Conservation of the *Toxoplasma* conoid proteome in *Plasmodium* reveals a cryptic conoid feature that differentiates between blood- and vector-stage zoite

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Abstract

The apical complex is the instrument of invasion used by apicomplexan parasites, and the conoid is a conspicuous feature of this apparatus found throughout this phylum. The conoid, however, is believed to be heavily reduced or missing from *Plasmodium* species and other members of the Class Aconoidasida. Relatively few conoid proteins have been previously identified making it difficult to address the question of how conserved this feature is throughout the phylum, and whether it is truly missing from some major groups. Moreover, parasites such as *Plasmodium* species cycle through multiple invasive forms raising the question of differential presence of the conoid between these stages. We have applied spatial proteomics methods and high-resolution microscopy to develop a more complete molecular inventory and organisation of conoid-associated proteins in the model apicomplexan *Toxoplasma gondii*. These data revealed conservation of the molecules of all substructures of the conoid throughout Apicomplexa including *Plasmodium* and even in allied Myzozoa such as *Chromera*. We reporter-tagged and observed the expression and location of several conoid proteins in the malaria model *P. berghei* and revealed equivalent structures in all zoite forms, as well as evidence of molecular differentiation between blood-stage merozoites and the ookinetes and sporozoites of the mosquito vector. Collectively we show that the conoid is a conserved apicomplexan element at the heart of the invasion mechanisms of these highly successful and often devastating parasites.

Introduction

It is hard to imagine a more insidious intrusion upon an organism's integrity than the penetration and occupation of intracellular spaces by a foreign organism. Apicomplexan parasites are masters of this transgression through actively seeking, binding to, and invading the cellular milieu of suitable animal hosts. From here they manipulate and exploit these cells to promote their growth and onward transmission to other cells and other hosts. The impacts of these infection cycles include major human and animal diseases, such as malaria, toxoplasmosis and cryptosporidiosis in humans, and spectrum of other diseases in livestock and wild animals (Montoya and Liesenfeld 2004; Striepen 2013; World Health Organization 2018; Rashid et al. 2019). The course of both human history and evolution has been shaped by these ubiquitous marauding parasites.

Key to the successful parasitic strategies of apicomplexans is the apical complex—a specialisation of the cell apical cortex that is the coordination centre of the interaction and penetration of host cells (Santos Pacheco et al. 2020). Most of the apicomplexan cell is encased in a pellicle structure of flattened membrane vesicles beneath the plasma membrane, as are all members of supergroup Alveolata including dinoflagellates and ciliates (Gould et al. 2008). These 'alveoli' sacs are supported

by robust proteinaceous networks and collectively this inner membrane complex (or IMC, as it is called in apicomplexans) provides shape and protection to the cell as well as other functions such as gliding motility in apicomplexans by IMC-anchored motors (Frénal, Dubremetz, et al. 2017). The IMC, however, is a general obstruction to other cellular functions that occur at the plasma membrane including exocytosis and endocytosis (Harding and Meissner 2014). Thus, the apical complex has evolved alongside the IMC to provide a site for these functions. When apicomplexans attack their host's cells the apical complex is the site of exocytosis of both initial host- recognition and -binding molecules, and then subsequent secretion of molecules injected into the host create a platform in its plasma membrane for parasite penetration (Kats et al. 2008; Lebrun et al. 2014). In infections such as those that humans suffer from, upon invasion further exocytosed molecules create a modified environment in the host cell that facilitate the parasites protection from the immune system, growth and reproduction. In many gregarine apicomplexans, on the other hand, only partial penetration of the host occurs and the parasite endocytoses host cytosol via their embedded apical complex (Schrével et al. 2016). Near relatives of Apicomplexa, such as colpodellids and some dinoflagellates, similarly feed on prey and host cells through their apical complex—such is the antiquity of this cell feature (Mylnikov 2009; Okamoto and Keeling 2014). The apical complex is thus a coordination of the cell cytoskeleton that defines an available disc of plasma membrane that is unobscured by the IMC, and vesicular trafficking machinery for delivery to and exchange with extracellular environment. A protuberance of the cell at the apical complex also provides mechanical properties to this important site.

Functional studies of the apical complex have occurred in select experimentally amenable taxa, mostly *Toxoplasma* and *Plasmodium*, but a mechanistic understanding of this cell feature or its conservation is still in its infancy. Rather, the apical complex is most broadly understood from ultrastructural studies, and apical rings are the basis of this structure. An apical polar ring (APR) acts as a microtubule organising centre (MTOC) for the subpellicular microtubules and in doing so seemingly coordinates the apical margin with the IMC (Russell and Burns 1984; Morrissette and Gubbels 2014). Within this opening created by the APR are further rings, a notably one being the 'conoid' that is conspicuous throughout much of Apicomplexa (Scholtyseck et al. 2020). The conoid is an open conical structure of variable length and cone pitch. It interacts intimately with secretory organelles including micronemes, rhoptries and other vesicles that penetrate and occupy its lumen (Paredes-Santos et al. 2012; Suarez et al. 2019). An open conoid (or 'pseudoconoid') in the related *Perkinsus* appears to even have microneme-like vesicles physically tethered to it, and in Coccidia a pair of intraconoidal microtubules is also lined by a chain of microvesicles (Perkins 1976; Paredes-Santos et al. 2012). In gregarines endocytosis occurs through the conoid aperture (Schrével et al. 2016). Thus, while the APR appears to play a chiefly structural organising role, the conoid is closely associated with the events and routes of vesicular trafficking, delivery and in some cases uptake. In most examples the conoid has a fibrous presentation by electron microscopy, a trait that is chiefly attributed to the presence of tubulin (Hu et al. 2002; Morrissette and Gubbels 2014). In the *Toxoplasma* conoid, this tubulin forms unusual open fibres with a comma-shaped profile (Hu et al. 2002). The ancestral state of conoid tubulin, however, is likely canonical microtubules as seen in gregarines, *Chromera*, and other apicomplexan relatives (Mylnikov 2009; Obornik et al. 2011; Schrével et al. 2016). It is unclear if the modified tubulin fibres of the *Toxoplasma* conoid arose specifically within coccidia or are more widespread in apicomplexans due to the limits of resolution or preservation of this dense structural feature. Nevertheless, this demonstrates a degree of plasticity of this tubulin element of the conoid. Electron microscopy also reveals further distinct rings at the apical end of the conoid, the preconoidal rings, where the conoid is in closest contact and perhaps interaction with the cell plasma membrane (Nichols and Chiappino 1987). Electron microscopy does not reveal any direct attachment fibres or structures from the conoid to the IMC. However, in *Toxoplasma* it is known that at least one protein links the conoid directly to the APR (Katris et al. 2014) and thus that there is architecture beyond that observed by ultrastructure.

A conspicuous structural deviation to the apical complex in Apicomplexa is the predicted loss of the conoid in some groups, a state enshrined within the class name Aconoidasida. This class contains two important groups: Haemosporida, such as *Plasmodium* spp., and Piroplasmida. Aconoidasida, are considered to have either completely lost the conoid (e.g. *Babesia*, *Theileria*), or at least lost it from multiple zoite stages, e.g all *Plasmodium* spp stages other than the ookinete. However, even while conoids have been attributed to micrographs of ookinete stages in some *Plasmodium* spp., in other studies these are alternatively labelled as apical polar rings (Aikawa 1967; Hanssen et al. 2013; Scholtyseck et al. 2020), and the prevailing understanding of many is that a conoid was lost outright.

The uncertainty over whether the conoid is present in Aconoidasida is a product of two problems. One is that we have little insight into the function of the conoid, so the consequences of its 'loss' are difficult to predict. The other is that we still know relatively little of the molecular composition of the conoid that would allow objectively testing for the presence of a homologous structure (Santos Pacheco et al. 2020). The conspicuous ultrastructure of conoids such as those of coccidia draw attention to tubulin being a major element, however it is known that there are at least other conoid proteins responsible for its assembly, stability, and function during invasion (Small et al. 2007; Katris et al. 2014; Graindorge et al. 2016; Leung et al. 2017; Long, Anthony, et al. 2017; Long, Brown, et al. 2017; Nagayasu et al. 2017; Leung et al. 2020; Tosetti et al. 2020; Back et al.). To test if a homologous conoid cell feature, that is cryptic by traditional microscopy, is present in Aconoidasida fuller knowledge of the molecules that characterise this feature in a 'classic' conoid model are needed. In our study we have sought such knowledge for the *Toxoplasma gondii* conoid using multiple proteomic approaches. We then asked if these conoid-specific proteins are present in similar locations within Aconoidsida, using the model *Plasmodium berghei* to investigate each of its zoite forms, ookinetes, sporozoites and merozoites. In doing so we address the question of what common machinery underpins the mechanisms of invasion and host exploitation that are central to these parasites' lifestyles and impact.

Results

Spatial proteomic methods identify new candidate conoid proteins.

To expand our knowledge of the proteins that contribute to conoid structure and function we applied multiple spatial proteomics discovery approaches. The primary method used was hyperplexed Localisation of Organelle Proteins by Isotope Tagging (hyperLOPIT) that we have recently applied to extracellular *T. gondii* tachyzoites (Christoforou et al. 2016; Barylyuk et al.). This approach entailed generating hyperLOPIT datasets from three independent samples. In each sample, mechanically disrupted cells were dispersed on density gradients and the distinct abundance distribution profiles formed by different subcellular structures and compartments were used to identify proteins that belong to common cellular niches. Combining the data from the three experiments provided enhanced discriminating power of protein location assignments, and from the 3832 proteins that were measured in each experiment we reported 63 proteins assigned to one of the two apical protein clusters, *apical 1* and *apical 2*, above a 99% probability threshold, and another 13 assigned to these clusters but below this high-confidence cut-off. These two clusters were verified as comprising proteins specific to the structures associated with the conoid, apical polar ring, and apical cap of the IMC (Barylyuk et al.). In addition to the 3832 proteins used in this high-resolution spatial proteome, a further 1013 proteins were quantified in either only two or one of the hyperLOPIT datasets. While assignment of these proteins had less data support, a further 16 proteins were assigned to the *apical* clusters from analysis of either the pairs of LOPIT experiments, or the single experiments. From these analyses, 92 proteins in total were assigned as putative apical proteins across these hyperLOPIT samples (Table S1).

