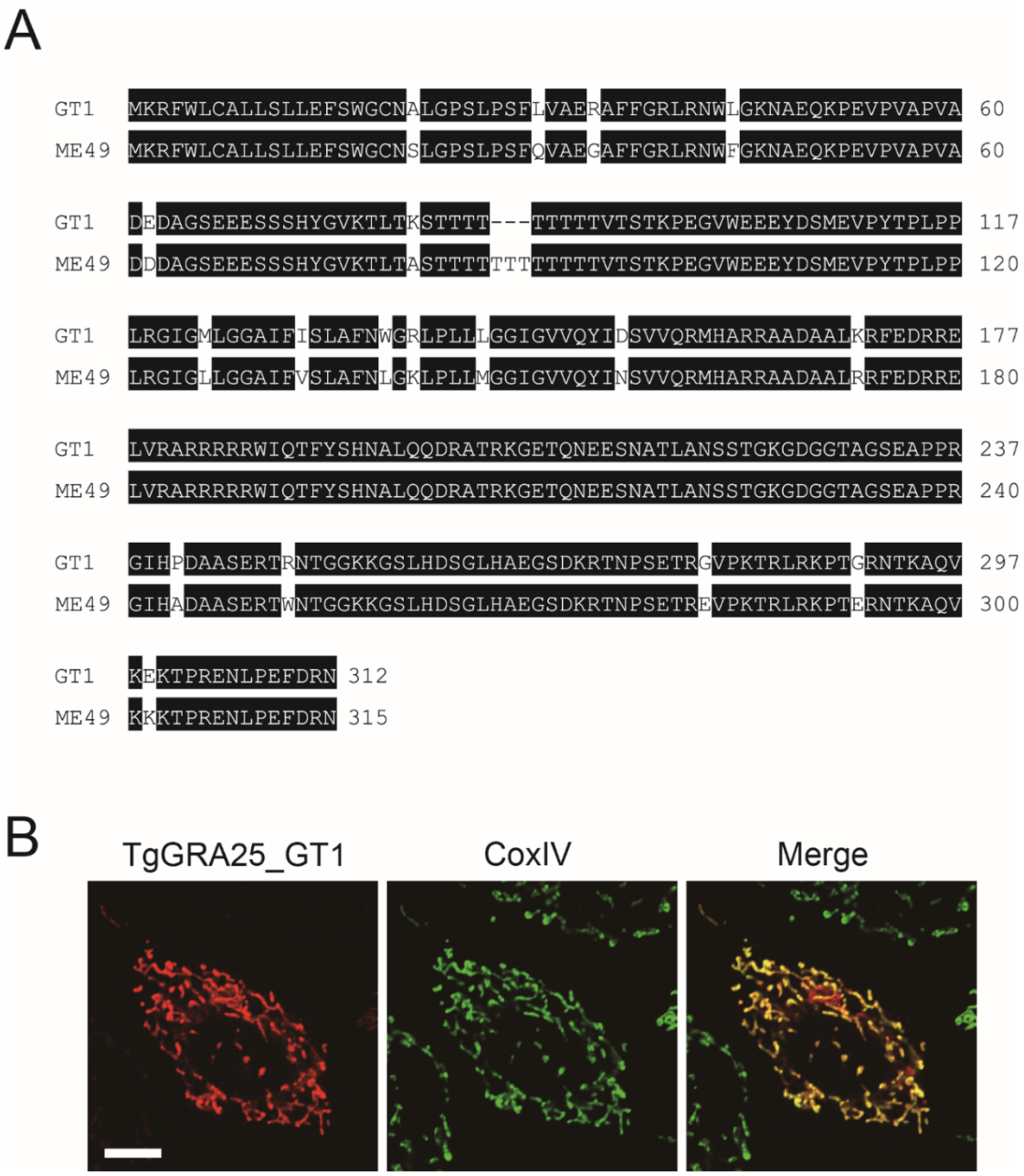


Figure S1. Comparing the type I and type II amino acid sequences of TgGRA25. (**A**) Alignment of TgGRA25 paralogs (type I_GT1 and type II_ME49). Black indicates 100% identity. (**B**) HeLa cells was transfected with a Myc-tagged version of the TgGRA25 plasmid, and the mitochondrial localization was monitored by immunofluorescence microscopy (red). Mitochondria in the same cells were stained with an anti-CoxIV monoclonal antibody (green). Scale bar, 10 μm.

