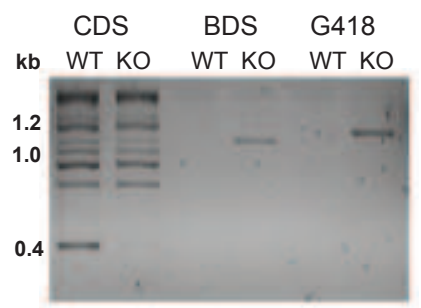
Clan CP C97 (LmxM.09.1310)



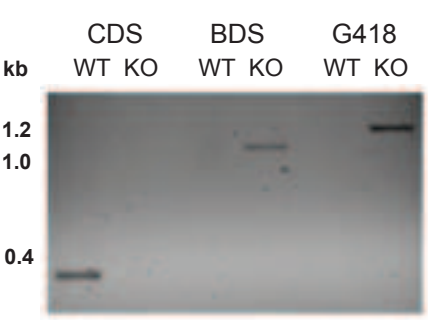
LmCALP25.1 (LmxM.25.1480)



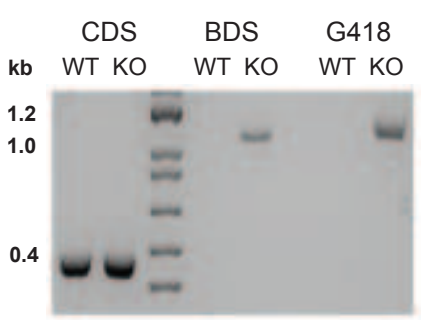
Clan CA C78 (LmxM.33.4000)



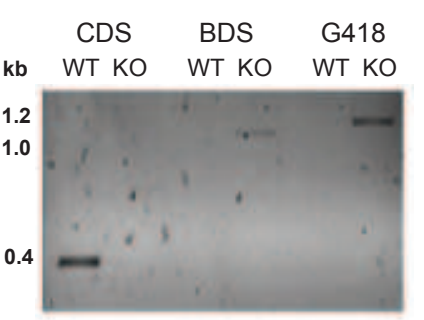
Clan CP C97 (LmxM.24.0650)



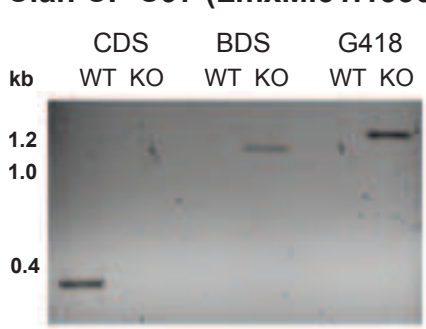
LmCALP31.6 (LmxM.30.0460a)



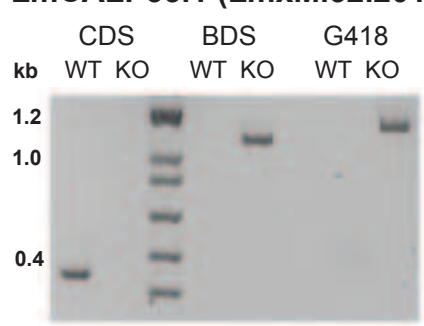
Clan CA C85 (LmxM.36.6020)



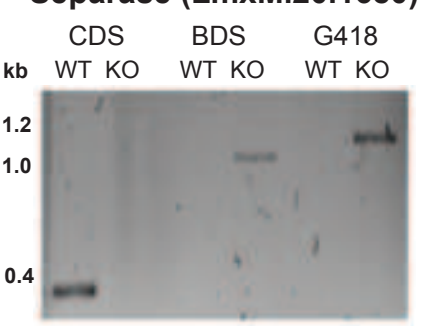
Clan CP C97 (LmxM.31.1330)



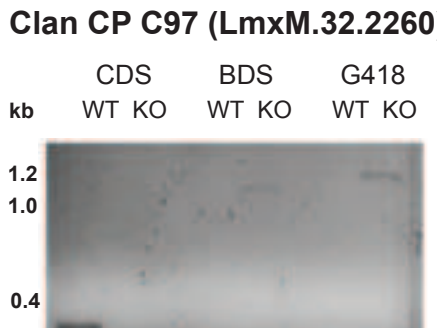
LmCALP33.1 (LmxM.32.2010)



Separase (LmxM.20.1680)