Of the 92 putative apical protein assignments by hyperLOPIT, 13 had be validated as being located to the very apex of the cell during our hyperLOPIT study (Barylyuk et al.), 23 by us and others previously, and 21 proteins were known to be specific to the apical cap or other IMC elements (Table S1 and refs therein). This left a further 35 new protein candidates for which there was no independent validation of their apical location. To bolster support for conoid-specific location we applied a second spatial proteomic strategy, proximity dependent biotinylating and pulldown, or BioID. We made three apical BioID 'baits' by endogenous gene 3' fusions with coding sequence for the promiscuous biotin-ligase BirA*. Two baits were known conoid markers: SAS6-like (SAS6L), a protein previously attributed to the preconoidal ring in *T. gondii* (de Leon et al. 2013) but by super-resolution imaging we observe across the entire conoid length (see below); and RNG2 where the C-terminus of this large protein is anchored to the APR that is in close proximity to the midpoint of the conoid in intracellular parasites (Katris et al. 2014). A third bait protein is an otherwise uncharacterised Membrane Occupation and Recognition Nexus (MORN) domain-containing protein (TGME49_245710) that we call MORN3. In an independent study of MORN proteins, we identified MORN3's location as throughout the IMC but with greatest abundance in a band at the apical cap although excluded from the very apex where the conoid is located (Fig 1a). Using these three BioID baits we rationalised that SAS6L and RNG2 proximal proteins would be enriched for those near the conoid, and MORN3 proximal proteins would be enriched for apical cap and IMC proteins but not for conoid-proximal proteins.

T. gondii cell lines expressing each of the BioID bait proteins were grown for 24 hours in host cells in elevated exogenous biotin. Streptavidin-detection of biotinylated proteins on Western blots showed unique banding patterns of each cell line and compared to wildtype controls (cells lacking Bir* fusions) (Fig 1). Biotinylated proteins from each cell line were then purified on a streptavidin matrix and analysed by mass spectrometry. Proteins enriched ≥3-fold compared to the control, or detected in the bait cell lines but not in the control, are indicated in Table S1. Of the hyperLOPIT-assigned *apical* proteins, 25 were also detected by BioID with both SAS6L and RNG2 but not MORN3, and these included known conoid-associated proteins (e.g. MyoH, CPH1, CIP2, CIP3, SAS6L, RNG2). Seven proteins were BioID-detected by MORN3 but not SAS6-L or RNG2, and these are all known apical cap or IMC proteins (AC4, AC8, AC9, AC10, AAP5, IMC9, IMC11). These data indicate that the BioID spatial proteomics indeed enrich for apical proteins with the differences between SAS6L/RNG2 and MORN3 labelling offers a level of discrimination for conoid-associated proteins compared to apical cap proteins.

Validation of conoid proteins and determination of substructural location

We confirmed the identification of new apical complex proteins in the region of the conoid in the hyperLOPIT study by endogenous 3'-tagging candidate genes with reporters (Barylyuk et al.). Imaging by wide-field fluorescence microscopy showed 13 of these proteins confined to a single small punctum at the extreme apex of the cell (Table 1). To test if our expanded hyperLOPIT analysis, including proteins with weaker hyperLOPIT *apical* assignment support, contained further proteins at the apical tip five further candidates were tagged by the same method. All of these proteins were observed to show the same extreme apical location (Fig S1, Table 1).

The conoid of *T. gondii* is a small structure (~400 x 500 nm) in close proximity to the APR and preconoidal rings, so widefield fluorescence microscopy is less able to distinguish between proteins at any of these specific structures. To determine the specific locations of our conoid-proximal proteins we employed 3D-SIM super-resolution imaging in cells co-expressing either SAS6L or RNG2 with C-terminal epitope reporters. By 3D-SIM we observe the V5 epitope-tagged version of SAS6L to locate to the entire barrel of the conoid (Fig 2a), rather than above the conoid in the region of the preconoidal rings as was previously reported for YFP-tagged SAS6L (de Leon et al. 2013). RNG2 C-

terminal reporters locate to the APR (Katris et al. 2014), and thus these two markers provide good definition of the relative positions of the new proteins.

Three of the new apical proteins, which were detected by BioID with baits SAS6L and RNG2 but not MORN3, all co-localised to the SAS6L pattern of the full body of the conoid (Fig 2a, Table 1). These labelled structures are situated within the APR defined by the RNG2 marker. A further three proteins, with this same pattern of BioID detection, all formed a ring at the posterior end, or base, of the conoid (Fig 2b, Table 1). These rings were posterior to the APR (RNG2) consistent with the retracted position of the conoid in intracellular parasites. We also examined the high-resolution location of one protein detected by all three BioID baits and this protein colocalised with the RNG2 marker (Fig 3a, Table 1) consistent with the convergence of the three bait proteins at the APR. We also examined further apical proteins that showed mixed BioID detection, including absence of any BioID positive signals. Four of these proteins formed rings at positions anterior to the conoid, and a further two proteins formed puncta in the same anterior location but too small to resolve further (Fig 3b, Table 1).

Collectively, these super resolution data confirm the identities of several new conoid-associated proteins with distinct protein positions at various sites on the conoid, and the enrichment for further conoid proteins by our spatial proteomic methods. During the hyperLOPIT validation of proteins assigned as *Plasma Membrane – peripheral 2* (on the cytosolic leaflet), one protein (MORN2) was found to be enriched at an apical dot (Barylyuk et al.). Given the close proximity of the conoid apex to the plasma membrane, and unknown molecular interactions between these cell structures that might occur here, we examined the location of MORN2 in high resolution (3D-SIM). MORN2 was seen as a small ring anterior to the conoid with a discernible gap between it and the SAS6L-labelled conoid body (Fig 3c). This location is consistent with MORN2 being associated with the plasma membrane and potentially forming a continuum of the annular structures through from the conoid, preconoidal rings, and to apical plasma membrane.

Evolutionary conservation of Toxoplasma conoid proteins throughout Myzozoa

With the expanded knowledge of conoid-associated proteins determined in this study, and using previously identified conoid-associated proteins, we next asked the following questions. How conserved is the *T. gondii* conoid proteome in other apicomplexan as well as related groups within the Myzozoa (chromerids, dinoflagellates)? Is there evidence of conoid loss from some Aconoidosida taxa as previously suggested? Presence or absence of conoid protein orthologues was tested for in genomic data by two methods. In the first, eukaryote-wide orthogroups defined by OrthoFinder from 79 evolutionary divergent taxa (Barylyuk et al.) were used to test for presence/absence of myzozoan orthologues in the orthogroups of *T. gondii* conoid proteins. The second method used reciprocal sequence searches including BLASTP and iterative HMMER searches. Orthologues identified by either method for select myzozoan taxa are shown in Table 1.

The orthology analysis shows that *T. gondii* conoid proteins are most highly conserved in other coccidia (*Sarcocystis*, *Eimeria*) where most (~80%) orthologues can be detected, although some are still seen to be *Toxoplasma*-specific. In other major apicomplexan groups, represented by *Plasmodium* spp., Piroplasmida, *Cryptosporidium* spp., *Gregarina*, approximately half of the conoid proteins are found, and this is also the case for the nearest apicomplexan relatives the chromerids. These reduced sets of identifiable conoid proteins could represent protein loss in these taxa, gain of novel proteins in the coccidia, or rapid evolution of relatively unconstrained primary protein sequence of some of these proteins resulting in failure of orthologue detection. Nevertheless, there is evidence of substantial conservation of the proteins associated with the conoid throughout all groups, including members of the Aconoidasida that show similar presence of orthologues to other known conoid-containing taxa (Table 1). The conserved proteins include those associated with all structural

components of the *T. gondii* conoid—conoid body, conoid base, and preconoidal rings. Indirect evidence that these proteins continue to function in conoid-type structures, rather than being repurposed for other cell functions, is provided by the absence of most of these proteins in the dinoflagellate *Symbiodinium microadriaticum* (Table 1) that is a coral symbiont and has apparently lost its feeding apparatus (peduncle) as homologue of the apical complex structures. There is further evidence of broadly conserved apical proteins detected by our spatial proteomic approaches but these remain to have their specific apical location determined (Table S1).

Conoid proteins locate to apical rings in Plasmodium zoites

To test if orthologues of *T. gondii* conoid-associate proteins occur in equivalent apical structures in *Plasmodium*, nine proteins were selected for reporter tagging in *P. berghei* (Table 1). This model provided ready access to all three invasive zoite forms of the parasite: the ookinete and sporozoite forms produced in the mosquito vector, and the merozoite form of the mammalian blood-staged infection. The nine proteins represented the three sites associated with the conoid (body, base, and preconoid rings) and one APR protein. GFP fusions of these proteins were initially observed in the large ookinete form by live cell widefield fluorescence imagining and an apical location was seen for all (Fig 4). Eight of these proteins were resolved only as a dot or short bar at the extreme apical end of the cell, whereas the APR orthologue presented as an apical cap. In addition, a tenth putative conoid protein (ICAP16) identified by Sidik et al (2016), that was not detected in our hyperLOPIT data but was by RNG2-BioID, also has a *Plasmodium* orthologue (Table 1). Live reporter-tagging of this protein reveals an apical punctum also (Fig 4). The locations of all ten of these proteins are consistent with equivalent functions in the apical complex to those of *T. gondii*.

To further resolve the location of the *P. berghei* apical proteins, 3D-SIM was performed on fixed cells for six proteins representing the different presentations found in *T. gondii*. The *P. berghei* orthologue of the conoid body protein (PBANKA_0310700) was resolved as a ring at the cell apex, and this structural presentation was also seen for orthologues of the conoid base (PBANKA_1216300) and preconoidal rings (PBANKA_1347000) (Fig 5). Further, two orthologues that are unresolved preconoidal puncta in *T. gondii* are seen in in *P. berghei* to mimic this presentation either as an apical punctum (PBANKA_1025300) or a barely resolved small ring (PBANKA_1313300) (Fig 5). The apical polar ring orthologue (PBANKA_1334800) that showed a broader cap of signal by widefield imaging was revealed as a ring of much larger circumference than the rings of the conoid orthologues (Fig 5). Furthermore, short spines that radiate from this ring in a posterior direction are resolved that account for the cap-like signal at lower resolution. The location of this protein is consistent with an APR function, although more elaborate in structure than what is seen in *T. gondii* (see Fig 3a). Finally, the ICAP16 orthologue (PBANKA_1419000) also resolved as ring (Fig 5) consistent with its predicted conoid location in *T. gondii* (Sidik et al. 2016). In all cases examined, the location and structures formed by the *Plasmodium* orthologues phenocopied those of *T. gondii*, strongly suggestive of conservation of function.

Conoid-type structures are present but compositionally distinct between vector and mammalian Plasmodium zoite forms

The presence of a possible homologous conoid structure in *Plasmodium* has been previously attributed to the ookinete stage but this structure is widely considered to be absent from blood-stage merozoites. With our new markers for components of an apparent conoid in *P. berghei*, and also the new APR protein (PBANKA_1334800), we tested for presence of these proteins in the other zoite stages: sporozoites and merozoites. To date we have tested for detectable presence of seven of these proteins in sporozoites, and eight in merozoites. In sporozoites all proteins tested for are detected at the cell apex (Fig 6a) and super resolution imaging of three of these again showed either a ring or unresolved punctum (Fig 6b).

In merozoites, of the eight proteins tested for, only six have been detected in this alternative zoite form of the parasite. The conoid body orthologue (PBANKA_0310700) is apparently not present in merozoites, and nor is the APR ring protein (PBANKA_1334800), suggesting differences in apical complex construction beyond even the conoid (Table 1). However, all six of the other conoid-associated orthologues are present in merozoites each forming an apical punctum juxtaposed to the nucleus consistent with apical location (Fig 7). These data support conservation of conoid constituents in the apical complex of both sporozoites and merozoites, but either a reduction in the complexity of this structure in merozoites or the possible substitution for other proteins yet to be identified.

