Supplemental Material

Supplemental methods

Plasmid construction. A plasmid expressing CAS9 and a single guide RNA (sgRNA) targeting the *UPRT* gene of *T. gondii* (pSAG1::CAS9-U6::sgUPRT) were obtained from Addgene (Cambridge, MA, USA). The sgRNAs were designed based with EuPaGDT (http://grna.ctegd.uga.edu). The CRISPR/CAS9 plasmids (pSAG1::CAS9-U6::sgNcGRA6, pSAG1::CAS9-U6::sgNcGRA7 pSAG1::CAS9-U6::sgNcGRA14, pSAG1::CAS9-U6::sgNcCyp, and pSAG1::CAS9-U6::sgNcUPRT) were generated with the Q5® Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA) from pSAG1::CAS9-U6::sgUPRT, changing the *UPRT*-targeting gRNA to other specific sgRNAs using the primers listed in Table 2. To generate a construct to express the FLAG-tag-fused *Neospora* genes, each gene was PCR amplified from *N. caninum* cDNA using the primers listed in Table 2, and cloned into p3XFLAG-CMV-14 (Sigma-Aldrich, St. Louis, MO, USA). The plasmids for the luciferase reporter assay are listed in Table 1 and were purchased from Promega (Madison, WI, USA). To construct a plasmid with which to generate the *NcGRA7*-complemented strain, the FLAG-tag-fused *NcGRA7* gene (*NcGRA7*–FLAG) was cloned into the *Eco*RV site of the pDMG plasmid (1), which expressed NcGRA7–FLAG with the *Toxoplasma* GRA1 5′-untranslated region (UTR) and *Toxoplasma* GRA2 3′-UTR (pDMG-NcGRA7FLAG). To generate constructs to express the recombinant NcGRA6 and NcGRA14 proteins (excluding their signal peptides and transmembrane domains), the genes (*NcGRA6* gene, nt 130–462; *NcGRA14*, nt 109–852) were PCR amplified from *N. caninum* cDNA using the

primers as listed in Table 2, and cloned into pGEX4T-1 (Amersham Pharmacia Biotech, Madison, CA, USA), generating pGEX4T-1/NcGRA6 and pGEX4T-1/NcGRA14, respectively.

Production of antisera, polyclonal antibodies, and monoclonal antibody against *N. caninum* proteins. An anti-NcSRS2 monoclonal antibody (1B8) (2) and a purified rabbit anti-rNcCYP polyclonal antibody (3) were prepared previously. To generate mouse antisera against NcGRA6 and NcGRA14, the recombinant proteins were prepared as described previously (3). Briefly, the *NcGRA6* gene (nt 130–462) and *NcGRA14* gene (nt 109–852) were amplified with PCR from cDNA of *N. caninum* tachyzoites and primers listed in Table 2, and cloned into the pGEX4T-1 plasmid (pGEX4T-1/NcGRA6 and pGEX4T-1/NcGRA14, respectively; Table 1). The recombinant NcGRA6 and NcGRA14 proteins were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* strain DH5α (Takara Bio Inc., Shiga, Japan). Each recombinant protein (50 μg) in Freund's complete adjuvant (Sigma) was injected intraperitoneally into 6-week-old female BALB/c mice on day 0. The same antigen in Freund's incomplete adjuvant (Sigma) was injected intraperitoneally into the mice on days 14, 28, and 42. The antisera were collected from the mice on day 56. To generate rabbit anti-NcGRA7 antiserum, 300 μg of recombinant NcGRA7 (4) in Freund's complete adjuvant was injected intradermally into a female Japanese white rabbit (Kitayama Labes, Nagano, Japan) on day 0. Recombinant NcGRA7 in Freund's incomplete adjuvant was injected intradermally into the rabbit on days 14, 28, and 42. IgG was purified from 2 ml of serum collected 7 days after the last

immunization using a protein A chromatography column (Econo-Pac® Protein A Kit, Bio-Rad Laboratories, CA, USA), according to the manufacturer's instructions.

The specificity of sera and antibodies used in this study was confirmed by western blotting and immunofluorescent antibody tests (IFAT) of wild-type *N. caninum* (Nc1) and each deficient parasite. Anti-NcGRA6 mouse serum, rabbit NcGRA7 antibody and rabbit NcCYP antibody showed specific reaction by western blots and IFAT. However, anti-NcGRA14 mouse serum did not react specifically with Nc1 by IFAT, but it could detect the specific band from Nc1 in western blots.

