Review

Evolution, Composition, Assembly and Function of the Conoid in Apicomplexa

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The phylum Apicomplexa has been defined by the presence of the apical complex, a structure composed of secretory organelles and specific cytoskeletal elements. A conspicuous feature of the apical complex in many apicomplexans is the conoid, a hollow tapered barrel structure composed of tubulin fibers. In *Toxoplasma gondii*, the apical complex is a central site of convergence for calcium-related and lipid-mediated signaling pathways that coordinate conoid protrusion, microneme secretion and actin polymerization, to initiate gliding motility. Through cutting-edge technologies, great progress has recently been made in discovering the structural subcomponents and proteins implicated in the biogenesis and stability of the apical complex and, in turn, these discoveries shed new light on the function and evolution of this definitive structure.

Highlights

Recent methods, such as proximity labeling, localization of organelle proteins by isotope tagging, and ultra-structure expansion microscopy, have greatly advanced the proteomic characterization of the different apical complex subcompartments.

The subpellicular microtubules (SPMTs) are decorated by unique microtubule associated proteins and emerge from the apical polar ring (APR) by an unknown mechanism. The conoid is composed of open tubulin fibers that are bent by the recently characterized DCX protein.

Recently characterized proteins, such as AC9, AC10, and ERK7, are essential for the stability of the APR, the conoid, and the SPMTs.

In addition to its structural complexity, the apical complex acts as a signaling hub by being the point of convergence for many regulatory pathways.

Broad evidence suggests that the conoid is derived from the flagellar root apparatus.

The Apical Complex Unifies the Phylum Apicomplexa

The Infrakingdom Alveolata is a major eukaryotic supergroup that comprises several groups of protists that share genetic and ultrastructural features. Among them, the phylum Apicomplexa groups thousands of diverse obligate intracellular, non-photosynthetic parasites able to infect a wide range of vertebrate and invertebrate hosts. They include pathogens of important medical and veterinary relevance although only a few of them have been intensively studied. The apical complex, which is composed of unique morphological structures at the apical pole of the parasites, has served as the basis for the classification of phylum members.

The apical complex can be divided into three structurally distinct groups of components that will be described in this review (Figure 1A). First, the apical cap comprises the most apical portion of the inner membrane complex (IMC) that extends along the length of the parasite. The IMC consists of membrane cisternae supported by a membrane skeleton of the proteinaceous alveolin network and the subpellicular microtubules (SPMTs). Collectively, the IMC confers structure to the parasite, and the apical portion contributes directly to apical complex function. In *T. gondii,* the apical cap consists of a single conical IMC cisterna and a series of enigmatic proteinaceous ‘annuli’ structures.

Second, the conoid is formed by a cone of spiraling tubulin-rich fibers with an open apical aperture that is often associated with preconoidal rings (PCRs) that, here, we collectively call the ‘conoid complex’. The conoid is located within the apical polar ring (APR) that serves as a unique apical microtubule-organizing center (MTOC) from which the SMPTs radiate [1]. The conoid is best described from the coccidian subgroup of Apicomplexa (*Toxoplasma*, *Besnoitia*, *Neospora*, *Eimeria*, and *Sarcocystis*); however, it is seen throughout the Apicomplexa, including in basal lineages, *Cryptosopridium* spp., and gregarines such as *Selenidium*. Haemosporidia (including *Plasmodium, Babesia* and *Theileria*), by contrast, have been generally thought to have lost the conoid although they retained an APR and an electron-dense ring structure at the apical tip. Third, the secretory organelles termed micronemes and rhoptries that discharge their contents in a regulated

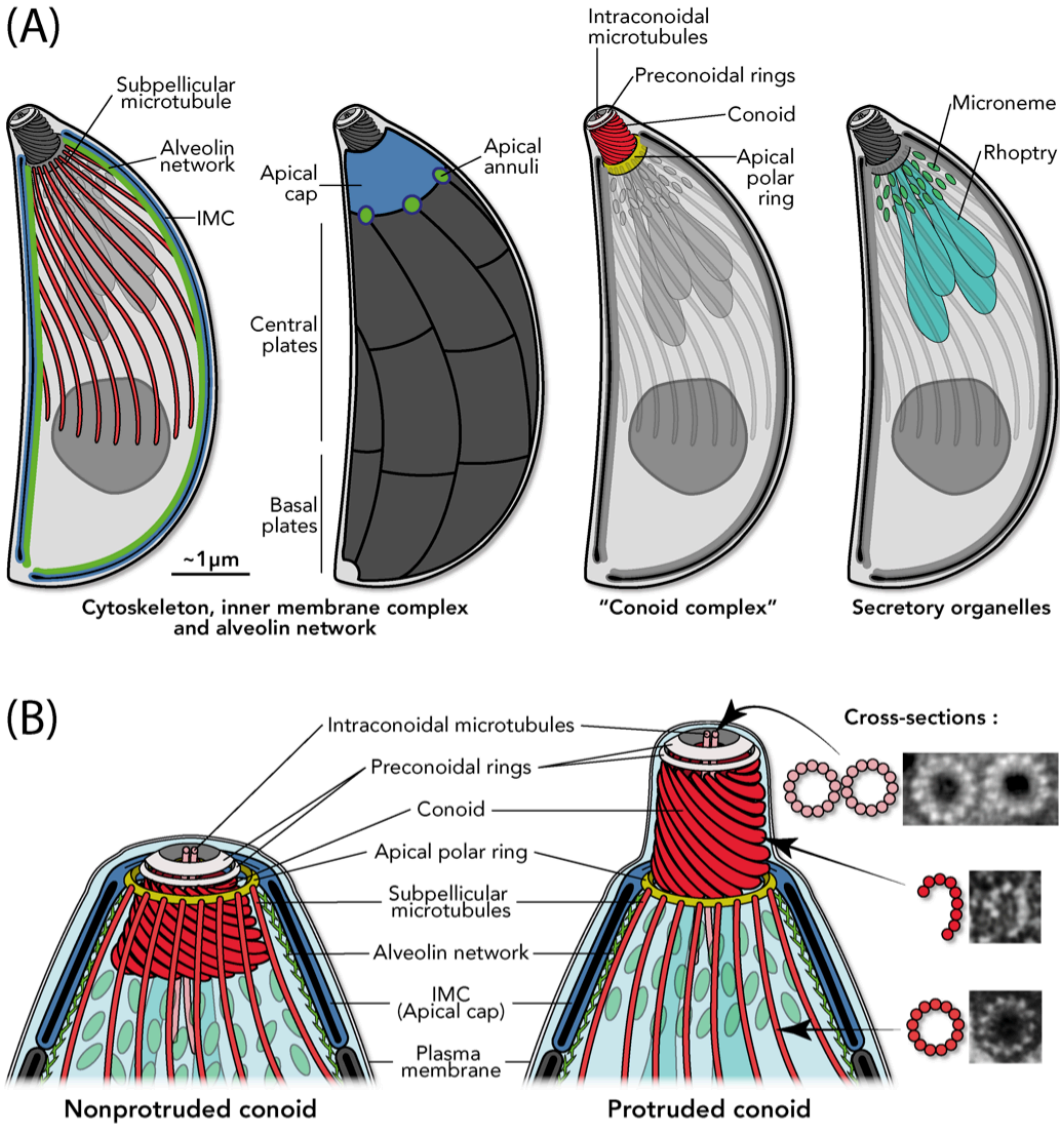

Figure 1. The Apical Complex of *Toxoplasma gondii*. **(A)** Structural and functional components of the apical complex: the cytoskeleton made of microtubules, alveolin network, and inner membrane complex (IMC), the 'conoid complex', and the secretory organelles. The different plates of the IMC are displayed on the second scheme, where the apical cap is at the top and the apical annuli are found at the level of the apical cap suture. **(B)** The apical complex is composed of three main tubulin-based structures: two intraconoidal microtubules, the conoid fibers of the conoid, and the subpellicular microtubules. The two intraconoidal microtubules and subpellicular microtubules are made of 13 protofilaments, as found in other organisms, while the tubulin fibers of the conoid are made of only nine protofilaments in a distinctive 'comma' cross sectional shape. Electron microscopy pictures are published in [27].

manner at the apical tip of the parasites and play critical roles in parasite dissemination [2].

