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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 up to a lifetime in mammals. Humans get infected by eating undercooked meat of animals containing the tissue cysts of this parasite. In immune-competent persons, *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 and dephosphorylation represent 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 (FCP/SCP) (Shi, 2009). PPMs are monomeric enzymes that specifically require Mg^{2+}/Mn^{2+} for their phosphatase activity. Based on the genomic datasets of apicomplexan (ToxoDB; https://toxodb.org/toxo/app), there is a notable expansion of PPM members in *T. gondii* (n = 33) compared with that in 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).

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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 orthologues of the mitochondrial phosphatases PTC5-PTC7 of yeast (*Saccharomyces cerevisiae*), and the mitochondrial human phosphatases PDPs and PPTC7 (*Homo sapiens*), which all have been reported to play crucial roles in mitochondrial function, cell growth, and response to environmental stresses (Guo et al., 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 endogenous labelling of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 in the tachyzoites of *T. gondii* (the developmental stage for dissemination during acute infection) using clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (CRISPR/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). 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 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).

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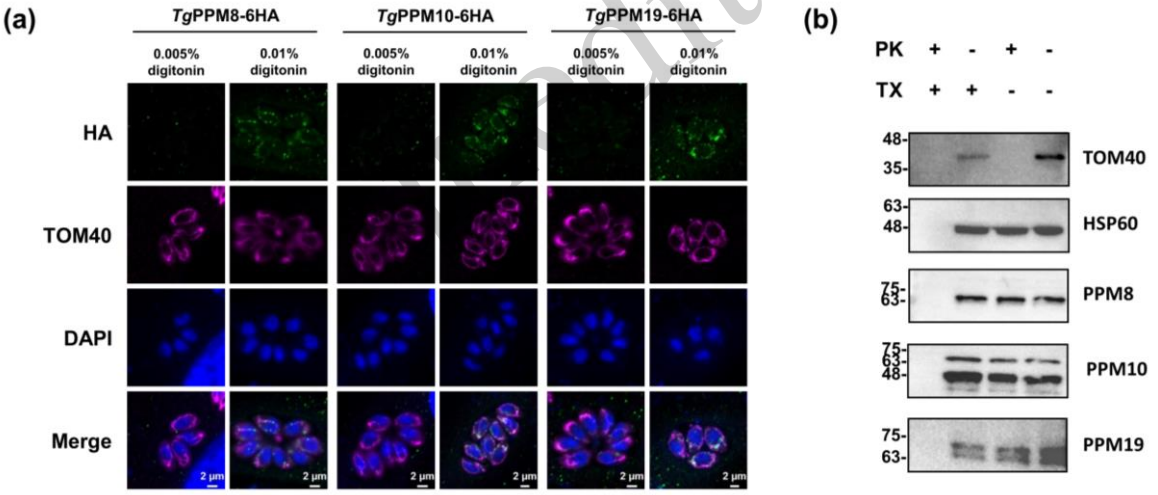

Fig. 1 Distribution of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 in the mitochondrion of *Toxoplasma gondii*. (a) After permeabilization with 0.005% or 0.01% digitonin, *Tg*PPM8-HA, *Tg*PPM10-HA, and *Tg*PPM19-HA are labeled with rabbit anti-HA antibody (green) in *T. gondii*. Mouse anti-TOM40 sera (fuchsia) and DAPI (blue) are used to indicate the location of mitochondrial membrane and nucleic acids, respectively. Scale bar: 2 μm. (b) After treatment with 0.5% Triton X-100 (TX), the digestion of *Tg*PPM8, *Tg*PPM10 and *Tg*PPM19 in mitochondria of *T. gondii* by 0.1 mg/ml protease K (PK). Detailed results are provided in the electronic supplementary materials of this paper.