Western blotting analysis. The protein lysates from purified tachyzoites (15 μg/10 μl) were mixed with 10 μl of 2 × SDS gel-reducing loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 140 mM 2-mercaptoethanol, 10% [w/v] glycerol, and 0.02% [w/v] bromophenol blue). The samples were heated at 95 °C for 5 min and separated on a 15% polyacrylamide gel. After SDS-polyacrylamide gel electrophoresis, the protein bands in the gel were transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). After the membranes were washed twice with PBS containing 0.05% (v/v) Tween 20, they were blocked with PBS containing 3% (w/v) skimmed milk (PBS-SM) for 12 h at 4 °C. After two further washes, the membranes were incubated with primary antibody or antiserum (1:500) for 1 h at room temperature. After the membranes were washed three times, they were incubated with horseradish-peroxidase-conjugated immunoglobulin G directed against the mouse or rabbit antibody (diluted 1:5,000 in PBS-SM; Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at 37 °C. After the membranes were washed

five times, the proteins were visualized with ECL™ Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK) with the VersaDoc™ Imaging System (Nippon Bio-Rad Laboratories, Tokyo, Japan), according to the manufacturer's instructions.

Infection rate, growth and egress of *N. caninum* lines. Parasites (1 ml/well) were added to Vero cells in a 12-well plate (parasites per host cell ratio = 2:1). At 2–3 h postinfection, the extracellular parasites were washed away and EMEM supplemented with 8% FBS was added. At 24 h postinfection, the infection rates were calculated with IFAT as follows: (number of NcSRS2-positive Vero cells/100 randomly selected Vero cells) × 100. To measure *N. caninum* replication in the Vero cells, the sizes of the PVs were determined by counting the number of parasites per PV (in a total of 100 randomly selected vacuoles) expressed as a percentage (%) in total PV at 48 h postinfection based on the NcSRS2 signal measured with IFAT. To measure *N. caninum* egress in the Vero cells, the percentage of egressed vacuoles was calculated by scoring at least 100 vacuoles as intracellular or egressed ones at 48 h postinfection based on the NcSRS2 signal measured with IFAT.

Pathological analysis. For the histopathological and immunohistopathological analyses, mice were killed and their brains rapidly removed 20 and 30 days after infection, respectively. The brains, livers, spleens, and lungs were fixed in 10% formalin and embedded in paraffin wax. Sections were cut to 4 μm and stained with hematoxylin and eosin (HE). The brains were cut in midsagittal sections through the cerebrum, cerebellum and brain stem. Immunohistochemistry was performed with rabbit anti-NcGRA7 antibody (diluted 1:3,000) or anti-*N. caninum* antibody (210-70 NC;

VMRD, Pullman, WA, USA; diluted 1:2,000) as the primary antibody and a secondary antibody conjugated with a horseradish-peroxidase-labeled polymer (EnVision+ kit, Dako, Copenhagen, Denmark) or with horseradish-peroxidase-labeled streptavidin–biotin (Universal LSAB+ Kit; Dako, Burlingame, CA, USA). Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 5 min at room temperature. The tissue sections were then incubated in goat serum for 30 min at room temperature to prevent nonspecific reactions. The sections were exposed to each primary antibody at 4 °C overnight and then incubated with the secondary antibody for 40 min at 37 °C. The signals were detected with diaminobenzidine (ImmPACT DAB®, Vector Laboratories Inc., Burlingame, CA, USA), followed by counterstaining with Mayer's hematoxylin.

References for supplemental methods

1. Nishikawa Y, Xuenan X, Makala L, Vielemeyer O, Joiner KA, Nagasawa H. 2003. Characterisation of *Toxoplasma gondii* engineered to express mouse interferon-gamma. Int J Parasitol 33:1525–1535.
2. Nishikawa Y, Xuan X, Nagasawa H, Igarashi I, Fujisaki K, Otsuka H, Mikami T. 2000. Monoclonal antibody inhibition of *Neospora caninum* tachyzoite invasion into host cells. Int J Parasitol 30:51–58.
3. Kameyama K, Nishimura M, Punsantsogvoo M, Ibrahim HM, Xuan X, Furuoka H, Nishikawa Y. 2012. Immunological characterization of *Neospora caninum* cyclophilin. Parasitology 139:294–301.

4. Nishikawa Y, Zhang H, Ikehara Y, Kojima N, Xuan X, Yokoyama N. 2009. Immunization with oligomannose-coated liposome-entrapped dense granule protein 7 protects dams and offspring from *Neospora caninum* infection in mice. Clin Vaccine Immunol 16:792–797.