The conoid has remained a mysterious dynamic organelle and a focus of interrogations regarding its origin, composition, and function. In recent years, comparative genomics, improved proteomics technologies, and reverse genetic strategies have contributed to address these questions. This review merges knowledge deduced from pioneering ultrastructural work decades ago with recent advances on the role, biogenesis, stability, and evolutionary history of the conoid at the center of the apical complex, primarily in *T. gondii*.

Keeping the Parasite in Shape: The IMC, Alveolin Network, and Cytoskeleton

The IMC and Alveolin Network

Phyla of the Alveolata, including Apicomplexa's major relatives, dinoflagellates and ciliates, are unified morphologically by the presence of flattened single-membrane alveolar sacs (alveoli) arranged beneath the plasma membrane. In association with proteinaceous networks, these give structure and support to the cells of all of these lineages. This system is called the inner membrane complex (IMC) in apicomplexan parasites, the amphiesma in dinoflagellates, and the epiplasm in ciliates [3,4].

In *T. gondii* the IMC is composed of three sets of alveolar plates sutured together along their margins (Figure 1A). At the apical pole, a single conical IMC plate forms the apical cap. The apical cap is also delimited at its base by five to seven apical 'annuli' (also referred to as 'peripheral annuli') embedded in the IMC suture [5]. Below it, a series of quadrilateral plates (called central plates) are found in the central subcompartment, and a third set (called basal plates) is found in the basal subcompartment. The functional relevance of these partitions is not understood.

The subcompartments are distinguished by the presence of specific proteins called ISPs (IMC subcompartment proteins), ISP1 being restricted to the apical cap at the level of the **pellicle** (see Glossary) [6]. Further proteomic understanding of the organization of the IMC comes from proximity labeling experiments identifying two novel sets of proteins called the IMC suture components (ISCs), localizing at the sutures between the plates, and the apical cap proteins(ACs) found exclusively at the apical cap [7,8] (Table 1). In addition to the ten ACs described so far, two more proteins were found at the apical cap, namely IMC11 and PhIL1 [9,10]. To date, five apical annuli proteins (AAPs) and one apical annuli methyltransferase (AAMT) have been localized to the annuli, adopting concentric circular structures [5] (AAP1, previously described as peripheral annuli protein 1, PAP1 [11,12]). AAP4 (described as PAP2 [12]) is the most conserved AAP among the coccidians, yet its contribution to parasite fitness *in vitro* appears to be modest and hence the role of the apical annuli remains elusive. Interestingly, most ACs and AAPs are apparently restricted to the coccidian subgroup of Apicomplexa, except for AC6 and AAP1.

The cytoplasmic side of the IMC is associated with the alveolin network, a rigid meshwork composed of a family of proteins called alveolins [13]. The proteins composing the alveolin network are also named IMC proteins and are implicated in parasite development [9,14]. Alveolins are defined by a common repetitive element ('alveolin repeat': EKIVEVP).Furthermore, both apicomplexan and ciliate alveolin networks have been shown to be composed of additional proteins with low complexity repeats enriched in K, E, Q, L, I, and V residues, collectively referred to as charged repeat motif proteins (CRMPs), or epiplastins in ciliates [15,16]. While the contribution of these alveolins and CRMPs has not been biochemically defined, it is hypothesized that they form the filamentous structure of the alveolin network; indeed, overexpression of some of these proteins results in filamentous structures [17].

The Subpellicular Microtubule-based Cytoskeleton

The distinctive crescent-like shape of *T. gondii* tachyzoites is conferred by an elaborate basket of 22 SPMTs that emerge and radiate from the unique MTOC called the apical polar ring (APR) (Figure 1A) [1,18]. The number of SPMTs varies with species and with the stage of the parasite's life cycle. In *Plasmodium berghei* for example, the sporozoite stage has 16 SPMTs [19]; however, interestingly, a recent study showed that the level of α-tubulin expression directly determines the number and

Glossary

Apico-basal flux: F-actin flux powered by myosin motors thought to be responsible for the motility of *T. gondii*. The flux starts at the apical pole of the parasite where actin is nucleated by Formin1. Then the F-actin is translocated toward the basal pole in between the IMC and the plasma membrane via the concerted action of MyoH at the conoid and MyoA along the length of the parasite.

Endodyogeny: a type of asexual reproduction in which two daughter cells are formed inside the mother cell and then exit from it, resulting in the loss of the mother cell.

Glideosome: the name of the molecular complex powering gliding motility; it is essential for movement and invasion. By itself, actin in *T. gondii* is involved in dynamic events such as motility, invasion, and organelle inheritance during division [2,107,108] but it does not have a structural role as it has in other types of eukaryotic cell.

Gliding motility: a type of motility that is independent of propulsion or traction appendages such as flagella and pili. In apicomplexans, a backward translocation of surface adhesins is used to propel the cell forward.

Moving junction: a complex of microneme and rhoptry proteins that keeps the parasite and host cell plasma membrane in tight apposition during invasion. During translocation of the moving junction toward the basal pole, the parasite is propelled inside the host cell, forming the parasitophorous vacuole.

Myzocytosis: a feeding strategy, found throughout the Myzozoa, in which the apical complex is used to 'suck' the cytoplasm of the prey directly into a food vacuole. It is also called 'cellular vampirism'.

Parasitophorous vacuole: a compartment derived from the host cell plasma membrane in which the parasite replicates following invasion.

Pellicle: peripherial cell structures consisting of the subpellicular microtubular and alveolin networks, IMC, and plasma membrane, that give the cell its structure and surface function.

Tachyzoite: the motile and rapidly replicating asexual stage of *T. gondii*. The tachyzoites are able to invade virtually all cell types. It is the most common stage studied in laboratory around the world.

length of SMPTs and, in turn, the shape of the parasite [20]. The SPMTs are composed of canonical microtubules (Figure 1B) and their stable length and left-handed twist are hypothesized to be dependent on their coating by specific microtubule-associated proteins (MAPs). Indeed, the SPMTs are heavily decorated with MAPs and display unusual properties compared to mammalian microtubules, such as an increased stability during cold treatment and in the presence of detergents, both of which normally result in depolymerization of microtubules [18].