Discussion:

The definition often used for the conoid in Apicomplexa is based on the observation of the tubulin-rich fibres observable by electron microscopy. But this cell feature is actually part of a continuum of structures better described as the 'conoid complex' (Santos Pacheco et al. 2020). This complex starts at the apical limits of the IMC which coincides approximately with, although often with some spatial separation from, the anterior end of subpellicular microtubules and the APR (Nichols and Chiappino 1987; Brockley Paterson and Desser 1989; Guttery et al. 2012). The conoid body is tethered within the APR and its anterior end continues as a series or apical rings (preconoidal rings) towards the apical plasma membrane. Proteins have been previously located to all of these 'substructures' including some linking one substructure to the next (Santos Pacheco et al. 2020). Indeed, the apparent spatial separation of compartments by ultrastructure is smaller than the size of many of the individual molecules that build it (Katris et al. 2014). Thus, at a molecular level it is unclear what the limits of any one substructure are, if this is even a biologically relevant notion.

In this study we have provided a substantial expansion of knowledge of the molecular composition of the conoid complex in *T. gondii* and the architecture of these molecules within this structure. Previous identification of conoid complex proteins used methods including subcellular enrichment, correlation of transcriptional expression, and proximity tagging (BioID) (Hu et al. 2006; Long, Anthony, et al. 2017; Long, Brown, et al. 2017). Amongst these datasets many components have been identified, although often with a high false positive rate. We have found the hyperLOPIT strategy to be a powerful approach for enriching in proteins specific to the apex of the cell, and BioID has further refined identification of proteins specific to the conoid complex region. Collectively, we now know of some 53 proteins that locate at the conoid complex (Table 1), with a dataset of many further proteins that await closer inspection (Table S1). Moreover, we have used high-resolution microscopy to define the specific locations of many and these show dedicated locations from the APR through to proteins tethered to the plasma membrane. These data reveal a molecularly complex feature well beyond its tubulin element.

The conservation of a large part of the conoid complex proteome throughout Apicomplexa argues that this is a cell feature maintained throughout this phylum. Conservation includes proteins from all substructural elements—conoid body, conoid base, APR, preconoidial rings and puncta—suggesting maintenance of this structure in its entirety rather than only select elements. Gene knockout studies in *T. gondii* indicate that these conserved elements play key roles in the viability of these parasites (Table 1). Moreover, the conservation of the conoid complex proteome extends to apicomplexan sister taxa such as *Chromera* which supports previous hypotheses that similarity of the ultrastructure of 'apical complexes' in these myzozoan allies represents genuine homologous structures. While the current sample of dinoflagellates indicates substantial absence of this feature, this could be secondary loss in this coral symbiont.

The interpretation of a conoid being absent in *Plasmodium* stages, and in other Aconoidasida, mostly stems from the lack of a conspicuous extended fibrous structure as seen in coccidia, *Cryptosporidium* spp. and gregarines. A tubulin component of the conoid, however, is reported from some Haemosporida such as in *Leucocytozoon* ookinetes although in dramatically reduced form (Brockley Paterson and Desser 1989). In this taxon only a few microtubules are seen in longitudinal section by TEM and only with difficulty due to the surrounding density of other molecules. With any further reduction in the tubulin component in *Plasmodium* spp. or other Aconoidasida detection of conoid tubulin by ultrastructural approaches would be even more challenged, and the dominance of the subpellicular microtubules confounds potential visualisation by specific staining of a potential highly reduced tubulin component.

Regardless of the outstanding question of whether there is a cryptic presence of tubulin in *Plasmodium* conoid-complexes, our data provide strong support for other compositional elements of this structure being present in all *Plasmodium* zoite forms. Reduction or even loss of the tubulin component seen in coccidia might reflect different mechanical properties and needs for these cells within their target host environments. It is presumed that the conoid in *Toxoplasma* provides a mechanical role in invasion, including the motility of the conoid that can be actively protruded from the cell apex. It is poorly known if this motility feature is seen more widely in coccidia or other apicomplexans, but it might be an adaptation in *Toxoplasma* to the tremendously wide host range and cell types that its zoites must invade, including penetrating thick mucilaginous layers at the gut epithelium. A reduction in this mechanical element of the *Plasmodium* conoid complex likely reflects either the different invasion challenges that its hosts present, or different solutions that these parasites have evolved. Such evolutionary change in the composition and architecture of the conoid complex is further supported by differentiation of its proteome between the different *Plasmodium* zoite forms, with the blood-stage merozoites seemingly further reduced or modified compared to ookinetes and sporozoites. This includes the absence of an APR protein in merozoites that in ookinetes forms an elaborate ring with radial posterior projections. We speculate that these proteins delineate so-called 'tines', translucent columns seen by TEM that occupy a dilation between the apical portion of the IMC and the subpellicular microtubules seen in *Plasmodium* ookinetes as well as other apicomplexans (Paterson et al. 1988; Brockley Paterson and Desser 1989; Guttery et al. 2012). This APR protein presents an even more elaborate structure than seen in *Toxoplasma* where it forms a simple ring. Perhaps this is a *Plasmodium* adaptation in lieu of the pronounced extendibility of the conoid complex displayed by *Toxoplasma*. In *Plasmodium* the different zoite stages' invasion requirements between merozoites entering erythrocytes and the multiple penetration events for the ookinetes to reach the mosquito gut basal lamina, or the sporozoites to reach this vector's salivary glands and then mammalian hepatocytes, have likely produced these compositional differences (Vlachou et al. 2006). Ookinete and sporozoite apical complexes might be under further selection by their need for high invasion competence. Each stage represents a transmission bottleneck with success among only one or few parasites is required for onward transmission. Increased investment in a robust and reliable apparatus might be justified at these important transmission points.

Evidence of conserved elements of the conoid and conoid-complex throughout Apicomplexa, despite differences in construction and ultrastructure, raise the question of what are the functions of this structure and are they common throughout the phylum? Indeed, even in *Toxoplasma* the function of the conoid is relatively poorly understood. Most studies of individual protein elements have identified molecules required for its the assembly and stability (Leung et al. 2017; Long, Anthony, et al. 2017; Nagayasu et al. 2017; Leung et al. 2020; Tosetti et al. 2020; Back et al.). But others have implicated roles in control processes, including activating motility and exocytosis, both of which are requirements for invasion as well as host egress events (Katris et al. 2014; Graindorge et al. 2016; Long, Brown, et al. 2017). Indeed, the conoid is intimately associated with both exocytic

vesicles and the apex of the plasma membrane, and this is a common trait throughout not just Apicomplexa but other myzozoans including perkinsids and dinoflagellates (Perkins 1976; Larsen 1988; Paredes-Santos et al. 2012; Suarez et al. 2019). The presence of conserved domains in the conoid-complex proteome is enriched for those that mediate protein-protein interactions as well as responses to regulatory molecules (e.g. Ca^{2+}, cyclic nucleotides) or regulatory protein modifications, and these features are seen in many of the proteins conserved widely amongst taxa (Table 1). This speaks to the conoid complex comprising an ordered regulatory platform for control of vesicle trafficking, fusion and fission, as well as initiation of cell motility. Such a feature as this seems unlikely to be superfluous and lost in these parasites so heavily dependent on mediating complex interactions with hosts through this portal in an otherwise obstructed elaborate cell pellicle. Recognising the common components and importance of the conoid complex throughout Apicomplexa is highly relevant to understanding the mechanisms of invasion and host interaction and the pursuit of better drugs and intervention strategies to combat the many diseases that they cause.

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Table 1: *Toxoplasma* conoid-associated proteins and presence of their orthologues in related taxa.

[a] *T. gondii* ME49	Protein Name	[b] Known Localization	* Ref for Localization	Proteomics: [c] LOPIT	Proteomics: BioID: SAS6-like	Proteomics: BioID: RNG2	Proteomics: BioID: MORN3	[d] Phylogenetic distribution of orthologues: *Sarcocystis neurona*	*Eimeria* spp.	*Plasmodium* spp.	*Theileria* spp.	*Babesia* spp.	*Cryptosporidium* spp.	*Gregarina niphandrodes*	Chromerids	*Symbiodinium microadriaticum*	*P. berghei* ANKA	[e] zoite stage: Ookinete	Sporozoite	Merozoite	[f] CRISPR KO Score	[g] Conserved Domains
222350		conoid body	LOPIT, 1, TS	+	●	●		●	●	●	●	●	●	●			1229900					
274120		conoid body	1, TS	+	●	●		●	●	●			●	●	●		0310700	●	●	○	0.64	
291880		conoid body	LOPIT, TS	+	●	●		●	●	●							0616200				1.77	
244470	RNG2	conoid+APR	7, 8, TS	+	●	●		●		●			●		●						-4.21	
256030	DCX	conoid body	2, 3	+	●	●	●	●	●	●	●	●	●	●	●		1232600				-5.03	UBQ, p25-α
295450	DIP13	conoid body	6					●	●	●		●	●		●	●	1141900				0.67	
301420	SAS6L	conoid body	4, 5, TS	+	●	●		●	●	●	●	●	●	●	●	●	1414900	●	●		-1.62	SAS6_N
226040	CAM3	conoid body	9	+					●												-3.25	EF
243250	MyoH	conoid body	10	+	●	●		●	●		●	●	●		●						-3.94	Myo, Cal, RCC1
246930	CAM1	conoid body	11, 10	+					●				●								1.09	EF
262010	CAM2	conoid body	11, 9	+				●	●						●						-0.81	EF
246720		conoid base	LOPIT, 1, TS	+	●	●		●	●	●			●		●	●	0109800	●	●	●	0.24	EF
258090		conoid base	LOPIT, 1, TS	+	●	●		●	●	●	●	●	●		●		1216300	●	?	?	-1.34	
266630	CPH1	conoid base	LOPIT, 1, TS	+	●	●		●	●	●	●	●	●	●	●		0620600				-4.16	ANK
208340		PCR	LOPIT, TS	+	●	●		●	●	●	●	●	●		●		0907700	●	?	●	-0.81	PH-like
253600		PCR	LOPIT, TS	+				●	●	●	●	●	●	●			0713200	●	●	?	-2.4	
306350		PCR	LOPIT, TS	+				●	●	●	●	●	●	●	●		1347000	●	●	●	-0.84	
250840	MLC3	PCR	10, TS	+		●		●	●		●	●	●	●							-1.91	EF
292120	MORN2	PCR	LOPIT, TS					●	●				●	●	●	●					-0.04	MORN
250340	Centrin 2	PCR+AA	12, 11, 13	+				●	●	●	●	●	●	●	●		1310400				-4.41	EF
219070		PCP	TS	+				●	●	●	●		●				1025300	●	?	●	-2.2	EF, Crp, CAP_ED
274160		PCP	TS	+				●	●	●	●	●	●		●		1313300	●	●	●	-2.8	
239300	ICMAP1	ICMT	14	+				●	●												-0.74	
267370	Kinesin A	APR	15	+	●	●		●	●	●				●	●	●					-2.7	Kinesin
320030		APR	LOPIT, 1, TS	+	●	●	●		●	●							1334800	●	●	○	-0.19	
243545	RNG1	APR	7, 16																		2.54	
315510	APR1	APR	15	+	●	●			●												-0.05	
202120	ICAP16	apex	18			●		●	●	●	●	●	●				1419000	●	●	●	-2.1	PH-like
206430	FRM1	apex	17	+				●	●	●	●	●	●	●			1245300				-3.24	TPR
209890	ICAP4	apex	18					●	●	●	●	●	●	●	●	●	1439000				-4.84	
216080	AKMT	apex	19		●	●		●	●	●	●	●	●	●	●		0932500				-4.3	SET
225020	CIP3	apex	1	+	●	●		●	●	●			●		●		1309800				-2.78	
227000		apex	LOPIT, 1	+	●	●		●	●	●				●			0510100				-3.17	
291020	MyoL	apex	20					●		●	●	●		●	●		1435500				-1.83	Myo head, RCC1
310070	AAMT	apex	21					●	●	●			●		●	●	1318900				-1.22	Methyltrans
312630	GAC	apex	22					●	●	●	●	●	●	●			1137800	●	●	●	-3.53	
315780	MLC7	apex	10	+					●	●		●	●		●	●	0514800				-0.12	EF
210810	CAP1	apex	23						●						●						-0.73	
226990		apex	1	+	●	●															1.41	
234250	CIP1	apex	1		●	●			●								1423000				-2.02	
234270		apex	1	+	●			●													-0.44	
239560	MyoE	apex	20					●	●												0.11	Myo head
252880	CRMP	apex	23	+	●	●		●													-2.35	Ax_dyn_light
254870		apex	1					●	●												0.64	TerD
255895		apex	1	+	●	●		●	●												0.23	
257300	CIP2	apex	1	+	●	●		●	●												-2.49	
278780		apex	LOPIT	+	●	●	●		●												-2.77	UBQ
284620		apex	TS	+				●	●												-1.02	
295420		apex	LOPIT, 1	+	●	●		●	●												-1.57	TerD
297180		apex	LOPIT	+	●	●		●	●												-1.52	
311260	MLC5	apex	10	+				●	●		●	●	●								-0.33	EF
313780		apex	1	+	●	●															0.71	
313780		apex	1	+	●	●															0.71	