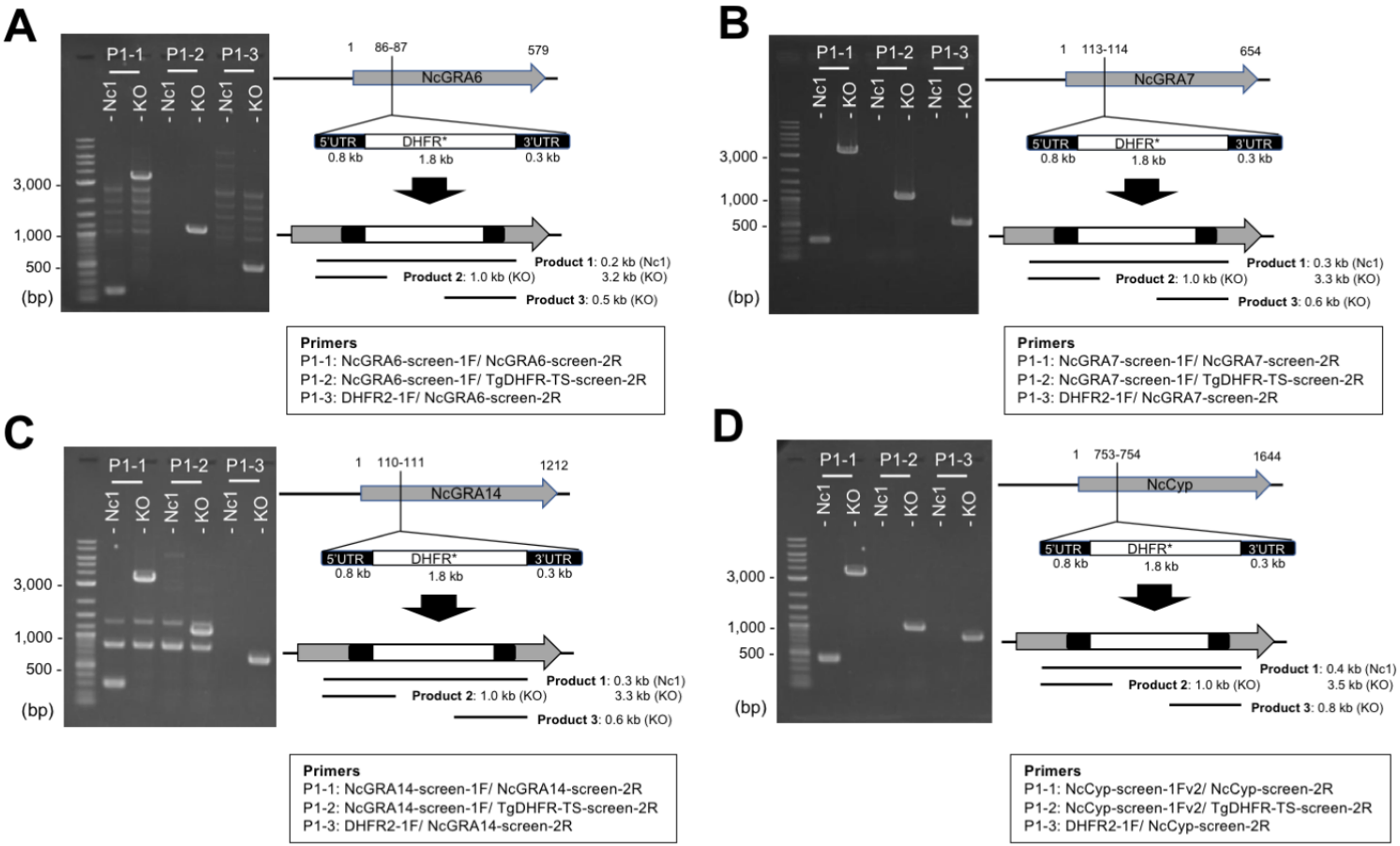

Fig. S1. Nishikawa et al.

Supplementary Figure 1 CRISPR/CAS9-mediated disruption of the *NcGRA6*, *NcGRA7*, *NcGRA14*, and *NcCYP* loci. Schematic representation of the CRISPR/CAS9 strategy used to inactivate the target genes by inserting the pyrimethamine-resistance DHFR cassette (DHFR*). Transfection of the CRISPR plasmid targeting one of these gene, together with an amplicon containing the DHFR*-expressing cassette flanked by regions homologous to the target gene, was used to disrupt the corresponding target gene by insertion. Diagnostic PCR demonstrated the homologous integration and gene disruption in a representative clone (KO) compared with the parental line Nc1. Product 1 (P1-1) is the fragment amplified in the wild-type cells that was lost during the insertion of DHFR* and the larger fragment was created by the insertion of DHFR* (2.9 kb). Product 2 (P1-2) and Product 3 (P1-3) provide evidence of homologous integration because the products were amplified between the DHFR* gene and regions in the target locus that lie outside the targeted amplicon. (A) Primer set for Product 1 (0.2 kb in Nc1 and 3.2 kb in *NcGRA6*KO): NcGRA6-screen-1F and NcGRA6-screen-2R. Primer set for Product 2 (no amplicon in Nc1 and 1.0 kb in *NcGRA6*KO): NcGRA6-screen-1F and TgDHFR-TS-screen-2R. Primer set for Product 3 (no amplicon in Nc1 and 0.5 kb in *NcGRA6*KO): DHFR2-1F and NcGRA6-screen-2R. (B) Primer set for Product 1 (0.3 kb in Nc1 and 3.3 kb in *NcGRA7*KO): NcGRA7-screen-1F and NcGRA7-screen-2R. Primer sets for Product 2 (no amplicon in Nc1 and 1.0 kb in *NcGRA7*KO): NcGRA7-screen-1F and TgDHFR-TS-screen-2R. Primer sets for Product 3 (no amplicon in Nc1 and 0.6 kb in *NcGRA7*KO): DHFR2-1F and NcGRA7-screen-2R. (C) Primer set for Product 1 (0.3 kb in Nc1 and 3.3 kb in *NcGRA14*KO): NcGRA14-screen-1F and NcGRA14-screen-2R. Primer set for Product 2 (no specific amplicon in Nc1 and 1.0 kb in *NcGRA14*KO): NcGRA14-screen-1F and TgDHFR-TS-screen-2R. Primer set for Product 3 (no amplicon in Nc1 and 0.6 kb in *NcGRA14*KO): DHFR2-1F and NcGRA14-screen-2R. (D)

