Cas9-mediated genome editing in *Giardia intestinalis*

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Abstract

CRISPR/Cas9 system is an extremely powerful technique that is extensively used for different genome modifications in various organisms including parasitic protists. *Giardia intestinalis*, a protozoan parasite infecting large number of people around the world each year, has been eluding the use of CRISPR/Cas9 technique so far which may be caused by its rather complicated genome containing four copies of each gene in its two nuclei. Apart from only single exception (Ebneter et al., 2016), without the use of CRISPR/Cas9 technology in its full potential, researchers in the field have not been able to establish knock-out cell lines to study the functional aspect of *Giardia* genes. In this work, we show the ability of *in-vitro* developed CRISPR/Cas9 components to successfully edit the genome of *G. intestinalis*. Moreover, we used 'self-propagating' CRISPR/Cas9 system to establish full knock-out cell lines for *mem*, *cwp1* and *mlf1* genes. We also show that the system function even for essential genes, as we knocked-down *tom40*, lowering the amount of Tom40 protein by more than 90%. Further, we tested the length of homologous arms needed for successful integration of homology recombination cassette used for genome editing. Taken together, our work introduces CRISPR/Cas9 to *Giardia* for routine use in the lab, further extending the catalogue of molecular tolls available for genetic manipulation of the protist and allowing researchers to study the function of *Giardia* genes properly for the first time.

Introduction

Giardia intestinalis is a binucleated protozoan parasite colonizing small intestine of various mammals (Einarsson, Ma'ayeh, et al., 2016). With exceptions (Tůmová et al., 2016, 2019), *Giardia* is considered tatraploid as both its nuclei harbor two copies of each gene (Morrison et al., 2007; Xu et al., 2020). Although this actual tetraploidy and lack of defined sexual cycle (Poxleitner et al., 2008) proved *Giardia* difficult to genetically manipulate, there has been handful of studies showing its possibility. Translational repression by morpholino oligonucleotides has been extensively used, e.g. (Hardin et al., 2017; Kim et al., 2014; Kim & Park, 2019; Krtková et al., 2016) and, very recently, catalytically inactive form of CRISPR/Cas9 system, CRISPRi, has been introduced to *Giardia* for successful knockdown of several genes (McInally et al., 2019). Moreover, classical CRISPR/Cas9 approach has

been also used recently to manipulate *Giardia* genome (Lin et al., 2019). Despite these aforementioned achievements, the only true knock-out in all four copies of the gene was achieved for *cwp1* using Cre/loxP system (Ebneter et al., 2016).

As the full knock-out of *cwp1* mentioned above took the authors months to establish and the fact that CRISPR/Cas-based systems work in *Giardia*, we used 'classical' CRISPR/Cas system to successfully knock-out several genes from *Giardia* genome. We used in-vitro developed Cas9-guideRNA ribonucleoprotein complex to edit *Giardia* genome. Furthermore, by introducing 'self-propagating' CRISPR/Cas system, we knocked-out *mem* gene (GL50803_16374) that is harbored only by one nucleus (Tůmová et al., 2019). We also used the same strategy to completely knock-out *cwp1* and *mlf1*, genes that were used as a targets for genomic manipulation previously (Ebneter et al., 2016; Lin et al., 2019) without achieving full knock-outs. Furthermore, we tested the system on *tom40* which is considered essential, causing massive knock-down of the gene. We further examined the length of flanking regions needed as a template for successful homologous recombination. Taken together, we show that CRISPR/Cas is suitable for genome editing in *Giardia intestinalis* and that it is capable of producing knock-out cell lines in various genes.

Results

Introducing the CRISPR/Cas system to *Giardia intestinalis*

So far, only *cwp1* had been knocked-out from *G. intestinalis* genome (Ebneter et al., 2016) which is why we chose this gene as a target for testing the CRISPR/Cas system. To assess whether the system is suitable for genome editing, we first electroporated the cells with modified pTG vector (Martincová et al., 2012), carrying gene for puromycin resistance (*pac*) flanked by 988 bp and 998 bp of both 5' and 3' upstream or downstream regions (UR/DR), respectively, of *cwp1* (Figure 1A) that would serve as template for future homology recombination of the homology recombination cassette (HRC) into *Giardia* genome. To these cells, we electroporated Alt-R® *S. p.* Cas9 Nuclease in complex with CWP1_96 or CWP1_492 guide RNAs, targeting Cas9 nuclease to 96 bp or 492 bp position from the start codon of *cwp1*, respectively, causing double-stranded break in the genomic DNA that would lead to homology recombination based on the template provided by pTG plasmid. After electroporation, the cells were grown to confluency and genomic DNA was extracted and tested for integration of the HRC into *Giardia* genome by PCR (Figure 1B). To test if the integration lead to complete deletion of *cwp1*, we analyzed isolated genomic DNA for the presence of the gene (Figure 1B).

Further, we tested if PCR product only may serve as a template for homology recombination. Thus, we amplified the HRC from pTG vector (Figure 1A) and electroporated 3 µg of the product together with Cas9-CWP1_96 or Cas9-CWP1_492 gRNA complex, respectively, into wild-type cells. After the cells reached confluency, we harvested genomic DNA and tested it by PCR for HRC integration and presence of the *cwp1* (Figure 1C).

As a control whether the CRISPR/Cas system is responsible for the genome editing, we electroporated wild type *Giardia* cells with 3 µg of the homology recombination cassette only and tested genomic DNA after electroporation. As a control, we used PCR product together with the Cas9

nuclease as described above. Surprisingly, the homology recombination cassette was integrated into *Giardia* genome even without the aid of Cas9 nuclease (Figure 1D).

To assess the efficiency of both approaches, the PUR gene was replaced by luciferase reporter gene (*nanouc*) in pTG plasmid (Figure 1A) and the cells were electroporated with (i) water as a control, (ii) 3 µg of HRC containing *nanoluc* (HRC-NanoLuc) or (iii) Cas9 nuclease in complex with CWP1_492 guide RNA or (iv) both the cassette and the RNP complex. The activity of NanoLuc was measured in all four strains 24 hours after electroporation, proving the last approach (iv) as the most efficient (Figure 1E).

Taken together, the CRISPR/Cas system is suitable for genome editing in *Giardia intestinalis*. However, by itself, the system is not sufficient to perform complete full-knock-out of the genes.

The full knock-outs of GOIs using 'self-propagating' CRISPR/Cas

We introduced plasmid pCas9 into *Giardia* cells. As a backbone of the plasmid, we used modified pONDRA vector (Dolezal et al., 2005) where the expression of HA-tagged, *Giardia* codon-optimized *S.p.* Cas9 nuclease is controlled by 5' UR and 3' DR of *mdh* and *α-tubulin* genes, respectively. The enzyme is targeted to the nuclei by SV40 nuclear localization signal (NLS) combined with NLS of GL50803_2340 gene as described in (McInally et al., 2019), (Figure 2A). The expression of Cas9 was confirmed by western blotting (Figure 2B) and its localization verified by immunofluorescence (Figure 2C). Apart from being targeted to the nucleoplasm, the enzyme is localized to distinct loci in both nuclei, resembling *Giardia* nucleoli (Jiménez-García et al., 2008; Tian et al., 2010). This localization corresponds to the work performed by the Dawson group where they targeted the CRISPRi by the same strategy (McInally et al., 2019) Further, the cells expressing Cas9 were sub-cloned to increase the number of cells expressing the nuclease, leading to 67,8 % (N=403) of cells with apparent expression.

G. intestinalis is considered tetraploid (Morrison et al., 2007; Xu et al., 2020), resulting in the need of knocking-out four copies of the gene to establish full knock-out. Thus, we first tested the system on the GL50803_16374 gene (*mem*), one of the few known genes present exclusively in one of *Giardia's* two nuclei (Tůmová et al., 2019). This would allow us the need to knock-out 'only' two copies in the same nucleus rather than four copies in two nuclei. For this, we engineered plasmid pTGuide which is derived from pTG vector (Martincová et al., 2012). It harbors PAC cassette and guide RNA cassette including gRNA insertion site (gRNA-IS) (McInally et al., 2019) flanked by 5' and 3' homologous arms of respective gene (Figure 3A). The homologous arms then serve as a template for homologous recombination, thus integrating the homology recombination cassette into the genome. In theory, once integrated into *Giardia* genome, the expression of respective gRNA would be continuous, further targeting Cas9 nuclease to other alleles of the respective gene.