[a] Proteins with location data by microscopy in this study shown in red.

[b] Known localization defined as 'apex' when low resolution imaging only has identified an apical punctum at the apex of the cell. PCR, preconoidal ring; AA, apical annuli; PCP, preconoidal punctum; ICMT, intraconoidal microtubules; APR, apical polar ring.

[c] Proteins with data in the hyperLOPIT datasets are indicated with '+'.

[d] Presence of orthologue by: ●, orthogroup analysis previously described (Barylyuk et al.); ●, reverse BLASTP analysis (Santos Pacheco et al. 2020) but absent in the orthogroup analysis; ●, reverse interative hmmer search but not in the other analyses.
[e] Expression and presence at the apex in each *P. berghei* zoite stage: ●, present; ○, absent; ?, untested.
[f] CRISPR KO phenotype scores where more strongly negative scores indicate increasing detrimental competitive growth in in vitro culture conditions (Sidik et al. 2016).
[g] Conserved domain abbreviations: EF, EF-hand; Myo, myosin; Cal, Calmodulin-binding motifs; TPR, Tetratricopeptide repeat; Methyltrans, Methyltransferase; Ax_dyn_light, Axonemal dynein light chain.
[*] References for localization data: TS, this study; LOPIT, (Barylyuk et al.); 1, (Long, Anthony, et al. 2017); 2, (Nagayasu et al. 2017); 3, (Leung et al. 2020); 4, (de Leon et al. 2013); 5, (Wall et al. 2016); 6, (Lévêque et al. 2016); 7, (Tosetti et al. 2020); 8, (Katris et al. 2014); 9, (Long, Brown, et al. 2017); 10, (Graindorge et al. 2016); 11, (Hu et al. 2006); 12, (Lentini et al. 2019); 13, (Leung et al. 2019); 14, (Heaslip et al. 2009); 15, (Leung et al. 2017); 16, (Tran et al. 2010); 17, (Tosetti et al. 2019); 18, (Sidik et al. 2016); 19, (Heaslip et al. 2011); 20, (Frénal, Jacot, et al. 2017); 21, (Engelberg et al. 2020); 22, (Jacot et al. 2016); 23, (Gould et al. 2011); 24, (Skariah et al. 2012).

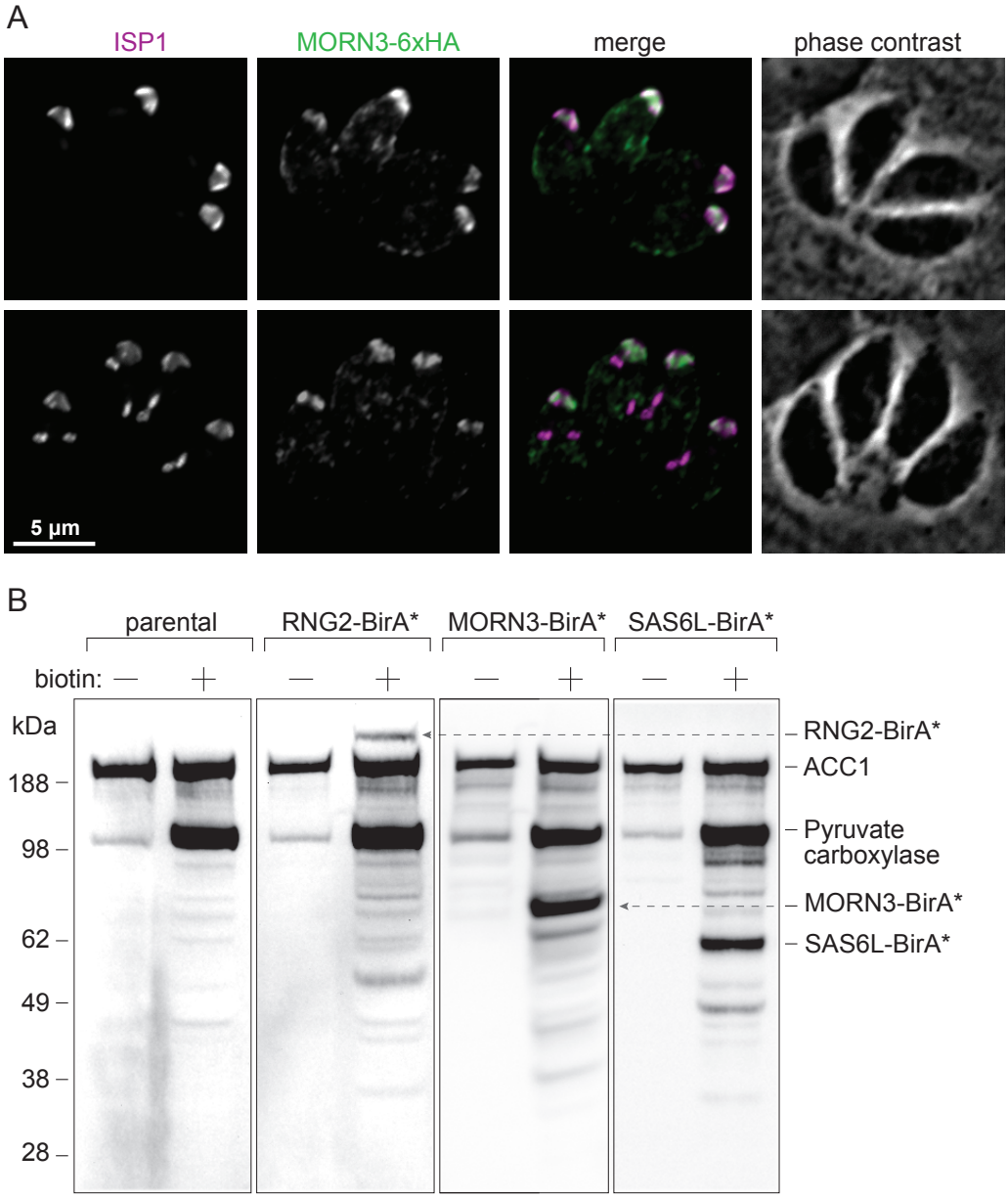

Figure 1: Apically targeted BioID using baits RNG2, SAS6L and new apical cap protein MORN3.
A. Immuno-detection of HA-tagged MORN3 in *T. gondii* intracellular parasites co-stained for apical cap-marker ISP1. Upper panels show mature cells, lower panels show internal daughter pellicle buds forming at the early stages of endodyogeny. Scale bar = 5 µm.
B. Steptavidin-detection of biotinylated proteins after 24 hours of growth either with or without elevated biotin. Native biotinylated proteins ACC1 and pyruvate carboxylase are seen in the parental control (lacking BirA*) and in the BioID bait cell lines. Additional biotinylated proteins are seen in each of the bait cell lines, including self-biotinylation of the bait fusion.

