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COMMENTARY

Serial Dissection of Parasite Gene Families

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Calcium ion signaling regulates central aspects of the biology controlling stage and life cycle transitions of apicomplexan parasites. In the current issue of *Infection and Immunity*, Long and coworkers (S. Long, Q. Wang, and L. D. Sibley, Infect Immun 84:1262–1273, 2016, http://dx.doi.org/10.1128/IAI.01173-15) describe a powerful genetic system enabling reliable serial genetic dissection of a large gene family encoding novel calcium-dependent protein kinases (CDPKs) that provides new insights into the roles of CDPKs during *Toxoplasma gondii* infection.

Intracellular calcium levels and calcium ion (Ca^{2+}) signaling regulate host cell invasion, egress, protein secretion, and differentiation in apicomplexan parasites (1, 2). The biology controlling central aspects of Ca^{2+} entry, storage, release, and signaling has come under increased scrutiny due to the central importance of Ca^{2+} signaling in mediating the cellular and developmental changes essential to apicomplexan parasitism. While a family of calcium (Ca^{2+})-dependent serine/threonine (S/T) protein kinases (CDPKs) is present in plants, ciliates, green algae, and the apicomplexan parasites (2), CDPKs are absent in the mammalian hosts of apicomplexan parasites, suggesting that this gene family could be a rich source of potential drug targets (3). Surprisingly, the CDPK gene family is greatly expanded in apicomplexan parasites, and *Toxoplasma gondii* carries genes that encode 14 distinct CDPKs, though the functions of most of these CDPK genes have not been previously elucidated. In this issue of *Infection and Immunity*, Long and coworkers (4) confirm the essentiality of calcium-dependent protein kinase 1 (CDPK1) (5, 6) and CDPK7 (7) and demonstrate that most CDPKs are not essential for the replicative stages of *T. gondii* infection in the intermediate host.

Ca^{2+} fluxes precede egress and are directly linked to parasite motility and invasion (8, 9). Moreover, Ca^{2+} signaling controls microneme secretion (10, 11) and is essential to trigger parasite motility, which is regulated by Ca^{2+}-mediated phosphorylation of parasite motility motor components (12, 13). Ca^{2+} binds directly to helix-loop-helix (EF hand) structures present in CDPKs to activate them. The canonical CDPK1 controls microneme secretion and is essential for parasite motility, invasion, and egress (5). The canonical CDPK3 controls parasite invasion (14) and egress (15) and regulates calcium homeostasis upstream of CDPK1 (16). Potent and selective inhibitors of *T. gondii* CDPK1 have been identified, and these inhibitors prevent parasite growth *in vitro* (17–19) or markedly reduce parasite burdens in an *in vivo* mouse infection model (20). However, many of the predicted *T. gondii* CDPKs are noncanonical and possess additional protein domains beyond the core canonical CDPK domains which consist of four EF hands in the C-terminal calcium activation domain and a single N-terminal S/T kinase domain (21). The importance and functions of the noncanonical CDPKs are largely unexplored in *T. gondii* and other apicomplexans.

Long and coworkers (4) deleted seven noncanonical CDPK genes (CDPK4, CDPK4a, CDPK4b, CDPK6, CDPK7a, CDPK8, and CDPK9) in both highly virulent type I and less-virulent cyst-forming type II *T. gondii* strains and engineered mutant strains with double or triple CDPK gene knockouts. Surprisingly, only one of the seven noncanonical CDPK knockouts exhibited any detectable phenotypic defect *in vitro* or *in vivo*. While deletion of CDPK6 did not alter acute virulence of the parasite, loss of CDPK6 reduced plaque formation and also reduced the number of brain cysts observed during chronic infection of the host *in vivo* (4). These results are intriguing in view of the fact that CDPK6 is the only noncanonical CDPK that is well conserved in the apicomplexan phylum (21).