Primer set for Product 1 (0.4 kb in Nc1 and 3.5 kb in *NcCYP*KO): NcCyp-screen-1Fv2 and NcCyp-screen-2R. Primer set for Product 2 (no amplicon in Nc1 and 1.0 kb in *NcCYP*KO): NcCyp-screen-1Fv2 and TgDHFR-TS-screen-2R. Primer set for Product 3 (no amplicon in Nc1 and 0.8 kb in *NcCYP*KO): DHFR2-1F and NcCyp-screen-2R.

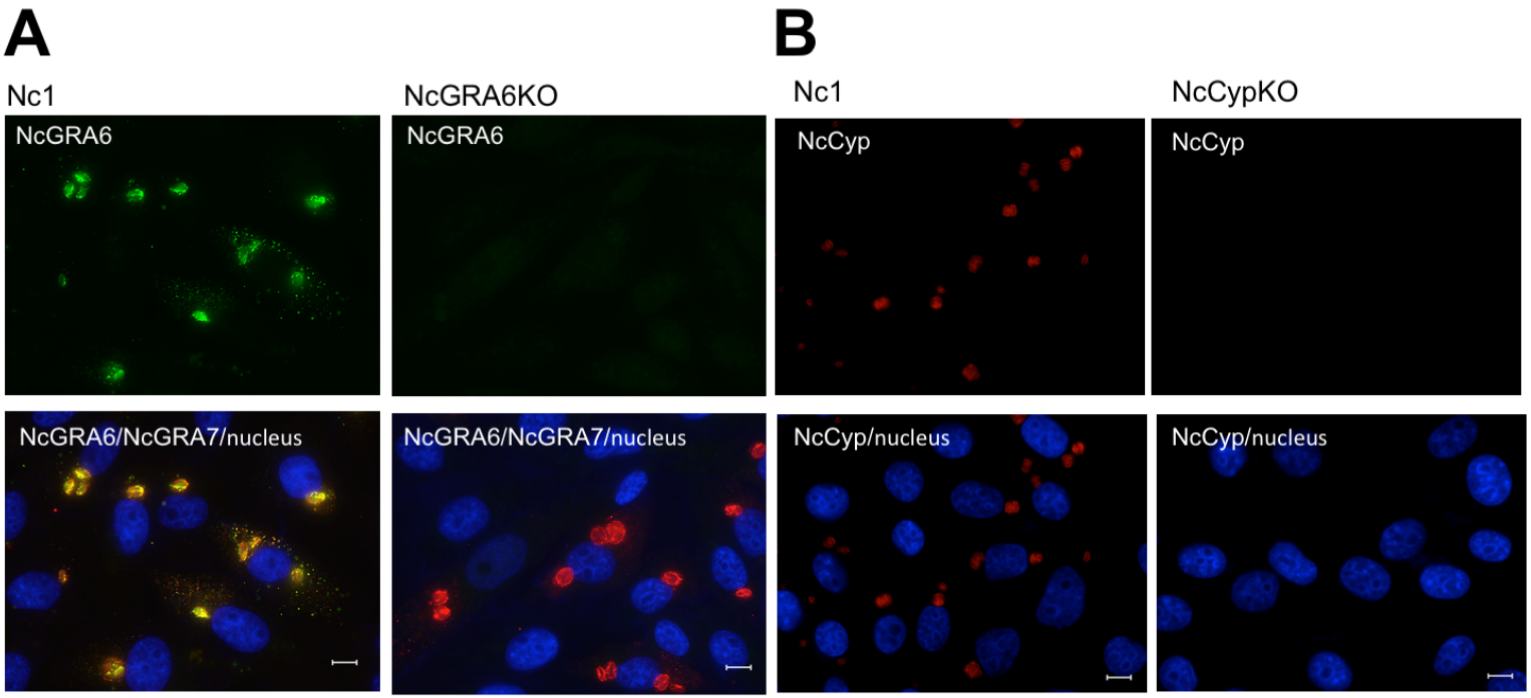

Fig. S2. Nishikawa et al.