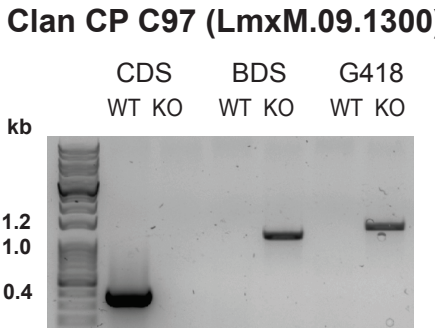


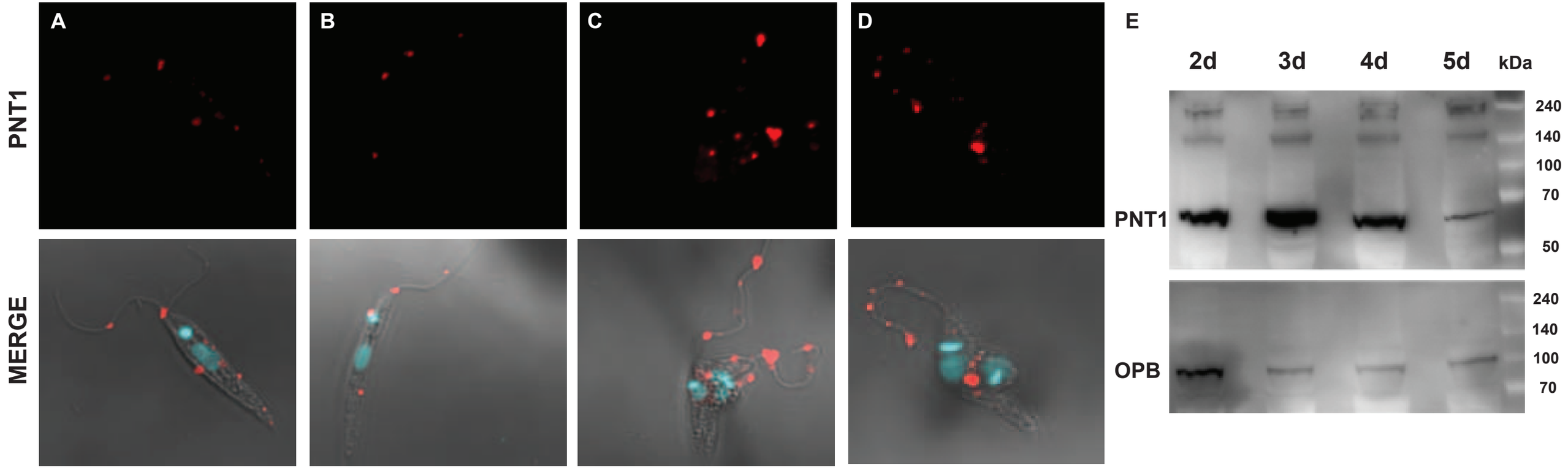
Clan CP C97 (LmxM.32.2260)