We cloned two different guide RNAs, mem_307 and mem_579 into the gRNA cassette of the pTGuide plasmid together with homologous arms for *mem* gene (GL50803_16374) which we electroporated into *Giardia* cells harboring pCas9 plasmid. After the cells reached confluency, we isolated genomic DNA and tested it for HRC integration as well as presence of *mem* in the genome (Figure 3B). As the HRC has been integrated and *mem* has not been present in genomic DNA, we

performed FISH on the same strain to check the completeness of the knock-out. By use of fluorescence in-situ hybridization, we were able to detect individual loci of GL50803_16374 in nuclear spreads of *Giardia* wild-type strain. We confirmed previously described observations that this gene is completely absent from one of the two nuclei (Figure 4A) (Tůmová et al., 2019). The observed hybridization frequency in nuclei was 50.66 % (n=454 nuclei). Up to four, usually three (82%) signals were detected in the positive nuclei of control cells (Figure 4A, insets). In contrast, in *Δmem* cells, the number of positive nuclei dropped to 7.41% (n=364) and these displayed only one signal per positive nucleus with poor hybridizing intensity (Figure 4B, insets). The average number of positive signals/loci per 100 cells hence dropped from 151.9 in control cells to 8 in knock-out cells, i.e. 19 times. The decrease was equivalent in two guide-RNA variants tested. Internal hybridization control with probe against GL50803_17023 bound with the same intensity in control and knock-out cells (Supplementary figure 1). Next, we isolated cDNA from *Δmem* cells and tested it for presence of corresponding RNA (Figure 3C). As the integration of the cassette occurred, the gene was deleted from the genome and we did not detect any signal from the cDNA, we decided to test the culture for presence of MEM protein by Mass spectrometry performed on whole-cell lysate. We did not receive any hits for MEM protein compared to the control cell line (Supplementary data S1). Taken together, our data prove successful knock-out of *mem* in *Giardia intestinalis*.

Next, we tested the system's capability of knocking-out 'normal' genes as well. First, we decided to knock-out *cwp1* as we knew from the literature the knock-out would not be lethal for the cells (Ebneter et al., 2016). Next, we chose *mlf1*, gene that is found only in mammals, *Drosophila* and *Giardia* (Bras et al., 2012; Su et al., 2007; Winteringham et al., 2004) where the gene is involved in *Giardia* encystation (Lin et al., 2019) and its expression is upregulated under the pressure of selection antibiotics (Su et al., 2007). Main criterion why we chose *mlf1* was the fact that we possess antibody recognizing respected protein which enables us to easily quantify the protein level in possible knock-out cells. For both genes, we chose two gRNAs, CWP1_96 and_492 and Mlf1_163rc and_491, targeting Cas9 to respective sequences in the genome. We cloned these guide RNAs together with respective 5' and 3' homologous arms (988 bp for *cwp1* and 1000 bp for *mlf1*) into pTGuide vector and electroporated the plasmids into *Giardia* cells harboring pCas9 plasmid. Due to *Giardia*'s tetraploidy and the fact that nuclear mixing has not been observed outside the encystation (Poxleitner et al., 2008), we expected the resulting *Giardia* populations to be a mix of cells possessing the HRC in (i) one nucleus, (ii) both nuclei or (iii) no nuclei at all. Thus, after the cells reached confluency, we subcloned both populations and tested the individual clones as well as starting populations for HRC integration and the presence of respective genes by PCR. In case of *Δcwp1* cells, HRC has been integrated into the genome in all resulting strains (Figure 5A). Moreover, the integration has very likely occurred in all four alleles of the gene as we did not detect any signal for *cwp1* in genomic DNA. Next, we encysted the strains for 22 h and tested those for presence of CWP1 protein by western blotting. As expected, the protein was not detectable in any of the strains (Figure 5B). Next, we tested 5 clones originating from 492_1 cells the same way as the original populations. In all the clones tested, HRC has been integrated into all alleles, as we did not receive any signal of *cwp1* in any strain (Figure 5C), nor we detected CWP1 protein in encysting cells (Figure 5D). To verify the results, we performed FISH analysis on a selected clone (*Δcwp1_1*) (Supplementary Figure 2), cDNA analysis on encysting cells (Figure 5E) and Mass spectrometry on encysting cells (Supplementary Data S1), showing the absence of *cwp1* and respected protein.

In ΔMlf1 cells, HRC has also been integrated into *Giardia* genome in all resulting populations, however, in contrast to *Δcwp1* cells, the gene has not been removed completely from the genomes as we detected the signal from *mlf1* in all the strains (Figure 6A). Nevertheless, we analyzed the cells for presence of Mlf1 protein by western blotting. In population 163, we detected only faint signal corresponding to Mlf1 protein (Figure 6B). After subcloning the 163 strain, we received only single clonal population which we tested the same way as mentioned above. The clonal population harbored the HRC in all the alleles as we did not detect *mlf1* signal (Figure 6C), nor we detected respected protein in cell lysate (Figure 6D). Again, the result was verified by Mass Spectrometry (Supplementary Data S1).

To test if the system is suitable for knocking-out essential genes as well, we cloned Tom40_474 gRNA together with 1000 bp of 5' and 3' UR and DR of *tom40* into pTGuide. The plasmid was than electroporated into Cas9-expressing cells. Resulting population was tested for HRC integration and presence of *tom40* in the genome (Figure 7A). Because all the analyzed strain still harbored *tom40*, we sub-cloned the population into individual clones which were than analyzed the same way as the original population. In all the clones, *tom40* was still present in the genome (Figure 7A). Nevertheless, we analyzed the clones by western blotting for Tom40 protein level. Surprisingly, we observed massive decrease of Tom40 protein level in clone 3 compared to other sub-populations (Figure 7B) which was then confirmed by Mass Spectrometry (Supplementary data S1). Surprisingly, the cells did not show any measurable phenotype.

Testing the homologous arms lengths needed for successful KO

Next, we tested the lengths of the homologous arms needed for successful recombination of HRC into *Giardia* genome. We chose *cwp1* for the testing as it showed the most convenient results in previous experiments. The schematic representation of the arms lengths tested is depicted in figure 8A. We detected the HRC integration as well as deletion of *cwp1* even when only 150 bp of 5' and 3' UR and DR lengths were used (Figure 8B). However, from all the experiments we performed, 600 bp+ length proved to be the most convenient as the HRC integration occurred at all times (Supplementary data S2).

Discussion

CRISPR/Cas system has been developed quite recently (Jinek et al., 2012), nevertheless, it has been enormously used since across various organisms including protozoan parasites (Grzybek et al., 2018) and is evolving rapidly (Broeders et al., 2020). *Giardia intestinalis* has been eluding the use of this powerful technique so far. This issue may be addressed to the facts that (i), with few exceptions (Tůmová et al., 2016, 2019), its genome harbors all the genes in four copies (Morrison et al., 2007; Xu et al., 2020) and (ii) its sex called diplomixis is limited exclusively to encystation (Poxleitner et al., 2008). Until recently (McInally et al., 2019), not even expression of full-length Cas9 nuclease in *Giardia* nuclei has been achieved (Ebneter et al., 2016). Thus, achieving full knock-out of any gene has been near-to impossible so far with one exception (Ebneter et al., 2016) where the authors used Cre/loxP system to subsequently delete all four copies of the gene in the genome, resulting in

encystation-deficient cells. This technique is efficient yet very tedious, thus unsuitable for routine use in the lab.

In this work, we introduced CRISPR/Cas9 to *Giardia*, first by transfecting the cells with *in-vitro* developed RNP complex of Cas9 and guide RNA together with a template (PCR or plasmid) for homologous recombination containing UR and DR of *cwp1*. We further proved that by co-expressing Cas9 nuclease together with plasmid harboring respective guide RNA surrounded by homologous arms, we were able to completely knock-out *mem*, *cwp1* and *mlf1* genes. According to immunofluorescence, using SV40 NLS combined with NLS of GL50803_2340 gene, Cas9 localizes not only to *Giardia* nuclei but to the nucleoli in particular. This localization matches the work by (McInally et al., 2019) where they used the same localization signals. It is caused by the nucleolus detention signal of *S. p.* Cas9 and may affect the enzyme stability (Tan et al., 2020), as the *Giardia* nucleolus shows 'functionally' similar protein composition as nucleoli of other eukaryots (Feng et al., 2020).

We further showed that the system is sufficient to decrease the expression level of essential genes as we knocked-down *tom40*, resulting in decrease of Tom40 protein level by more than 90 %. We further tested the length of homologous arms needed for successful knock-out. Taken together, this work introduces CRISPR/Cas9 technique for routine use in *Giardia intestinalis*, thus extending the catalogue of molecular tolls usable for genetic manipulation of the protist and allowing researchers to study the function of various genes properly for the first time.