Supplementary Figure 2 IFAT analysis of Vero cells infected with Nc1, *NcGRA6*-deficient, and *NcCYP*-deficient parasites at 24 h postinfection. Expression of NcGRA6 and NcCYP was lost in the *NcGRA6*- and *NcCYP*-deficient parasites (KO), respectively. (A) Expression of NcGRA6 and NcGRA7 was visualized with anti-NcGRA6 mouse serum (green) and rabbit NcGRA7 antibody (red). Yellow indicates colocalization of NcGRA6 and NcGRA7. (B) Expression of NcCYP was visualized with rabbit NcCYP antibody (red). The nuclear DNA was stained with Hoechst (blue). Scale bar, 10 μm.

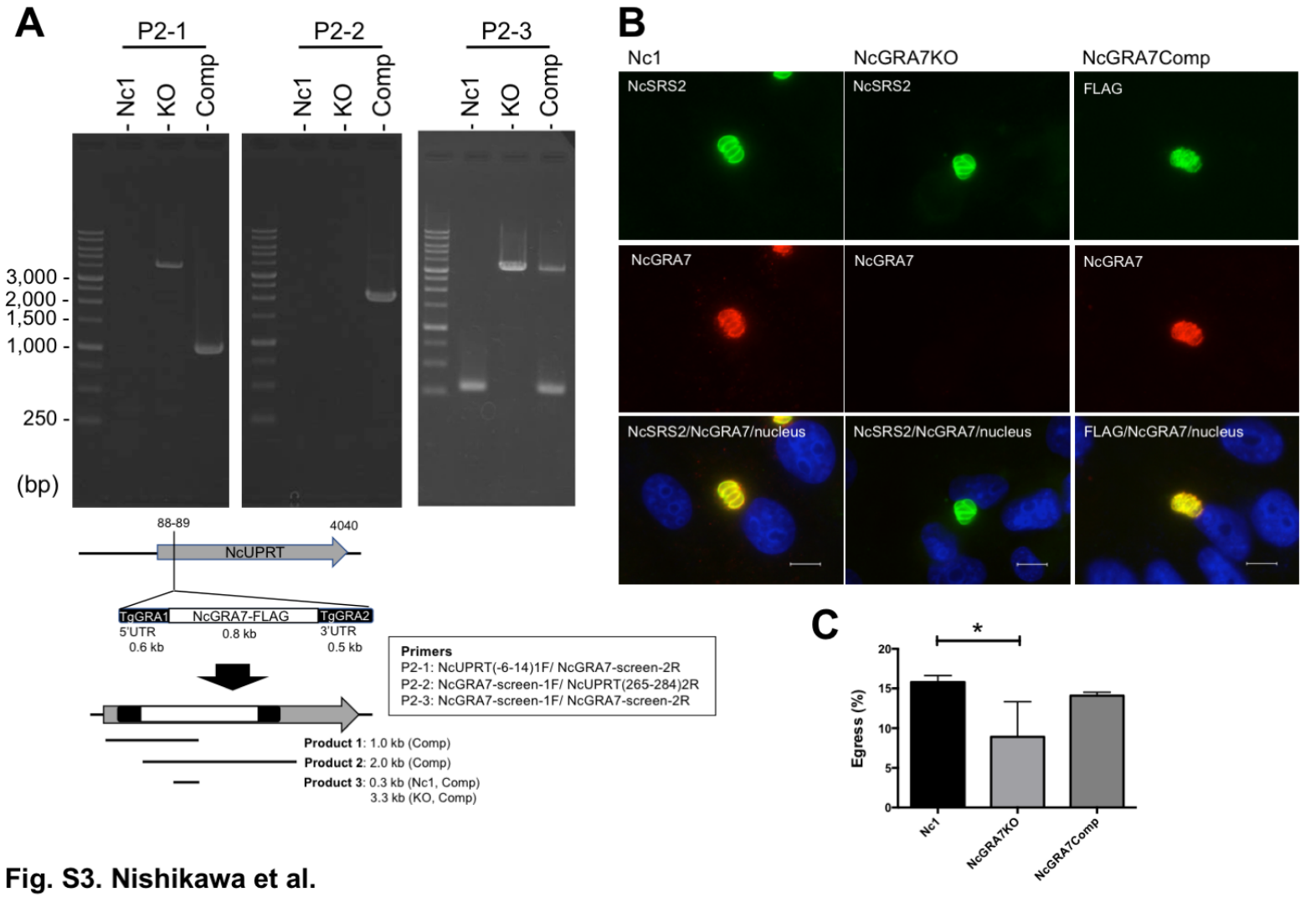

Fig. S3. Nishikawa et al.