Proteomic studies based on mass spectrometry analysis of the *T. gondii* cytoskeleton identified a number of detergent-insoluble proteins that belong to the SPMT network [21]. SPM1 and SPM2 were subsequently characterized as MAPs. In particular, SPM1 contributes to the unusual stability and resistance to detergent extraction of the SPMTs [22]. Additionally, the thioredoxin-like proteins 1 and 2 (TrxL1 and TrxL2) were shown to decorate the SPMTs and a TrxL1 pull-down assay was used to identify four other SPMT-associated proteins called TLAP1–4 (thioredoxin-like-associated proteins 1–4) [23,24]. TLAP3 selectively coats the SPMTs in the apical cap region and the intraconoidal microtubules of both mother and daughter cells. TLAP2 is present all along the SPMTs (except for a region close to the APR), while TLAP4 also associates with the mitotic spindle microtubules. Double and triple knockouts did not cause defects in SPMT biogenesis or arrangement but resulted in increased susceptibility to cold treatment [24]. Of relevance, the SPMTs are polyglutamylated toward the apical pole [25]. This reversible post-translational modification, preferentially targeting α-tubulin, changes the electric charges on the carboxy-terminal tail which is believed to regulate electrostatic microtubule–MAP interactions [26].

Conoid Complex: The Conoid and Associated Structures

The Conoid Is a Cone of Tubulin Fibers

As with SPMTs, the conoid is composed of tubulin fibers harboring unique features. Canonical microtubules are made of 13 protofilaments of α-and β-tubulin dimers arranged in a hollow tube [27]. By contrast, the conoid of T. gondii is composed of 10–14 tightly curved and tilted tubulin fibers of nine protofilaments that form a comma-shaped strip in cross-section, rather than a hollow tube (Figure 1B). It is hypothesized that this unusual conformation is required to achieve the tight curve that these fibers assume, but it is currently unknown if this conformation occurs in conoids of other apicomplexans. The tubulin element of the conoid is made of the same tubulin isoforms as the canonical microtubules (α-and β-tubulin), so it is likely that tubulin-binding proteins specific to these fibers confer this unusual conformation. One such protein, DCX, also referred to as apicortin/doublecortin [28], contains two tubulin-binding domains, P25-α and DCX, and was shown to localize exclusively at the conoid, contributing to its stability and consequently to parasite fitness [29]. Strikingly, DCX can generate and stabilize curved microtubules in a heterologous system (i.e., *Xenopus laevis* S3 cells) and even 'open' microtubules reminiscent of the comma shape of the conoid fibers [30]. Incidentally, the tubulin that composes the conoid is not polyglutamylated [25].

The Preconoidal Rings

In *T. gondii*, the conoid is topped by two rings, the PCRs. The role of these two PCRs is still unknown and their close proximity to the conoid and APR complicates their study by standard microscopy techniques (Box 1). Only four proteins at or proximal to the PCRs have been described so far. The dynein light chain 8a (DLC8a) [21] is found in part at the PCRs and was shown to be important for microneme secretion and rhoptry positioning [12]. A structural analysis of DLC8a highlights a β-strand that is essential for the homodimerization and localization of the protein [31]. Centrin 2 (CEN2) is a multifunctional protein that localizes to the apical annuli, the centrosomes, and the basal pole of the parasite, in addition to the PCRs. CEN2 is reported to participate in microneme secretion and invasion, although dissecting which population of CEN2 performs these functions is challenging given their multiple locations [12,21,32]. The two other proteins, SAS6L and the SSNA1/DIP13 homolog, have been reported near or at the PCRs, and these are discussed in the next sections of this review. Additionally, the spatial proteomic method referred to as localization of organelle proteins by isotope tagging[i] (LOPIT) (Box 2) has recently identified further proteins located as either rings or puncta above the conoid (Table 1); however, these proteins await further functional characterization [33].

The Apical Polar Ring

The conoid sits within the APR and, in fact, protrudes through it during egress and invasion events (Figure 1B). The APR is hypothesized to serve as an MTOC for the generation of the SPMTs. It is closely associated with the apical end of the ‘apical cap’ of the IMC, although this is typically slightly more anterior than the APR, and this makes it difficult to confidently assign proteins to either of these locations [34] and this is even more evident in hematozoans [35].Nevertheless, proteins in close proximity to this site of microtubule origin are typically ascribed APR proteins. The first such protein identified in *T. gondii* was RNG1, a small protein appearing at this site late during daughter cell formation [36]. RNG2, an unrelated large protein, was subsequently found near the APR by C-terminal reporter protein tagging [15]. Curiously, when RNG2 was also tagged at its N terminus, it was found that the location of this part of the protein was at the base of the conoid [37]. This tethered position of RNG2, connecting the conoid base to the APR, is evident when the conoid is extruded and the orientation of RNG2 terminal markers flips as the conoid passes through the APR. In contrast to RNG1, RNG2 appears very early during the biogenesis of daughter cells, associated with the premitotic duplicated centrosomes. It was subsequently implicated in the cGMP/PKG-based regulation of microneme secretion

