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Running title: Evolution of cell polarity

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Abstract

A signature feature of the animal kingdom is the presence of epithelia: sheets of polarized cells that both insulate the organism from its environment and mediate interactions with it. Epithelial cells display a marked apico-basal polarity, which is highly conserved across the animal kingdom, both in terms of morphology and of molecular regulators. How did this architecture first evolve? Although the last eukaryotic common ancestor almost certainly possessed a simple form of apico-basal polarity (marked by the presence of one or several flagella at a single cellular pole), comparative genomics and evolutionary cell biology reveal that the polarity regulators of animal epithelial cells have a surprisingly complex and stepwise evolutionary history. Here, we retrace their evolutionary assembly. We suggest that the "polarity network" that polarized animal epithelial cells evolved by integration of initially independent cellular modules that evolved at distinct steps of our evolutionary ancestry. The first module dates back to the last common ancestor of animals and amoebozoans and involved Par1, extracellular matrix proteins, and the integrin-mediated adhesion complex. Other regulators, such as Cdc42, Dlg, Par6 and cadherins evolved in ancient unicellular opisthokonts, and might have first been involved in F-actin remodeling and filopodial dynamics. Finally, the bulk of "polarity proteins" as well as specialized adhesion complexes evolved in the metazoan stem-line, in concert with the newly evolved intercellular junctional belts. Thus, the polarized architecture of epithelia can be understood as a palimpsest of components of distinct histories and ancestral functions, which have become tightly integrated in animal tissues.

Introduction

Every cell lives in an asymmetric world. This applies just as well to unicellular microbes as to the cells that compose multicellular organisms. Unsurprisingly, the asymmetries imposed by environment or lifestyle are often reflected in the ultrastructure of cells (Bornens, 2018). Mobile microbes that explore their environment by swimming, gliding or crawling are often polarized in their preferred direction of motion: think of the front-to-back polarity of a sperm cell, or of the leading-to-trailing edge axis of an amoeba (Bray, 2001). Even immobile cells are not free of asymmetry: sessile cells are often polarized in accordance with unilateral attachment to the substrate, and floating cells (such as planktonic diatoms) experience gradients in abiotic parameters such as gravity, light or chemicals that are reflected in the inner ultrastructure (Arrieta et al., 2020; A. G. Larson et al., 2022). Perfectly isotropic spherical cells are just as imaginary as the "spherical cow" sometimes postulated by physicists.

The best-characterized example of cell polarity is probably the one of animal epithelial cells. Since epithelia outline the boundaries of organs or organisms, the two sides of the cells that compose them face radically different environments: inside versus outside. Accordingly, epithelial cells have a markedly polarized ultrastructure, with the apical side (facing the outside world or the lumen of internal organs) comprising several characteristic organelles (cilia, microvilli), while the basal side (facing the primary body cavity) faces a self-secreted sheet of extracellular matrix, the basal lamina, to which epithelial cells attach via specialized integrin-based adhesion complexes (Figure 1A). Between those two poles, intercellular adhesion complexes – often also regionalized along the apico-basal axis – ensure the cohesion of cells and the sealing of the epithelium, and several intracellular organelles (Golgi apparatus, MTOC, nucleus) also have a polarized distribution along the apico-basal axis (Figure 1A). The molecular basis of apico-basal polarity in animal epithelia has been dissected in deep detail thanks to remarkable studies in fruit flies, nematode worms, and mammalian cells. A self-organized interplay of protein complexes demarcates the apical, junctional and basal domains, ensuring both the correct positioning of individual structures and the large-scale coherence and maintenance of polarity (Buckley & St Johnston, 2022; Campanale et al., 2017; Gibson & Perrimon, 2003; Lang & Munro, 2017; Munro, 2006; Rodriguez-Boulan & Macara, 2014). These complexes appear conserved between mammals, flies and nematodes, supporting an ancient evolutionary origin of polarized epithelia at least within bilaterian animals.

How did apico-basal polarity evolve? At first sight, the answer might seem straightfoward: the apical flagella and microvilli of epithelial cells have clear parallels in the closest unicellular relatives of animals, the choanoflagellates, which have a conspicuous morphological apico-basal axis (Brunet and King, 2017). Flagella are similarly present and restricted to one pole of the cell in many other single-celled eukaryotes (Cavalier-Smith, 1978; Mitchell, 2007). Because flagella and MTOC are ancient eukaryotic features (Azimzadeh, 2014; Nabais et al., 2020), some sort of apico-basal polarity axis must be as ancient as eukaryotes, and one could have expected the polarity complex of animal epithelial cells to be of ancient eukaryotic ancestry.

However, comparative genomics and evolutionary cell biology reveal an entirely different picture. None of the metazoan "polarity proteins" is of ancient eukaryotic ancestry: all of them therefore evolved much later than the generic apico-basal polarity of the flagellum and the MTOC. Moreover, many proteins that we are used to considering as part of the "same complexes" in animals evolved at markedly different times, their successive origins spanning hundreds of millions of years. Overall, the pictures that emerges is one of stepwise assembly of polarity complexes, correlated with successive evolutionary innovations in the mechanisms of cell-substrate and cell-cell adhesions, which were only combined and integrated into a single network in early animal ancestors as they evolved sealed epithelial sheets. Below, we retrace the molecular steps of the origin of animal epithelial polarity proteins and offer hypotheses on the cellular phenotypic innovations that might have accompanied them.

I. The apico-basal polarity of animal epithelial cells and its molecular control

The molecular network that organizes the apico-basal polarity of metazoan epithelial cells was largely discovered thanks to mutant studies in two invertebrate model organisms: the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. A screen for mutants affecting the segregation of cell fate determinants during the asymmetric first division of the *C. elegans* embryo led to the discovery of six genes with "polarity-defective" phenotypes numbered from *par-1* to *par-6* (Hung and Kemphues, 1999; Kemphues et al., 1988; Watts et al., 1996). PAR genes are thus united by a common class of mutant phenotypes, but they are not a gene family as defined by common sequence and domain composition. The same line of research in *C. elegans* also led to the discovery of the apical determinant atypical protein kinase C (aPKC) (Tabuse et al., 1998). Other conserved polarity proteins were discovered thanks to *Drosophila* mutants: Scribble (Scrib) (Bilder and Perrimon, 2000), Lethal giant larvae (Lgl) (Klämbt and Schmidt, 1986), Discs large (Dlg) (Woods and Bryant, 1991), Crumbs (Crb) (Tepass et al., 1990; Tepass and Knust, 1993) and Stardust/MPP5/PALS1 (Bachmann et al., 2001; Hong et al., 2001). It was later found that these proteins have three important additional properties: (1) they are broadly conserved in bilaterian animals with a crucial role in regulating the apico-basal polarity of epithelia (although mutant phenotypes in mammals were often more subtle than in flies and nematodes); (2) they themselves have a polarized distribution; and (3) they all interact together, by assembling into a handful of mutually antagonistic complexes limiting each other's influence (reviewed in (Bonello & Peifer, 2019; Buckley & St Johnston, 2022; Gibson & Perrimon, 2003; Henrique & Schweisguth, 2003; Lang & Munro, 2017; Munro, 2006)). The initial symmetry breaking event that defines apico-basal polarity generally either comes from early developmental cues (maternal egg geometry, sperm entry point) or from the extracellular matrix.