Supplementary Figure 3 Generation of *NcGRA7*-complemented parasite. CRISPR/CAS9-mediated gene insertion of FLAG-tag-fused *NcGRA7* into the *Neospora UPRT* (*NcUPRT*) locus. Transfection of the CRISPR plasmid targeting the *NcUPRT* gene, together with an amplicon containing the FLAG-tag-fused *NcGRA7* cassette flanked by regions homologous to the *NcUPRT* gene, was used. Diagnostic PCR demonstrated the homologous integration and gene insertion in a representative clone (Comp) compared with the Nc1 and *NcGRA7*-deficient parasites (KO). Product 1 (P2-1) was amplified in the complemented cells after the insertion of the FLAG-tag-fused *NcGRA7* cassette (1.9 kb). Product 2 (P2-2) and Product 3 (P2-3) provide evidence of homologous integration because they were amplified from between the FLAG-tag-fused *NcGRA7* cassette and regions in the *NcUPRT* locus that lie outside the targeted amplicon. (A) Primer set for Product 1 (1.0 kb in *NcGRA7*-complemented parasite): NcUPRT(-6-14)1F and NcGRA7-screen-2R. Primer set for Product 2 (2.0 kb in *NcGRA7*-complemented parasite): NcGRA7-screen-1F and NcUPRT(265-284)2R. Primer set for Product 3 (0.3 kb in Nc1 and *NcGRA7*-complemented parasite, 3.3 kb in *NcGRA7*KO and *NcGRA7*-complemented parasites): NcGRA7-screen-1F and NcGRA7-screen-2R. (B) IFAT analysis of Vero cells infected with Nc1, *NcGRA7*-deficient, and *NcGRA7*-complemented parasites at 24 h postinfection. Expression of NcGRA7 was lost in the *NcGRA7*-deficient parasite (KO). FLAG-tag-fused NcGRA7 colocalized with NcGRA7 in the complemented parasite (Comp). The shapes of the parasites were visualized with an anti-NcSRS2 antibody, and the nuclear DNA was stained with Hoechst (blue). Scale bar, 10 μm. (C) Egress rates of the different parasite lines in Vero cells at 72 h postinfection. Each bar represents ta mean ± standard deviation (n = 3 for all groups). *Statistically significant differences relative to the value for Nc1, according to one-way ANOVA and a Tukey–Kramer post hoc analysis ($P < 0.05$).