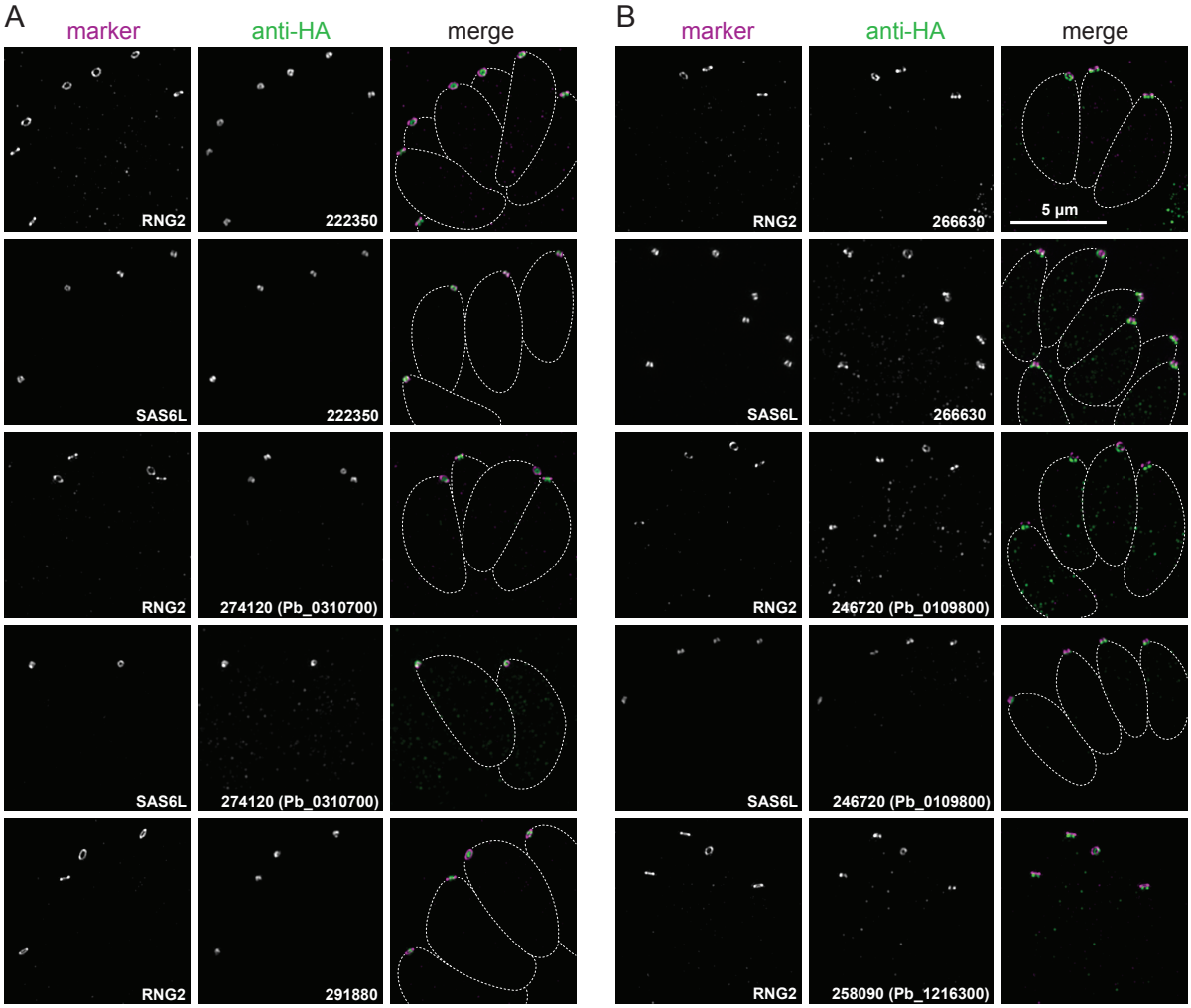

Figure 2: Super-resolution imaging of *T. gondii* proteins at the conoid body and base.
Immuno-detection of HA-tagged conoid proteins (green) in cells co-expressing either APR marker RNG2 or conoid marker SAS6L (magenta). All images are at the same scale, scale bar = 5 µm.
A. Proteins specific to the conoid body.
B. Proteins specific to the conoid base.

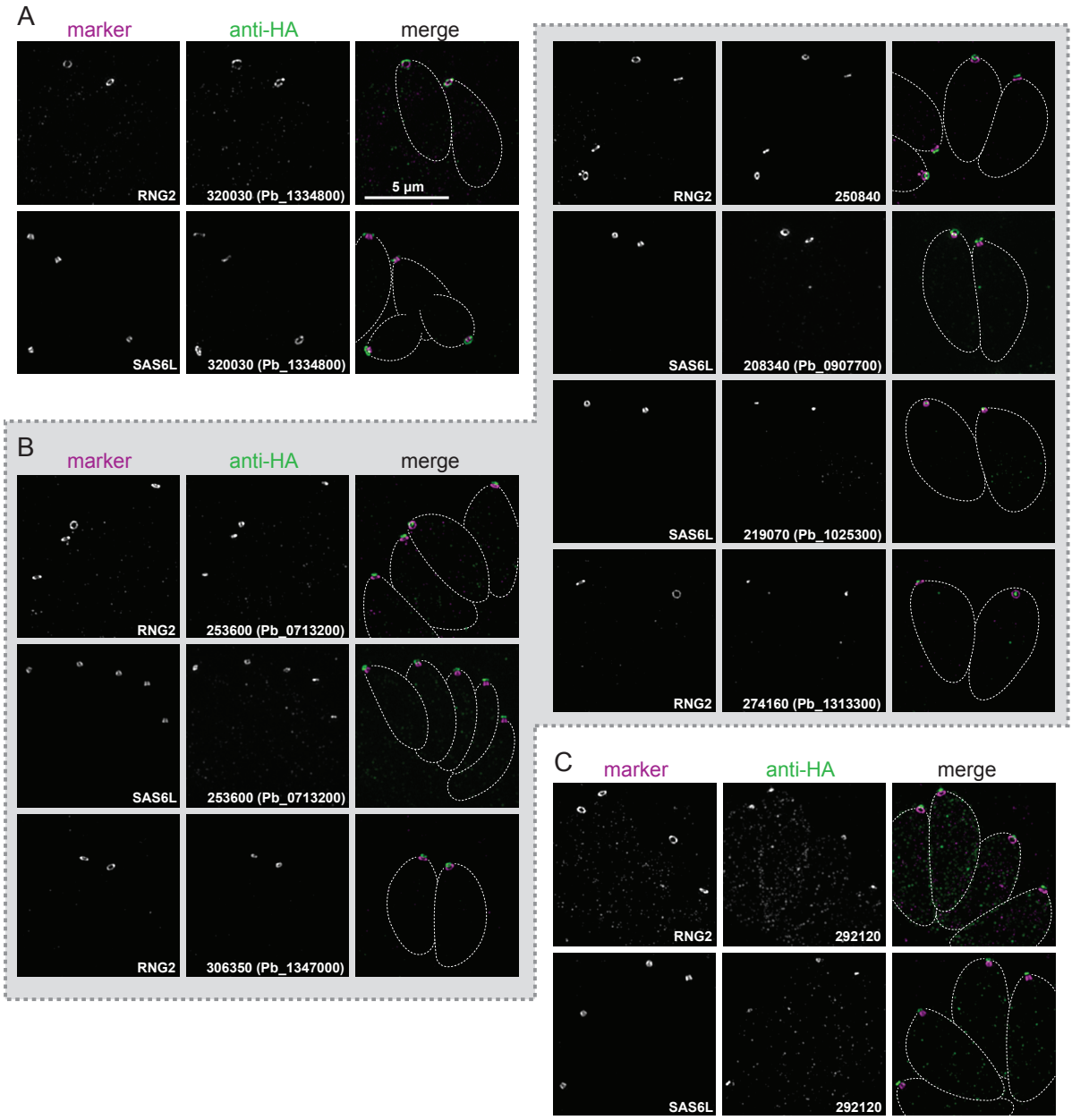

Figure 3: Super-resolution imaging of *T. gondii* proteins at the APR, preconoidal rings, and apical plasma membrane.

Immuno-detection of HA-tagged proteins as for Figure 2.

A. Protein specific to the apical polar ring (APR).

B. Proteins specific structures at the anterior end of the conoid body.

C. A peripheral protein (MORN2) of the inner leaflet of the plasma membrane.

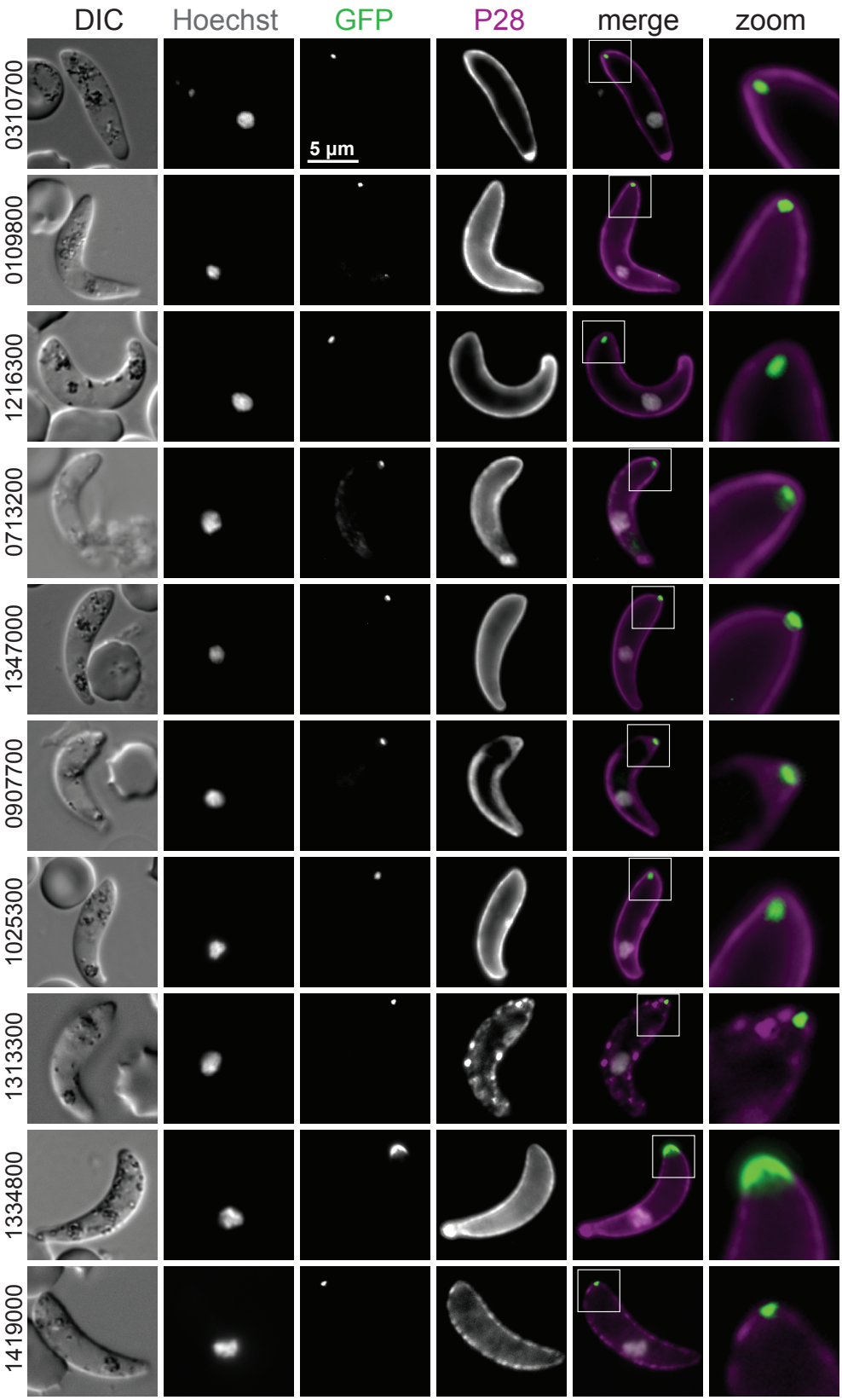

Figure 4: Live cell imaging of *P. berghei* ookinetes expressing GFP fusions of conoid complex orthologues.

Widefield fluorescence imaging showing GFP (green), Hoechst 33342-stained DNA (grey), and live cy3-conjugated antibody staining of ookinete surface protein P28 (magenta). All images are at the same scale, scale bar = 5 µm shown.