The polarized architecture of epithelial cells is broadly conserved across animal lineages and likely dates back to the last common ancestor of animals (Leys and Riesgo, 2012). Broadly conserved polarized elements include an apical flagellum/cilium (scaffolded by microtubules and supported by a basal body doubling as the MTOC) and apical microvilli (supported by F-actin); an apical Golgi apparatus; lateral intercellular junctions; and a basal layer of extracellular matrix called a "basal lamina" (Fig. 1A) (Brunet and King, 2017; Leys et al., 2009; Leys and Hill, 2012; Leys and Riesgo, 2012). Mirroring this ultrastructural conservation, "polarity proteins" are broadly conserved in animals: the nine "polarity proteins" shown in Fig. 1A are all found in bilaterians, cnidarians, placozoans and sponges, and only two (Crb and Scrib) are absent in ctenophores (Belahbib et al., 2018; Fahey and Degnan, 2010; Riesgo et al., 2014). (The topology of the animal tree of life is currently uncertain, and it is unknown whether sponges or ctenophores diverged first from all other animals (King and Rokas, 2017). Depending on the answer, the absence of these genes in ctenophores might reflect loss or ancestral absence.)

However, outside of animals, this conservation generally breaks down. Indeed, five of nine polarity proteins are unique to metazoans, only two (Cdc42 and Par1) are conserved in yeast, and none of them can be traced back to the last eukaryotic common ancestor. This pattern contrasts with the ancient eukaryotic ancestry of the flagellate cell architecture and of the apico-basal polarity of the centrosome and the microtubule cytoskeleton. As we will discuss below, successive ancestors of animals appear to have taken advantage of ancient cell polarity mechanisms and to have progressively added elements that dynamically coordinate microtubule, actin microfilaments, and intracellular trafficking to ensure adhesion first to the surrounding extracellular matrix and eventually to other cells.

II. The first polarized elements from the LECA: flagella, microtubules, basal bodies

The most ancient element of the apico-basal polarity of animal epithelial cells are the apical cilium/flagellum and its underlying centriole/basal body doubling as an MTOC, which are ancestral eukaryotic features. Their homology across eukaryotes is supported by a shared ultrastructure observable by electron microscopy (nine doublets of microtubules surrounding a central pair for the flagellum ("9+2" structure), and nine triplets of microtubules for the centriole/basal body (Margulis, 1981)). Moreover, extensive molecular similarities in centriolar and flagellar composition among eukaryotes have been uncovered by comparative genomics (see (Azimzadeh, 2021; Nabais et al., 2020) for reviews) and proteomics (Pazour et al., 2005; Sigg et al., 2017).

Although the eukaryotic tree of life remains incompletely resolved (Burki et al., 2020; Keeling and Burki, 2019), a polarized flagellate phenotype (most likely with two apical flagella anchored by basal bodies) is widespread among extant lineages and was likely present in the last eukaryotic common ancestor (LECA). Interestingly, flagellates belonging to diverse eukaryotic groups also show a polarized array of cortical microtubules forming a cell-wide "umbrella" radiating from the MTOC (Fig. 2B-G), suggesting a similar architecture might have been present in the LECA. Finally, a complex array of microtubular roots likely supported the basal body of the LECA (Yubuki and Leander, 2013). Thus, some aspects of apico-basal polarity are likely of ancient eukaryotic ancestry. On the other hand, some aspects of cell polarity that may seem ancient may not be. Although the Golgi apparatus likely dates back to the LECA (Barlow et al., 2018) and can display a polarized localization upon its association with the centrosome or

MTOC in animal cells (Azimzadeh, 2014), the position of the Golgi varies widely in unicellular flagellates (Harris, 2009), casting doubt that a polarized Golgi apparatus is ancient.

The flagellate phenotype was not necessarily the only phenotype present in the LECA (or in early eukaryotes): crawling motility might be ancient as well (Brunet et al., 2021; Fritz-Laylin, 2020). Crawling cells are also polarized, and the crawling cell types of animals tend to "recycle" polarity proteins in the context of leading-to-trailing edge polarity (Etienne-Manneville, 2008). However, although the centrosome is the most consistently polarized element of flagellated and epithelial cells, it bears no consistent relationship to the direction of crawling in protists or in metazoan cells: both "centrosome-forward" and "centrosome-back" migration have been described in *Dictyostelium* (Daunderer et al., 1999) and in crawling mammalian cells (Kopf and Kiermaier, 2021), depending on contexts. This suggests that the polarity of crawling cells (though potentially also ancient) is best understood as distinct from the polarity of flagellated and epithelial cells, although it can reuse some of its components in a variable way depending on contexts.

III. Elements of the basal lamina and of focal adhesions in the amoebozoan/opisthokont (amorphean) ancestor

The most ancient "polarity protein" is Par1/MARK, a kinase that notably plays a key role in regulating microtubule polarity and dynamics (Drewes et al., 1997; Tassan and le Goff, 2004). In epithelial cells, Par1 promotes basolateral identity by orienting the plus-ends of microtubules toward the basal pole (Doerflinger et al., 2003). In mammalian cells, Par1 also promotes polarized secretion of basal lamina proteins (such as laminin and collagen IV (Daley et al., 2012; Lewandowski and Piwnica-Worms, 2014)) and of ECM receptors such as integrins and dystroglycan (Cohen et al., 2004; Masuda-Hirata et al., 2009; Yamashita et al., 2010). This link between Par1, microtubule orientation, and polarized secretion of cell/matrix adhesion components is not restricted to epithelial cells: Par1 also plays an important role in crawling cells (McDonald, 2014), where it orients microtubules toward the leading edge (Nishimura et al., 2012) and activates FAK at focal adhesions, thus promoting cell/matrix attachment at the lamellipodium (Pasapera et al., 2022). Interestingly, the yeast Par1 homolog kin2 controls polarized secretion during budding (Elbert et al., 2005; Yuan et al., 2016).

Par1 is found as far back as amoebozoans (the group that includes most unicellular amoebae, such as *Dictyostelium*) and thus dates back to the last common ancestor of Amorphea (the clade that includes amoebozoans and opisthokonts; Figure 2A). Interestingly, this phylogenetic distribution mirrors that of the integrin-mediated adhesion complex: integrins themselves are found in some amoebozoans (though not *Dictyostelium*) (Kang et al., 2021) as well as in apusozoans, filastereans and certain choanoflagellates (Richter et al., 2018; Sebé-Pedrós et al., 2010). Most other components of the adhesion complex are also of amorphean ancestry and broadly conserved in amoebozoans (including *Dictyostelium*), including talin, paxillin and VIN (which is homologous, and equally related, to both vinculin and α-catenin in metazoans (Miller et al., 2013)) (Figure 2B). Finally, predicted secreted proteins containing EGF/laminin and fibronectin III have also been detected in the genomes of *Dictyostelium* (Eichinger et al., 2005) and of other unicellular amorpheans (King et al., 2008).

Although functional data in unicellular amorpheans remain spotty, available evidence supports a pre-metazoan role for components of the integrin-mediated adhesion complex in cell/substrate adhesion. In *Dictyostelium*, Paxillin and talin are predicted to form complexes with VIN based on their sequence (Huber and O’Day, 2012; Nagasaki et al., 2008), co-localize at cell/substrate adhesion sites (Bukharova et al., 2005; Patel et al., 2008), and are required for adhesion and motility (Bukharova et al., 2005; Patel et al., 2008). Although *Dictyostelium* lacks integrins, it is worth noting that mammalian cells knocked-out for integrins still use vinculin to ensure coupling of the cytoskeleton to the substrate, presumably via non-specific friction (Reversat et al., 2020). The ECM secreted by *Dictyostelium* facilitates crawling, notably during aggregation (Huber and O’Day, 2017) and enhancement of crawling by secreted EGF/laminin III domain proteins depends on talin and paxillin (Suarez et al., 2011). Extracellular matrix in amoebozoans does not only mediate migration, but can also be shaped into stalks that mediate attachment of spores to substrates – either as unicellular “sporocarps” (which were proposed to have been present in the last common amoebozan ancestor (Kang et al., 2017)) or multicellular fruiting bodies (as in *Dictyostelium* and other slime molds).

