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Functional redundancy of three mitochondrial Mg^{2+}/Mn^{2+}-dependent protein phosphatases (PPMs) in *Toxoplasma gondii*

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Toxoplasma gondii is a single-celled parasite that infects nearly all warm-blooded animals, including humans (Montoya and Liesenfeld, 2004). It occurs worldwide and can persist for a lifetime in mammals. Humans get infected by eating undercooked meat of animals containing the tissue cysts of this parasite. In immune-competent individuals, *T. gondii* infection usually does not cause significant clinical symptoms, whereas in pregnant or immunocompromised individuals, *T. gondii* infection (toxoplasmosis) can cause more serious problems like abortion and even death (Dunn et al., 1999; Wang et al., 2017). A combination of pyrimethamine and sulfadiazine is usually used to treat toxoplasmosis, although it is generally inefficient and causes side effects (Alday and Doggett, 2017). Worse still, there is a lack of vaccines to prevent *T. gondii* infection in humans or animals.

Phosphorylation/dephosphorylation represents one of the most common and important post-translational modifications of proteins in lifeforms, including unicellular parasites (Yang and Arrizabalaga, 2017). Serine/threonine phosphatases are the major proteins exerting dephosphorylation, and include three major families: phosphoprotein phosphatases (PPPs), Mg^{2+}/Mn^{2+}-dependent protein phosphatases (PPMs), and aspartate-based phosphatases (TFIIF-associating component of C-terminal domain (CTD) phosphatase (FCP)/small CTD phosphatase (SCP)) (Shi, 2009). PPMs are monomeric enzymes that specifically require Mg^{2+}/Mn^{2+} for their phosphatase activity. Based on the genomic dataset of apicomplexan (ToxoDB; https://toxodb.org/toxo/app), there is a notable expansion of the PPM member in *T. gondii* (n=33) compared with other protozoan parasites such as *Cryptosporidium parvum* (n=14), *Plasmodium falciparum* (n=13), and *Babesia bovis* (n=4), suggesting a crucial role for this protein family in *T. gondii*. Hence, we focused on PPMs that have a mitochondrial localization in *T. gondii*, as mitochondrial components have been demonstrated to be essential for this parasite and represent target candidates for intervention in toxoplasmosis (Vercesi et al., 1998; Macrae et al., 2012).

Here, we identified three PPMs with a specific distribution in the mitochondrion of *T. gondii*, namely *Tg*PPM8 (TGGT1_218590), *Tg*PPM10 (TGGT1_254410), and *Tg*PPM19 (TGGT1_275840). These PPMs showed moderate sequence similarities (45%–49%) and were predicted to be orthologs of the mitochondrial phosphatases *Ptc5–Ptc7* of yeast (*Saccharomyces cerevisiae*), and the mitochondrial human phosphatases pyruvate dehydrogenase phosphatase (*Pdp*) and protein phosphatase *Ptc7* homolog (*Pptc7*) (*Homo sapiens*) (Fig. S1), which have all been reported to play crucial roles in mitochondrial function, cell growth, and response to environmental stresses (Guo et al.,

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2017; Niemi et al., 2019). However, little is known about the role of PPMs in the mitochondrion of *T. gondii*, which hinders the development of these molecules as intervention targets.

To address this issue, we first performed 6× hemagglutinin (HA)-tagged endogenous labeling of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 (*Tg*PPM8-6HA, *Tg*PPM10-6HA, and *Tg*PPM19-6HA) in the tachyzoites of *T. gondii* (the developmental stage for dissemination during acute infection) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) genome editing system, which enabled us to observe the subcellular distribution of these PPMs in this parasite by an immunofluorescence assay. It was shown that *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 all exhibited an intracellular lasso shape around the nucleus and stretched under the plasma membrane (the typical distribution of the mitochondrion in *T. gondii* tachyzoites) (Fig. S2). By permeabilizing the tachyzoites with digitonin (a non-ionic detergent that selectively lyses eukaryotic cells), we were able to detect the mitochondrial outer membrane protein translocase of the outer mitochondrial membrane 40 (TOM40), whereas *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 were no longer detected; neither *Tg*PPM8, *Tg*PPM10, nor *Tg*PPM19 in the tachyzoites was digested by proteinase K unless pretreated with Triton X-100. These results clearly showed that *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 are independent of the mitochondrial membrane and distributed in the mitochondrial matrix of *T. gondii* tachyzoites (Fig. 1).