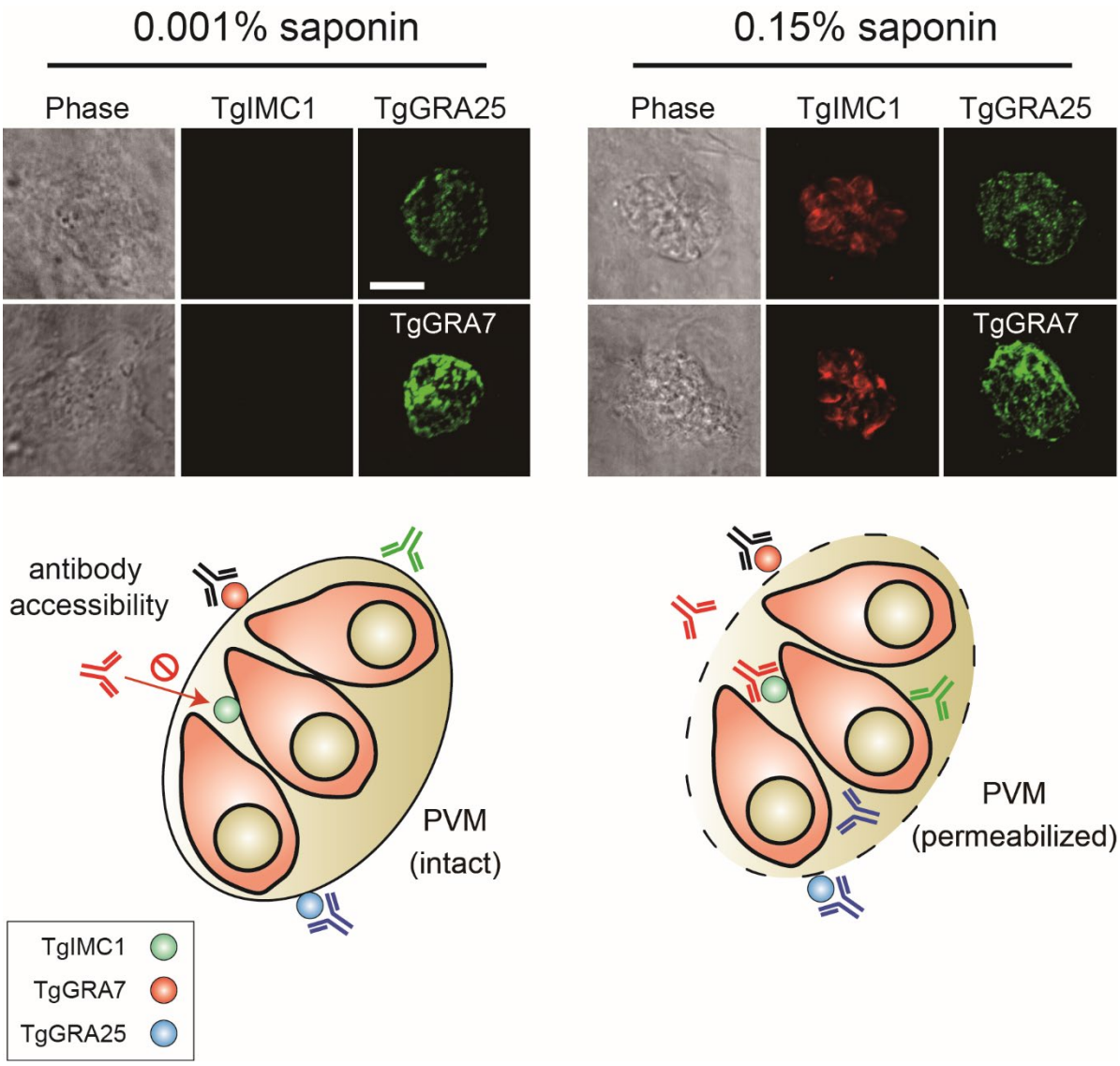


Figure S2. Topology of T. gondii proteins. Different permeabilization conditions with 0.001% saponin (left) or 0.15% saponin (right) used in IFATs of Vero cells infected with the Pru strain. All cells were stained with TgIMC1 (red, control for permeabilization of the PMV; Hu et al. 2002) as well as with either anti-TgGRA25 (green) or anti-TgGRA7 (green, control for the cytoplasmic face of the PMV; Terkawi et al. 2013) polyclonal antibodies. In both conditions, TgGRA7 and TgGRA25 were stained with their specific antibodies, but TgIMC1 was negative in the 0.001% saponin, indicating that TgGRA25 traffics to the cytoplasmic face of the PMV (bottom shows the topology of T. gondii proteins).