Materials and Methods

Cell lines, cultivation and electroporation

For all the experiments, *Giardia intestinalis* WB clone C6 (ATCC 50803) cells, a kind gift from prof. Scott Dawson, UC Davis, CS, USA, were used. The cells were cultivated under anaerobic conditions in TYI-S-33 medium supplemented with 0.1 % bovine bile (Sigma-Aldrich) and 10 % heat-inactivated adult bovine serum (Sigma-Aldrich) (Keister, 1983) and appropriate antibiotics at 37 °C. The cells were electroporated using Bio-Rad Gene Pulser (BioRad) as described in (Voleman et al., 2017). Subcloning of *G. intestinalis* culture was performed as follows: Full grown *Giardia* culture was put on ice for 10 min do detach. After that, 20 µl of cells were put to 180 µl of TYI-S-33 media in 96-wells Tissue Culture Plate (VWR) and the same dilution step was performed 5 times. A drop of 2 µl from each dilution were checked for *Giardia* cell number in three different wells to estimate optimal dilution where only one to two cells per well were present. Then, twenty times 2 µl of cells from selected dilution were put to another 96-wells plate into individual wells and checked for cell number. The wells where only one cell had been found were filled with growth medium and grown under anaerobic conditions at 37 °C. After forming a monolayer, the cells from individual wells were put to cultivation tubes and grown normally. To ensure the cloning was performed correctly, the whole procedure was repeated twice.

The cells were subjected to encystation according to Uppsala encystation protocol (Einarsson, Troell, et al., 2016) in TYI-S-33 media supplemented with 10 % heat-inactivated adult bovine serum (Sigma-Aldrich) and 5 mg/ml bovine bile (Sigma-Aldrich).

Guide RNA design and cloning

Guide RNAs (20 nt) were designed with EuPaGDT design tool (http://grna.ctegd.uga.edu/) using NGG PAM sequence. Off-target hits were checked against *G. intestinalis* Assemblage A *Giardia*DB-28 genome. gRNA oligonucleotides with four-base overhangs complementary to the vector sequence were annealed and cloned into *BbsI*-digested pTGuide as described in (McInally et al., 2019). The annealing step was performed at 95 °C for 5 minutes, then the samples were left in cooling heat-block for two hours at RT to allow proper annealing.

Cloning and plasmid construction

pTGuide plasmid was constructed as follows: pTG vector (Martincová et al., 2012) was modified by inserting the guide RNA expression cassette containing guide RNA insertion site (gRNA-IS) surrounded by U6 5' and 3' UR and DR, respectively, into pTG vector. The cassette was amplified from dCas9g1pac vector (kind gift form prof. Scott Dawson, UC Davis, CS, USA) (McInally et al., 2019) using gRNAcassette-F/R primers containing *KpnI* and *ClaI* restriction sites (Table S1). After cleavage with respective enzymes, the cassette was ligated into *KpnI*/*ClaI*-linearized pTG vector. Further, *MluI* and *AvrII* and *ClaI* and *PacI* sites were inserted 5' and 3' of the PAC cassette and the RNA expression cassette, respectively, for further homologous arms cloning.

pCas9 was constructed as follows: Giardia codon-optimized *S. p.* Cas9 nuclease followed by SV40 NLS, 3 x HA tag and NLS from GL50803_2340 together with 5' UTR from *mdh* and 3' UTR from *tubulin* were amplified from pCas9U6g1pac vector (McInally et al., 2019) using primers Cas9-F/R. After that, the product was cleaved with *KpnI* and *MluI* restriction enzymes and cloned into *KpnI/MluI*-linearized pONDRA vector (Dolezal et al., 2005).

pTGuide-Tom40 was constructed as follows: At first, Tom40_474-F/R primers (Table S1) were annealed to create Tom40-474 guide RNA that was inserted into pTGuide vector via *BbsI* restriction sites, forming pTGuide-474 plasmid. Then, 1000 bp of 5' UR and 3' DR of *Tom40* (GL50803_17161) were amplified from *G. intestinalis* genomic DNA by Tom40-5-F/R and Tom40-3-F/R primers, respectively (Table S1). Resulting sequences were cleaved with respective enzymes and subsequently cloned into pTGuide-474 plasmid via *MluI*/*AvrII* and *ClaI*/*PacI* restriction sites, respectively.

pTGuide-CWP1 was constructed as follows: First, two guide RNAs (CWP1_96 and CWP1_492) were created by annealing CWP1_96-F/R and CWP1_492-F/R primers, respectively, and inserted into pTGuide vector via *BbsI* restriction sites. Then, 988 bp of 5' UR and 998 bp of 3' DR of *cwp1* were amplified from *G. intestinalis* genomic DNA using cwp1-5-F/R and cwp1-3-F/R primers, respectively. Resulting sequences were cleaved by *MluI*/*AvrII* and *ClaI/PacI* restriction enzymes, respectively, and inserted into *MluI/AvrII*- or *ClaI*/*PacI*-linearized plasmid.

pTGuide-MEM was constructed as follows: First, two guide RNAs (MEM_307 and MEM_579) were created by annealing mem_307-F/R and 579-F/R primers, respectively, and inserted into pTGuide vector via *BbsI* restriction sites. Then, 1000 bp of 5' UR of *mem* was amplified from *G. intestinalis* genomic DNA using mem-5-F and R primers. Resulting sequence was cleaved by *MluI* and *AvrII* restriction enzymes and inserted into *MluI/AvrII*-linearized plasmid. 3´ flanking region was created by joining two PCR products by overlap extension polymerase chain reaction (OE-PCR). First, pac cassette together with mem-guide RNA cassette was amplified from pTGuide using primers pac cassette-F and gRNA cassette-R. Then, 1000 bp of 3' DR of *mem* was amplified from *Giardia* genomic DNA using mem-3-F and R primers. PCR products from reaction 1 and 2 were used as a template for

3rd PCR reaction without adding any primers. Based on the overhangs created in reactions 1 and 2, complete 3' flanking region was created and resulting sequence was cleaved with *AvrII* and *PacI* restriction enzymes and inserted into *AvrII*/*PacI*-digested plasmid.

pTGuide-Mlf1 was constructed as follows: First, two guide RNAs (Mlf1_163rc and Mlf1_491) were created by annealing mlf1_163rc-F/R and mlf1_491-F/R primers, respectively, and inserted into pTGuide vector via *BbsI* restriction sites. Then, 1000 bp of 5' UR and 3' DR of *mlf1* were amplified from *G. intestinalis* genomic DNA using mlf-5-F/R and mlf-3-F/R primers, respectively. Resulting sequences were cleaved by *MluI*/*AvrII* and *ClaI*/*PacI* restriction enzymes, respectively, and inserted into *MluI*/*AvrII*- or *ClaI*/*PacI*-linearized plasmid.

Homology recombination cassette containing NanoLuc was amplified from pTG-NLuc (Figure 1A) where *pac* was replaced by *nanoluc*, amplified from pNL1.1.PGK (Promega).

mRNA from encysting and non-encysting KO strains was isolated by NucleoTrap mRNA Mini Column kit (Macherey-Nagel) according to manufacturer's protocol, with additional step of passing the cell lysates through 0.9 mm needle (20 gauge) 6 times. Then, 60 ng of purified mRNA was used for preparation of cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen) in 20 µl reaction volume according to manufacturer's protocol. For subsequent PCR, 1 µl of cDNA was used. PCR was performed using SaphireAmp Fast PCR Master Mix (Takara) in 50 µl volume.

For testing the integration of individual HRC-cassettes, primers were used as follows: for HRC, primers CWP-int-F and pTGuide-int-R were used, for HRC-MEM, HRC-CWP1 and HRC-Mlf1, primers mem-int-F and pTGuide-int-R, CWP-int-F and pTGuide-int-R and Mlf-int-F and pTGuide-int-R were used, respectively. For HRC-Tom40, primers Tom40-int-F and pTGuide-int-R were used. For testing the homologous arms lengths, primers CWP-int-F and pTGuide-int-R were used (Table S1).

For testing the presence of individual genes in *Giardia* genome, following primers were used: For *mem*, *cwp1* and *mlf1*, mem-genomic-F/R, CWP-genomic-F/R and mlf-genomic-F/R were used, respectively. For *tom40*, primers Tom40-F/R were used (Table S1).

For testing homologous arms lengths, primers HA-X-F/R were used (Table S1).

Genome Editing by exogenous Cas9 RNP complexes

Alt-R® CRISPR-Cas9 crRNA (CRISPR RNA) (CWP1_96: GAAATCTACGATGCCACTGA, CWP1_492: ACAGCTGATTGCAGTCTAGG) and tracrRNA (trans-activating crRNA) were synthesized by Integrated DNA Technologies (IDT) and Alt-R® *S. pyogenes* Cas9 Nuclease V3 was delivered by the same company. Cas9-RNP complexes were prepared according to the company protocol (0.6 µl 100 µM crRNA, equal amount of tracrRNA and 1.3 µl 62 µM Cas9 nuclease were used for each reaction). Cas9-RNP complexes were delivered to *G. intestinalis* by standard electroporation protocol (Voleman et al., 2017). In case of simultaneous electroporation of Cas9-RNP together with the homology recombination cassette or the cassette itself, DNA was amplified from pTG plasmid (Figure 1A) using HRC-F and HRC-R primers (Table S1). For the electroporation, 3 µg of DNA were used. Successful recombination of HRC was detected by PCR on isolated genomic DNA (Geneaid) using

CWP-int-F and CWP-int-R primers. Native GiCWP1 gene was amplified using CWP genomic-F and CWP genomic-R primers.