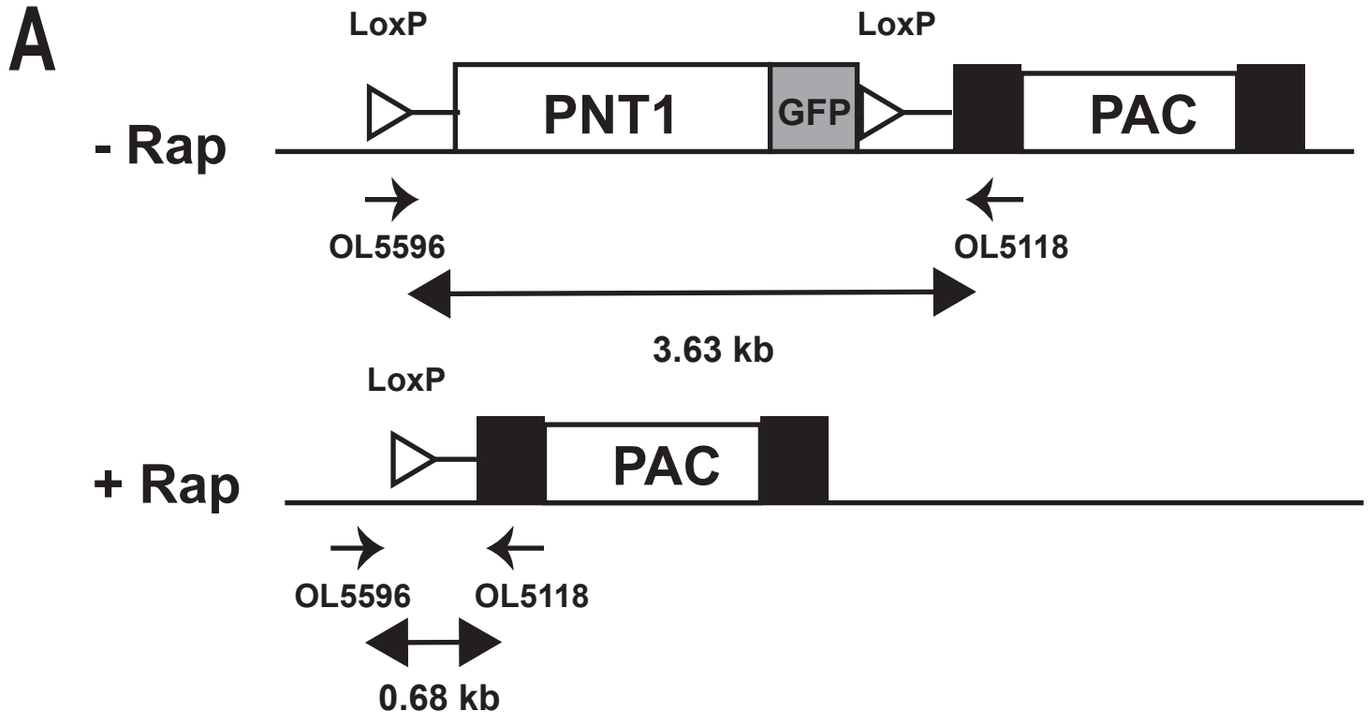


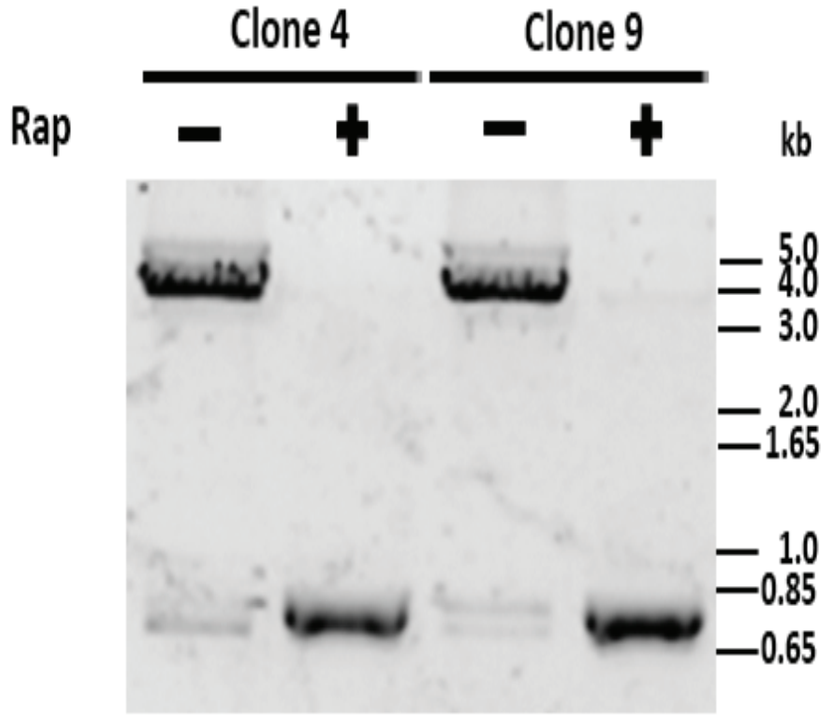
Clan CA C51 (LmxM.32.2830)

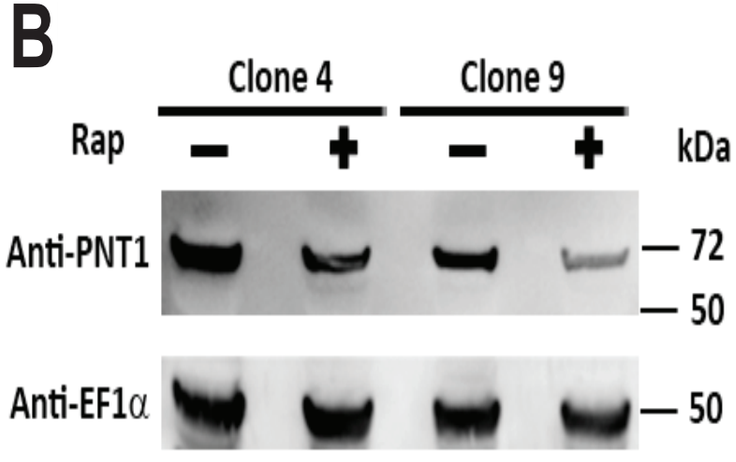
Clan CP C97 (LmxM.09.1300)