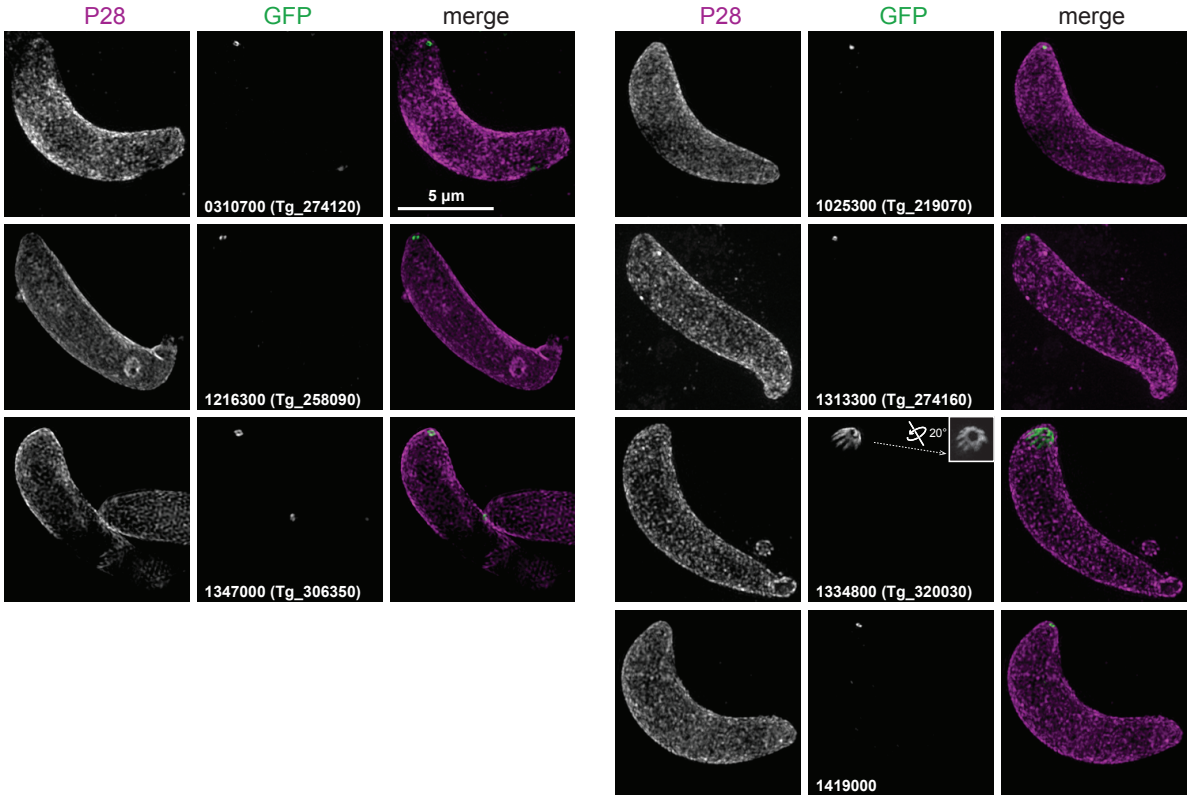

Figure 5: Super-resolution imaging of *P. berghei* conoid complex orthologues in ookinetes.
GFP-fused conoid complex proteins (green) shown with the cell surface stained for ookinete surface protein P28 (magenta). Inset for APR protein (1334800) shows rotation of the 3D-reconstruction to view the parasite apex face on. All images are at the same scale, scale bar = 5 µm.

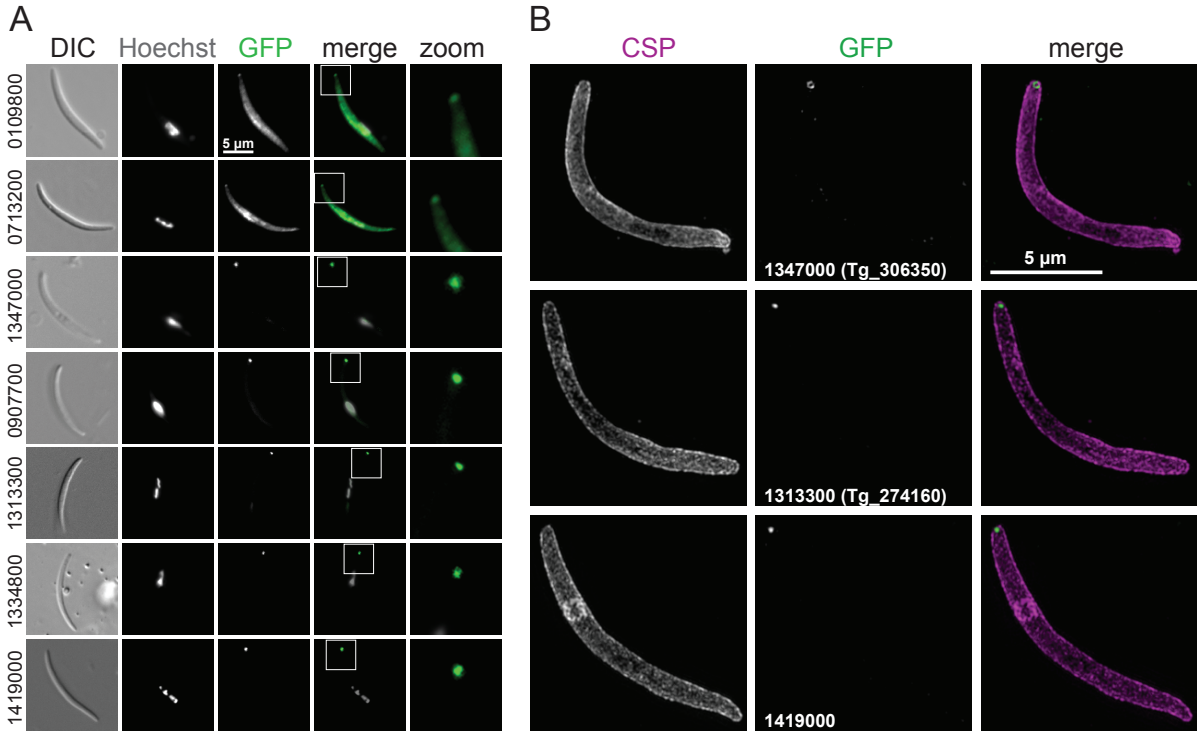

Figure 6: Live cell widefield, and super-resolution imaging of *P. berghei* sporozoites expressing GFP fusions of conoid complex orthologues.

A. Widefield fluorescence imaging showing GFP (green) and Hoechst 33342-stained DNA (grey). All images are at the same scale, scale bar = 5 µm shown.

B. Super-resolution imaging of GFP-fused conoid complex proteins (green) in fixed cells shown with the cell surface stained for sporozoite surface protein 13.1 (magenta). All images are at the same scale, scale bar = 5 µm shown.

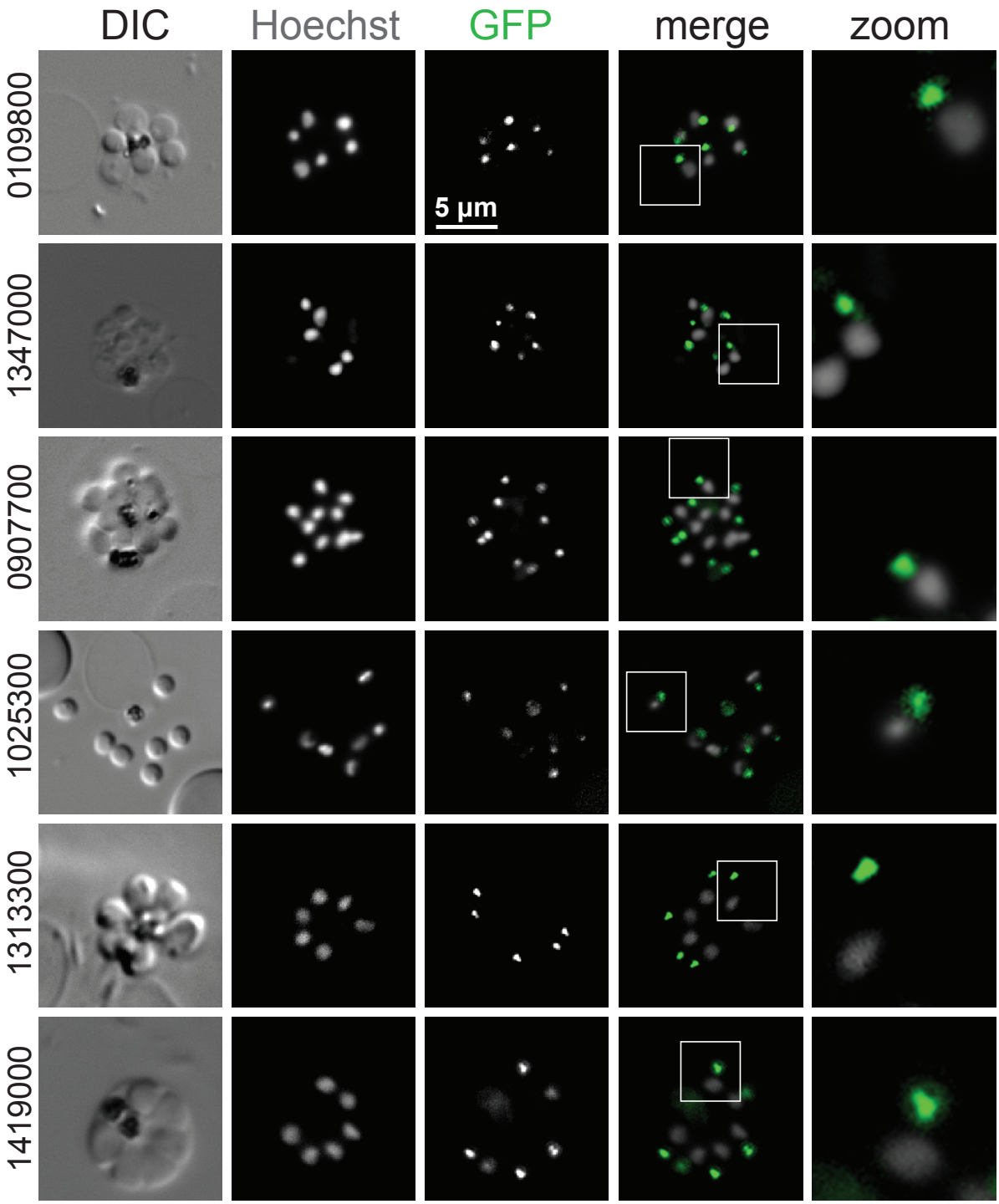

Figure 7: Live cell imaging of *P. berghei* merozoites expressing GFP fusions of conoid complex orthologues.

Widefield fluorescence imaging showing GFP (green) and Hoechst 33342-stained DNA (grey) with come parasites seen pre-egress from the erythrocyte and others post egress. All images are at the same scale, scale bar = 5 µm shown.

Supplemental Material

Table S1: *Toxoplasma* candidate apical proteins identified by hyperLOPIT and BioID methods, and presence of their orthologues in related taxa

Footnotes:

[a] Known localization defined as 'apex' when low resolution imaging only has identified an apical punctum at the apex of the cell. PCR, preconoidal ring; AA, apical annuli; PCP, preconoidal punctum; ICMT, intraconoidal microtubules; APR, apical polar ring; SPMT, subpellicular microtubules.