Luciferase assay

NLuc luminescence was monitored using Nano-Glo® Luciferase Assay System (Promega) according to manufacturer's protocol. Briefly, the cells were collected 24 hours after transfection and mixed with equal volume of Nano-Glo® Luciferase Assay reagent and the relative luminescence (RLU) was measured after 7 minutes using GloMax® 20/20 Luminometer (Promega). The identical cell lysate was then used for protein concentration determination using Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) according to manufacturer's protocol. The sample absorbance measurements were carried out on Shimadzu UV-1601 Spectrophotometer using UV Probe 1.10 software.

Fluorescence *in situ* hybridization

The FISH protocol and probe construction were carried out according to (Tůmová et al., 2019). Briefly, the chromosome spreads were prepared as described by (Tůmová et al., 2016). The FISH hybridization mixture (20 ng of the labeled probe, 10 μg of salmon sperm, 50% of deionized formamide (Sigma-Aldrich)) in 2 x SSC was incubated on slides at 82 °C for 5 min. Single-color FISH was developed by a TSA-Plus TMR System according to the manufacturer's protocol (PerkinElmer) using a digoxigenin-labeled probe and an anti-dig-HRP antibody (Roche Applied Science). In two-color FISH, a sequential double hybridization signal development was processed according to the manufacturer's protocol (PerkinElmer), as a combination of (i) a digoxigenin-labeled probe, anti-dig-HRP antibody, and TSA-Plus TMR and (ii) a biotin-labeled probe, streptavidin-HRP, and TSA-Plus Fluorescein. For the FISH probe design, primers mem-fish-F/R and cwp1-fish-F/R were used, respectively. For internal hybridization control, a probe against GL50803_17023 was used in combination with *mem* probe and GL50803_17495 in combination with *cwp1* probe (Tůmová et al., 2016, 2019). The PCR products of approximately 2000 bp inside the ORFs of the above listed genes (in case of *cwp1* the whole ORF of 726 bp was used) were cloned into a pJET 1.2/blunt cloning vector (Fermentas) and transformed into chemically competent TOP10 *E. coli* cells (Invitrogen). Purified PCR products amplified from plasmids isolated from a single bacterial colony (QIAprep Spin MiniprepKIT, Qiagen) were labeled by random priming with digoxigenin-11-dUTP (Roche) or biotin-11-dUTP (PerkinElmer) using a DecaLabel DNA Labeling Kit (Fermentas). Controls for FISH accuracy were conducted as described in (Tůmová et al., 2016, 2019).

Mass Spectrometry

Protein Digestion

Cell pellets were homogenized and lysed at 95°C for 10 min in 100 mM TEAB (Triethylammonium bicarbonate) containing 2 % SDC (sodium deoxycholate), 40 mM chloroacetamide, 10 mM TCEP (Tris(2-carboxyethyl)phosphine) and further sonicated (Bandelin Sonoplus Mini 20, MS 1.5). Protein concentration was determined using BCA protein assay kit (Thermo) and 30 µg of protein per sample was used for MS sample preparation.

Samples were further processed using SP3 beads according to (Hughes et al., 2019). Briefly, 5 µl of SP3 beads was added to 30 µg of protein in lysis buffer and filled to 50 µl with 100 mM TEAB. Protein binding was induced by addition of ethanol to 60 % (vol./vol.) final concentration. Samples were mixed and incubated for 5 min at RT. After binding, the tubes were placed into magnetic rack and the unbound supernatant was discarded. Beads were subsequently washed two times with 180 µl of 80 % ethanol. After washing, samples were digested with trypsin (trypsin/protein ration 1/30) reconstituted in 100 mM TEAB at 37°C overnight. After digestion, samples were acidified with TFA to 1% final concentration and peptides were desalted using in-house made stage tips packed with C18 disks (Empore) according to (Rappsilber et al., 2007).

nLC-MS2 analysis

Nano Reversed phase columns (EASY-Spray column, 50 cm x 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size) were used for LC/MS analysis. Mobile phase buffer A was composed of water and 0.1% formic acid. Mobile phase B was composed of acetonitrile and 0.1% formic acid. Samples were loaded onto the trap column (C18 PepMap100, 5 µm particle size, 300 µm x 5 mm, Thermo Scientific) for 4 min at 18 µl/min. Loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. Peptides were eluted with Mobile phase B gradient from 4% to 35% B in 120 min. Eluting peptide cations were converted to gas-phase ions by electrospray ionization and analyzed on a Thermo Orbitrap Fusion (Q-OT- qIT, Thermo Scientific). Survey scans of peptide precursors from 350 to 1400 m/z were performed in orbitrap at 120K resolution (at 200 m/z) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1,5 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS2 ion count target was set to 10^4 and the max injection time was 35 ms. Only those precursors with charge state 2–6 were sampled for MS2. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles (Hebert et al., 2014).

Data analysis

All data were analyzed and quantified with the MaxQuant software (version 1.6.3.4) (Cox & Mann, 2008). The false discovery rate (FDR) was set to 1% for both proteins and peptides and we specified a minimum peptide length of seven amino acids. The Andromeda search engine was used for the MS/MS spectra search against the *G. intestinalis* protein database (downloaded from https://giardiadb.org on July 11th of 2020). Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds and a maximum of two missed cleavages. Dithiomethylation of cysteine was selected as fixed modification and N- terminal protein acetylation and methionine oxidation as variable modifications. The “match between runs” feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min) and this was also used in quantification experiments. Quantifications were performed with the label-free algorithm in MaxQuant (Cox et al., 2014). Data analysis was performed using Perseus 1.6.1.3 software (Tyanova et al., 2016).

Immunofluorescent labelling and microscopy analysis

For immunofluorescence, the cells were fixed by 1 % paraformaldehyde for 30 minutes at 37 °C. The cells were then centrifuged at 900 x g for 5 minutes at RT and washed in PEM buffer (200 mM PIPES, 2 mM EGTA, 0.2 mM $MgSO_4$, pH 6.9). Then, the cells were resuspended in PEM buffer and transferred to poly-L-lysine-coated coverslips and let to attach for 15 minutes. The cells were then permeabilized by 0.2 % Triton X-100 in PEM buffer for 20 minutes and washed three times with PEM buffer. Then, the cells were blocked by PEMBALG (PEM supplemented with 1 % BSA, 0.1 % NaN_3, 100 mM lysine and 0.5 % gelatin) for 30 minutes. All blocking and immunolabeling steps were performed in humid chamber at room temperature. After blocking, the cells were incubated in PEMBALG containing monoclonal anti-HA antibody produced in rat (1:1000 dilution, Roche) for one hour. The coverslips were then washed three times for 15 minutes in 0.1 % Tween-20 in PBS and incubated in PEMBALG containing monoclonal anti-rat antibody conjugated with Alexa488 (1:1000 dilution, Life Technologies) for one hour. The coverslips were then washed three times for 15 minutes in 0.1 % Tween-20 in PBS and mounted in Vectashield containing DAPI (Vector Laboratories).

Static images were acquired on Leica SP8 FLIM inverted confocal microscope equipped with 405nm and white light (470-670 nm) lasers and FOV SP8 scanner using HC PL APO CS2 63x/1.4 NA oil-immersion objective. Laser wavelengths and intensities were controlled by a combination of AOTF and AOBS separately for each channel. Emitting fluorescence was captured by internal spectrally-tunable HyD detectors. Imaging was controlled by the Leica LAS-X software. Maximum intensity projections and brightness/contrast corrections were performed in FIJI ImageJ software.

For FISH experiments, Olympus BX51 fluorescence microscope equipped with a DP70-UCB camera was used; imaging sensitivity was set up equally for controls and knock-out cell lines.