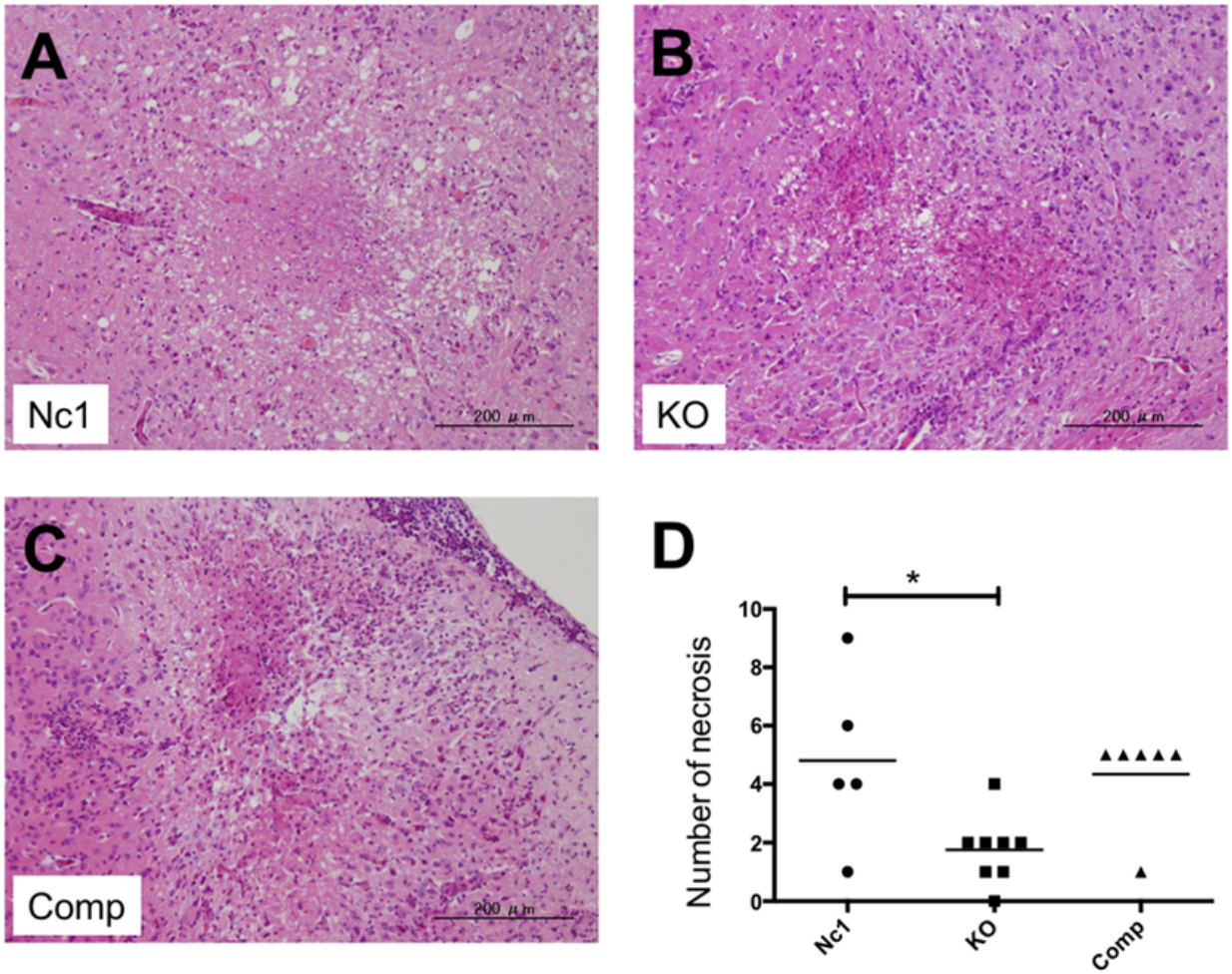

Fig. S4. Nishikawa et al.

Supplementary Figure 4 Pathological analysis of brain tissues from mice 20 days after their infection with Nc1, *NcGRA7*-deficient parasite (KO), or *NcGRA7*-complemented parasite (Comp). (A–C) Focal necrosis associated with gliosis and lipid-laden macrophages. (D) Number of necrotic lesions in the brain tissue. Number of necrotic lesions per individual (symbols) and mean levels (horizontal lines) are indicated ($n = 5$ for Nc1; $n = 8$ for KO; $n = 6$ for Comp). *Statistically significant differences detected with one-way ANOVA and a Tukey–Kramer post hoc analysis ($P < 0.05$).

