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Calcium Builds Strong Host-Parasite Interactions

Oliver Billker[1,*] and Julian C. Rayner[1,*]
[1]Malaria Programme, Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge CB10 1SA, UK
*Correspondence: oliver.billker@sanger.ac.uk (O.B.), julian.rayner@sanger.ac.uk (J.C.R.)
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Apicomplexan parasite invasion of host cells is a multistep process, requiring coordinated events. In this issue of *Cell Host & Microbe*, Paul et al. (2015) and Philip and Waters (2015) leverage experimental genetics to show that the calcium-regulated protein phosphatase, calcinuerin, regulates invasion in multiple parasite species.

Calcium—A Critical Second Messenger Regulating Parasite Invasion

Single cell eukaryotes from the family Apicomplexa comprise some of the most widely distributed and successful parasites on the planet,and include *Plasmodium* species, the causative agents of malaria. Apicomplexan parasites often have complex lifecycles, and *Plasmodium* parasites are no exception, transitioning through several stages in both vertebrate and mosquito hosts. All vertebrate stages must invade a host cell in order to replicate. Invasion is a highly complex process, relying on multiple ligands that must be correctly processed and trafficked to function, as well as an actin-myosin motor that allows them to physically force their way into the host cell (Cowman et al., 2012). Unsurprisingly, given the number of convoluted molecular steps that occur in succession, invasion is tightly regulated, in part through dynamic protein post-translational modifications that are in turn governed by complex signaling networks (Doerig et al., 2015).

An array of studies over the last decade has established that calcium is as a key second messenger regulating invasion. Calcium plays a role in an array of invasion-related processes, including egress of newly developed parasites from their previous host cell, release of invasion ligands on to the parasite surface, and regulation of parasite motility and host cell entry (Lourido and Moreno, 2015). In attempting to understand how calcium signals are translated into cellular processes, much attention has focused on the protein kinases that add phosphorylation moieties to invasion-related proteins. By contrast, the role of the protein phosphatases that remove these moieties has remained largely unexplored. Two papers in this issue address this gap by performing detailed functional studies of the calcium-regulated protein phosphatase calcineurin (Paul et al., 2015; Philip and Waters, 2015).

Calcineurin Regulates Host Cell Attachment in Apicomplexan Parasites

Calcinuerin functions as a heterodimer of catalytic (CnA) and regulatory (CnB) subunits. In *Plasmodium* parasites, CnA and CnB are both expressed at multiple stages where host cell invasion occurs, indicating a possible invasion-related function. Paul et al. (2015) and Philip and Waters (2015) used conditional genetic technologies to deplete the amount of CnA or CnB protein present in a cell and worked across multiple apicomplexan species to identify a very specific function for calcineurin during the invasion process.

Depleting CnB in tightly synchronized blood stage *P. falciparum* parasites had no effect on intraerythrocytic development but had a very strong effect on erythrocyte invasion; CnA depletion had a similar impact (Paul et al., 2015). Critically, depletion of CnB had no impact on the exocytosis of invasion ligands from either micronemes or rhoptry organelles, even when CnB depletion was combined with drug inhibition to potentiate the effect in a chemical genetic approach. Instead of impacting these earlier stages, where a role for calcium regulation has previously been well established, CnB depletion severely inhibited the attachment of merozoites to erythrocytes. In *P. falciparum*, merozoite-erythrocyte recognition and attachment can be mediated by a number of largely interchangeable receptor-ligand interactions. By employing a range of antibody- and enzyme-mediated inhibition approaches, Paul et al. (2015) established that calcineurin functions at one specific step during attachment, when members of the two main superfamilies of invasion ligands, the PfRhs and PfEBAs, are engaging their host cell receptors. Depleting calcineurin does not affect the level of these proteins, but inhibits their ability to form productive contacts with their target receptors.

Depletion of CnA in blood stages of the rodent model *P. berghei* had similar effects, with no effect on intra-erythrocytic development or protein processing in merozoites, but a very marked effect on erythrocyte invasion in general and erythrocyte attachment in particular (Philip and Waters, 2015). One of the key advantages of working with the rodent *Plasmodium* species *P. berghei* is the relative ease with which other life cycle stages can be investigated, meaning that Philip and Waters (2015) could also study the role of calcineurin more broadly. After the blood stages, the next motile and invasive stage in the life cycle occurs in the mosquito, when highly motile ookinetes are formed after fertilization and burrow their way through the midgut wall to form oocysts on the luminal side. CnA-depleted ookinetes developed and moved completely normally in vitro, but when fed to mosquitoes were not able to form oocysts, indicating a defect in host cell attachment and traversal across the midgut wall. CnA depletion also had an impact on the ability of *P. berghei* sporozoites to invade and develop inside hepatocytes, although not as marked as the effect at other stages.