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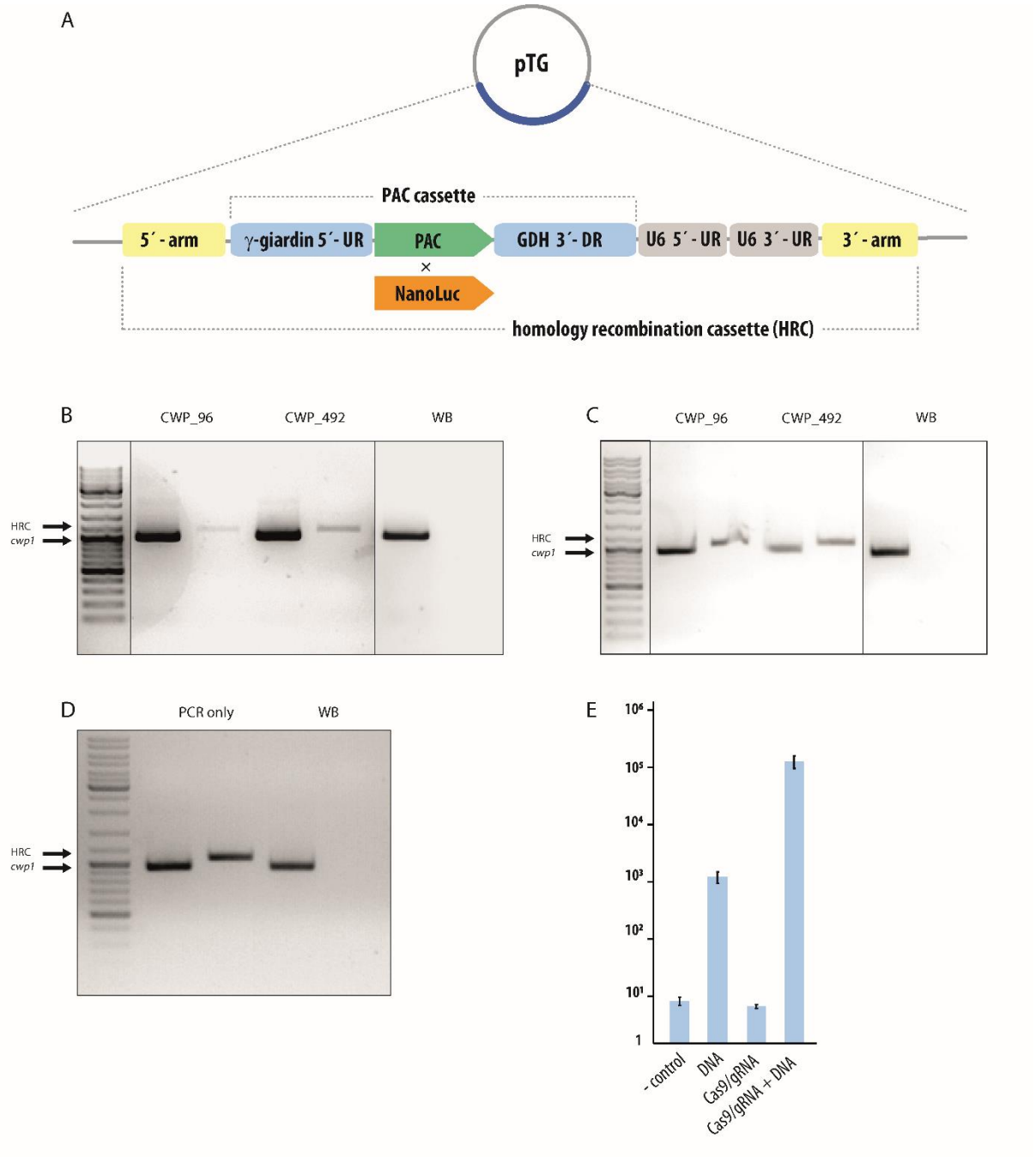
A
pTG
PAC cassette
5´- arm
γ-giardin 5´- UR
PAC
GDH 3´- DR
U6 5´- UR
U6 3´- UR
3´- arm
×
NanoLuc
homology recombination cassette (HRC)
B
CWP_96
CWP_492
WB
HRC
cwp1
C
CWP_96
CWP_492
WB
HRC
cwp1
D
PCR only
WB
HRC
cwp1
E
10⁶
10⁵
10⁴
10³
10²
10¹
1
- control
DNA
Cas9/gRNA
Cas9/gRNA + DNA

Figure 1

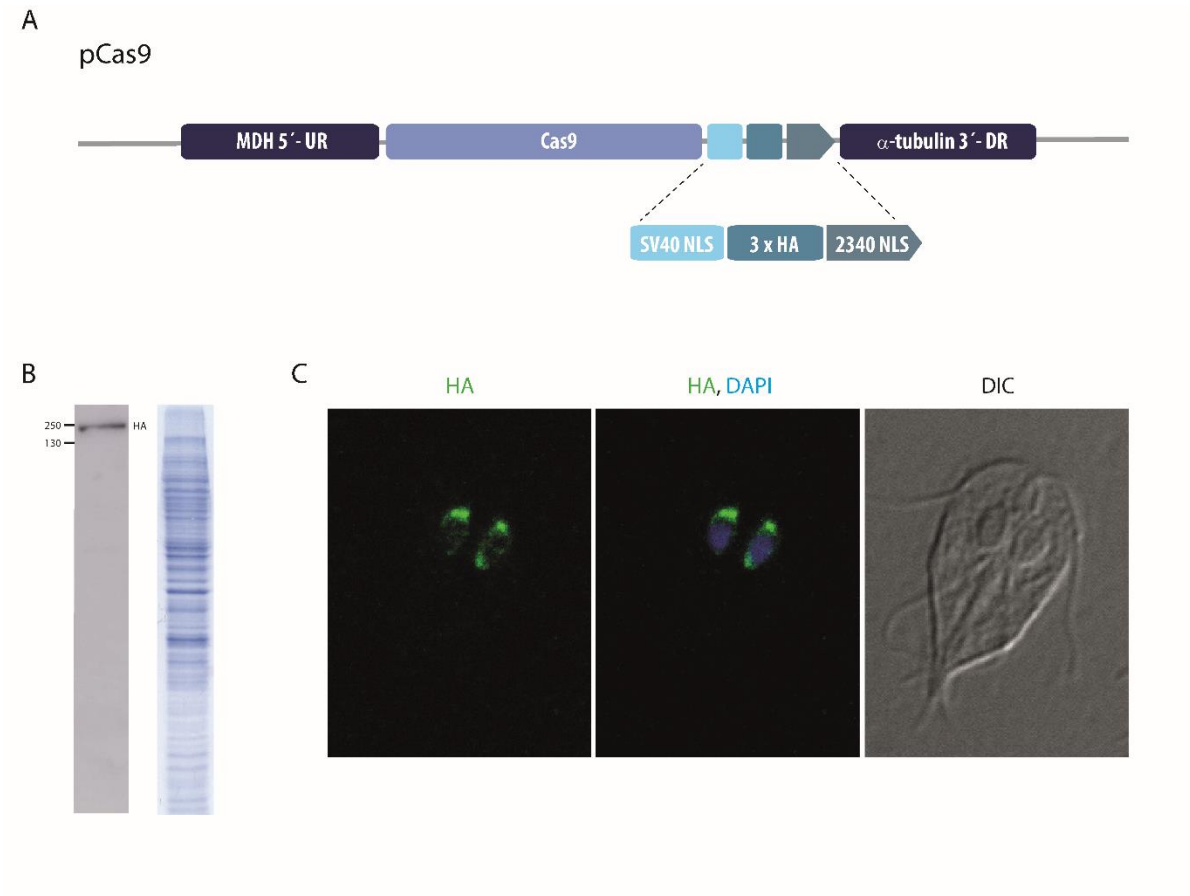
A
pCas9
MDH 5´- UR
Cas9
α-tubulin 3´- DR
SV40 NLS
3 x HA
2340 NLS
B
250
130
HA
C
HA
HA, DAPI
DIC