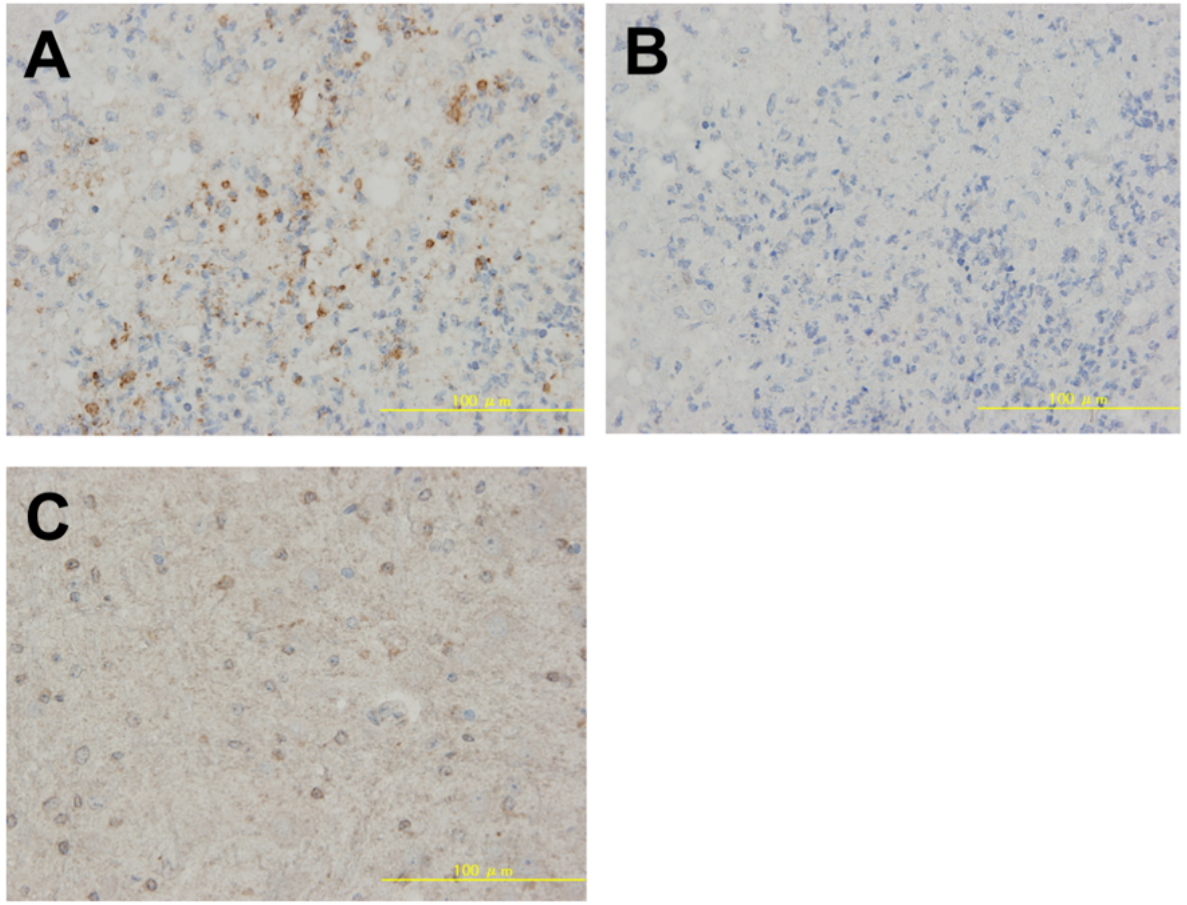

Fig. S5. Nishikawa et al.

Supplementary Figure 5 (A) Immunohistochemical analysis of brain tissues of *N. caninum*-infected mouse with rabbit NcGRA7 antibody. (B) Immunohistochemical analysis of brain tissues of *N. caninum*-infected mouse with preimmune rabbit antibody. (C) Immunohistochemical analysis of brain tissues of uninfected mouse with rabbit NcGRA7 antibody.

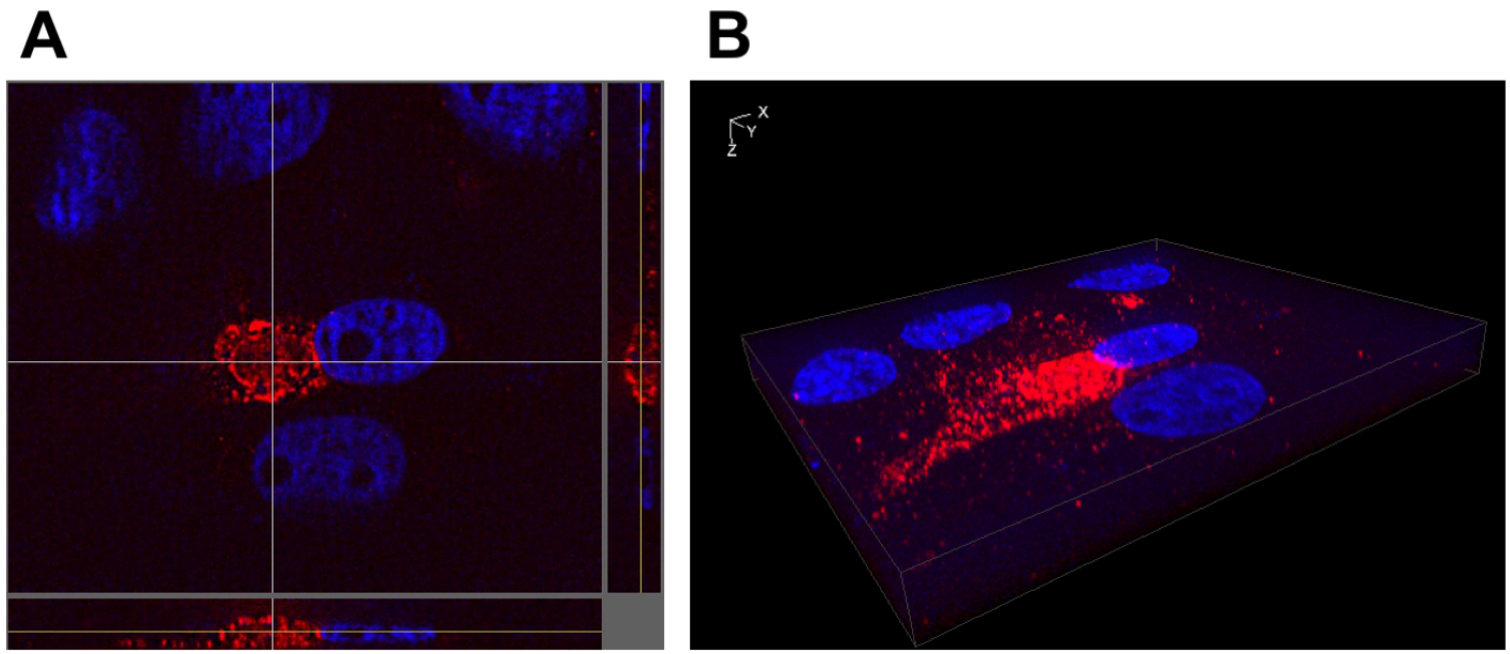

Fig. S6. Nishikawa et al.

Supplementary Figure 6 IFAT analysis of Nc1-infected Vero cells at 48 h postinfection with rabbit NcGRA7 antibody. Secretion of NcGRA7 into the host cell cytosol was visualized (red). The nuclear DNA was stained with Hoechst (blue). (A) 2D image. (B) 3D image.