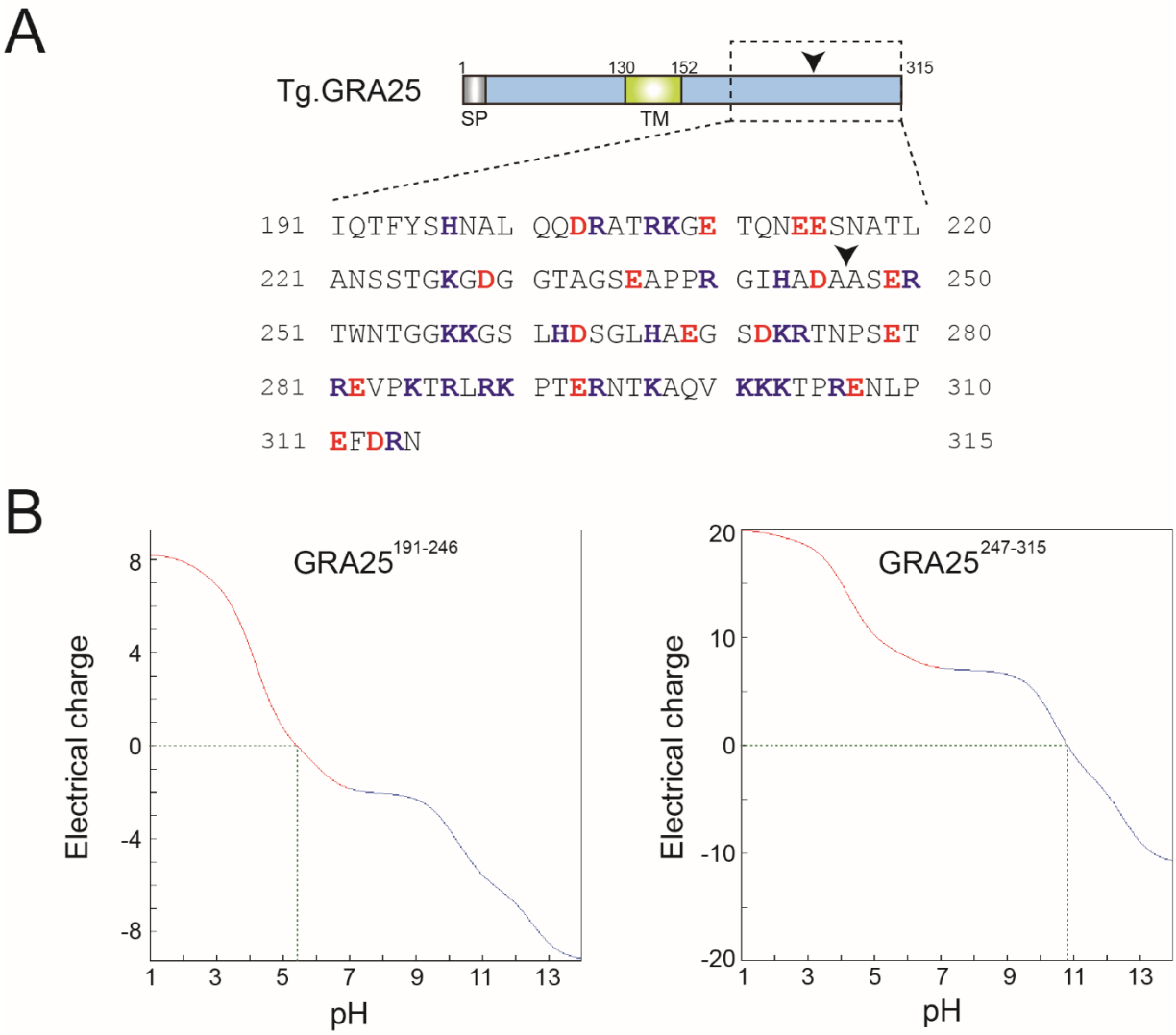


Figure S3. Amino acid sequence and its electrostatics of the C-terminal region in TgGRA25. (**A**) The amino acid sequence (from 191–315) of C-terminal region in TgGRA25 (Type II: ME49). Blue and red characters indicate basic and acidic residues, respectively. Arrowhead shows position between 246 and 247 in TgGRA25. (**B**) Electrostatics of two polypeptides (TgGRA25$^{191\text{-}246}$ and TgGRA25$^{247\text{-}315}$) used in the study are shown.