In other non-animal amorpheans, molecular data are less extensive but adhesion of cells to surfaces (either environmental substrates or self-secreted ECM) is widespread. In chytrids (early-branching fungi), anchorage is mediated by rhizoids (Laundon et al., 2022; Medina et al., 2020), filamentous protrusions that resemble filopodia and have been proposed to be the evolutionary precursors of multicellular hyphae (Laundon et al., 2020) (Figure 3D,E). In the filasterean *Capsaspora owczarzaki*, integrins localize at filopodia that mediate anchorage onto external substrates and are necessary for attachment based on antibody blockade assays (Parra-Acero et al., 2020) (Figure 3F). Similarly, during choanoflagellate settlement, basal filopodia allow "walking" onto external substrates before being replaced by a structure of extracellular matrix called a "theca", that consists in an anchoring stalk topped by a cup housing the cell (Dayel et al., 2011; Leadbeater, 2015) (Figure 3G). Choanoflagellates inside the theca retain short basal filopodia (Sebé-Pedrós et al., 2013a) .

Importantly, all known choanoflagellate species have the ability to secrete (at least in one phase of their life history) complex extracellular structures that provide anchorage or protection to the cells – which, depending on species, can be a simple stalk, a stalk topped with a cup housing the cell (called a "theca") or an elaborate mineralized basket (called a "lorica") (Leadbeater, 2015). In some choanoflagellates, that basal ECM appears to have been co-opted to ensure cohesion of cells in multicellular colonies, which can be sessile branching colonies or swimming, spherical rosettes of cells anchored onto a shared core of ECM. In the model choanoflagellate *Salpingoeca rosetta* (Booth and King, 2022), central ECM is fundamental to the structural stability and morphology of rosettes, as demonstrated both by functional genetics (Booth and King, 2020; Levin et al., 2014; Wetzel et al., 2018) and biophysical characterization (B. T. Larson et al., 2020). Finally, the basal lamina appears universally present in metazoan epithelial cells (Leys and Riesgo, 2012) (Figure 3H,I).

Overall, adhesion of cells to external substrates was likely already present in the last common amorphean ancestor. It relied both on adhesion complexes containing integrin, talin, paxillin and vinculin, as well as on secretion of an ECM rich in EGF/laminin and fibronectin III domains. Par1 might already have had a function in regulating secretion of cell/ECM adhesion components; however, functional data outside animals and yeast are lacking and will be key to test that hypothesis. Importantly, cell/ECM adhesions were not necessarily yet integrated in the apico-basal polarity of the cell (as defined by the flagellum): in crawling amoebozoans and breviates that retain a flagellum, its position appears lateral compared to the surface of cell/substrate adhesion, rather than diametrically opposed to it (indeed, the flagellum directly adheres to the substrate and mediates gliding in breviates). Thus, Par1-mediated cell/ECM adhesion might be best understood as a module having originated independently of apico-basal polarity, but that became integrated with it when it was restricted to the basal side during the evolution of animal epithelia.

Co-option of cell-matrix adhesion mechanisms in a multicellular context might have occurred more than once, and might have taken place in at least two other amorphean lineages besides animals. In the multicellular form of the social amoea *Dictyostelium discoideum,* elements of the F-actin cytoskeleton and of the integrin-mediated adhesion complex seem involved in structuring the external cell layer of the fruiting body: both the vinculin/α-catenin homolog VIN and F-actin (as well as the β-catenin-like protein Aardvark) are enriched at cell-cell junctions and necessary for tissue sealing (Dickinson et al., 2012, 2011; Miller et al., 2013). Since multicellularity is probably not an ancestral feature of amoebozoans (Kang et al., 2017), this likely reflects convergence between the molecular basis of multicellularity in *Dictyostelium* and animals (Parfrey and Lahr, 2013). In another intriguing parallel, in the ichthysoporean *Sphaerforma arctica* (that develops by cellularization of a large multinucleated cell, followed by cell release), components of both the F-actin cytoskeleton (Arp2/3, formins, septins, myosins, Rho and profilins) and of the integrin-mediated adhesion complex (integrins, Pinch, parvin, paxillin, talin and VIN) are strongly upregulated at late cellularization stages, when intercellular adhesion might be present (Dudin et al., 2019).

IV. Obazoan and filozoan ancestors: origin of Cdc42 and Par6: coordinating polarity and actin polymerization

One of the most important apical determinants of epithelial cells is the small GTPase Cdc42, which has been extensively characterized in yeasts and metazoans. It notably orchestrates actin and microtubule remodeling, intercellular junction assembly, and membrane trafficking (Etienne-Manneville, 2004).

Cdc42 can be traced as far back at least as the last common ancestor of obazoans (opisthokonts, breviates and apusozoans; Fig. 1B (Sebé-Pedrós et al., 2013a)). It is a member of the Rho family of small GTPases, which is of ancient eukaryotic ancestry and is abundantly represented in most eukaryotic genomes (Boureux et al., 2007). Although the exact phylogeny of Rho GTPases remains uncertain (Eliáš and Klimeš, 2012; Filić et al., 2021), it is classically considered that Rac is the most ancient and founding member of the family, and that Cdc42 originated as a paralog of Rac in ancestral obazoans (Boureux et al., 2007). Besides its role in epithelial polarity, Cdc42 co-operates with Rac to regulate many F-actin-supported structures, including filopodia (Krugmann et al., 2001; Nakamura et al., 2000), lamellipodia (Etienne-Manneville et al., 2005; Etienne-Manneville and Hall, 2001; Kurokawa et al., 2004; Nishimura et al., 2005) and phagocytic cups (Cox et al., 1997; Hoppe and Swanson, 2004; Massol et al., 1998). All three of these phenotypes might have predated obazoans (Velle and Fritz-Laylin, 2019), as they are found in *Dictyostelium* (Moores et al., 1996; Wessels et al., 1994) and *Naegleria* (Velle and Fritz-Laylin, 2020). Because of this functional promiscuity, the exact nature of the first division of labor between Rac and Cdc42 (and, thus, the ancestral function of Cdc42) is unclear. The most important difference might be that Cdc42 is frequently involved in polarized vesicle trafficking (Harris and Tepass, 2010), while Rac seems to more exclusively control actin remodeling.

What cellular innovation might have accompanied the evolution of Cdc42? A unique feature of obazoans might be the nature of their attachment to external substrates: besides relying on punctate adhesions and on broad lamellipodia, obazoans often walk or anchor onto external substrates using a basal array of filopodia-like protrusions organized into a “pedestal”, a feature observed in apusozoans (Yabuki et al., 2013), breviates (Walker et al., 2006), chytrid fungi (Laundon et al., 2020; Medina et al., 2020) (Fig. 3D-E), filastereans (Parra-Acero et al., 2018; Sebé-Pedrós et al., 2013b) (Fig. 3F) and choanoflagellates (Dayel et al., 2011) (Fig. 3G). Indeed, one of the many functions of Cdc42 in animal cells is promoting filopodial outgrowth (Krugmann et al., 2001; Nakamura et al., 2000), notably during motility of axonal growth cones which relies on a similar “bouquet” of exploratory filopodia (Brown et al., 2000; Chen et al., 2006; Kim et al., 2002). The control of polarized trafficking by Cdc42 might have facilitated directional growth and/or movement of filopodia in ancestral obazoans. Interestingly, the yeast *S. cerevisiae* has lost Rac but retains Cdc42 as a central regulator of budding – which is thought to have evolved from the hyphal tip growth of multicellular fungal ancestors (Wendland, 2001), itself possibly derived from the extension of filopodia-like rhizoids (Laundon et al., 2020).