Table 1. Apical Complex Proteins with Known Subcompartment Localization

Protein Name	ToxoDB[II] TgME49 ID	Localization		Phenotype		Conservation				Refs
						Myzozoa				
		LOPIT[I] Assignment	Experimental Evidence	Phenotype Score	Experimental Evidence	Pf.	Cp.	Cv.	Pm.	
Centrin2	250340	Apical 2	PCR + AA	-4.41	Essential (iKD)	●	●	●	●	[12, 21, 32]
DLC8a	223000	Inconclusive	PCR + APR + Apical Cap	-5.38	Essential (iKD)	●	●	●	●	[12, 21, 31]
SAS6L	301420	Apical 1	PCR + Conoid	-1.62	Small Defect (KO)	●	●	●	●	[102]
SSNA1/DIP13	295450	Inconclusive	PCR + Conoid	0.67	No Defect (KO)	●	●	●	●	[103]
-	219070	n/a	Dot above conoid	-2.2	n/a	●	●	○	○	[39]
-	274160	Outlier	Dot above conoid	-2.8	n/a	●	●	○	○	[39]
-	253600	Apical 1	Ring above conoid	-2.4	n/a	●	●	○	○	[33]
-	306350	Apical 1	Ring above conoid	-0.84	n/a	●	●	○	○	[33, 39]
-	208340	Apical 1	Ring above conoid	-0.81	n/a	●	●	○	○	[33, 39]
DCX	256030	Apical 1	Conoid	-5.03	Small Defect (KO)	●	●	●	○	[29, 30]
MyoH	243250	Apical 2	Conoid	-3.94	Essential (iKD)	○	●	●	○	[55]
CAM1	246930	Apical 2	Conoid	1.09	No Defect (KO)	○	○	○	○	[21, 56]
CAM2	262010	Apical 2	Conoid	-0.81	No Defect (KO)	○	○	○	○	[21, 56]
CAM3	226040	Apical 2	Conoid	-3.25	Essential (iKD)	○	○	○	○	[56]
-	222350	Apical 1	Conoid	-1.31	No Defect (KO)	●	●	○	○	[33, 38]
-	274120	n/a	Conoid	0.64	No Defect (KO)	●	●	●	○	[38]
-	291880	Apical 1	Conoid	1.77	n/a	●	○	○	○	[33, 39]
CPH1	266630	Apical 1	Base of conoid	-4.16	Essential (iKD)	●	●	●	○	[33, 38]
-	246720	Apical 2	Base of conoid	0.24	No Defect (KO)	●	●	●	○	[33, 38]
-	258090	Apical 1	Base of conoid	-1.34	No Defect (KO)	○	○	●	○	[33, 38]
ICMAP1	239300	Apical 2	ICMT	-0.74	n/a	○	○	○	○	[42]
RNG1	243545	n/a	APR	n/a	No Defect (iKD)	○	○	○	○	[25, 36]
RNG2	244470	Apical 1	APR	-4.21	Strong Defect (iKD)	●	●	●	○	[25, 37]
KinesinA	267370	Apical 1	APR	-2.7	Small Defect (KO)	○	○	○	○	[40]
APR1	315510	Apical 1	APR	-0.05	Small Defect (KO)	○	○	○	○	[40]
-	320030	Apical 1	APR	-0.19	No Defect (KO)	●	○	○	○	[33, 38, 39]
KinesinB	273560	Inconclusive	SPMT	-0.94	n/a	●	○	○	○	[40]
SPM1	263520	Tubulin Cytoskel.	SPMT	1.21	Defect (KO)	●	●	●	○	[22]
SPM2	286590	Tubulin Cytoskel.	SPMT	1.34	No Defect (KO)	●	○	○	○	[22]
TrxL1	232410	Tubulin Cytoskel.	SPMT + ICMT	0.99	No Defect (KO)	●	○	●	○	[23]
TrxL2	225790	Tubulin Cytoskel.	SPMT	1.98	n/a	○	○	○	○	[23]
TLAP1	286600	Tubulin Cytoskel.	SPMT	0.43	n/a	●	○	○	○	[23]
TLAP2	232130	Tubulin Cytoskel.	SPMT	-0.82	No Defect (KO)	●	○	●	○	[23, 24]
TLAP4	201760	Apical 1	SPMT	0.54	n/a	○	○	○	○	[23, 24]
-	248740	Inconclusive	SPMT	-4.02	n/a	○	○	○	○	[8]
ISP1	260820	Apical 1	Apical Cap	0.36	No Defect (KO)	●	●	●	○	[6]
IMC11	239770	Apical 1	Apical Cap	1.53	n/a	○	○	○	○	[9]
PhIL1	258410	IMC	Apical Cap	1.74	n/a	●	○	○	○	[9, 10]
AC1	311480	IMC	Apical Cap	0.15	n/a	○	○	○	○	[7]
AC2	250820	Apical 1	Apical Cap	0.5	n/a	○	○	○	○	[7]
AC3	308860	Apical 1	Apical Cap	-0.37	n/a	○	○	○	○	[7]
AC4	214880	Apical 1	Apical Cap	0.01	n/a	○	○	○	○	[7]
AC5 / TLAP3	235380	Apical 1	Apical Cap (SPMT+ICMT)	1.44	n/a	○	○	○	○	[7, 23, 24]
AC6	251850	Cytosol	Apical Cap	0.05	n/a	●	○	●	○	[7]
AC7	225690	Apical 1	Apical Cap	-0.02	n/a	○	○	○	○	[7]
AC8	229640	Apical 1	Apical Cap	-0.1	n/a	○	○	○	○	[8]
AC9	246950	Apical 1	Apical Cap	-3.88	Essential (iKD)	○	○	○	○	[8, 25]
AC10	292950	Apical 1	Apical Cap	-2.58	Essential (iKD)	○	○	○	○	[25]
-	293190	Apical 1	Apical Cap	1.18	n/a	○	○	○	○	[33, 39]
-	241880	Inconclusive	Apical Cap	0.52	n/a	○	○	○	○	[39]
-	230490	Inconclusive	Apical Cap	-5.81	n/a	●	●	●	●	[39]
AAP1 - PAP1	242790	Apical 2	Apical Annuli	n/a	No Defect (KO)	○	○	○	○	[5, 11, 12]
AAP2	295850	Apical 2	Apical Annuli	-1.58	No Defect (iKD)	●	○	○	○	[5, 33]
AAP3	313480	Apical 2	Apical Annuli	-1.68	n/a	○	○	○	○	[5]
AAP4 - PAP2	230340	Apical 1	Apical Annuli	-1.14	Defect (KO)	●	○	○	○	[12]
AAP5	319900	Apical 1	Apical Annuli	-1.47	n/a	○	○	○	○	[5]

[a]Abbreviations: AA, apical annuli; APR, apical polar ring; Cp, *Cryptosporidium parvum*; Cv, *Chromera velia*; ICMTs, intraconoidal microtubules; iKD, inducible knockdown; KO, knockout; n/a, not available; PCR, preconoidal rings; Pf, *Plasmodium falciparum*; Pm, *Perkinsus marinus*; SPMTs, subpellicular microtubules.

[b]Conservation: ortholog presence (black circle) or absence (open circle) in selected myzozoan taxa is shown. Presence of orthologs was assessed by reverse BLAST analysis where a taxon's best matching protein as putative ortholog then retrieved the Toxoplasma query sequence as its best BLAST match.

[c]Phenotype scores are published in [110]. LOPIT data were extracted from [33].

during invasion and egress [37]. RNG1 and RNG2 are apparently absent outside of the coccidian subgroup, although within the Sarcocystidae RNG2 is fast evolving so detection of distant orthologs might be challenging. TGME49_320030, which was first localized at the tip of the parasite [33,38] and then confirmed to be localized at the APR [39], is also conserved in *Plasmodium* spp. Finally, two other proteins, APR1 and KinesinA were localized at the APR and contribute to its stability [40].

The Intraconoidal Microtubules

The conoid complex of *T. gondii* contains a pair of intraconoidal microtubules. The intraconoidal microtubules traverse the PCRs and the conoid and are lined with an enigmatic row of evenly spaced small vesicles [41]. One or more rhoptry necks are also often closely associated with the intraconoidal microtubules which might assist with the discharge of this organelle [34]. ICMAP1 (intraconoidal microtubule-associated protein 1) is restricted to the coccidian subgroup and decorates preferentially the intraconoidal microtubules [42]. When overexpressed, ICMAP1 binds to regular microtubules, suggesting that its selectivity to the intraconoidal microtubules is conferred by association with other partners. The SPMT-binding proteins TrxL1 and TLAP3 are also visualized at the intraconoidal microtubules while being absent from the conoid [23,24].

Specialized Secretory Organelles Discharge Their Contents Apically in Response to Local Signaling Events

The Apical Secretory Organelles Are Essential for Invasion and Egress

The invading apicomplexan zoites, such as the *T. gondii* **tachyzoite**, are highly polarized cells harboring a dedicated arsenal of secretory organelles at the apical pole that participate in multiple steps of the parasite's lytic cycle (Figure 1A). The micronemes are abundant rod-like organelles that secrete adhesins required for **gliding motility** and invasion, as well as perforins and proteases necessary for parasite egress from the infected host cells [43]. The rhoptries are arranged in a bunch of approximately ten club-shaped organelles, delineated by a neck and a bulb region. The discharge of their membranous and proteinaceous contents (RONs from the neck and ROPs from the bulb) occurs exclusively upon contact with host cells. These organelles critically contribute to invasion via the formation of the **moving junction** [44] and the **parasitophorous vacuole** membrane [45]. Moreover, the rhoptries deliver effector proteins into the host cells to subvert cellular functions and, notably, to neutralize the cell-autonomous defense mechanisms [46].