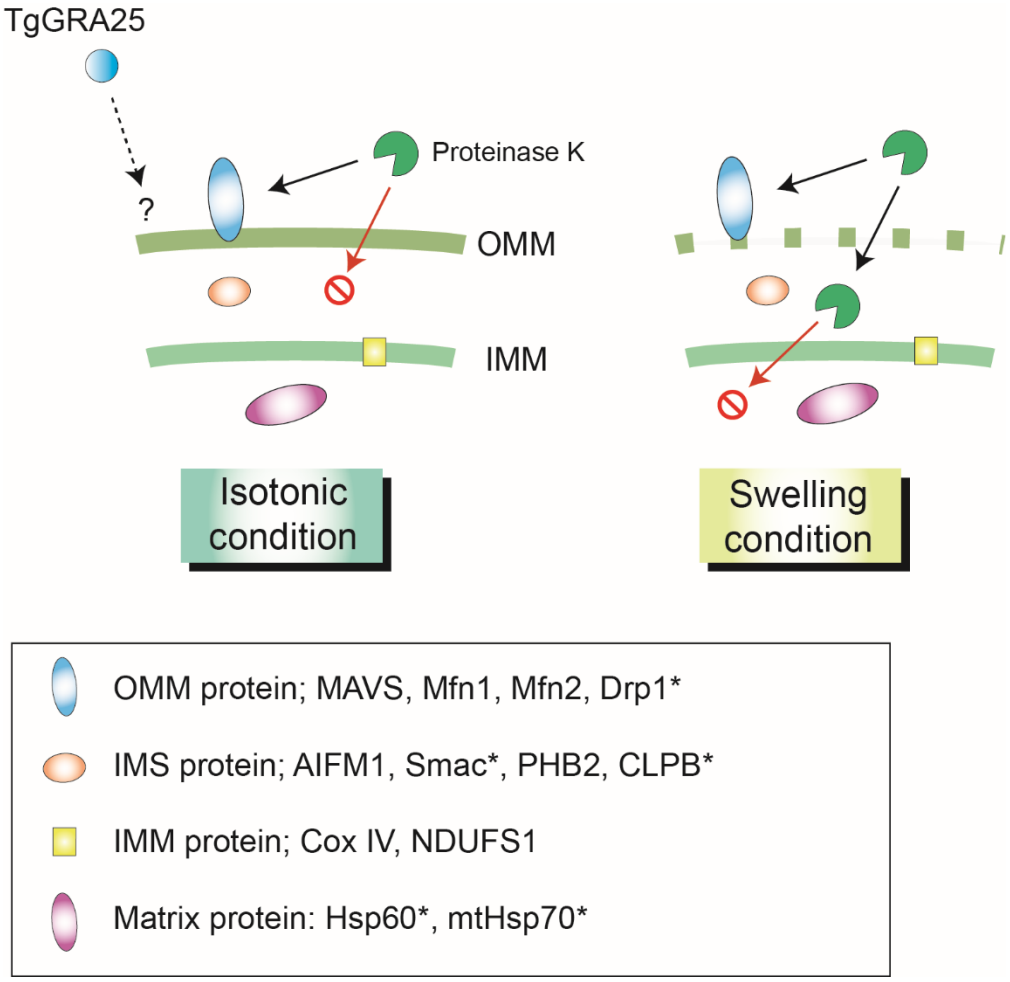


Figure S4. Schematic diagram of isolated mitochondria under isotonic and swelling conditions. Schematics of an isotonic (left) and swelling (right) conditions used in the present study. Under the isotonic condition, proteinase K can access to OMM proteins but not IMS, IMM, or matrix proteins because of membrane barriers. Besides, mitochondria under the swelling condition can allow the protease to reach into the intermembrane space (IMS) followed by cleavage of both OMM and IMS proteins. The inset shows mitochondrial proteins we monitored as markers for each category, and the asterisk indicates unanchored proteins.