Another notable loss is that of Par6 in choanoflagellates. Par6 dates back at least to the filozoan node, as indicated by the presence of a homolog in *Capsaspora* (NCBI identifier: CAOG_08417), but appears lost in all choanoflagellates (Richter et al., 2018; Richter and King, 2013). In animal cells, Par6 directly binds Cdc42 (Buckley and St Johnston, 2022) and often polarizes microtubules, notably during lamellipodia-based migration (Etienne-Manneville, 2013; Etienne-Manneville et al., 2005; Garrard et al., 2003). Choanoflagellates are capable of bleb-based crawling but seem to have lost lamellipodia-based migration (Brunet et al., 2021), which might be tentatively linked to the loss of Par6.

Testing hypotheses on the early function of Cdc42 will require functional experiments in metazoan outgroups such as chytrid fungi, filastereans or choanoflagellates. Importantly, in early obazoans, Cdc42 might not have been preferentially associated with the apical pole of the cell: filopodial pedestals are usually basal (when a flagellum is present) and, in yeast, Par1 and Cdc42 are active at the same pole (the budding site), consistently with apico-basal segregation of these two markers having evolved later in metazoan ancestors.

V. Filozoan ancestor: MAGUK proteins, cadherins, filopodial contacts, and the ancestry of cell-cell adhesions

Filozoa is the clade that comprises filastereans (such as *Capsaspora owczarzaki*), choanoflagellates and metazoans. Regarding polarity regulators, the filozoan node was marked by three important molecular innovations: cadherins (Nichols et al., 2012), spectrin (Linden and King, 2021)[1], the MAGUK proteins (the earliest members of which were Dlg and MPP (de Mendoza et al., 2010)), and Par6 (see above).

Cadherins are fundamental to intercellular adhesion in epithelia, but evolved before animal multicellularity: indeed, many cadherins are found in choanoflagellates, including species that seem strictly unicellular. The function of choanoflagellate cadherins is unknown, but possibilities include inter-microvillar adhesions (Brunet et al., 2019; Dayel and King, 2014), bacterial capture (Abedin and King, 2008), or transient cell-cell adhesions (for example during mating (Levin and King, 2013; Woznica et al., 2017)). A single cadherin has been described in *Capsaspora*, but no cadherins have been found in filozoan outgroups (except in oomycetes, a distant eukaryotic lineage which might have acquired them by horizontal transfer (Lévesque et al., 2010)).

MAGUK proteins are fundamental to the assembly of diverse protein complexes in animals. Their architecture consists of 3 PDZ domain, one SH3 domain, and one GUK (inactive guanylate kinase) domain (de Mendoza et al., 2010). All three domain types function in "protein-protein interactions" – so MAGUK proteins seem to essentially serve as scaffolds to assemble complexes.

In animals, MAGUK proteins (including Dlg, MPP and ZO) are often necessary for the assembly of diverse types of intercellular adhesions, including adherens junctions, tight junctions, and synapses (Firestein and Rongo, 2001; Funke et al., 2005; Hough et al., 1997). In epithelia, Dlg/PSD-95 generally localizes at the level of junctional belts, including adherens junctions in *C. elegans* embryos (Bossinger et al., 2001; Firestein and Rongo, 2001; McMahon et al., 2001), adherens junctions in mammalian cells (Laprise et al., 2004), and both septate (Woods et al., 1996) and adherens junctions (Bonello et al., 2019) in *Drosophila*. It also has a key role in in the post-synapse where it scaffolds the (metazoan-specific) adhesion proteins neurexin and neuroligin (Guan et al., 1996) (Arendt, 2020). The sister-family of Dlg among MAGUK, the MPP proteins, similarly scaffold diverse protein complexes, including the post-synapse or ternary complexes at the erythrocyte membrane (Chytła et al., 2020). One metazoan-specific MPP paralog, MPP5/Stardust/PALS1 (de Mendoza et al., 2010), interacts with Crb and is an important part of the apical polarity complex (see section VII). In vertebrates, ZO proteins (which are metazoan-specific variations on the MAGUK theme; Figure 4A) have recently been suggested to form phase-separated condensates which are developmental precursors to tight junctions (Beutel et al., 2019; Schwayer et al., 2019).

While choanoflagellates and filastereans lack metazoan-like intercellular adhesion belts, they do possess a Dlg ortholog and can display some labile intercellular adhesions. For example, contacts between the filopodia of different cells mediate aggregative multicellularity in *Capsaspora* ((Phillips et al., 2022); and Sebastian Najle and Nuria Ros, personal communication) and similar filopodial contacts have been reported in the center of choanoflagellate rosettes (Naumann and Burkhardt, 2019) (although structural maintenance of choanoflagellate multicellularity seems to rely more on extracellular matrix and on cytoplasmic bridges). Interestingly, during the formation of metazoan epithelia, adherens junctions often develop from filopodial contacts of more loosely packed cells (Martín-Blanco and Knust, 2001). This has been extensively documented in primary epithelial cell cultures (Vasioukhin et al., 2000; Vasioukhin and Fuchs, 2001) but also *in vivo* in several contexts, including ventral zippering in nematode embryos (Raich et al., 1999), mouse embryo compaction (Fierro-

González et al., 2013), vascular endothelium formation (Almagro et al., 2010), *Drosophila* dorsal closure (Jacinto et al., 2002) and *Drosophila* heart tube formation (Zhang et al., 2018). A similar developmental sequence has been observed in synaptogenesis: Dlg/PSD-95 already occurs in the dendritic filopodia that are the earliest developmental precursors of the post-synapse (Fiala et al., 1998; Takahashi et al., 2003) and promotes their maturation into dendritic spines (Lee et al., 2006).

In addition to adhesion, Dlg also organizes the orientation of the microtubule spindle during division in epithelial cells and stem cells, notably by interaction with the spindle-orienting factor Pins (Bergstralh et al., 2017). Both Dlg and Pins are present in the choanoflagellate *S. rosetta* but might be incapable of interacting in that species (Anderson et al., 2016), suggesting that the function of Dlg in spindle orientation might have only evolved in metazoans, possibly in concert with the evolution of adhesion belts (see section VI).

VI. Choanozoan ancestors and the origin of apical microvilli

The closest unicellular relatives of animals are choanoflagellates. A shared feature of choanoflagellates and animals are apical microvilli filled with F-actin (Peña et al., 2016; Sebé-Pedrós et al., 2013a). In choanoflagellates and many animal cell types (such as sponge choanocytes), microvilli are organized as a circle around the flagellum, forming a "collar", which mediates bacterial capture in sponges and choanoflagellates but performs other functions (such as mechanosensation or filtration) in other choanozoans (Brunet and King, 2017). Microvilli have a similar ultrastructure to filopodia: both are long, thin, membrane-boud cellular protrusions supported by bundled F-actin. However, microvilli differ from filopodia in having a stable and consistent length, and being generally linked into tight bundles (Delacour et al., 2016). Both types of protrusions are scaffolded by a common set of ultrastructural proteins (including fascin, villin and ezrin/radixin/moesin), which suggests that microvilli might have evolved from modified filopodia in the choanozoan stem-line. Consistent with that putative "sister-organelles" relationship, microvilli-specific myosins (myosin XV and myosin VII) and the filopodia-specific myosin X appear to be paralogs (Houdusse and Titus, 2021; Sebé-Pedrós et al., 2014).

Like animal epithelial cells (Pelaseyed and Bretscher, 2018), choanoflagellates display a dense and specialized apical cytoskeleton of F-actin – which includes microvilli, an apical ring of F-actin at the base of the collar, and phagocytic cups which mediate capture and consumption of bacterial prey (Dayel and King, 2014; Karpov and Leadbeater, 1998). This suggests that the association of actin regulators such as Cdc42 and Par6 with the apical pole might have evolved in that context in the choanozoan stem-line.