Calcineurin therefore plays a key role at multiple invasive steps in the *Plasmodium* parasite life cycle, but is its role conserved more broadly? Paul et al. (2015) also studied the function of calcineurin in

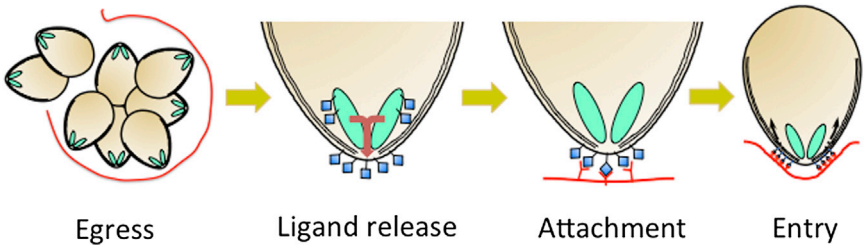

Figure 1. Calcium Regulates Multiple Stages in the Apicopmlexan Invasion Cascade
Acting through an array of signaling networks that influence kinase and phosphatase effectors, the secondary messenger calcium is known to play a role in parasite egress, release of invasion ligands from intracellular organelles, and physical entry into the host cell. Paul et al. (2015) and Philip and Waters (2015) now add an additional step: control of parasite-host attachment through the calcium-regulated phosphatase, calcineurin.

the related parasite *Toxoplasma gondii*, this time by downregulating CnA gene expression using a tetracycline-inducible system. As in both *Plasmodium* species, calcineurin depletion had no effect on parasite intracellular development, egress from the host cell, or protein trafficking and secretion during the early stages of invasion. However, free *T. gondii* tachyzoites with CnA depleted had a marked reduction in adherence and invasion when added to fibroblast monolayers, indicating that the central role of calcineurin in regulating host-parasite attachment is indeed conserved across apicomplexan parasites.

These studies therefore indicate an important role for calcium during invasion, regulating not only the early stages when parasites emerge and invasion ligands are released and proteolytically processed, but also the very final step, when the parasites must form a tight and productive attachment to their target host cell (see Figure 1). It is also worth noting that while *T. gondii* and *P. berghei* are extensively and successfully used as models for different aspects of human malaria parasite biology, there are still very few studies that show protein functions that are conserved across all apicomplexan species. Cross-species biology of this manner is incredibly powerful and will hopefully be embraced more broadly.

The Power of Conditional Genetics and the Need for Scale

Both studies leverage the power of conditional genetic technologies, and in their study of calcineurin, Philip and Waters (2015) provide an important addition to the genetic toolkit for *P. berghei*, an inducible protein degradation system. Protein destabilization domains, such as DD, have been used with some success to regulate protein levels in *T. gondii* and *P. falciparum*, and this approach was employed in the study of *P. falciparum* calcineurin by Paul et al. (2015). The DD domain is structurally unstable and promotes ubiquitination and rapid degradation of itself and the protein to which it is fused, unless it is stabilized by the Shld1 compound (Armstrong and Goldberg, 2007). However, the DD degron is impractical for knocking down essential genes in *P. berghei* blood stages because the Shld1 compound would need to be maintained at high concentrations in mice throughout the period needed to select mutant parasites, raising issues of cost and toxicity. Philip and Waters (2015) therefore adopted a different degron system that relies on the plant hormone auxin, which is significantly cheaper. It also only requires auxin to be added for a short amount of time, because rather than stabilizing the degron as in the Shld1-DD system, addition of auxin actually induces degradation by targeting the degron and attached protein to the auxin receptor TIR1 and recruiting a ubiquitin ligase that is conserved in eukaryotes (Nishimura et al., 2009). The system therefore requires a parent *Plasmodium* line expressing TIR1 and an auxin-inducible degron (AID) fused to the protein of interest.

Philip and Waters (2015) validated the AID system using two other target proteins in addition to CnA and found that it enables rapid, specific, and remarkably complete protein knockdown in asexual blood stages and across sexual development, suggesting it will be a highly versatile system to study gene functions around the *Plasmodium* life cycle. Some additional validation and optimization of the AID in mosquito and liver stages will be required, and the system will share some of the drawbacks of other protein-based conditional systems—above all, the requirement that the fusion tag must not interfere with protein function and that the target protein must be in a subcellular location where it can be targeted for degradation by the proteasome. Furthermore, so far, only cultured parasites have been treated with auxin, and it remains to be seen whether the system works in mice or mosquitoes, given the relatively high concentration of auxin that seems to be required. The AID system has also been developed for *P. falciparum* (Kreidenweiss et al., 2013) and joins an increasing range of conditional knockout or knockdown systems that are now available for apicomplexan parasites, each with its own advantages and limitations (de Koning-Ward et al., 2015). A systematic head-to-head comparison of different conditional and inducible approaches on a larger set of genes would clearly be desirable and may soon become feasible in *P. berghei*, where barcoded alleles now allow the massive parallel phenotyping of large numbers of mutants in the same mouse (Gomes et al., 2015).

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