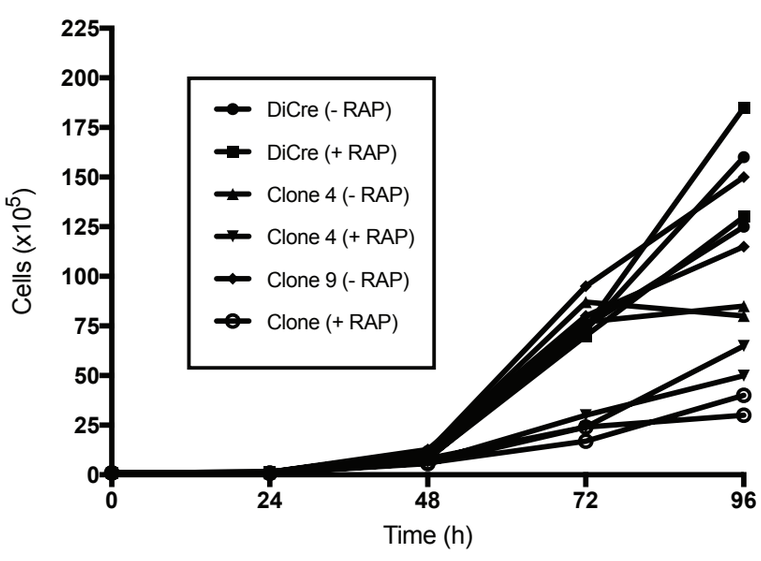


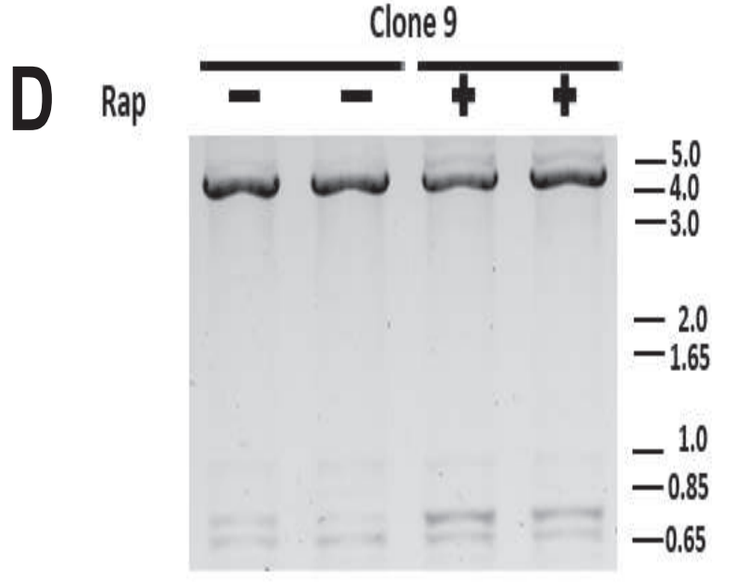

A
B
C
D
PNT1
MERGE
E
2d
3d
4d
5d
kDa
PNT1
OPB
240
140
100
70
50
240
140
100
70


A
LoxP
LoxP
PNT1
GFP
PAC
- Rap
OL5596
OL5118
3.63 kb
LoxP
PAC
+ Rap
OL5596
OL5118
0.68 kb


Clone 4
Clone 9
Rap
kb
5.0
4.0
3.0
2.0
1.65
1.0
0.85
0.65


B
Clone 4
Clone 9
Rap
kDa
Anti-PNT1
72
50
Anti-EF1α
50


C
DiCre (- RAP)
DiCre (+ RAP)
Clone 4 (- RAP)
Clone 4 (+ RAP)
Clone 9 (- RAP)
Clone (+ RAP)
Cells (x10^5)
Time (h)
0
24
48
72
96