VII. Metazoan ancestors: origin of epithelial architecture and the explosion of polarity proteins

Epithelia are an evolutionary novelty of metazoans (Leys et al., 2009; Leys and Riesgo, 2012). Layers of polarized cells defined by sealing abilities, found even in sponges (Adams et al., 2010). Structurally, epithelia rely on adherens junctions and on occluding junctions (called "tight junctions" in chordates and "septate junctions" in other animals). Both types of junctions are likely ancestral for metazoans, since both have been reported in sponges (Leys and Hill, 2012; Leys and Riesgo, 2012), ctenophores (Hernandez-Nicaise, 1991), cnidarians (Ganot et al., 2015), placozoans (Grell and Ruthmann, 1991) and bilaterians (Abedin and King, 2010). In most cases, occluding junctions are apical to adherens junctions and found in the same cells, although in ctenophores they seem segregated to different cell types (adherens junctions are found between ciliated cells of the comb rows, apical organs and tentacular epidermis; while occluding junctions called "belt junctions" occur in other epithelia (Hernandez-Nicaise, 1991)). Consistently, some key molecular components of both types of junctions are universally conserved in metazoans: claudins for occluding junctions (Ganot et al., 2015), and α- and β-catenin and classical cadherins for adherens junctions (Nichols et al., 2012, 2006). Intriguingly, β-catenin may not associate with adherens junctions in ctenophores (Salinas-Saavedra et al., 2019)) – but does so in sponges (Schippers and Nichols, 2018). (Intriguingly, *Dictyostelium discoideum* seems to have converged on a similar molecular machinery for sealing the exterior cell layer of the fruiting body – see section II above). Other canonical junction components have a more restricted distribution: neuroglian is absent in ctenophores and certain sponges (Ganot et al., 2015), and ZO only evolved after ctenophores and sponges branched off from other animals, by C-terminal addition of a ZU5 domain to the canonical MAGUK architecture (de Mendoza et al., 2010)). Importantly, the stability of junctional belts relies on coupling with more ancient components of the cytoskeleton, such as actin (which is pan-eukaryotic) and myosin II (Sebé-

Pedrós et al., 2014), as well as some of their regulators such as Rho (which is at least of opisthokont ancestry (Boureux et al., 2007; Eliáš and Klimeš, 2012; Filić et al., 2021)). Finally, in line with the evolution of cohesive epithelial sheets, the basal lamina was also a locus of important innovation in stem-metazoans: although several of its components can be traced back to the last amorphean common ancestor, some crucial proteins – such as fibrillar collagen and collagen IV – are metazoan innovations (Fidler et al., 2017; Linden and King, 2021).

Besides intercellular junctions and sealed epithelia, the metazoan stem-line was also marked by the evolution of novel proteins involved in polarity complexes: aPKC, Par3, Lgl, Std/PALS1/MPP5, Crb and Scrib are all specific to metazoans (de Mendoza et al., 2010; Richter et al., 2018) (and appeared early in metazoan evolution: aPKC, Par3 and Lgl are universally conserved in animals (Fahey and Degnan, 2010; Riesgo et al., 2014), and Crb, Std and Scrib are only absent from ctenophores (Belahbib et al., 2018; Salinas-Saavedra and Martindale, 2020)). Interestingly, Lgl and Par3, which evolved at the same time as aPKC, are both aPKC substrates (Buckley and St Johnston, 2022). These proteins, like many other developmental regulators (King et al., 2008), seem to have largely evolved by combination of pre-existing domains. aPKC is part of the larger and older Protein Kinase C superfamily, and Par3, Lgl, Crb, and Scrib are all largely composed of protein-protein interaction domains of ancient eukaryotic (or pre-eukaryotic) ancestry, such as WD40 (Jain and Pandey, 2018), SH3 (Tatárová et al., 2012) and PDZ (Ponting, 1997). Yet, the conserved domain topology in these proteins may hint that their domains are functional integrated – as for MAGUK proteins (Laursen et al., 2020) – rather than just 'beads on a string.'

It has long been noted that one of the most important functional outputs of the "ballet" of polarity complexes is the adequate positioning and assembly of the junctional belts (Gibson and Perrimon, 2003; Tepass et al., 2001). One selective driving force for the rapid evolution of animal-specific polarity regulators might thus have been the emerging necessity to establish and maintain epithelial sealing. Consistently with that hypothesis, the endodermal epithelium of the sea anemone *Nematostella vectensis* (Cnidaria) develops without ever expressing any Par proteins, aPKC, or Lgl, and does form morphologically canonical apical and basal domains (including apical cilia, apical microvilli and a basal lamina) but lacks septate junctions and is permeable to extracellular fluid (Salinas-Saavedra et al., 2018). By contrast, the *Nematostella* ectoderm, which develops at the same time, remains impermeable due to the expression of the canonical complement of Par (Salinas-Saavedra et al., 2018). These results suggest that, at least in *Nematostella*, the main function of Par proteins is the establishment and maintenance of cell-cell adhesions and epithelial sealing.

Polarity proteins that might first have served mostly to “clear” junctional components from the apical and basal domains would then have easily acquired interactions with pre-existing apical (Cdc42, Par6) and basal (Par1) regulators, and eventually ended up intertwined with them in tight mutual feedback loops. Eventually, in many species and cell types, metazoan-specific polarity proteins acquired a central role in the definition of apico-basal polarity as a whole. For example, in MDCK cells, Par3 is required for ciliogenesis (Sfakianos et al. 2007) and aPKC/Par6 for microvillar formation (Zihni et al., 2017) – even though Par3, Par6 and aPKC all evolved much later than the structures they regulate. It is worth noting, however, that in several systems, loss-of-function mutants for polarity proteins are less severe than one could expect: the loss of polarity gene function in mammalian cells often leads to subtle phenotypes, perhaps due to functional redundancy between multiple paralogs (Buckley and St Johnston, 2022)), and Par proteins appear dispensable for the development of polarity and junctions in the *Drosophila* midgut (Chen et al., 2018). Thus, the extent of the “takeover” of apico-basal polarity by complexes first involved in defining the position of the junctional belt (aPKC/Par6/Par3 and Dlg/Scrib/Lgl) might vary between species and organs.

The alignment of the apico-basal axis between cells within epithelial sheets has also provided an opportunity for tissues to harness asymmetries across epithelial layers to generate planar cell polarity. Although a collective phenomenon, the evolution of planar cell polarity (PCP) may have relied on pre-existing polarization of individual cells in a plane orthogonal to the apico-basal axis. Eukaryotic flagella often beat in a plane, and the flagella of choanoflagellates and of choanocytes in sponges even bear a modification, called a vane, that acts like a paddle to generate irregular fluid flows, breaking the radial symmetry around the cell (Nielsen et al., 2017; Pinskey et al., 2022). Indeed, unicellular flagellates often display planar asymmetry of flagellar beating (Wang et al., 2021). Such asymmetric flagellar beating, integrated and aligned over epithelia, is critical for animals – as in ciliomotor cells mediating gliding or swimming in many aquatic invertebrates (Arendt et al., 2015), adult mammalian airways (Ramirez-San Juan et al., 2020), or in the embryonic mammalian node cavity which possesses motile cilia to produce asymmetric morphogen gradients (Babu and Roy, 2013). Intercellular alignment and coordination of planar cell-polarity is mediated by a set of multi-pass transmembrane and cytosolic proteins: Frizzled, Flamingo, Van Gogh, Dishevelled, Prickle, and Inversin/Diego – which also control many other planar polarized aspects of metazoan cell biology (Devenport, 2014; Wallingford, 2012; Wu and Mlodzik, 2009). Most "PCP proteins" are metazoan innovations, and are under the control of the animal-specific Wnt signaling pathway (Richter et al., 2018). Nonetheless, the choanoflagellate *S. rosetta* possesses clear homologs of Prickle and Inversin. Their function in choanoflagellates is difficult to surmise: similar to apico-basal polarity proteins, these proteins are entirely composed of protein-protein interaction domains, and their known metazoan interacting partners are absent from *S. rosetta*. Nonetheless, given the frequent role of planar cell polarity in controlling the orientation of cilia, animals might have built upon pre-existing modules that regulated the beating orientation of flagella in the last choanozoan common ancestor.