In *T. gondii* the discharge of the rhoptries and micronemes presumably happens at the very tip of the parasite after channeling through the conoid to reach the parasite's plasma membrane (PPM). In support of this view, electron microscopy images show the presence of micronemes [43] and rhoptries [41,47] inside the conoid. Alternatively, micronemes have been postulated to exocytose between the base of the conoid (once protruded) and the APR [41]. The lack of certainty between these options reflects the difficulty of capturing such dynamic events.

Similarly, there is a considerable gap of knowledge regarding the machineries implicated in microneme and rhoptry fusion to the PPM. A conserved pleckstrin-homology (PH) domain-containing protein (APH), acylated

Box 1. Toward the Localization of Apical Complex Proteins at a High Level of Resolution

Resolved localization of proteins to the subcompartments of the apical complex, and notably the conoid complex, has historically been achieved only by immunoelectron microscopy. While proteins of the subpellicular microtubules are easy to localize (thanks to the large sizes and characteristic shapes of the microtubules), localizing a protein at the APR, conoid, preconoidal rings, or intraconoidal microtubules can be much more difficult. For example, the conoid-associated protein 1 (CAP1) was claimed to be present at the conoid but unfortunately no colocalization with a known marker of the conoid was performed. This protein was identified via a genetic screen selecting proteins important for parasite resistance to reactive nitrogen intermediates of macrophages [109]. Another protein belonging to the so-called Charged Repetitive Motifs Protein (CRMP) family was localized in mature tachyzoites as a small apical dot and discovered by comparison with a pellicular protein of the ciliate *Tetrahymena* [15]. Finally, other proteins were localized at the apical complex but not studied in detail – for example, ICAP4, ICAP16 (Indispensable Conserved Apicomplexan Protein) [110], and other unnamed proteins [33,38].

How can proteins be more precisely assigned to the different subcompartments of the apical complex? A few methods can be used to achieve this goal, and some are not particularly costly in time or reagents. For example, in extracellular parasites, substructures of the conoid can be distinguished by using the phenomenon of induced conoid extrusion with ethanol or phosphodiesterase inhibitors [55]. Under these conditions, proteins localized to the apical polar ring appear immobile. By contrast, proteins localized to the conoid, preconoidal rings, and intraconoidal microtubules appear extruded from the parasite's body [21,25]. Also, colocalization with a known marker of each subcompartment can be performed in order to determine the relative location of the protein of interest. Finally, if an antibody against the protein of interest is available, it is possible to use the high-resolution technique of immunoelectron microscopy to localize a protein in a specific subcompartment of the apical complex. Emerging technologies such as ultrastructure expansion microscopy (U-ExM) might also be used to distinguish two tightly apposed subcompartments by physically expanding the sample size without unduly affecting the ultrastructure [25].

Box 2. Localization of Organelle Proteins by Isotope Tagging (LOPIT)

LOPIT is a method for determining the subcellular locations of a cell's proteome en masse[i] [111]. It utilizes mechanical disruption of cells followed by fractionation of subcellular compartments, structures, and complexes by differential and equilibrium density-gradient centrifugation. Protein abundance distribution profiles across these fractions are accurately quantified by mass spectrometry for all proteins simultaneously, and cofractionation of proteins across the gradients is then analyzed by machine-learning methods to map several thousand proteins to different subcellular niches on a cell-wide scale. The basis for these assignments is that colocated proteins will share the same abundance distribution profile, and the output of this method is the steady-state location of up to thousands of proteins and protein complexes throughout the cell.

at the surface of the micronemes and binding to phosphatidic acid, and a C2 domain containing protein (DOC2.1), are both known to critically participate in microneme exocytosis [48–50]. At the surface of the rhoptries, another acetylated protein called ARO (armadillo repeat only protein) ensures the positioning of these organelles to the apical pole of the parasite and their organization as a bundle [51–53]. In addition, a protein of the ferlin calcium sensor family (FER2) was shown to be critical for rhoptry secretion [54]. Recently, a lipid-binding rhoptry protein called RASP2 was described as the most direct mediator of rhoptry discharge in *Toxoplasma* and *Plasmodium* [47]. Nevertheless, this limited knowledge of the molecular machinery associated with parasite exocytosis reflects our lack of understanding of these key events.

The Conoid Hosts Actomyosin Machinery That Initiates Gliding Motility

Most apicomplexans rely on the **glideosome**, a conserved actomyosin-based machine, to power gliding motility for invasion, egress, and dissemination [2]. In *T. gondii*, gliding involves the concerted action of at least two myosin motors and the apical exocytosis of transmembrane adhesins that are translocated, along with the F-actin, toward the posterior pole of the parasites. MyoA is distributed at the periphery of the parasites, whereas MyoH is firmly anchored to the conoid; both are required for motility, invasion, and egress [55]. MyoH interacts with three MLCs (myosin light-chain proteins) at the conoid, namely MLC3/5/7, and possibly with three calmodulin-like proteins, namely CaM1/2/3, that implicate Ca^{2+} in activating the motor [55,56]. Interestingly, while MyoE and MyoL are also found at the conoid, no specific role has been assigned to these motors to date [57]. Also, despite the absence of a conspicuous conoid, *Plasmodium falciparum* also has a myosin (MyoB) that is concentrated to the apical tip of the parasite [58].

Parasite motility and host cell invasion cannot be achieved without the instrumental contribution of the glideosome-associated connector (GAC) [59]. The GAC is predicted to bridge the microneme transmembrane adhesins to the actomyosin system. GAC localizes throughout the cell, including the conoid; interestingly, the recruitment of the GAC at the conoid is dependent on the activity of the apical complex lysine methyltransferase (AKMT) [60]. The apical localization of AKMT is itself dependent on its SET [Su(var)3–9, Enhancer-of-zeste and trithorax] and C-terminal domains [61]. AKMT not only plays a key role in GAC recruitment at the conoid but also regulates actin polymerization and/or its posterior translocation [62]. Indeed, gliding motility relies on an **apico-basal flux** of F-actin, and the formin FRM1 localized at the apical tip is the only nucleator of actin required for motility [62]. FRM1, GAC, and AKMT are all essential for parasite survival and are conserved across the entire phylum, indicating the fundamental importance of these conoid-associated activities in apicomplexan motility (Table 2).

The Conoid Is a Signaling Hub That Controls Gliding Motility, Invasion, and Egress

The conoid is a dynamic organelle that responds to changes in intracellular Ca^{2+} levels by enigmatically protruding through the APR [63,64] (Figure 1B). The cytosolic rise in intracellular Ca^{2+} mobilized from internal stores is sufficient to induce conoid protrusion [65] and to concomitantly trigger microneme secretion and activation of the actomyosin system to generate motion [66]. Electron microscopy images suggest that conoid protrusion and microneme secretion are intimately linked [64]. Currently, only a small-molecule screen identified Conoidin A, a compound that uncouples the two events by inhibiting conoid protrusion without affecting microneme secretion [67]. Several calcium-responsive proteins have been implicated in these processes, such as the calcium-dependent protein kinase 1 (CDPK1), a key mediator of microneme secretion [68], which is also required for conoid protrusion and generation of the apico-basal flux of F-actin [62].The substrates of CDPK1 associated with these events remain to be identified. In addition to a phosphorylation cascade, Ca^{2+} is also able to directly modulate protein function via calcium-binding domains present on proteins. Among them, the calmodulin-like proteins (CAMs) are localized at the conoid and have been shown to be essential for parasite motility [21,56] and DOC2.1-harboring C2 domains that is instrumental for microneme secretion [50].