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 (ΔPPM8), *Tg*PPM10 (ΔPPM10), or *Tg*PPM19 (ΔPPM19) using a CRISPR/CAS9 strategy. Compared with the parental RHΔ*ku80*, ΔPPM8 resulted in a decreased number ($P <$ 0.01) but increased size of plaques, whereas both ΔPPM10 and ΔPPM19 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 ΔPPM8 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 (ΔPPM8) resulted in a significantly ($P < 0.001$) higher mRNA level of *Tg*PPM10; deletion of *Tg*PPM10 (ΔPPM10) resulted in significant upregulation of *Tg*PPM8 and *Tg*PPM19; and deletion of *Tg*PPM19 (ΔPPM19) resulted in significant upregulation of *Tg*PPM8 and *Tg*PPM10 in the mutant tachyzoites of *T. gondii* compared with their levels in RHΔ*ku80* (the parental strain for mutants). 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 ΔPPM8ΔPPM10 and ΔPPM8ΔPPM19 resulted in a significantly decreased size ($P < 0.0001$ and $P < 0.0001$, respectively) and number ($P < 0.001$ and $P < 0.0001$, respectively) of plaques. However, ΔPPM8ΔPPM10 showed no difference from ΔPPM8 or ΔPPM10 in terms of the number of plaques, although ΔPPM8ΔPPM19 resulted in a somewhat decreased number ($P < 0.01$) of plaques compared with ΔPPM8 or ΔPPM19 (Fig. 2). Nonetheless, we could not obtain ΔPPM8ΔPPM10ΔPPM19 or ΔPPM10ΔPPM19 as stable strains, possibly due to a lethal phenotype.

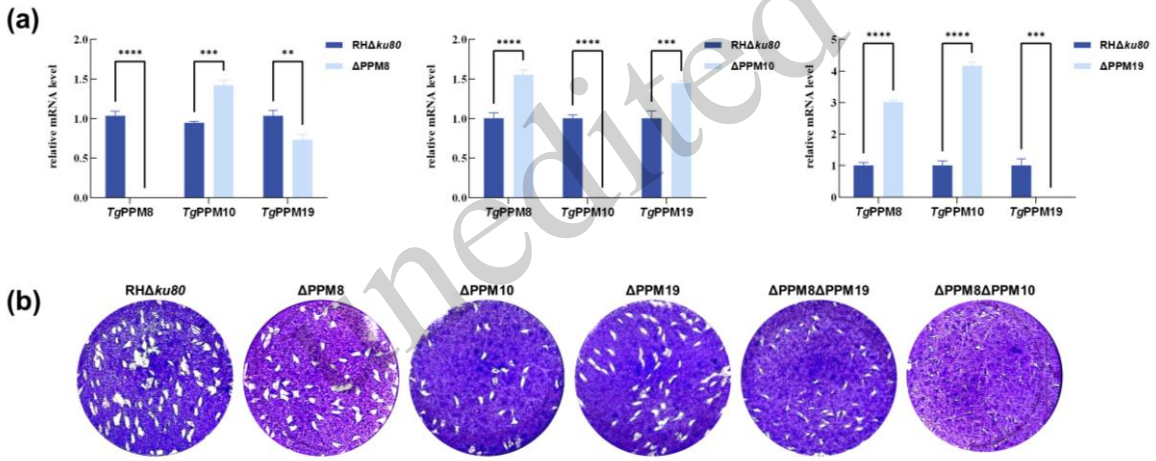

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Fig. 2 Deletion of PPM8, PPM10, and/or PPM19 and the phenotype in *Toxoplasma gondii* tachyzoites. (a) Relative mRNA levels of *Tg*PPM8, *Tg*PPM10, and *Tg*PPM19 in the RHΔ*ku80*, ΔPPM8, ΔPPM10, or ΔPPM19 tachyzoites, with β-tubulin used as the internal reference. The statistical analysis is performed using a $2^{-\Delta\Delta Ct}$ method and the data are shown as the mean of three independent experiments. ****, $P \le 0.0001$; ***, $P \le 0.001$; **, $P \le 0.01$, by unpaired *t* tests. (b) Plaques caused by ΔPPM8, ΔPPM10, ΔPPM19, ΔPPM8ΔPPM10, or ΔPPM8ΔPPM19 tachyzoites in human foreskin fibroblasts (HFFs), compared with that by RHΔ*ku80*. Detailed results are provided in the electronic supplementary materials of this paper.

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, or *Tg*PPM19 are promising vaccine strains. However, due to the functional redundancy among the three mitochondrial PPMs and their different essentialities in *T. gondii in vitro*, only the double-deletion mutants ΔPPM8ΔPPM10 and ΔPPM8ΔPPM19 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.

Acknowledgments

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Author contributions

Kaiyin SHENG and Xueqiu CHEN performed the experimental research and data analysis, 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; reviewed and edited the manuscript. All authors read and approved the final manuscript and, therefore, had 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 conflict of interest.

This article does not contain any studies with human. 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 the People's Republic of China and with the Helsinki Declaration of 1975, as revised in 2013.

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Supplementary information
Detailed results; Materials and methods

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