Conclusion

Like any complex biological structure, the apico-basal polarity of animal cells is a palimpsest of components that originated in different contexts at different times. The first polarized elements – flagella and their basal body – likely evolved in the context of directed locomotion by flagellar swimming or gliding. Despite being restricted to one pole of the cell, the position of flagella conditioned cell-wide polarity of cortical microtubules, with the basal body doubling as an MTOC. We propose that the second element that was later incorporated in animal epithelial polarity was cell/substrate adhesion involving integrin-mediated adhesion complexes and secretion of a specialized ECM, which might have facilitated lamellipodium-mediated crawling in stem-amorpheans. Importantly, in unicellular eukaryotes, lamellipodia are not necessarily located diametrically opposed to the flagellum (when they coexist), but appear often rather lateral to it. Cell modules that were eventually segregated to "basal" and "apical" poles in animal epithelial cells thus might not have always been located at opposite cellular poles. Even the polarized architecture of budding yeasts or of mesenchymal crawling cells from modern animals cannot be straightforwardly aligned to that of epithelial cells, despite using some of the same molecular components. The cell/ECM adhesion module might have been repositioned opposite to the flagellum before the origin of choanozoans, as ECM mediating anchorage to the substrate (or to other cells) is generally secreted at the basal pole of choanoflagellates, opposite to the flagellum. The intermediate elements – cell-cell adherens and tight junctions – appear to have evolved only in the metazoan stem-lineage, in part via evolution of animal-specific molecules, but also by co-opting pre-existing components such as cadherins and Dlg. In spite of its mosaic origin, once established, the architecture of epithelia has been universally conserved across animals – with basal lamina ensuring common anchorage of the cells, junctional belts providing cohesion and sealing, and apical microvilli, flagella, and actin meshwork allowing interactions with the environment.

The reconstruction of these evolutionary events, however, still carries much uncertainty, with an obvious constraint being the relative paucity of functional data of "polarity proteins" in many single-celled relatives of animals. Most of the functional insights we relied on indeed come either from the amoebozoan *Dictyostelium*, budding yeast, or bilaterian animals – with many unexplored branches in between. Thanks to fast ongoing progress in functional genetics in a broad range of protists (Booth et al., 2018; Booth and King, 2020; Faktorová et al., 2020; Parra-Acero et al., 2018; Phillips et al., 2022), we do not expect this gap to persist for long. We thus look forward to our reconstitution of the evolutionary roots of cell polarity being soon refined and replaced by a better-informed, higher-resolution version.

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Figure legends

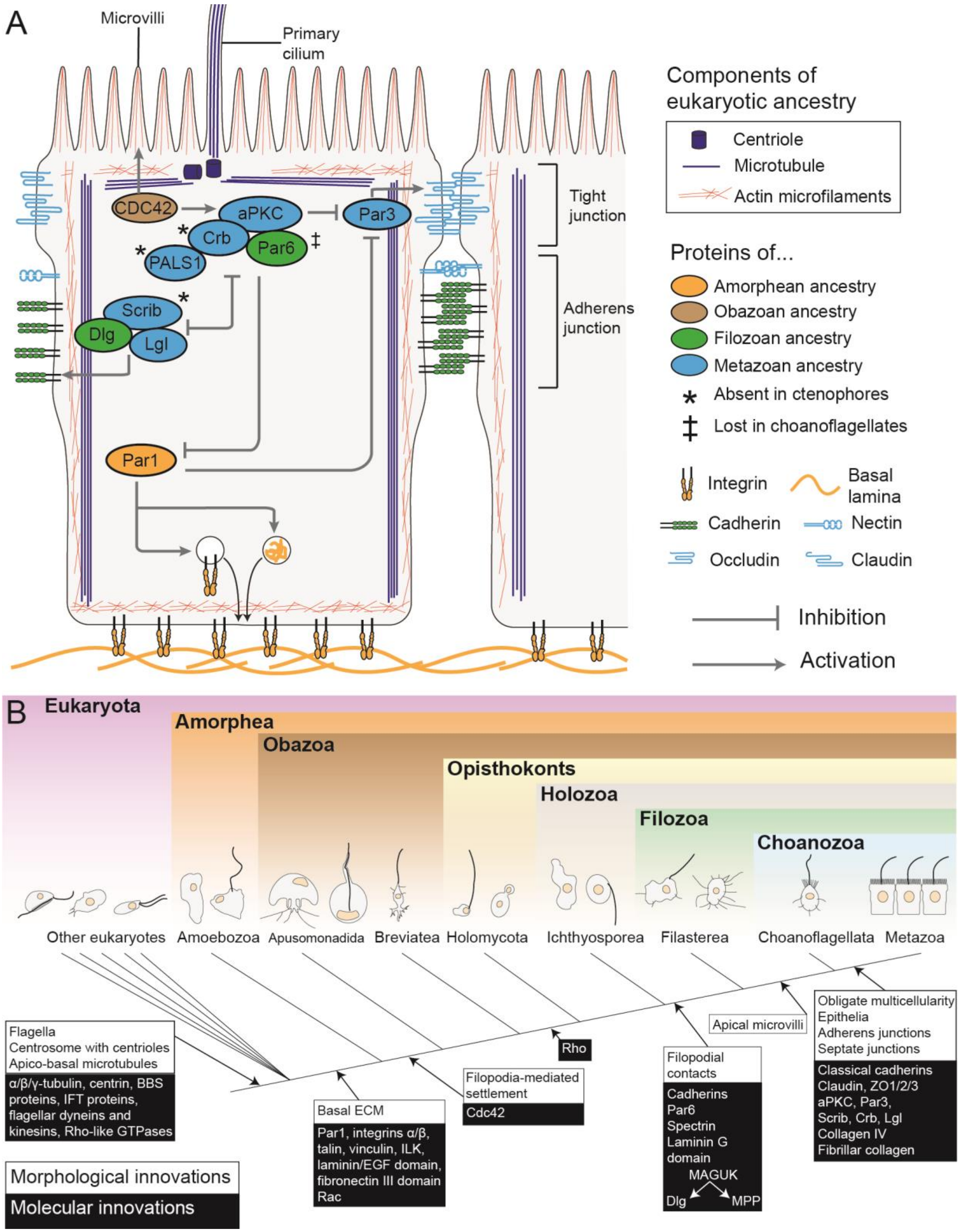

Figure 1: The apico-basal polarity network of a mammalian epithelial cell and its evolutionary origin. A: The polarized architecture of a mammalian epithelial cell and the protein complexes that regulate it. Time of evolutionary origins are color-coded (see text). Redrawn with modification from (Buckley and St Johnston, 2022). The basal lamina is coded as being of amorphean ancestry as that is the origin of some key domains, but some important basal lamina proteins evolved later (see text). **B:** Timing of origin of metazoan polarity proteins, and of corresponding morphological features, during eukaryotic evolution.