Figure 2

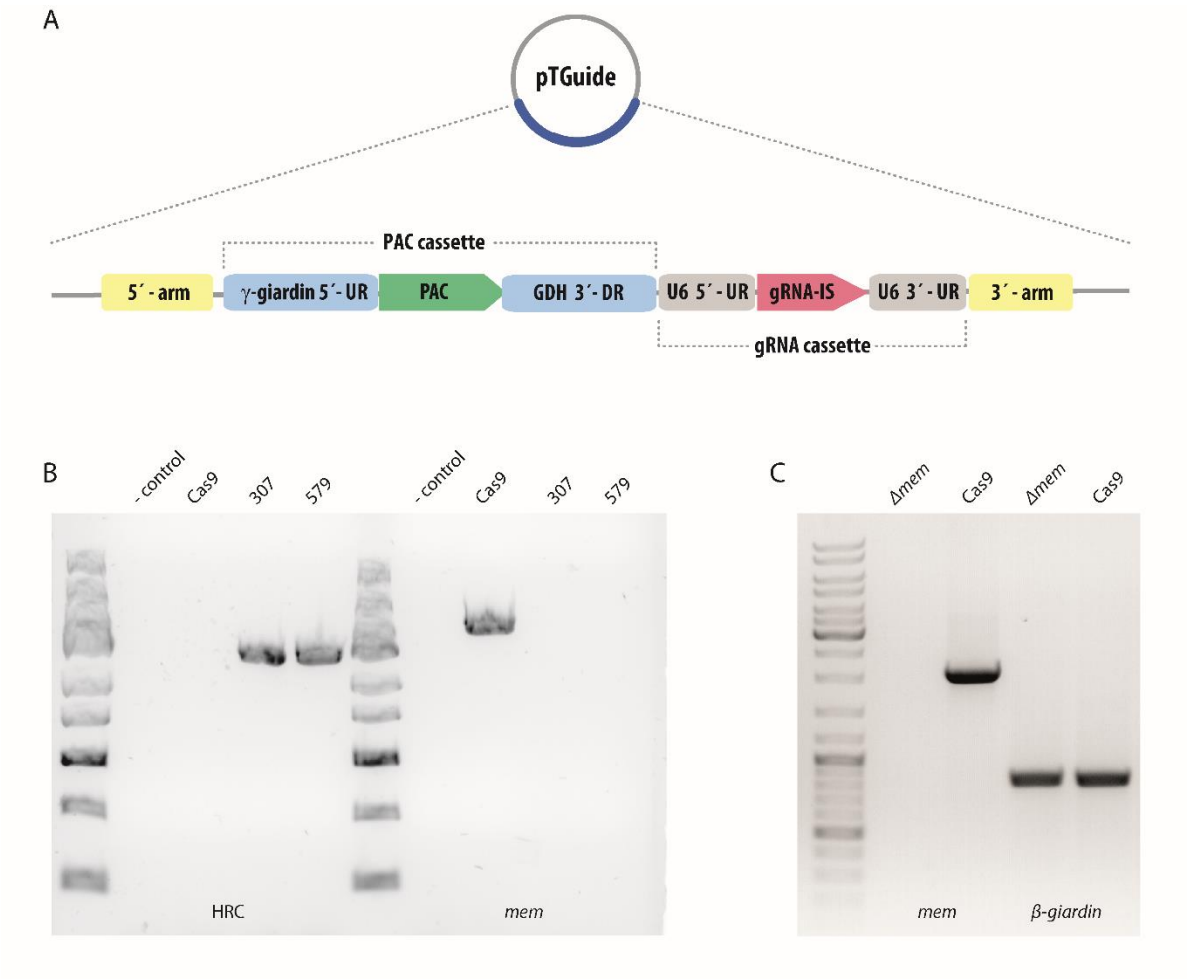
A
pTGuide
PAC cassette
5´-arm
γ-giardin 5´-UR
PAC
GDH 3´-DR
U6 5´-UR
gRNA-IS
U6 3´-UR
3´-arm
gRNA cassette
B
- control
Cas9
307
579
- control
Cas9
307
579
HRC
mem
C
Δmem
Cas9
Δmem
Cas9
mem
β-giardin

Figure 3

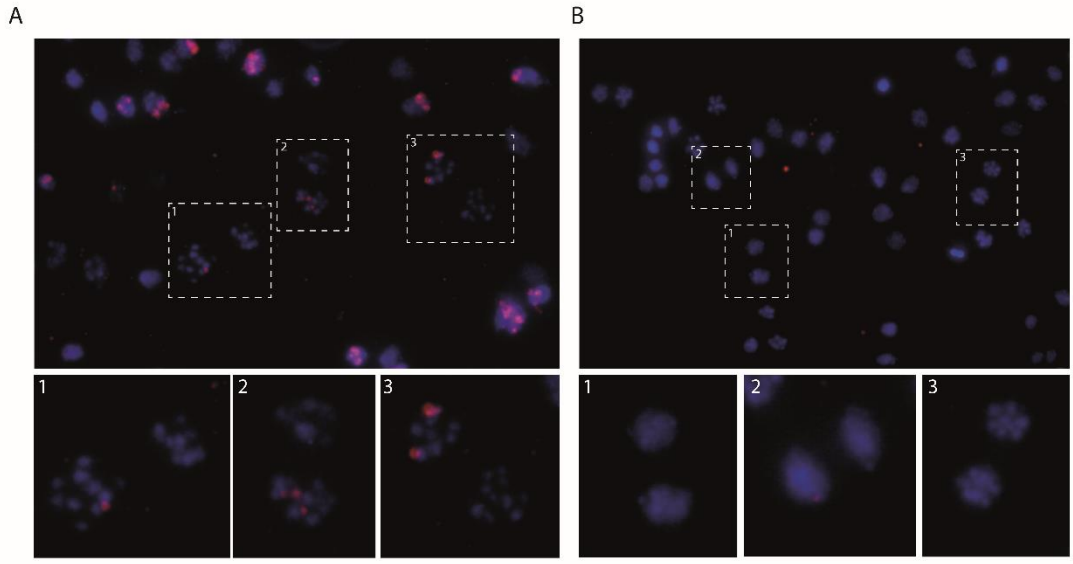

Figure 4

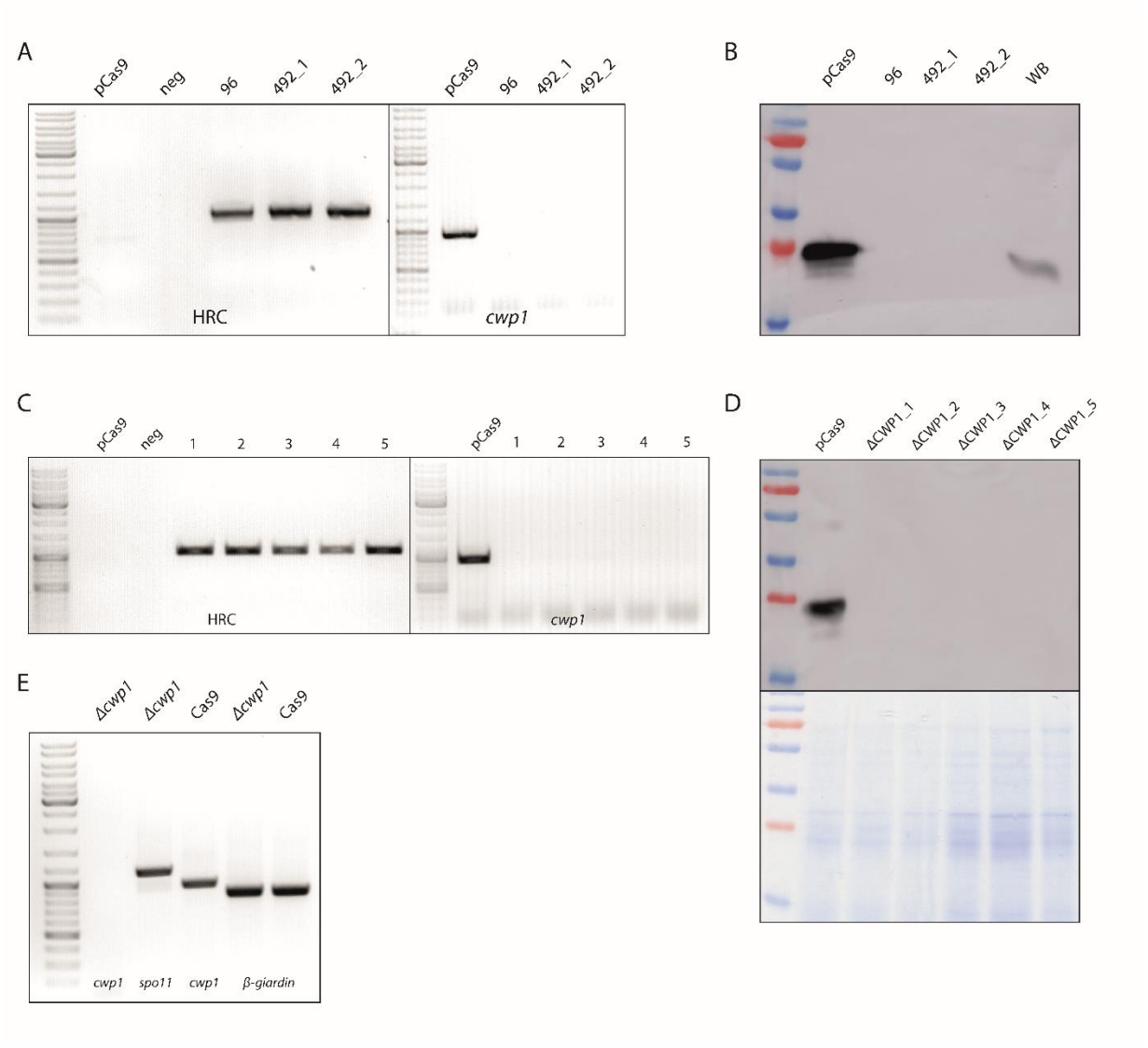
A
pCas9
neg
96
492_1
492_2
pCas9
96
492_1
492_2
HRC
cwp1
B
pCas9
96
492_1
492_2
WB
C
pCas9
neg
1
2
3
4
5
pCas9
1
2
3
4
5
HRC
cwp1
D
pCas9
ΔCWP1_1
ΔCWP1_2
ΔCWP1_3
ΔCWP1_4
ΔCWP1_5
E
Δcwp1
Δcwp1
Cas9
Δcwp1
Cas9
cwp1
spo11
cwp1
β-giardin