Besides calcium signaling, the apical complex is the site of extensive lysine methylation on nonhistone proteins, as shown by immunofluorescence with antimethylated lysine antibodies [62]. AKMT is a main contributor of methylation at the apical complex, although no substrate has been formally identified. The recently characterized signaling platform composed of the guanylate cyclase complex (CDC50/GC/UGO), which integrates intrinsic and extrinsic signals to trigger egress, is localized at the PPM but restricted to the apical cap region [69,70]. Similarly, in *Plasmodium*, GCβ/CDC50A is redistributed at the apical

Table 2. Apical Complex Proteins with Unknown Subcompartment Localization

Protein Name	ToxoDB[II] TgME49 ID	Localization		Phenotype		Conservation				Ref
						Myzozoa				
		LOPIT[I] Assignment	Experimental Evidence	Phenotype Score	Experimental Evidence	Pf.	Cp.	Cv.	Pm.	
AAMT	310070	Inconclusive	Apical	-1.22	n/a	●	●	●	●	[5]
CIP1	234250	Inconclusive	Apical	-2.02	No Defect (KO)	○	○	○	○	[38]
CIP2	257300	Apical 1	Apical	-2.49	No Defect (KO)	○	○	○	○	[38]
CIP3	225020	Apical 1	Apical	-2.78	Defect (KO)	●	●	●	○	[38]
MLC3	250840	n/a	Apical	-1.91	n/a	○	○	○	○	[55]
MLC5	311260	Apical 2	Apical	-0.33	No Defect (KO)	○	○	○	○	[55]
MLC7	315780	Apical 2	Apical	-0.12	No Defect (KO)	●	●	○	○	[55]
MyoE	239560	n/a	Apical	0.11	No Defect (KO)	○	○	○	○	[57]
MyoL	291020	n/a	Apical	-1.83	No Defect (KO)	●	●	○	○	[57]
GAC	312630	Cytosol	Apical + Cytosol	-3.53	Essential (iKD)	●	●	○	○	[59]
FRM1	206430	n/a	Apical	-2.8	Essential (iKD)	●	●	●	○	[62]
AKMT	216080	Inconclusive	Apical + Cytosol	-4.3	Strong Defect (KO)	●	●	●	●	[60]
CRMP	252880	Apical 1	Apical	-2.35	No Defect (iKD)	○	○	○	○	[15]
CAP1	210810	Inconclusive	Apical	-0.73	n/a	○	○	●	○	[110]
ICAP4	209890	Inconclusive	Apical + Cytosol	-4.84	Strong Defect (sgRNA)	●	●	●	●	[111]
ICAP16	202120	Inconclusive	Apical	-2.1	Strong Defect (sgRNA)	●	●	○	○	[111]
-	278780	Apical 1	Apical	-2.77	No Defect (iKD)	○	○	○	○	[33]
-	313780	n/a	Apical	0.71	No Defect (KO)	○	○	○	○	[38]
-	255895	Apical 1	Apical	0.23	n/a	○	○	○	○	[38]
-	254870	Inconclusive	Apical	0.64	No Defect (KO)	○	○	○	○	[38]
-	226990	Apical 1	Apical	1.41	No Defect (KO)	○	○	○	○	[38]
-	234270	Apical 2	Apical	-0.44	No Defect (KO)	○	○	○	○	[38]
-	295420	Apical 2	Apical	-1.57	n/a	○	○	○	○	[33, 38]
-	227000	Apical 1	Apical	-3.17	n/a	●	○	○	○	[33, 38]
-	297180	Apical 1	Apical	-1.52	No Defect (iKD)	○	○	○	○	[33]
-	284620	n/a	Apical	-1.02	n/a	○	○	○	○	[39]

[a]Abbreviations: Cp, *Cryptosporidium parvum*; Cv, *Chromera velia*; iKD, inducible knockdown; KO, knockout; n/a, not available; Pf, *Plasmodium falciparum*; Pm, *Perkinsus marinus*; sgRNA, single guide RNA.
[b]Conservation: ortholog presence (black circle) or absence (open circle) in selected myzozoan taxa is shown. Presence of orthologs was assessed by reverse BLAST analysis where a taxon's best matching protein as putative ortholog then retrieved the *Toxoplasma* query sequence as its best BLAST match.
[c]Phenotype scores are published in [110]. LOPIT data were extracted from [33].

pole of the ookinete by interacting with the IMC protein ISP3. This retains the complex in an ideal spatial position to control microneme exocytosis and activation of the actomyosin system [71].

Biogenesis, Protein Composition, and Stability of the Conoid

Cell division in *T. gondii* tachyzoites entails an unusual process, called **endodyogeny**, in which two daughter cells are formed in the cytoplasm of the mother cell. The development of the daughter apical complex and IMC are the first conspicuous signs of this process, and ultimately all essential organelles segregate into these nascent structures and the mother cell plasma membrane eventually delineates these new cells. Thus, biogenesis of the conoid and associated structures occupies a critical point in new cell formation.

The earliest events of daughter cell formation are the duplication of the centrosome, that will subsequently form the mitotic spindle, and the recruitment of IMC protein such as FBXO1 to the centrosomes, RNG2 to the conoid complex, and AC9, AC10, and IMC15 to the alveolin network [9,25,37,72]. In addition, MAPK-L1 and ZFP2 were shown to regulate the daughter cell assembly via the regulation of centrosome duplication and its coordination with mitosis [11,73]. Further IMC proteins are recruited to these developing daughter buds and, as they elongate, they separate from the centrosome, although they remain tethered to it by a striated fiber joined to the conoid complex (see below). Although the IMC is formed mainly *de novo* in the daughter cells, some of the IMC proteins from the mother cell are recycled as these structures are disassembled relatively late in this process [74]. A series of glideosome-associated protein (GAP) and GAP with multiple-membrane spans (GAPM) are anchored in the IMC and participate in its biogenesis and stability [75]. GAPM1 was also shown to be essential for the stability of the SPMTs, indicating a physical connection between the two structures [76].

Recent progress in proteomics and proximity labeling, using the BioID strategy [77], identified numerous further components of the alveolin network. These efforts have established the existence of a hierarchy in the assembly of the components of the apical cap and advances in understanding how its elements contribute to its

maintenance and stability [78]. While AC9 and AC10 are detectable very early during daughter cell formation, other ACs appear later during daughter cell development [8,25,40]. AC9 and AC10 are the only two members of the AC family reported to be fitness-conferring when individually conditionally destabilized, resulting in severe impairment in motility, invasion and egress and loss of the conoid and APR in mature parasites [25,79]. Deoxycholate extraction and ultrastructure expansion microscopy (U-ExM) experiments provided further evidence that, when SPMTs are disorganized, the APR is lost in mature parasites of AC9 and AC10 mutants. The kinase ERK7 was recently reported to use AC9 as a scaffold and pseudosubstrate. A direct link between APR and integrity of the conoid was previously reported with the double deletion of the two APR proteins, KinesinA and APR1, that led to a destabilization of the APR, detachment of the conoid, and dispersion of the SPMTs upon deoxycholate extraction [40]. Of relevance also, the ankyrin repeat-containing protein CPH1 (conoid protein hub 1), localized at the base of the conoid, has been shown to contribute to conoid maintenance in extracellular parasites as, in CPH1-depleted parasites, the conoid was shortened and partially collapsed [38].