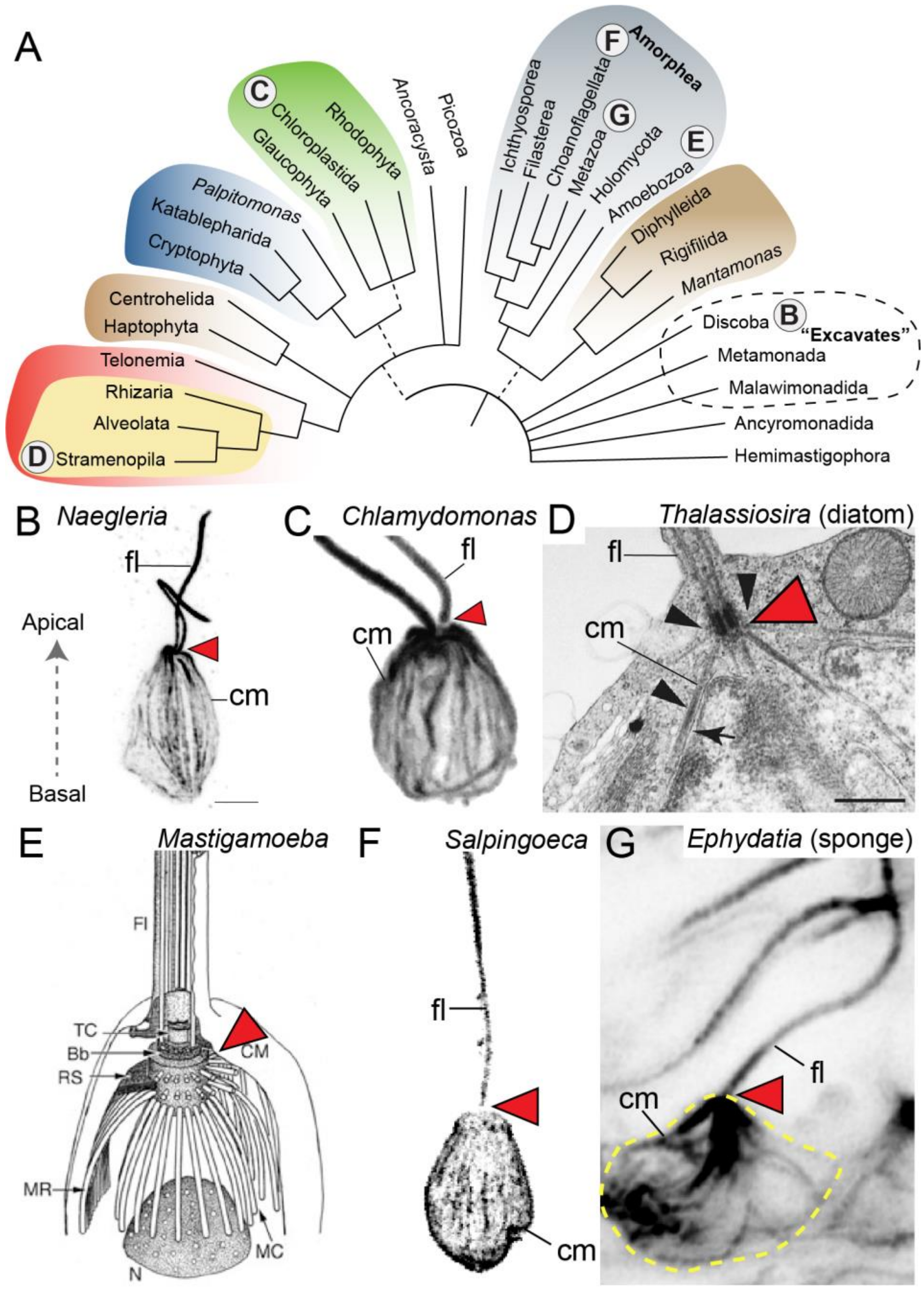

Figure 2: Polarized architecture of the microtubule cytoskeleton in flagellates across the eukaryotic tree of life. A: Phylogenetic tree of eukaryotes (modified from (Burki et al., 2020)). Flagellate taxa shown in following panels are indicated by letters. **B-G:** Tubulin cytoskeleton of diverse flagellates. In all panels: fl: flagellum, cm: cortical microtubules, red arrowhead: microtubule-organizing center. **B:** Tubulin immunostaining of *Naegleria gruberi*, reproduced

from (Velle et al., 2022). **C:** Tubulin immunostaining of *Chlamydomonas reinhardtii*, reproduced from (Ranjan et al., 2019). **D:** Transmitted electron micrograph of a sperm cell of the diatom *Thalassiosira*, reproduced from (Idei et al., 2013). **E:** Schematic of the microtubule cytoskeleton in the amoebozoan *Mastigamoeba*, reproduced from (Walker et al., 2017). **F:** Tubulin immunostaining of the choanoflagellate *Salpingoeca rosetta* (T. Brunet, unpublished data). **G:** Tubulin immunostaining of a choanocyte in the sponge *Ephydatia muelleri* (T. Brunet, unpublished data). Dashed yellow line: outline of the cell body.