Figure 5

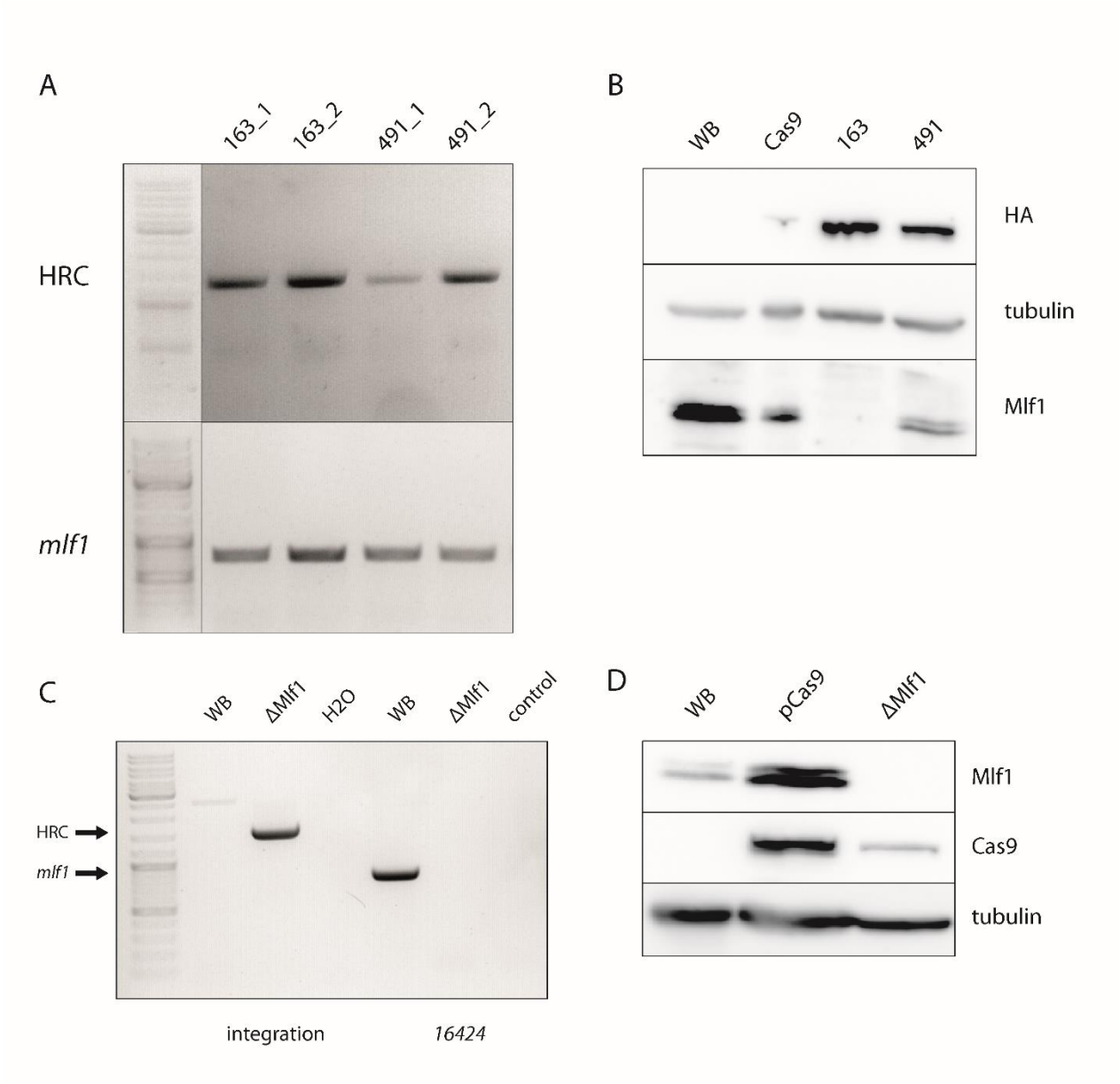
A
163_1
163_2
491_1
491_2
HRC
mlf1
B
WB
Cas9
163
491
HA
tubulin
Mlf1
C
WB
ΔMlf1
H2O
WB
ΔMlf1
control
HRC
mlf1
integration
16424
D
WB
pCas9
ΔMlf1
Mlf1
Cas9
tubulin

Figure 6

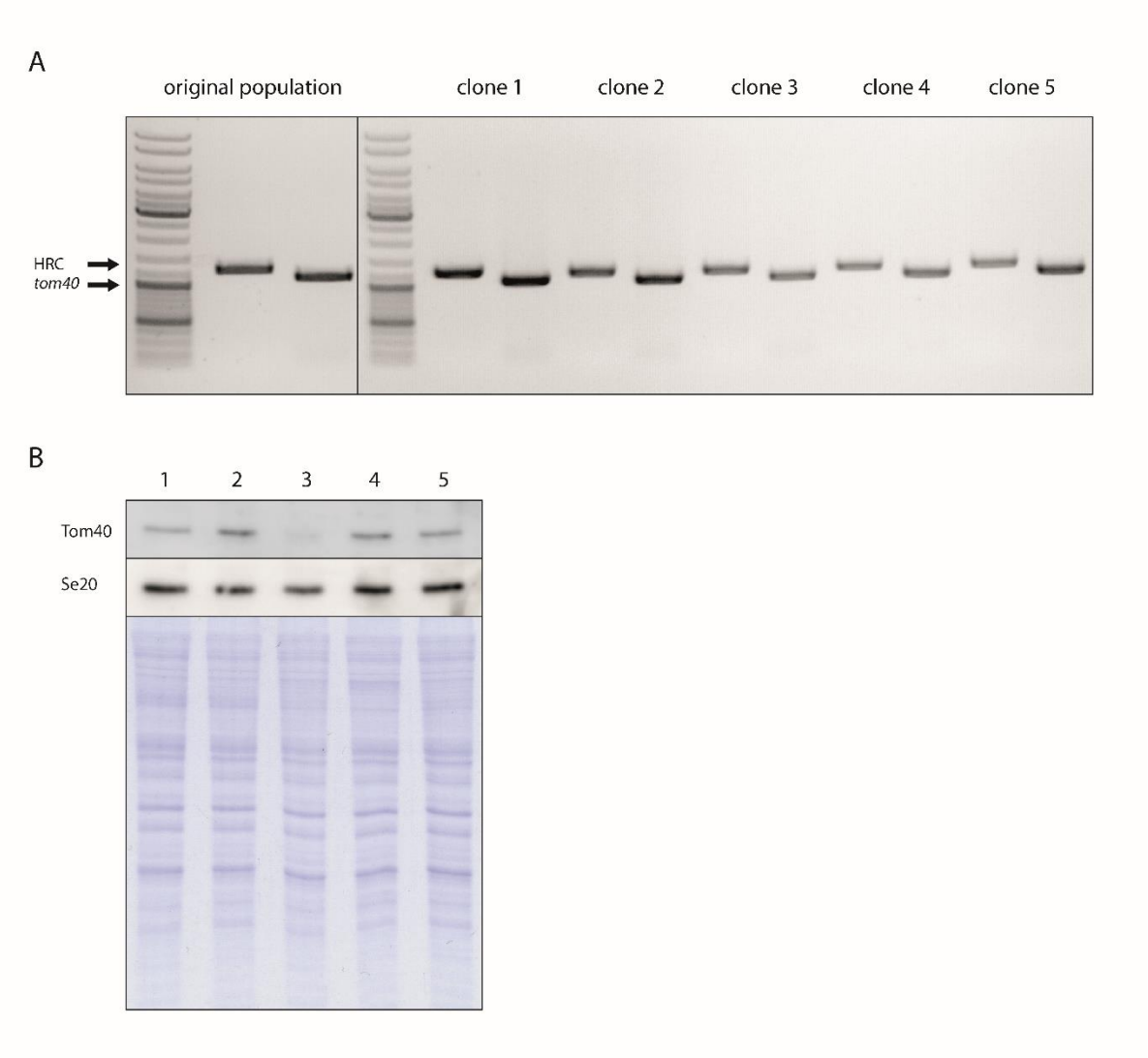
A
original population
clone 1
clone 2
clone 3
clone 4
clone 5
HRC
tom40
B
1
2
3
4
5
Tom40
Se20