[b] LOPIT assignment strength: ●, Protein assigned to one of the two *apical* clusters at >99% probability in (before '/') the analysis of concatenated three or two datasets where the protein was full quantified or (after '/') the analysis of individual hyperLOPIT datasets; ●, as for above except apical assignment probability is below the 99% threshold; ●, assignment to a cluster other than *apical* in one non-catenated hyperLOPIT datasets.

[c] Presence of orthologue by: ●, orthogroup analysis previously described (Barylyuk et al.); ●, reverse BLASTP analysis (Santos Pacheco et al. 2020) but absent in the orthogroup analysis; ●, reverse interative hmmer search but not in the other analyses.

[d] CRISPR KO phenotype scores where more strongly negative scores indicate increasing detrimental competitive growth in in vitro culture conditions (Sidik et al. 2016).

[*] References for localization data: TS, this study; LOPIT, (Barylyuk et al.); 1, (Long, Anthony, et al. 2017); 2, (Nagayasu et al. 2017); 3, (Leung et al. 2020); 4, (de Leon et al. 2013); 5, (Wall et al. 2016); 6, (Lévêque et al. 2016); 7, (Tosetti et al. 2020); 8, (Katris et al. 2014); 9, (Long, Brown, et al. 2017); 10, (Graindorge et al. 2016); 11, (Hu et al. 2006); 12, (Lentini et al. 2019); 13, (Leung et al. 2019); 14, (Heaslip et al. 2009); 15, (Leung et al. 2017); 16, (Tran et al. 2010); 17, (Tosetti et al. 2019); 18, (Sidik et al. 2016); 19, (Heaslip et al. 2011); 20, (Frénal, Jacot, et al. 2017); 21, (Engelberg et al. 2020); 22, (Jacot et al. 2016); 23, (Gould et al. 2011); 24, (Skariah et al. 2012); 25, (Chen et al. 2015); 26, (Chen et al. 2017); 27, (Beck et al. 2010); 28, (Anderson-White et al. 2011); 29, (Suvorova et al. 2015); 30, (Liu et al. 2013); 31, (Liu et al. 2016); 32, (Frénal et al. 2010).

Table S1: Oligonucleotide primers used for P. berghei gene tagging

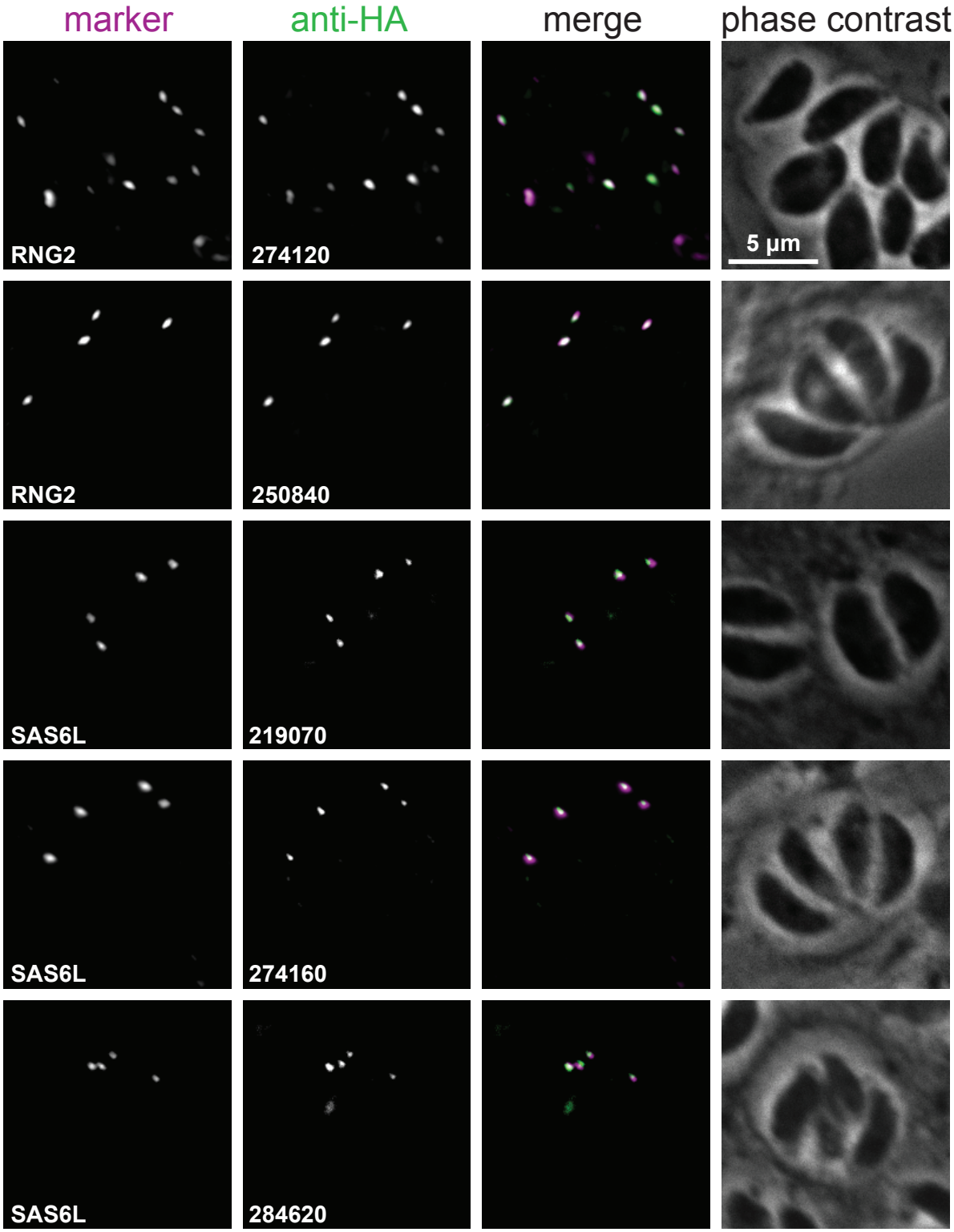

Figure S1: Immuno-detection of candidate conoid complex proteins, identified by hyperLOPIT and BioID approaches, in *T. gondii* intracellular parasites

Widefield fluorescence imaging of HA-tagged candidates (green) co-expressing either APR marker RNG2 or conoid marker SAS6L (magenta). All images are at the same scale, scale bar = 5 µm.

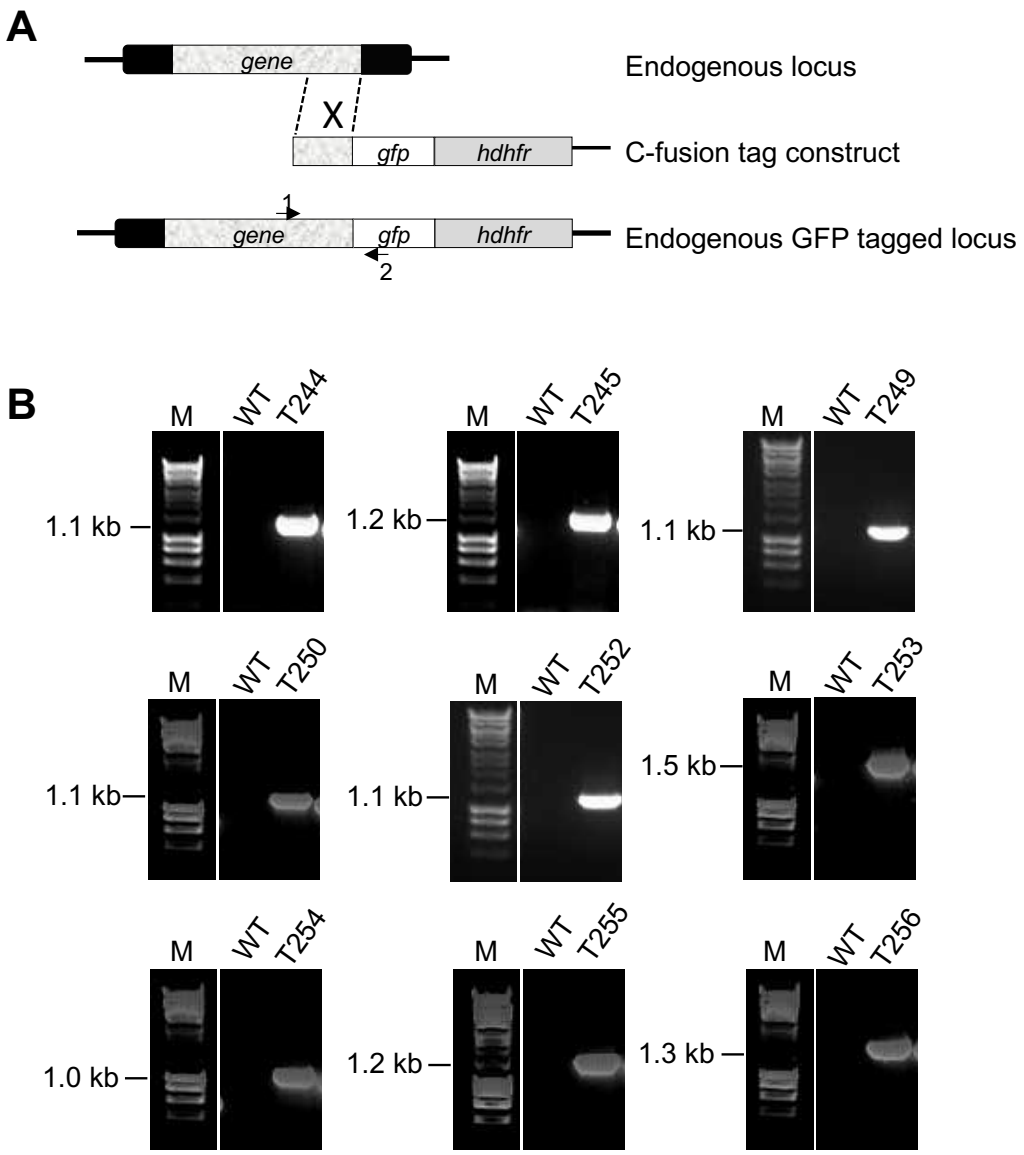

Figure S2:

A. Schematic representation of the endogenous gene locus, the constructs and the recombined gene locus.

B. Validation of correct gene tagging by diagnostic PCR

Materials and Methods:

Growth and generation of transgenic T. gondii

T. gondii tachyzoites from the strain RH and derived strains, including RH Δku80/TATi (Sheiner et al., 2011) (a kind gift from Lilach Sheiner and Boris Striepen, The University of Georgia), were maintained at 37°C with 10% CO_2 growing in human foreskin fibroblasts (HFFs) cultured in Dulbecco's Modified Eagle Medium supplemented with 1% heat-inactivated fetal bovine serum, 10 unit ml^{-1} penicillin and 10 µg ml^{-1} streptomycin, as described elsewhere (Roos et al., 1994). When appropriate for selection, chloramphenicol was used at 20 µM and pyrimethamine at 1 µM. Scaling up of the parasite culture for hyperLOPIT experiments was done according to the method described by Roos et al. (Roos et al., 1994).