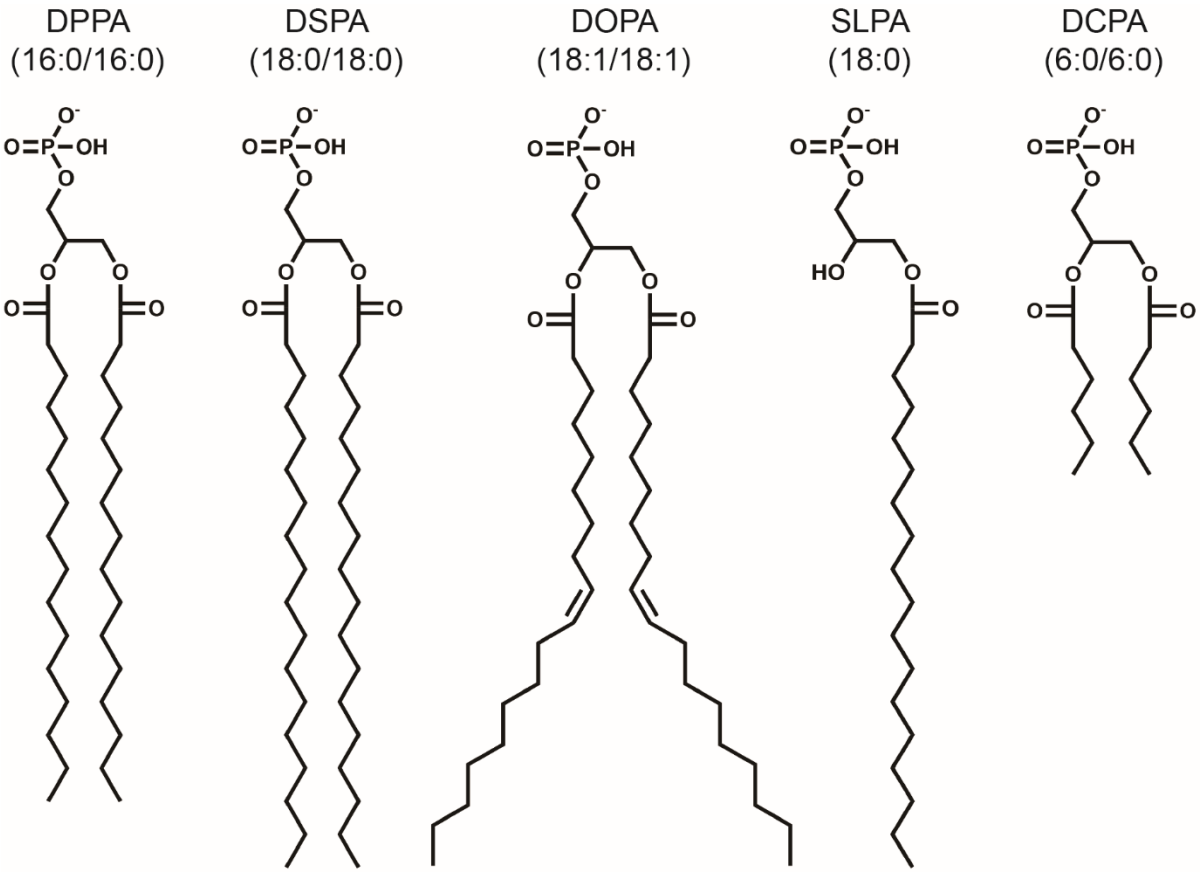


Figure S5. Molecular structure of PA derivatives. TgGRA25 preferentially binds to saturated PAs (DPPA and DSPA), but not to unsaturated (DOPA), saturated