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Succinctus

Toxoplasma CRISPR/Cas9 constructs are functional for gene disruption in *Neospora caninum*

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ABSTRACT

Herein we describe, to our knowledge for the first time the use of the clustered regularly interspaced short palindromic repeats/CRISPR-associated gene 9 (CRISPR/Cas9) system for genome editing of *Neospora caninum*, an apicomplexan parasite considered one of the main causes of abortion in cattle worldwide. By using plasmids containing the CRISPR/Cas9 components adapted to the closely related parasite *Toxoplasma gondii*, we successfully knocked out a green fluorescent protein (GFP) in an Nc-1 GFP-expressing strain, and efficiently disrupted the Nc*GRA7* gene in the Nc-Spain7 isolate by insertion of a pyrimethamine resistance cassette. The successful use of this technology in *N. caninum* lays the foundation for an efficient, targeted gene modification tool in this parasite.

Neospora caninum is a protozoan parasite from the phylum Apicomplexa, and is closely related to *Toxoplasma gondii*. As the causative agent of bovine neosporosis, it is considered one of the main causes of abortion and neonatal mortality in cattle worldwide, entailing significant economic losses (Dubey et al., 2007; Dubey and Schares, 2011; Reichel et al., 2013). At present, there is an urgent need to develop control measures that protect cattle from abortion and vertical transmission. In order to find new vaccines and drug targets, it is imperative to increase knowledge of the molecular factors and genes involved in the parasite's biology. Thus, developing specific tools for genetic manipulation of *Neospora* will help us understand host-parasite interactions, and possibly aid in the development of vaccine and treatment strategies. While a wide array of tools has been developed for the closely related parasite *T. gondii*—including an assortment of promoters, fluorescent proteins, selectable markers, and genome editing tools—few such methods are available for *N. caninum* (reviewed in Suarez et al., 2017). Indeed, the genetic manipulation of *N. caninum* is limited to a few studies such as the introduction of the *LacZ* gene, the expression of bradyzoite (the semi-dormant stage of the parasite that is present in tissue cysts) antigens such as Nc*SAG4*, the expression of antigenic and secreted *Toxoplasma* proteins (e.g. TgSAG1, TgGRA2, TgGRA15 or TgROP16), the mutation of dihydrofolate reductase-thymidylate synthase (DHFR-TS; conferring resistance to pyrimethamine) or the insertion of drug selectable markers such as chloramphenicol acetyl transferase (CAT; resistance to chloramphenicol) or Ble (resistance to phleomycin) (Howe et al., 1997; Beckers et al., 1997; Howe and Sibley, 1997; Zhang et al., 2010; Marugán-Hernández et al., 2011; Pereira et al., 2014; Pereira and Yatsuda, 2014; Mota et al., 2017).

Recently, the adaptation of the clustered regularly interspaced short palindromic repeats/CRISPR-associated gene 9 (CRISPR/Cas9) technology—a naturally occurring DNA recognition system used as a defense mechanism in bacteria and archaea—has led to extremely efficient gene editing in a variety of organisms (Jinek et al., 2012; Mali et al., 2013; Shen et al., 2014; Sidik et al., 2014). Briefly, this system relies on the introduction of site-specific double-strand DNA breaks (DSBs) by the endonuclease Cas9 in a target sequence that is homologous to the single guide RNA (sgRNA, or gRNA hereafter). These DSBs are subsequently repaired by the organism either by the non-homologous end joining (NHEJ) pathway, leading to insertion and deletion mutations in the targeted genes ("indels"), or by homologous direct repair (HDR) in the presence of a DNA donor template. A gRNA complex will be

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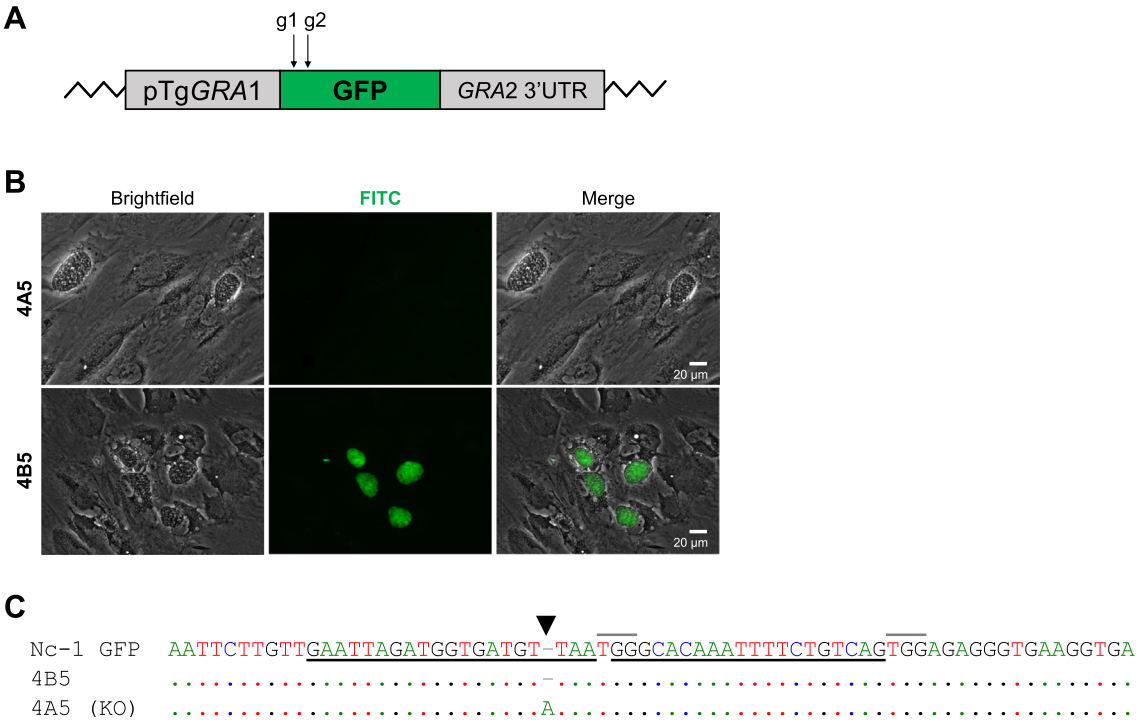