Long and coworkers (4) initially used a single-guide-RNA CRISPR/Cas9 insertional mutagenesis strategy to disrupt noncanonical CDPKs; however, they found that this genome engineering approach failed to prevent transcriptional expression of the CDPK gene sequences downstream of the targeted insertion site. Consequently, CRISPR/Cas9-mediated insertional mutagenesis or deletion/insertion of just a few nucleotides may be insufficient to reliably disrupt the functions of multiexonic genes or of other targeted genes. Moreover, the potential off-target effects of using CRISPR/Cas9 mutagenesis in *T. gondii* remains uncharacterized and is a potential concern for the validation of mutant strains. To circumvent these issues and challenges in validation of mutant strains and to also address the biological functions of the large CDPK gene family in *T. gondii*, Long and coworkers (4) devised an elegant genetic approach to serially dissect gene families.

A double-guide-RNA approach was used previously to precisely target complete single-gene deletions in *T. gondii* using a CRISPR/Cas9 system (22). The new scheme devised by Long and coworkers additionally incorporates several features to enable more-reliable development of mutant parasite strains that possess multiple targeted gene deletions. This approach is broadly important to address parasite biology of large gene families such as the CDPKs where biological functions are likely to be redundant and may not become visible until several genes have been serially disrupted. To reduce the possibility of off-target effects and to in-

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crease targeting efficiency, Long and coworkers used type I (23, 24) and type II (25) *T. gondii* strains with deletions in the nonhomologous end joining gene (Δ*ku80* strains) and CRISPR/Cas9 systems that were recently reported for *T. gondii* (26, 27). To generate complete gene deletions, a double-guide-RNA strategy was used to target the first guide close to the ATG codon and to target the second guide close to the stop codon of the CDPK gene of interest. This double-guide-RNA plasmid is cotransfected with a plasmid expressing a selectable marker and mCherry, and this cassette is flanked by loxP sites and flanked again on the 5′ and 3′ ends with homology regions matched with the specific CDPK gene targeted for disruption. Drug-resistant mCherry-positive parasites are selected and genotyped to verify targeted gene deletion. Flanking the selectable marker with loxP sites permits rapid and reliable excision and recovery of the selectable marker following transfection of the mutant strain with a plasmid expressing Cre-GFP (GFP stands for green fluorescent protein) (28). Once excision is validated (drug sensitive and mCherry negative), the mutant strain is targeted for a second gene knockout, and this serial gene deletion strategy can be continued until the desired mutant strain containing multiple targeted gene deletions is isolated.

Previously, Fox and coworkers (23, 25, 29, 30) established an efficient strategy to develop mutant strains containing multiple targeted gene deletions through the development of type I and type II Δ*ku80* strains. This strategy used the *HXGPRT* selectable marker that can be positively or negatively selected (31). However, while forward selection with *HXGPRT* is extremely reliable and efficient, the excision of this marker in negative selection conditions depends on protein expression level and ~10% of mutant strains carrying this marker turn out to be difficult or refractory to the subsequent targeted removal of *HXGPRT* (32). The current scheme employed by Long and coworkers (4) used the pyrimethamine-resistant dihydrofolate reductase-thymidylate synthase (DHFR-TS) selectable marker (33), which is not the ideal selectable marker for wide use of this genetic system because pyrimethamine is currently used as a treatment for *T. gondii* infection in humans. However, *HXGPRT* and other available genetic markers can be easily adapted for use in this new genetic model.

One current limitation of the new scheme used by Long and coworkers (4) is the need to develop and cotransfect two distinct targeting plasmids to isolate one gene knockout. Emerging technologies, such as direct availability of guide RNAs, rather than expression of guides, may bypass the need for two plasmids in the future. Nonetheless, the use of guide RNA accelerates the reliable development of gene knockouts, the use of Cre/loxP to excise the selectable marker accelerates the speed and reliability of selectable marker recovery, and the use of the Δ*ku80* background decreases off-target effects and increases overall gene targeting efficiency. In addition, as new genetic tools and CRISPR/Cas9 technologies emerge, these will be adapted to improve the genetic tool kit available for serial dissection of gene families. Importantly, the work reported by Long and coworkers (4) clearly emphasizes again that deletion of the complete protein encoded by a parasite gene remains the panacea to study loss of gene function (34). Moreover, elegant systems have already been devised that permit the conditional depletion of essential proteins in *T. gondii* (35, 36). Thus, it is highly likely that similar CRISPR/Cas9 strategies to mediate conditional expression of essential genes will quickly emerge. Together, these powerful approaches will enable unprecedented genetic access to dissect the unique and complex biology expressed by apicomplexan parasites.

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