D
Clone 9
Rap
5.0
4.0
3.0
2.0
1.65
1.0
0.85
0.65

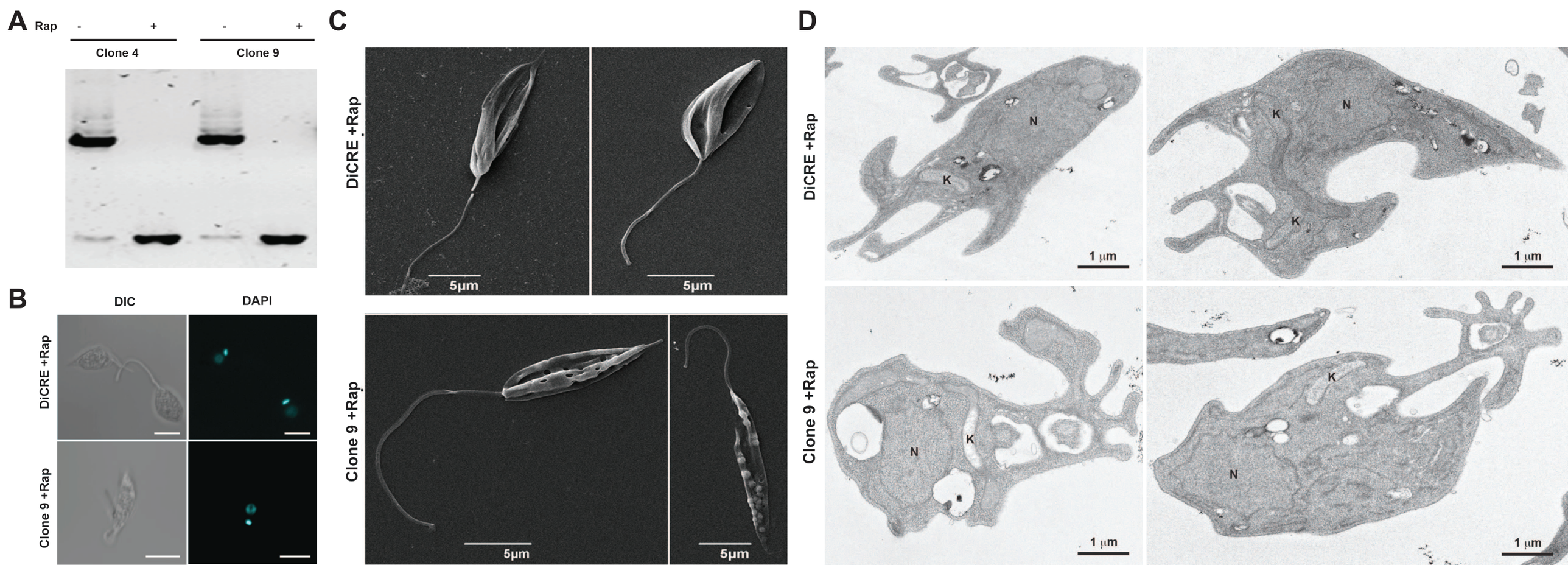

A
Rap
-
+
-
+
Clone 4
Clone 9
B
DIC
DAPI
DiCRE +Rap
Clone 9 +Rap
C
DiCRE +Rap
Clone 9 +Rap
5µm
5µm
5µm
5µm
D
DiCRE +Rap
Clone 9 +Rap
N
K
1 µm

Table 1 – Summary of 16 cysteine peptidases, their localisation and essentiality.

Gene ID	Name	Family	Localisation	Gene deletion status
LmxM.04.0450	LmCAL4.1	C2	Flagellum	Null mutant
LmxM.25.1480	LmCAL25.1	C2	Endomembrane - lysosome	Null mutant
LmxM.27.0490	LmCAL27.1	C2	Flagellar pocket	No growth*
LmxM.30.0460	LmCAL31.6		Small cytoplasmic organelles - lysosome	Extra CDS**
LmxM.32.2010	LmCAL33.1	C2	Endomembrane - dots	Null mutant
LmxM.32.2830	D-alanyl-glycyl endopeptidase-like	C51	Small cytoplasmic organelles	Null mutant
LmxM.32.2850	D-alanyl-glycyl endopeptidase-like	C51	Small cytoplasmic organelles	Null mutant
LmxM.33.4000	ubiquitin modifier-specific peptidase 1	C78	No detectable signal	Null mutant
LmxM.36.6020	OTU-Like cysteine peptidase	C85	Small cytoplasmic organelles	Null mutant
LmxM.09.1310	PPPDE 1 putative peptidase domain containing protein, putative	C97	Cytoplasm (low expression)	Null mutant
LmxM.09.1300	PPPDE 2 putative peptidase domain containing protein, putative	C97	*Failed endogenous tagging*	Null mutant
LmxM.24.0650	PPPDE 3 putative peptidase domain containing protein, putative	C97	Cytoplasm (low expression)	Null mutant
LmxM.31.1330	PPPDE 4 putative peptidase domain containing protein, putative	C97	Endomembrane - endocytic	Null mutant
LmxM.32.2260	PPPDE 5 putative peptidase domain containing protein, putative	C97	Endomembrane – endocytic, lysosome	Null mutant
LmxM.20.1680	Separase	C50	Nucleus	Null mutant
LmxM.11.0720	PUF 9 Target 1 (PNT1)	C11	Cell body and flagellum (foci), data from antibody staining.	Essential, validated using CRISPR-Cas9 and Di-Cre.

* No double resistant transfectants recovered

** Double resistant mutants retained at least one copy of the gene.