Figure 7

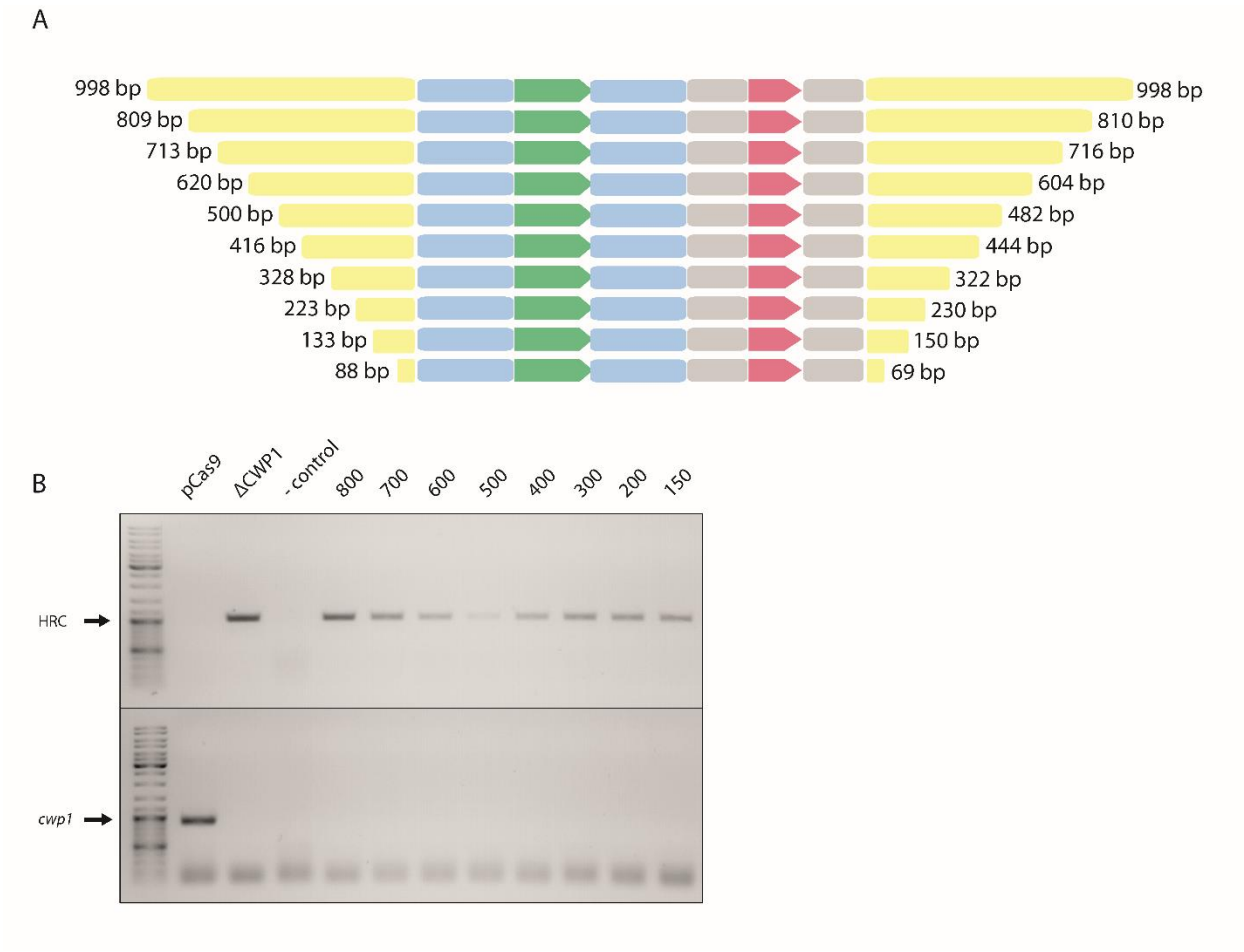
A
998 bp
809 bp
713 bp
620 bp
500 bp
416 bp
328 bp
223 bp
133 bp
88 bp
998 bp
810 bp
716 bp
604 bp
482 bp
444 bp
322 bp
230 bp
150 bp
69 bp
B
pCas9
ΔCWP1
-control
800
700
600
500
400
300
200
150
HRC
cwp1

Figure 8

Figure legends

Figure 1: (A) Schematic diagram of modified pTG vector. (B-D) Integration of homology recombination cassette (HRC) into *Giardia* genome. (B) *Giardia* cells carrying modified pTG were electroporated with Cas9-gRNA RNP complexes harboring two different guide RNAs (CWP1_96 and CWP1_492). The strains were tested for HRC integration and presence of *cwp1* in the genome. (C) Wild-type *Giardia* cells were electroporated with 3 µg of HRC together with Cas9-gRNA RNP complexes (96 and 492) and tested as described above. (D) Wild-type *Giardia* cells were electroporated with 3 µg of HRC only and tested accordingly. Wild-type cells were used as a control in all three experiments (WB, B-D). The technique is not sufficient to achieve full knock-out as the *cwp1* is still present in the genome. (E) Comparison of two techniques used for genome editing by *nanoluc* activity measurement. *Giardia* cells were electroporated with 3 µg of HRC carrying *nanoLuc* (DNA) or 3 µg of HRC-NanoLuc together with Cas9-gRNA-492 RNP (Cas9/gRNA + DNA). Cas-gRNA RNP and water (- control) were used for controls.

Figure 2: Expression of S. p. Cas9 in *Giardia intestinalis*. (A) Schematic diagram of pCas9 used for transfection of *Giardia* cells. To target the protein into *Giardia* nuclei, we used nuclear localization signals from SV40 followed by NLS from GL50803_2340 as described in (McInally et al., 2019). (B) The expression of Cas9 was verified by western blotting. Protein profile of the strain is also shown. (C) HA-tagged Cas9 (green) is targeted to *Giardia* nuclei (blue) which are stained with DAPI.

Figure 3: Knock-out of *mem*. (A) Schematic diagram of pTGuide plasmid carrying PAC cassette and guide RNA cassette including gRNA insertion site (gRNA-IS) used for all knock-out experiments. (B) Cells carrying pCas9 were transformed with pTGuide-MEM_307 or _579 and tested for HRC integration and presence of *mem* in the genome. (C) Strain 579 from Figure 3B was used as *Δmem* and tested for presence of *mem*-RNA by isolation of corresponding cDNA. Cas9 was used as a control cell line. β-giardin was used as a control of isolated cDNA.

Figure 4: FISH analysis of *Δmem* cells. (A) In wild-type cells, *mem* is present only in one nucleus as described previously (Tůmová et al., 2019). In the positive nucleus, usually (82 %, N=454) three distinct loci can be detected (inset 2), however, the number ranges in individual nuclei (insets). (B) In *Δmem* cells, the probe signal is barely visible (insets), pointing to a successful knock-out of *mem*.

Figure 5: Knock-out of *cwp1*. (A) Cells carrying pCas9 were transformed with pTGuide-CWP1_96 or _492 and tested for HRC integration and presence of *cwp1*. Two strains carrying CWP1_492 gRNA (491_1 and _2) were tested. (B) The same *Giardia* strains were tested for CWP1 protein level by western blotting using anti-CWP1 antibody. The cells were subjected to encystation 22h prior to the analysis. (C+D) Subcloning of *Δcwp1* cells. 5 different clones originated from cells carrying

pTGuide-CWP_492 were tested as described above. (E) Δcwp1 cells were tested for presence of mem-RNA by PCR on corresponding cDNA. *β-giardin* was used as a control gene. Cells carrying pCas9 and wild-type cells (WB) were used as control strains (A-E).

Figure 6: Knock-out of *mlf1*. (A) Cells carrying pCas9 were transformed with pTGuide-Mlf1_163 or _491 and tested for HRC integration and presence of *cwp1*. Two *Giardia* populations of each gRNA were tested (_1 and _2). (B) The same *Giardia* strains were tested for Mlf1 protein level by western blotting using anti-Mlf1 antibody. Expression of Cas9 was also verified using anti-HA antibody. Tubulin was used as a loading control. (C+D) Subcloning of *Δmlf1* cells. One clone originated from cells carrying pTGuide-Mlf1_163 was tested as described in 'A'. Cells carrying pCas9 and wild-type cells (WB) were used as control strains (A-D).

Figure 7: (A) Integration of HRC-Tom40 into *G. intestinalis* genome. *Giardia* cells expressing pCas9 were transformed with pTGuide-Tom40. The original population harbors both the HRC-Tom40 and native *tom40* in its genome. Five sub-clones from the original population were tested for HRC-Tom40 integration and *tom40* presence. (B) Clone 3 shows decreased level of Tom40 protein. Sec20 protein was used as a loading control. Protein profiles of individual clones are shown.

Figure 8: Testing of homologous arms lengths needed for successful recombination into *G. intestinalis* genome. (A) Schematic diagram showing different lengths used in the HRC. (B) *Giardia* cells carrying pCas9 plasmid were transfected by pTGuide-CWP1 vector containing homologous arms of different lengths. Resulting strains were tested for successful integration of the HRC and presence of *cwp1*. Water was used as a PCR template in -control, *Δcwp1* cells were used as a control of successful knock-out. Cells carrying pCas9 were used as the control strain for *cwp1* presence in the genome.