Fig. 1. Analysis of the *Neospora caninum* Nc-1 GFP knockout (KO) generated with clustered regularly interspaced short palindromic repeats/CRISPR-associated gene 9 (CRISPR/Cas9). (A) Schematic representation of the *GFP* gene disruption achieved by employing two guide RNAs (gRNAs; g1 and g2) in the Nc-1 GFP strain. (B) Images (20×) from cell culture flasks containing human foreskin fibroblast cells infected with two different clones obtained after transfection and limiting dilution (4A5 and 4B5) showing green fluorescence. Note the absence of green fluorescence in clone 4A5. White bars represent scale bars (20 µm). (C) Sequence alignment of the parental strain (Nc-1 GFP) and two clones, 4B5 and 4A5 (KO). Identical nucleotides are presented as dots. The two gRNA sites are underlined in black. Protospacer adjacent motif (PAM) sites are indicated by grey lines above the sequence. The cutting site is indicated by an arrowhead. UTR, untranslated region. pTg*GRA*1, promoter region of the *Toxoplasma* dense granule 1 gene.

Table 1
Guide RNAs (gRNAs) and primers used in the present work.

Region	Primer	Sequence	Used for
GFP	GFP gRNA1	GAATTAGATGGTGATGTTAA	Disrupt *GFP* gene in the N-terminal end
	GFP gRNA2	GGGCACAAATTTTCTGTCAG	
	GFP-Fw	TGGCCAAATCAAAGGCTATT	Amplify and sequence the gRNA targeted region of the *GFP* gene
	GFP-Rv	TGGGTATCTTGAAAAGCATTGA	
GRA7	GRA7 gRNA1	GTTTGTGGACTGGCAATCGC	Disrupt *GRA7* gene in both N- and C-terminal ends
	GRA7 gRNA2	GCAGTGATCCGGAACAGGAG	
	GRA7-Fw (P1)	TCGCTGTTCCTGTAGGCTTT	Amplify and sequence the gRNA targeted region of the *GRA7* gene together with repair template
	GRA7-Rv (P2)	CTGTCATCTGGGACACGAAA	
Loxp-DHFR-mCherry plasmid	Plasmid-Fw (P4)	GGCGTGAAGATCTGGGACAA	Amplify and sequence the gRNA targeted region of the *GRA7* gene together with repair template
	Plasmid-Rv (P3)	ACGACCTACACCGAACTGAGAT	

able to guide the Cas9 only if an appropriate protospacer-adjacent motif (PAM) is located immediately after the target sequence. The utility of using gRNAs designed to target specific genes, combined with the high efficiency of disruption by Cas9, has proven useful for gene manipulation in many model organisms (Cong et al., 2013). In apicomplexan parasites, to date, this has only been achieved in *Plasmodium*, *Cryptosporidium* and *Toxoplasma* (Suarez et al., 2017). In the latter, the CRISPR/Cas9 system was recently adapted to produce efficient targeted gene disruption and site-specific insertion of selectable markers (Shen et al., 2014; Sidik et al., 2014). Since then, it has been widely employed as an efficient and powerful means of testing the role of specific genes in diverse genetic backgrounds and even to perform a genome-wide screen of *Toxoplasma* genes conferring fitness in human foreskin fibroblasts (HFFs) (Sidik et al., 2016).

In the present work we describe, to our knowledge, the first successful use of the CRISPR/Cas9 system in *N. caninum* by transfecting plasmids developed for *Toxoplasma*. We disrupted two different genes, the green fluorescence protein (*GFP*) expressed in Nc-1, and the Nc*GRA7* in the wild-type Nc-Spain7 isolate, demonstrating the utility of the *Toxoplasma*-adapted plasmids and CRISPR/Cas9 technology in *N. caninum*. To disrupt the genes, we used the universal pU6 plasmid (Addgene plasmid # 52694), which contains Cas9 with a nuclear localisation sequence driven by the Tg*TUB1* promoter and a gRNA expression site driven by the *T. gondii* U6 promoter (Sidik et al., 2014). Using the *Bsa*I-specific sites in the

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