lysoPA (SLPA), or saturated PAs with short acyl chains (DCPA).

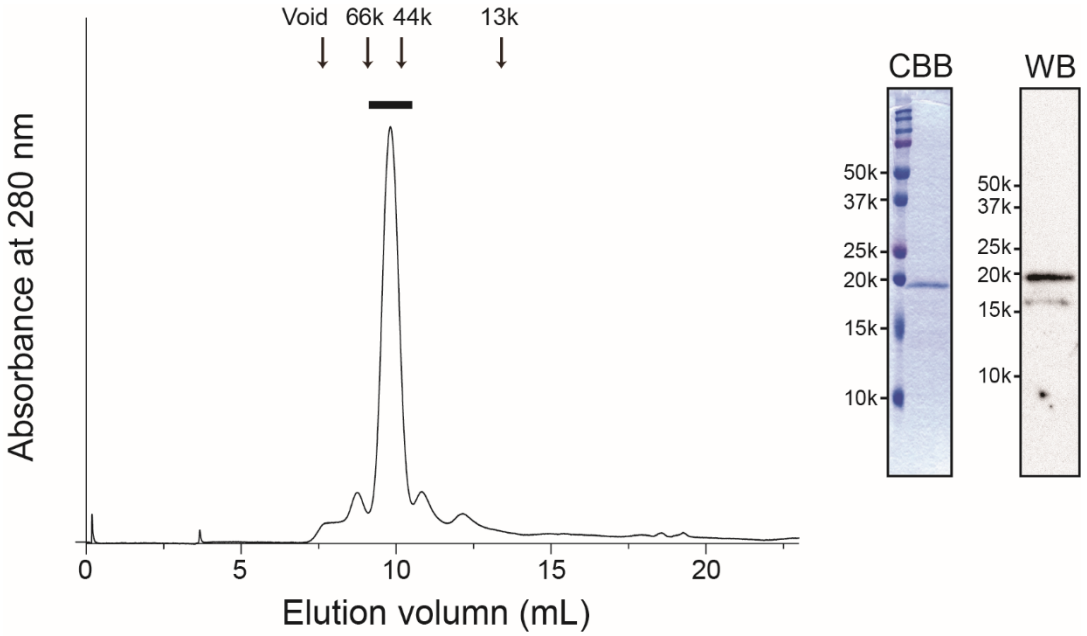
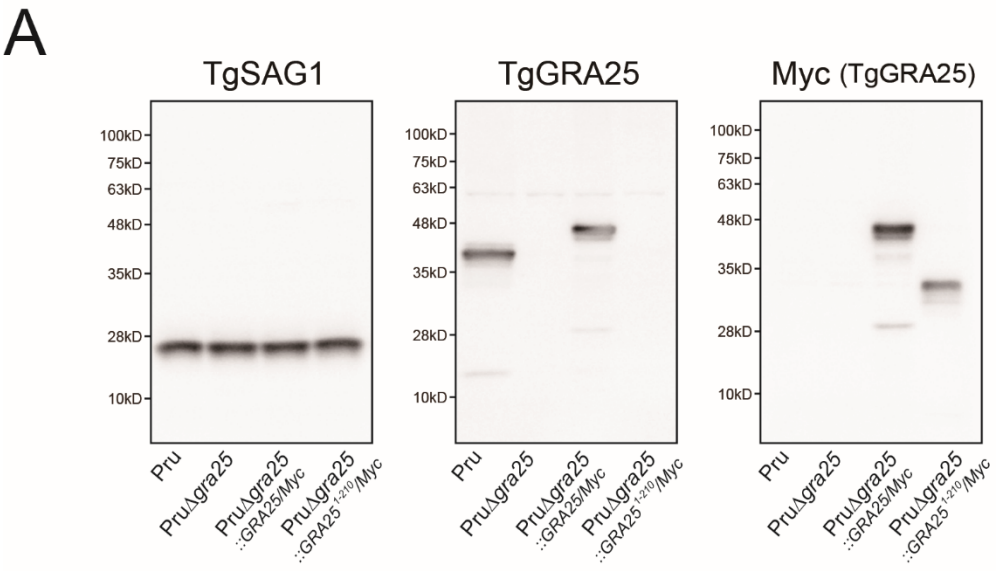
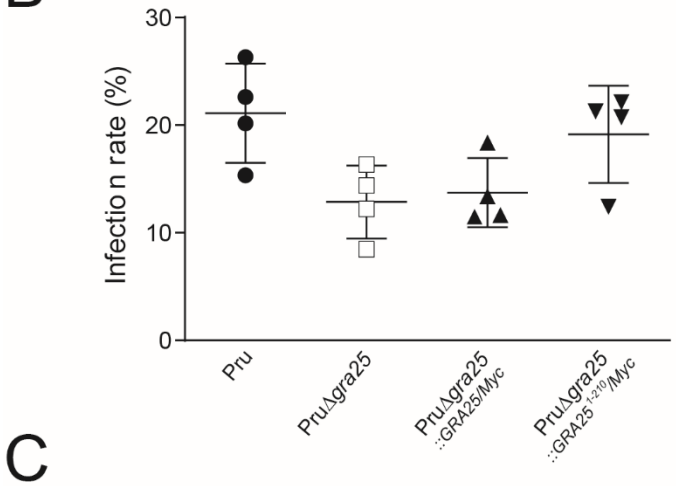