Remarkably, depletion of ERK7 led to a defect in conoid assembly without impacting on the APR or on microneme secretion [79,80]. This is the first functional evidence that the presence of the conoid is not a prerequisite for microneme exocytosis. The absence of the conoid implies that the machinery for actin polymerization is missing and explains the severe defect in motility and invasion.

The Evolutionary History and Diversity of the Apical Complex

The namesake of Apicomplexa, and the basis of their success as intracellular parasites, is the presence of the apical complex. All of this begs the question: what is the origin of this cell feature? The solution to this puzzle is not immediately accessible in apicomplexans because the parasite cell forms that bear the apical complex lack one or more flagella. Flagella, and their associated flagellar root apparatus of microtubules and fibers, form the central organizing centers for the cytoskeleton of most eukaryotes [81]. Their absence in the apicomplexans, with the only exception being male gametes that lack the apical complex, deprive us of a cellular context to interpret the apical complex structures and features. Many relatives of Apicomplexa, however, possess strikingly similar structures to the apical complex that, importantly, occur in the presence of flagella and their elaborate root apparatus.

Myzozoa encompasses apicomplexans and their nearest relatives: chrompodellids, perkinsids, and dinoflagellates (Figure 2). This group is named after the mode of feeding, **myzocytosis** ('cell sucking' or 'cellular vampirism'), that is present in most myzozoan lineages and is thought to represent an ancestral behavior of the group [82]. The apparatus of myzocytosis bears strong ultrastructural similarity to the apicomplexan's apical complex. Within the nearest relatives of apicomplexans, the chrompodellids, *Colpodella* spp. are free-living micropredators that attach to their prey cells and feed through an apical structure supported by an open-sided tube of microtubules referred to as a 'pseudoconoid' [83,84]. Focused into the lumen of this pseudoconoid is a population of densely staining elongated vesicles described as micronemes and rhoptries owing to their similar appearance to those of apicomplexans. Very similar structures are seen in the related *Chromera velia* in which, moreover, a central pair of intraconoidal microtubules runs down the middle of the pseudoconoid, as is seen in *Toxoplasma* [85,86]. This apical complex is in close proximity to the flagella, and some pseudoconoid microtubules are continuous with, and seemingly part of, the flagellar rootlet fibers [87]. Unlike *Colpodella*, *Chromera* is photosynthetic, but while it is associated with coral communities [88] any direct interactions with animals or other organisms are poorly understood. Therefore, the function of the apical complex in *Chromera* is unknown.

Perkinsids, the sisters to dinoflagellates, are exclusively parasitic and they bear the most striking ultrastructural similarities to apicomplexan apical complexes (Figure 2). *Perkinsus* spp. are mollusk parasites and possess a conical pseudoconoid and an array of rhoptries and micronemes, some of which are tethered directly to the pseudoconoid [89,90]. As for apicomplexans, the tip of the pseudoconoid is centered within an apical polar ring that serves as an MTOC for a corset of microtubules below the IMC. The role of the apical complex in *Perkinsus* spp. is again unclear as parasite uptake by their hosts is thought to be mediated via phagocytosis by host hemocytes [91]. However, the related *Parvilucifera* spp. are intracellular parasites of dinoflagellates and, while their pseudoconoid is relatively reduced compared to that of *Perkinsus*, they apparently do use this apparatus to invade their hosts [92–94]. In both groups a constituent sheet of microtubules runs from the pseudoconoid to close proximity with the flagellar basal bodies and root apparatus [84].

Dinoflagellates are seemingly the most unlike apicomplexans in cell organization and lifestyle (with the exceptions of some basal members [95]). Despite half of its members being photosynthetic, micropredatory heterotrophy is widespread in this clade and many feed by myzocytosis via a specialized tubular structure called a peduncle [96]. The peduncle is located next to the basal bodies that typically occupy a lateral position on the cell. The peduncle is a membrane-bound appendage that is structurally supported by either a single curved sheet of microtubules or, in larger examples, by multiple articulated curved sheets [97]. Associated with these microtubular sheets are a variety elongate and darkly staining membrane-bound vesicles [96,98–100]. Further, the peduncle microtubules are connected to the flagellar basal bodies by a striated fiber band [98] (S.C.F.C.M. Calado, PhD thesis, Universidade de Aveiro, 2010). This is strikingly similar to the *Toxoplasma* band of striated fiber assemblin (SFA) that, during new cell formation, temporarily anchors the conoid to its centrioles, the basal