Then, we tested the essentiality of these mitochondrial PPMs during the lytic cycle of *T. gondii* (i.e., host cell invasion, parasite replication, and egress from the invaded cells, which usually form plaques in in vitro experiments) by deleting *Tg*PPM8 (Δ*ppm8*), *Tg*PPM10 (Δ*ppm10*), or *Tg*PPM19 (Δ*ppm19*) using a CRISPR/Cas9 strategy (Fig. S3). Compared with the parental RHΔ*ku80*, Δ*ppm8* resulted in a decreased number (P<0.01) but increased size of plaques, whereas both Δ*ppm10* and Δ*ppm19* were linked to a decreased number (P>0.05 and P<0.01, respectively) and size (P<0.01 and P>0.05, respectively) of plaques, suggesting their essentiality in *T. gondii* infection and pathogenesis. PPM8 appeared to play a role in adhesion to and invasion of host cells rather than replication of *T. donii* (Sidik et al., 2016), as Δ*ppm8* resulted in increased size but fewer plaques. However, surprisingly, neither *Tg*PPM8, *Tg*PPM10, nor *Tg*PPM19 significantly affected the pathogenicity of *T. gondii* mutants in mice.

By additional explorations, we demonstrated that deletion of *Tg*PPM8 (Δ*ppm8*) resulted in a significantly (P<0.001) higher messenger RNA (mRNA)

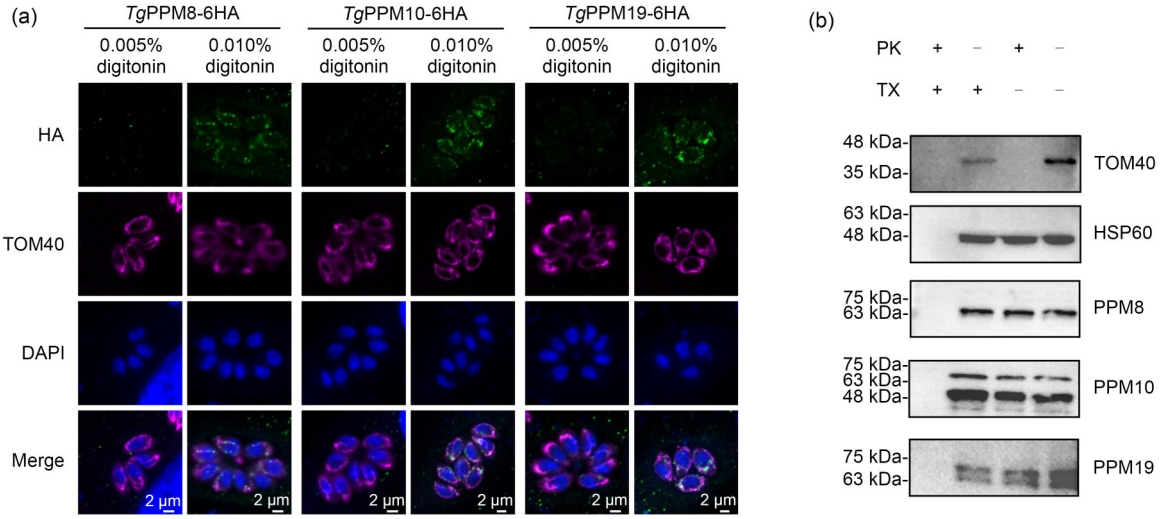

Fig. 1 Distribution of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 in the mitochondrion of *Toxoplasma gondii*. (a) After permeabilization with 0.005% (0.05 g/L) or 0.010% (0.10 g/L) digitonin, *Tg*PPM8-6HA, *Tg*PPM10-6HA, and *Tg*PPM19-6HA were labeled with a rabbit anti-HA antibody (green) in *T. gondii*. Mouse anti-TOM40 sera (fuchsia) and 4',6-diamidino-2-phenylindole (DAPI) (blue) were used to indicate the locations of mitochondrial membrane and nucleic acids, respectively. (b) After treatment with 0.5% (volume fraction) Triton X-100 (TX), *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 in the mitochondrion of *T. gondii* were digested by 0.1 mg/mL protease K (PK) with or without the treatment with 0.5% TX. HA: hemagglutinin; HSP60: heat shock protein 60.

level of *Tg*PPM10, deletion of *Tg*PPM10 (Δ*ppm10*) resulted in a significant upregulation of *Tg*PPM8 and *Tg*PPM19 ($P<0.0001$ and $P<0.001$, respectively), and deletion of *Tg*PPM19 (Δ*ppm19*) resulted in a significant upregulation of *Tg*PPM8 and *Tg*PPM10 (both $P<0.0001$) in the mutant tachyzoites of *T. gondii* compared with their levels in RHΔ*ku80* (the parental strain for mutants) (Fig. 2a). These results imply a functional redundancy of these mitochondrial PPMs in *T. gondii*. This idea was confirmed by deleting two or more of these PPMs in this parasite. Specifically, compared with parental RHΔ*ku80*, both Δ*ppm8*Δ*ppm10* and Δ*ppm8*Δ*ppm19* resulted in a significantly decreased size (both $P<0.0001$) and number ($P<0.001$ and $P<0.0001$, respectively) of plaques. However, Δ*ppm8*Δ*ppm10* showed no difference from Δ*ppm8* or Δ*ppm10* in terms of the number of plaques, although Δ*ppm8*Δ*ppm19* resulted in a somewhat decreased number ($P<0.01$) of plaques compared with Δ*ppm8* or Δ*ppm19* (Figs. 2b and S4). Nonetheless, we could not obtain Δ*ppm8*Δ*ppm10*Δ*ppm19* or Δ*ppm10*Δ*ppm19* as stable strains, possibly due to a lethal phenotype.