Figure S6. Gel filtration chromatography of recombinant TgGRA25$^{191\text{-}315}$ protein. The elution positions of the molecular weight markers and void volume are indicated. Right, the peak top (bar) of eluted protein was analyzed by Coomassie brilliant blue staining or Western blotting against anti-His tag.


A
TgSAG1
TgGRA25
Myc (TgGRA25)
100kD
75kD
63kD
48kD
35kD
28kD
10kD
Pru
PruΔgra25
PruΔgra25 ::GRA25/Myc
PruΔgra25 ::GRA25 1-210/Myc


B
Infection rate (%)
30
20
10
0
Pru
PruΔgra25
PruΔgra25 ::GRA25/Myc
PruΔgra25 ::GRA25 1-210/Myc

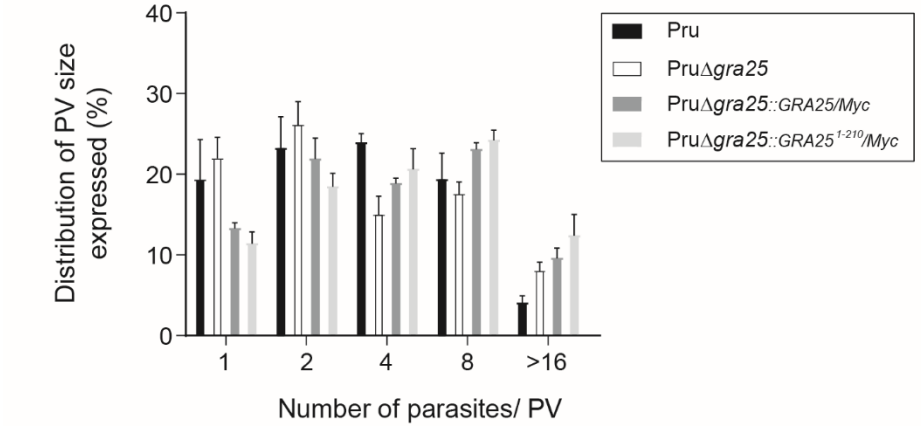

C
Distribution of PV size expressed (%)
40
30
20
10
0
1
2
4
8
>16
Number of parasites/ PV
Pru
PruΔgra25
PruΔgra25::GRA25/Myc
PruΔgra25::GRA25 1-210/Myc