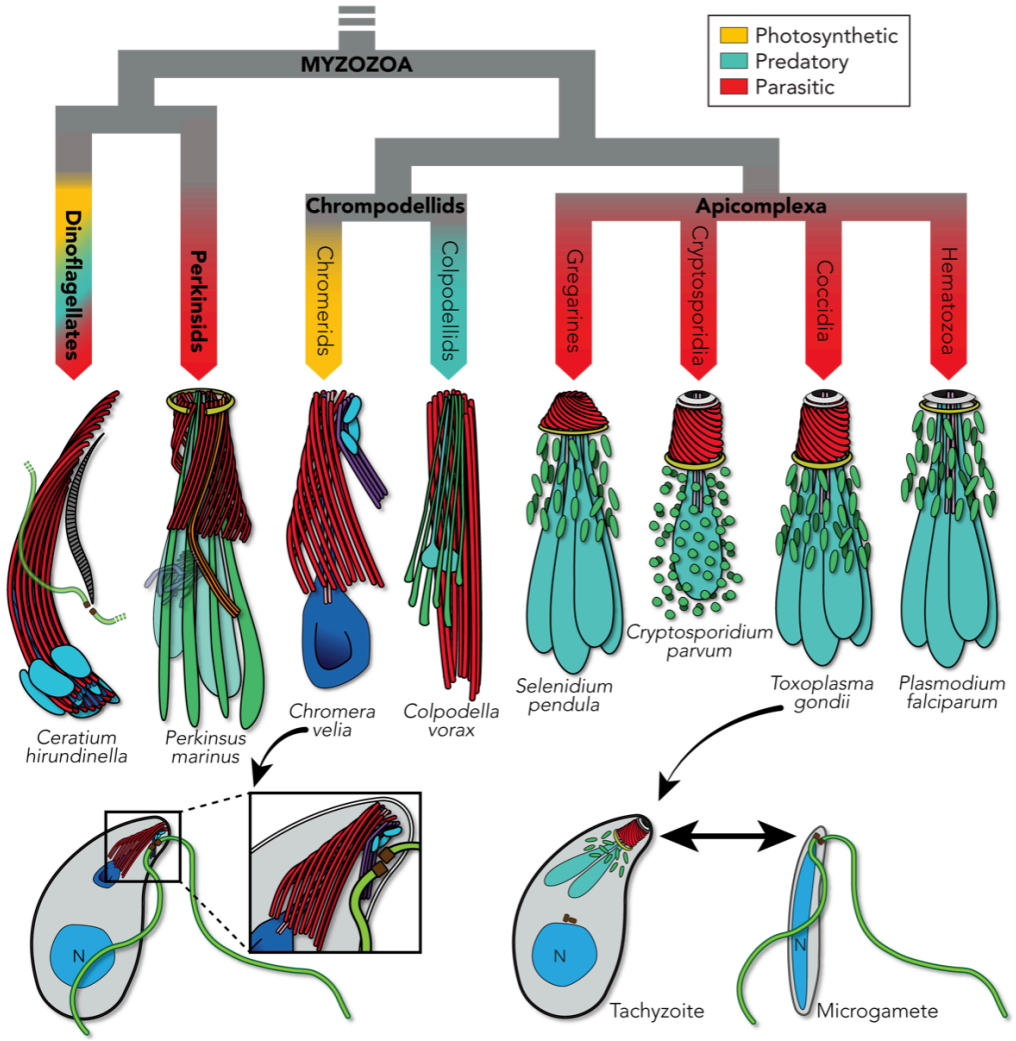

Figure 2. Evolution of the Apical Complex in the Myzozoa Superphylum. Myzozoans Are Part of the Alveolata Infrakingdom. The color of the phylogenetic tree branches represents the trophic strategy(ies) of the lineages: yellow, photosynthetic; cyan, predatory; red, parasitic. For each branch, the apical complex of a representative species is shown. In red are the tubulin-based elements of either a closed conoid or an open conoid-like structure (called a peduncle in dinoflagellates). Rhoptries are represented in blue-green and micronemes in green. The apical polar ring is represented in yellow, the intraconoidal microtubules in light pink, and the preconoidal rings in light gray. For *Ceratium*, the striped gray band connecting the peduncle to the flagellar bases represents the striated fiber, and in dark blue and cyan are the membrane-bound organs reminiscent of rhoptries and micronemes. For *Perkinsus*, some extended microtubules of the conoid are represented in orange, and associated with them are additional membrane-bound vesicles. For *Chromera*, an intralumenal endomembrane system is represented in dark blue, the extralumenal endomembrane system is represented in cyan, and the anterior flagellar rootlet microtubules in violet. All cartoons are based on electronmicrographs. At the bottom, the relative position of the apical complex and flagellar apparatus is shown for selected organisms. The basal bodies of flagella or centrioles are represented in brown and the flagella in green. Adapted from [102].

body equivalents in these cells [101]. Furthermore, similar to the *T. gondii* conoid, the peduncle is a retractable structure able to extend up to 100μm in the largest examples as the cell actively seeks and penetrates its prey [96]. Despite a reorientation of the flagellar apparatus and peduncle within the dinoflagellate cell, and the greater diversity of microtubular peduncle structures that scale with prey size, all other features of this feeding apparatus are consistent with those of the apical complex in related myzozoan linages.

Collectively, the consistent picture that emerges from myzozoan diversity of the evolution of the apical complex is of an elaboration of the flagellar root apparatus (rather than the flagellum itself, as others have suggested [87]) that has specialized in directed uptake from, delivery to, and mechanical interaction with, other organisms. With the reduction of the flagellum to centrosomal centrioles in most apicomplexan cell stages, the apical complex is the most conspicuous retained element of the associated flagellar root structures. It is common in other protists for adaptations of the flagellar root fibers and bands to be implicated in feeding [81], and even in mammalian cells centrioles are often associated with site-directed exocytosis. While it is currently not known what the protein contents of the non-apicomplexan 'microneme' and

'rhoptry' structures are, nor the secretory behaviors of these compartments, their consistent presence associated with conoid-like structures suggests similar exocytic functions in modulating host/prey cell interaction. The association of the 'pseudoconoids' with the IMC, including an APR seen in *Perkinsus*, is likely also integral to the apical complexes in these cells but currently we know nothing of the proteins that contribute to these features. By contrast, there are proteins known from the apicomplexans that reinforce a structural link with the flagellar apparatus. In addition to the SFA band, a truncated paralog of the formative basal body protein SAS6, called SAS6-like, is associated with the *T. gondii* conoid [102]. A *T. gondii* homolog of the SSNA1/DIP13 coiled-coil protein family that localize with microtubule structures, including centrosomes and flagellar structures, is also present in Apicomplexa [103]. DIP13 associates with the tip and base of the conoid, as well as the posterior elongating edge of the developing cell IMC during daughter formation. While these examples indicate some repurposing of flagellar proteins, they are consistent with ongoing evolution of such a key component of all eukaryotes. The diversity of apical complex structures in the broader Myzozoa is also consistent with that seen within Apicomplexa. An apicomplexan synapomorphy is the emergence of a closed conoid that is seen from gregarines onward. However, there is variation in number, size, presentation, and molecular contents of exocytic organelles, micronemes, and rhoptries (e.g., [104]). There is also diversity with respect to the presence or absence of conoid-association of rings (e.g., [105]), although it is prudent to be cautious about inferences drawn solely from microscopically observed ultrastructure. The long-standing interpretation of hematozoans and piroplasms lacking a conoid is based on images of select taxa such as *Plasmodium* spp., and notably often only on pathogenic life stages (conoids are suggested in lesser studied examples, e.g., *Leukocytozoon* [35]). But recent data suggest that proteins specific to the conoid in *T. gondii* are present in taxa such as *Plasmodium* (Table 1) [106]. Thus, we may be on the threshold of a significant new interpretation of the roles and relevance of apical complex structures even within the better studied groups.

Concluding Remarks

The progress in proteomics and super-resolution imaging technologies have considerably expanded our knowledge of the composition of the apical complex but a lot of questions remain to be addressed (see Outstanding Questions). A much more comprehensive inventory of the protein composition of the apical complex has recently been achieved by the global LOPIT method. This approach allows a unique opportunity to assign hypothetical proteins to precise subcellular compartments en masse. Powerfully complementary are the advanced cryogenic electron microscopy and U-ExM technologies that can resolve protein locations to higher, and even molecular-level, resolution (Box 1). These tools can also be applied to the less-studied organisms, allowing questions of conservation, diversity, and evolution of apical complex construction and function to be asked across the many taxa that possess this feature.

Outstanding Questions

What is the biological relevance and role of conoid protrusion?

What are the mechanisms and proteins implicated in conoid protrusion through the APR?

Is conoid protrusion necessary for microneme exocytosis?

Can the signaling of microneme exocytosis and conoid protrusion be uncoupled?

Is DCX the only protein conferring the specific shape to the conoid fibers?

What controls the tight bend of the conoid fibers?

How do the SPMTs get their particular twist, strict length (only two-thirds of the parasite's length), and consistent number/symmetry?

How does the APR act as an MTOC?

What is the role of the intraconoidal microtubules?

What is the function of the preconoidal rings?

What governs the number of apical annuli?

What is the function of the apical annuli?

What features of the T. gondii apical complex are specific to this taxon, and which features are widely shared by other apicomplexans and even myzozoans?

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Resources

[i] https://proteome.shinyapps.io/toxolopittzex
[ii] http://ToxoDB.org

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