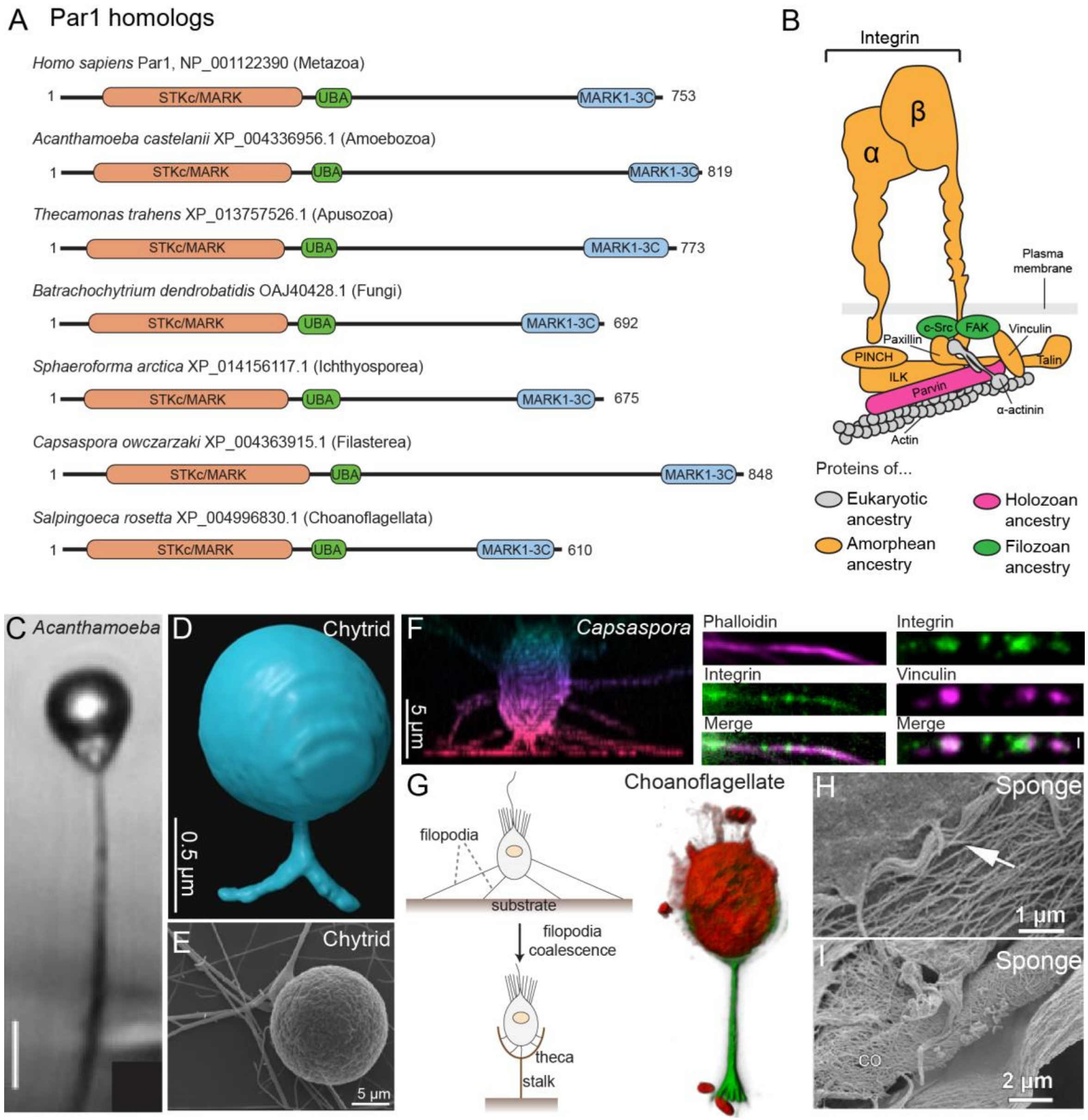

Figure 3: The integrin-mediated adhesion complex, Par1, and cell-substrate adhesions date back to the last common ancestor of opisthokonts and amoebozoans. A: Structure of Par1 orthologs in selected amorpheans with their NCBI accession numbers. Domain architecture was predicted by NCBI CD-Search, except the UBA domain in unicellular amorpheans (which was identified by manual inspection of ClustalX alignment to the *Homo sapiens* ortholog). **B:** Components of the integrin-mediated adhesion complex color-coded by time of ancestry. Modified from (Kang et al., 2021). **C:** Sporocarp of the amoebozoan *Acanthamoeba pyriformis*, with a unicellular spore on top of a stalk of extracellular material. From (Kang et al., 2021). **D,E:**

Developing sporangium of the chytrid fungus *Rhizoclosmatium globosum* at the germling (D) and thallus (E) stages attached to the substrate by rhizoids. From (Laundon et al., 2022, 2020). **F:** Left: the filasterean *Capsaspora owczarzaki* adhering to the substrate (bottom) via a "pedestal" of filopodia. From (Parra-Acero et al., 2018). Right: presence of F-actin (phalloidin; middle column), integrin (middle and right columns) and vinculin (right column) in the filopodia of *Capspora* revealed by immunofluorescence. Modified from (Parra-Acero et al., 2020). **G:** Left: settlement in choanoflagellates is first mediated by basal filopodia that ensure cell-substrate attachment before the theca is secreted. Modified from (Brunet and King, 2017). Right: The choanoflagellate *Salpingoeca rosetta* attached to the substrate by a stalk (theca) made of extracellular matrix. Red: cell body stained by LysoTracker Red, green: extracellular material stained by fluorescent wheat germ agglutinin (Alexa 488-WGA). Imaris 3D rendering of a confocal stack acquired on a Zeiss LSM780 with AiryScan (T. Brunet). **H, I:** Basal lamina underlying the exopinacoderm of the sponge *Ephydatia*. Co: collagenous mesh, arrow: filopodia by which exopinacocytes anchor onto the basal lamina. From (Leys et al., 2009).

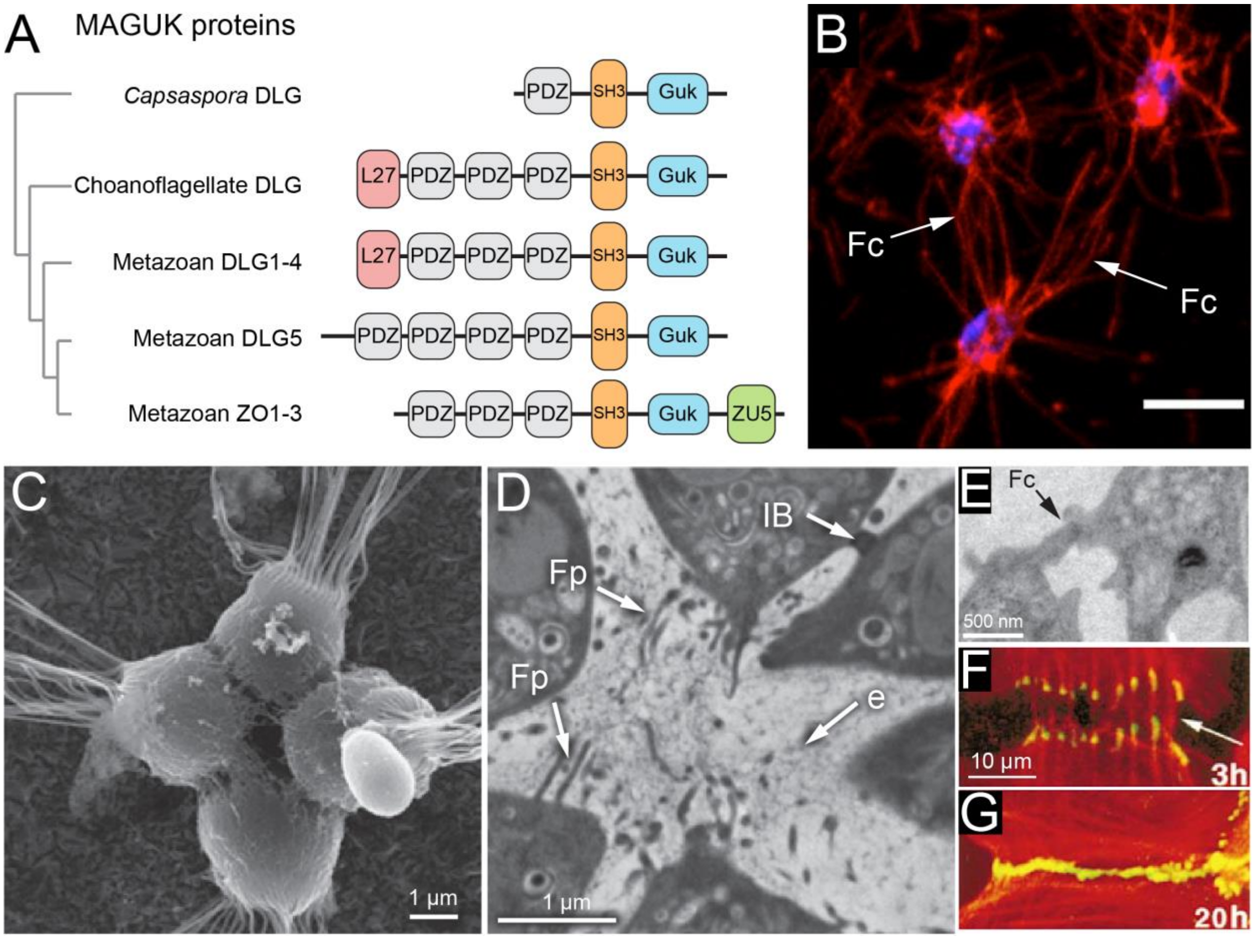

Figure 4: Filozoan innovations: MAGUK proteins, cadherins, and intercellular filopodial contacts. A: Structure of MAGUK proteins in filozoans. Modified from (de Mendoza et al., 2010). **B:** Filopodial contacts allowing cell aggregation in the filasterean *Capspora owczarzaki*, from (Phillips et al., 2022). **C-E:** Rosette colonies of the choanoflagellate *S. rosetta*. **C:** Scanning electron micrograph of a rosette. **D:** Cells extend abundant filopodia in the core of the rosette. C,D from (Dayel et al., 2011). **E:** Filopodial contact at the core of an *S. rosetta* rosette, from (Naumann and Burkhardt, 2019). **F,G:** Filopodial contacts maturing into adherens junctions in a primary culture of epidermal keratinocytes. Red: F-actin, green: E-cadherin. From (Vasioukhin et al., 2000).

Footnotes

1. To pinpoint the evolutionary emergence of spectrin, we explored the proteome-wide InterProScan analysis in the supplementary data of the study cited. We defined spectrins as proteins with more than 5 consecutive spectrin repeats. Spectrins could readily be identified acros metazoans, choanoflagellates, and in all three filasterean genera studied (*Capsaspora*, *Ministeria* and *Pigoraptor*), but not in other eukaryotes.