In summary, we identified and functionally characterized three mitochondrial PPMs (*Tg*PPM8, *Tg*PPM10, and *Tg*PPM19) in *T. gondii*, which play important roles in the tachyzoite growth and proliferation of this parasite in vitro. As a single, indispensable organelle, a mitochondrion is an ideal target for controlling the *T. gondii* parasite and resultant toxoplasmosis. This is of global importance (Melo et al., 2000). The loss-of-function mutants of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 are promising vaccine strains. However, owing to the functional redundancy among the three mitochondrial PPMs and their different essentialities in *T. gondii* in vitro, only the double-deletion mutants Δ*ppm8*Δ*ppm10* and Δ*ppm8*Δ*ppm19* appear to have potential for the development of an attenuated strain of this parasite. Of course, further investigations, particularly in terms of host-cell invasion, parasite replication, and pathogenicity in vivo, are warranted. A better understanding of these aspects should provide insights into the mitochondrial biology of *T. gondii* and possibly intervention in toxoplasmosis in humans and animals.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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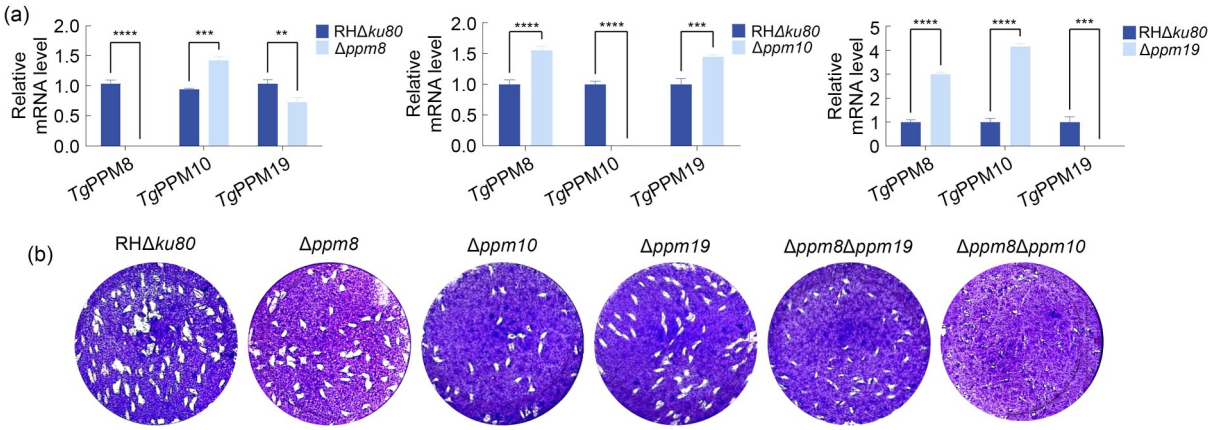

Fig. 2 Deletion of PPM8, PPM10, and/or PPM19 and the phenotype in *Toxoplasma gondii* tachyzoites. (a) Relative mRNA levels of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 in the RHΔ*ku80*, Δ*ppm8*, Δ*ppm10*, or Δ*ppm19* tachyzoites, with β-tubulin used as the internal reference. The statistical analysis was performed using $2^{-\Delta\Delta C_T}$ method and the data are shown as mean±standard deviation of three independent experiments. $^{****}P<0.0001$, $^{***}P<0.001$, and $^{**}P<0.01$, by unpaired *t*-test. (b) Plaques caused by Δ*ppm8*, Δ*ppm10*, Δ*ppm19*, Δ*ppm8*Δ*ppm19*, or Δ*ppm8*Δ*ppm10* tachyzoites in human foreskin fibroblasts (HFFs), compared with that caused by RHΔ*ku80*.

Author contributions

Kaiyin SHENG and Xueqiu CHEN performed the experimental research and data analysis, and wrote and edited the manuscript. Yimin YANG, Jie XIA, and Kaiyue SONG contributed to the study visualization and investigation. Chaoqun YAO revised the manuscript. Yi YANG provided funding acquisition. Aifang DU and Guangxu MA contributed to the study conceptualization, supervision, and funding acquisition, and reviewed and edited the manuscript. All the authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Kaiyin SHENG, Xueqiu CHEN, Yimin YANG, Jie XIA, Kaiyue SONG, Chaoqun YAO, Yi YANG, Aifang DU, and Guangxu MA declare that they have no conflicts of interest.

The use of the experimental animals in this study was approved by the Experimental Animal Ethics Committee of Zhejiang University (No. ZJU201308-1-10-072). All procedures followed were in accordance with the Guidelines for the Use of Experimental Animals of China.

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Supplementary information

Figs. S1–S4; Materials and methods