BioID

Sample preparation: For the proximity biotinylation assay, we generated three different cell lines (*T. gondii* tachyzoites RH Δku80 TATi) by *in situ* genomic C-terminal tagging of one of the three bait proteins (SAS6L, RNG2 or MORN3) with the promiscuous bacterial biotin ligase, BirA*. Our protein tagging protocol is described in our previous work (Barylyuk et al.). We then followed the BioID protocol of Chen et al. (Chen et al. 2015). The biotinylation of the proximal proteins by the BirA* enzyme was triggered by addition of 150 µM biotin into the ED1 growth media 24 h prior to parasite egress. The non-tagged parental cell line was used as a negative control for background biotinylation.

The BirA* activity was validated by a western blot detection of the biotinylated proteins. $1x10^7$ tachyzoites were lysed in the NuPAGE™ LDS Sample Buffer (ThermoFisher) and separated in the NuPAGE™ 4-12% Bis-Tris gel (ThermoFisher) and blotted on a nitrocellulose membrane, which was then blocked by 3% BSA for 1h. The membrane was then incubated in the presence of streptavidin-HRP conjugate (ThermoFisher; 1:1000 dilution in 1% BSA) for 1h, followed by five 5-minute washes in TBST buffer. The HRP chemiluminescent signal was visualised by the Pierce™ West Pico kit (ThermoFisher) and a digital camera.

For the proteomic analysis ~ 2×10^9 tachyzoites were harvested after 24-h biotinylation and egress. The parasites were separated from the host-cell debris by passing through 3 µm filter and washed 5x in phosphate-buffered saline. The cell pellets were lysed in RIPA buffer and the volume of each sample was adjusted to 1.8 mL and 5 mg of total protein content. 200 µL of unsettled Pierce™ Streptavidin Magnetic Beads (ThermoFisher) were first washed in RIPA buffer and then incubated with the cell lysates for 1h at room temperature with gentle rotation of the tubes. The beads were then washed 3x in RIPA, 1x in 2M UREA 10mM Tris-HCl pH 8.0 and again 3x in RIPA, reduced in 10 mM DTT 100 mM ammonium bicarbonate solution for 30 min at 56°C, alkylated in 55 mM iodoacetamide 100 mM ammonium bicarbonate for 45 min at room temperature in dark, and finally washed in 50 mM ammonium bicarbonate for 15 min at room temperature with gentle rotation. The peptides were digested off the beads by an overnight 37°C incubation in 1 µg of trypsin dissolved in 50 mM ammonium bicarbonate and the remaining peptides were extracted by 10% formic acid. Both peptide extractions were combined, desalted using C18 solid-phase extraction cartridges (SepPak C18, 100 mg sorbent, Waters), dried in a vacuum centrifuge (Labconco), and resuspended in 0.1% (vol.) aqueous formic acid.

Peptide analysis by liquid chromatography and mass spectrometry: Peptide fractions were analysed in a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled on-line with a nanoflow ultrahigh-performance liquid chromatography (nano-UPLC) system Dionex UltiMate 3000 RSLCnano (Thermo Fisher Scientific Inc., Waltham, MA, USA) through an EASY-Spray nano-electrospray ionization (nano-ESI) source (Thermo

Fisher Scientific Inc., Waltham, MA, USA). Samples were loaded onto an Acclaim PepMap 100 C18 trapping column (300 µm i.d. × 5 mm length, 5 µm particle size, 100 Å pore size; Thermo Fisher Scientific) from the UltiMate 3000 autosampler with 0.1% aqueous formic acid for 3 minutes at a flow rate of 10 µL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. The trapped peptides were then separated on an analytical reverse-phase nano EASY-Spray RSLC C18 column (75 µm i.d. × 50 cm length, 2 µm particle size, 100 Å pore size; Thermo Fisher Scientific) at a flow rate of 300 nL/min using a gradient elution program. Solvent A was 0.1% (vol.) formic acid in water (HPLC-grade, Fisher). Solvent B was 0.1% (vol.) formic acid in water-acetonitrile (HPLC-grade, Rathburn) blend (20% : 80%, vol.). The linear gradient employed was 2-40% B in 30 minutes. Further wash and equilibration steps gave a total run time of 60 minutes.

The peptides eluting from the nano-LC column were ionized by applying a positive voltage of 2.1 kV to the nano-ESI emitter electrode. The mass spectrometer was operated in the data-dependent acquisition mode (DDA). The precursor survey mass spectra (MS1) were acquired between 380 and 1500 *m/z* at a 70,000 resolution with an AGC target of 1e6 and a maximum C-trap fill time of 250 ms. The top twenty most intense precursor ions were selected in the quadrupole using an isolation window of 1.5 *m/z* and subjected to fragmentation in the HCD mode at a normalised collision energy of 25. A dynamic exclusion limit of 20 s was applied. The fragment ion spectra (MS2, or MS/MS) were acquired between 100 and 1600 m/z at a 17,500 resolution with an AGC target of 5e4 and a maximum C-trap fill time of 120 ms. The raw data were managed in XCalibur v2.1 (Thermo Fisher Scientific).

LC-MS/MS data processing and protein identification: The raw LC-MS/MS data were interpreted, and peak lists were generated using Proteome Discoverer 1.4 (Thermo Fisher Scientific). The peak lists were exported as Mascot generic files and searched against the annotated protein sequences of *Toxoplasma gondii* strain ME49 (ToxoDB.org release 29, retrieved on 20 December 2016; 8,322 sequences) and common contaminant proteins (cRAP, 115 sequences) with Mascot v2.6.0 (Matrix Science) assuming digestion by trypsin with up to two missed cleavage sites. The precursor and fragment ion tolerances were set to 5 ppm and 0.1 Da, respectively. Carbamidomethylation of cysteine was set as a static modification. Oxidation of methionine and deamidation of asparagine and glutamine were allowed as variable modifications. Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller et al. 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Total spectrum counts were used to calculate the enrichment of proteins in the BioID pulldown samples relative to the wild-type control sample. The contaminant proteins were not considered for the analysis.

hyperLOPIT Spatial proteomics

The hyperLOPIT spatial proteomics data were acquired and analysed as described in (Barylyuk et al.). Three independent hyperLOPIT datasets contained 4,189, 4,547, and 4,292 proteins identified at FDR < 5% based on at least one unique high-confidence peptide (FDR < 1%) and quantified across all ten TMT quantification channels. These three 10plex datasets were considered individually. In addition, the data on proteins shared across pairs or all three individual datasets were concatenated affording three 20plex (4,021, 3,916, and 4,078 proteins for $1 \cap 2$, $1 \cap 3$, and $2 \cap 3$, respectively) and one 30plex (3,832 proteins), thus, yielding seven datasets in total. For each dataset, the normalised

protein abundance distribution profiles were derived from the TMT reporter ion intensity values. A Bayesian machine-learning classification method based on t-augmented Gaussian mixture models with maximum *a posteriori* prediction (TAGM-MAP) (Crook et al. 2018) was applied to probabilistically attribute proteins to 26 subcellular niches defined by 718 marker proteins. The protein localisation probability was calculated as the probability of a protein belonging to the most likely subcellular class and not being an outlier. Assignments at a localisation probability above 99% were accepted.

Immunofluorescence microscopy

T. gondii-infected HFF monolayers grown on glass coverslips were fixed with 2% formaldehyde at room temperature for 15 min, permeabilized with 0.1% TritonX-100 for 10 min and blocked with 2% BSA for 1 h. The coverslips were then incubated with a primary antibody for 1 h, followed by 1 h incubation with a secondary antibody. Coverslips were mounted using ProLong® Diamond Antifade Mountant with DAPI (Invitrogen). Images were acquired using a Nikon Eclipse Ti widefield microscope with a Nikon objective lens (Plan APO, 100x/1.45 oil), and a Hamamatsu C11440, ORCA Flash 4.0 camera.

3D-Structured Illumination Microscopy (3D-SIM) was implemented on a DeltaVision OMX V4 Blaze (Applied Precision) with samples prepared as for widefield immunofluorescence assay (IFA) microscopy expect High Precision coverslips (Marienfeld Superior, No1.5H with a thickness of 170 µm ± 5 µm) were used in cell culture and Vectashield (Vector Laboratories) was used as mounting reagent. Samples were excited using 405, 488 and 594 nm lasers and imaged with a 60x oil immersion lens (1.42 NA). The structured illumination images were reconstructed in softWoRx software version 6.1.3 (Applied Precision). All fluorescence images were processed using Image J software (http://rsbweb.nih.gov./ij/).

Ethics statement

The animal work performed in the UK passed an ethical review process and was approved by the United Kingdom Home Office. Work was carried out in accordance with the United Kingdom 'Animals (Scientific Procedures) Act 1986' and in compliance with 'European Directive 86/609/EEC' for the protection of animals used for experimental purposes. The permit number for the project licence is 40/3344.

Generation of transgenic Plasmodium berghei and genotype analyses

To observe the location of *P. berghei* proteins, the C-terminus of the gene was tagged with green fluorescent protein (GFP) sequence by single crossover homologous recombination. To generate the GFP-tag line, a region of these genes downstream of the ATG start codon was amplified, ligated to p277 vector, and transfected as described previously (Guttery et al. 2012). The p277 vector contains the human *dhfr* cassette, conveying resistance to pyrimethamine. A schematic representation of the endogenous gene locus, the constructs and the recombined gene locus can be found in Fig S2. A list of primers used to amplify these genes can be found in Table S2. For the parasites expressing a C-terminal GFP-tagged protein, diagnostic PCR was used with primer 1 (Int primer) and primer 2 (ol492) to confirm integration of the GFP targeting construct. The primer details can be found in Table S2**.**

P. berhei phenotype analyses

Blood containing approximately 50,000 parasites of the GFP-tagged lines was injected intraperitoneally (i.p.) into mice to initiate infections. Parasitaemia was monitored by microscopy on Giemsa stained thin smears. Four to five days post infection blood was collected with 10-15% parasitaemia and allowed to further develop in schizont and ookinete culture medium as described previously (PMID: 29138437). The schizonts/merozoites and ookinetes were examined after 24 h of

culture with a Zeiss AxioImager M2 microscope (Carl Zeiss, Inc) fitted with an AxioCam ICc1 digital camera. To examine the location of these proteins in sporozoites, 30–50 *Anopheles stephensi* SD 500 mosquitoes were allowed to feed for 20 min on anaesthetized, infected mice who's asexual parasitaemia had reached 15% and were carrying comparable numbers of gametocytes as determined on Giemsa stained blood films. To assess mid-gut infection, approximately 15 guts were dissected from mosquitoes on day 14 post feeding and oocysts were observed on an AxioCam ICc1 digital camera fitted to a Zeiss AxioImager M2 microscope using a 63x oil immersion objective. On day 21 post-feeding, another 20 mosquitoes were dissected, and their guts and salivary glands crushed separately in a loosely fitting homogenizer to release sporozoites, which were then used for imaging.

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