Figure S7. Generation and complementation of parasites deficient in *TgGRA25*. **(A)** The parental (Pru), knock-out (PruΔ*gra25*), or its complemented (PruΔ*gra25::GRA25/Myc* and PruΔ*gra25::GRA25*$^{1\text{-}210}$*/Myc*) parasite lysates were analyzed by immunoblotting with antibodies against TgGRA25 (serum), Myc, or TgSAG1 (loading control) as indicated. (**B**) Vero cells were infected with indicated parasites at an MOI of 1 for 24 h. The cells were fixed and immunofluorescence with antibody against the TgSAG1 was used to detect the infection. The total number of cells was estimated by counting the cells stained with DAPI. The data are representative of 4 replicates. (**C**) Similar to those in (B), except that cells were incubated for 48 h post-infection and the parasite numbers in individual PVs in cells were scored. The data are representative of 4 replicates.

Supplementary Table

The following primers were used in the study.

Primer	Sequence	Detail
TK1229	5'-aaagctagcgtttaaaccatgaagcgtttctggttgtgcgcgctgc	5' oligo for TgGRA25_ME49 (A.A. 1)
TK1230	5'-tttgaattcgtttctatcaaattccgggagg	3' oligo for TgGRA25_ME49 (A.A. 315)
TK1267	5'-tttagatcttttcctgagccgcgttttcgg	3' oligo for TgGRA25_ME49 (A.A. 290)
TK1268	5'-tttagatctcctttctgacgcagcgtctgc	3' oligo for TgGRA25_ME49 (A.A. 250)
TK1269	5'-tttagatctctcccttccttgtcgcgcgg	3' oligo for TgGRA25_ME49 (A.A. 210)
TK1272	5'-aaaagatctatccagacgttctactcccacaacgc	5' oligo for TgGRA25_ME49 (A.A. 191)
TK1274	5'-tttagatctgtttctgtcgaattccgggagg	3' oligo for TgGRA25_GT1 (A.A. 312)
TK1276	5'-gaacgcggagcagaagccaggttttagagctagaaatagc	TgGRA25_138 gRNA (ME49)
TK1279	5'-gttgggaaagaacgcggagcagaagcaagcttcgccaggctgtaaa	5' oligo for DHFR-TgGRA25_138 (ME49)
TK1280	5'-gctacgggagcgaccggcacctctgggaattcatcctgcaagtgcatag	3' oligo for DHFR-TgGRA25_138 (ME49)
TK1288	5'-tgtggcgtctcgattgtgagactagtcgaccgaaggctgtagtac	5' oligo for TgGRA1-5'UTR-TgUPRT
TK1289	5'-tttccatcgactcgccagctactagctagagtcgactggaactac	3' oligo for TgGRA2-3'UTR-TgUPRT
TK1290	5'-gtttcgacacgtctgtatagacgc	5' sequencing oligo for pDXF
TK1359	5'-aaagctagcaccatgggacggttgagttggcaggtggcggcc	5' oligo for hMitoPLD (A.A.1)
TK1360	5'-tttggtaccggtttggctttcgctggaggtgccgc	3' oligo for hMitoPLD (A.A.252)
Cas9/U6	5'-aacttgacatccccatttac	3' oligo for pSAG1::CAS9-U6::sgUPRT
gRNA/seq	5'-actctcgaatgtcttctctga	5' sequencing oligo for the gRNA region

Supplementary References

Hu, K., Mann, T., Striepen, B., Beckers, C.J., Roos, D.S., and Murray, J.M. Daughter cell assembly in the protozoan parasite *Toxoplasma gondii*. *Mol. Biol. Cell*, **13**, 593-606 (2002)

Terkawi, M.A., Kameyama, K., Rasul, N.H., Xuan, X., and Nishikawa, Y. Development of an immunochromatographic assay based on dense granule protein 7 for serological detection of *Toxoplasma gondii* infection. *Clin. Vaccine Immunol.*, **20**, 596-601 (2013)