Title:

Unique composition and hyperstability of the ventral disc, *Giardia*'s complex microtubule organelle

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Abstract

Giardia is a common protistan parasite that causes diarrheal disease worldwide. Motile trophozoites colonize the small intestine, attaching to the villi with the ventral disc, a unique and complex microtubule (MT) organelle. Attachment to the host epithelium allows *Giardia* to resist peristalsis during infection of the host gastrointestinal tract. Despite our emerging view of the complexity of ventral disc architecture, we are still in the very preliminary stages of understanding how specific structural elements contribute to disc stability or generate forces for attachment. The ventral disc is a large, dome-shaped, spiral MT array decorated with microribbon-crossbridge protein complexes (MR-CB) that extend upward into the cytoplasm. Using a new high salt method for disc biochemical fractionation followed by shotgun proteomic analyses and validation by GFP-tagging, we identified 54 new disc-associated proteins (DAPs). Of 87 DAPs confirmed to date, 54 localize only to the disc, and the remainder localize to additional structures such as the flagella, basal bodies, or median body. Almost one third of DAPs lack any homology to proteins in other eukaryotes and another one third simply contain ankyrin repeat domains. Many DAPs localize to specific structural regions of the disc, including the ventral groove region and disc margin. We also demonstrate that the disc is a stable structure lacking canonical MT dynamic instability and show that the disc structure and composition remain intact after detergent extraction in up to 2M potassium chloride. Future genetic, biochemical, and functional analyses of DAPs will be central toward understanding disc architecture, assembly and dynamics.

Introduction

Microtubules (MTs) in protists assemble into cytoskeletal arrays and organelles that adopt shapes, functions, or regulatory mechanisms not present in model eukaryotic systems (Dawson and Paredez, 2013). The ubiquity and diversity of unique cytoskeletal organelles in microbial eukaryotes underscores the fact that cytoskeletal variation is the norm rather than the exception (Russell et al., 2017). Protistan cytoskeletal organelles are often composed of conserved MAPs as well as proteins that lack homology to proteins in other eukaryotes (Hagen et al., 2011; Hu et al., 2006; Preisner et al., 2016), and thus may be an untapped reservoir of non-canonical MT-binding proteins governing MT assembly, nucleation, or dynamics (Hampl et al., 2009).

One such unique microtubule organelle is present in *Giardia,* a widespread protistan parasite that causes diarrheal disease in humans and livestock (Einarsson et al., 2016). Commonly ingested from contaminated water sources, *Giardia* cysts excyst into flagellated trophozoites and proliferate in the proximal small intestine (Nosala and Dawson, 2015). There they attach to the intestinal microvilli using the ventral disc (Elmendorf et al., 2003; Nosala and Dawson, 2015), a microtubule (MT) organelle (Crossley and Holberton, 1983a; Crossley and Holberton, 1985; Feely et al., 1982; Friend, 1966; Holberton, 1973; Holberton, 1981) 8 µm in diameter with parallel, uniformly spaced MTs that spiral one and a quarter turns to form a dome. An overlap zone occurs between the upper and lower portions, and the ventral groove region extends over the ventral flagella (Figure 1A, B).

The overall architecture of the ventral disc was first described by Cheissin over 50 years ago (Cheissin, 1964), and abundant MT-associated proteins termed 'giardins' were isolated from the disc two decades later (Crossley and Holberton, 1983a). Early work described the overall structural elements of associated with the ventral disc MT spiral array (Friend, 1966), and the first 3D high-resolution structure of the ventral disc was obtained recently using cryo-electron tomography (cryo-ET) (Schwartz et al., 2012). Cryo-ET of whole isolated ventral discs defined an elaborate cytoskeletal architecture with dense protein complexes coating nearly every protofilament of the microtubule spiral array (Figure 1A). In sum, the disc contains more than 1.2 mm of tubulin forming roughly one hundred MTs that vary in length from 2 to 18 µm (Brown et al., 2016). Associated with the entire length of the MT spiral are unique repetitive substructural elements – the trilaminar microribbons – that extend 150-400 nm dorsally into the cytoplasm and are believed to stabilize the overall disc structure (Holberton, 1973; Holberton, 1981) (see Figure 1C, D). Regularly spaced "crossbridge" structures link adjacent microribbons (Holberton, 1973) and repeat every 16 nm (Figure 1D), corresponding to the distance of two alpha/beta tubulin dimers. Repeating every 8 nm are sidearms and paddles that are spaced at the distance of a single alpha/beta tubulin dimer. Other repetitive MT-associated substructures include three MT-associated proteins (gMAPs 1-3) and three MT inner proteins (gMIPS 5, 7 and 8). An associated structure at the disc periphery, the lateral crest (Figure 1C), forms a seal with surfaces in early attachment (Feely, 1990; Feely et al., 1982).

Despite our new understanding of the complexity of ventral disc architecture (Brown et al., 2016), we still know very little about the composition of the disc or the contribution of specific structural elements to disc stability and the generation of attachment forces. In a

comprehensive proteomic analysis of detergent-extracted, isolated ventral discs, we previously identified over twenty new disc-associated proteins (now referred to as DAPs, rather than giardins) that specifically localize to regions of the ventral disc or lateral crest (Hagen et al., 2011). The complexity of the disc architecture as revealed by cryo-ET implied that there could be disc proteins that were still undiscovered.

Here we have identified 54 additional disc-associated proteins (DAPs) using a new method of disc biochemical fractionation in high salt followed by shotgun proteomic analyses. Localization of these candidate DAPs to the ventral disc was confirmed by GFP tagging. We also demonstrate that the complex disc is a "hyperstable" structure in that drugs that normally affect MT dynamic instability have no effect on disc microtubules (Dawson et al., 2007) and no turnover of disc-associated proteins (DAPs) has been observed (Dawson et al., 2007; Hagen et al., 2011). The overall ventral disc structure remains intact after detergent extraction in up to 2M potassium chloride. Further dissection of the mechanisms underlying the disc's unusual stability will first require thorough knowledge of the functional roles of unique disc-associated proteins.

Materials and Methods

Giardia culture conditions

Giardia strains were cultured in sterile 16 ml screw-capped disposable tubes (BD Falcon) containing 12 ml modified TYI-S-33 medium supplemented with bovine bile and 5% adult and

5% fetal bovine serum [56]. Cultures were incubated upright at 37°C without shaking and reached confluence at ~48 hr. Puromycin (50 µg/ml) was added when required to maintain episomal plasmids in GFP strains.

C-terminal GFP tagging of candidate disc-associated proteins

Giardia GFP strains were created using Gateway cloning as previously described (Hagen et al., 2011). To add C-terminal GFP tags to candidate DAPs, PCR forward primers were designed to bind 200 bp upstream of the gene to include the *Giardia* native promoter. Full-length genes lacking stop codons were amplified from *Giardia lamblia* strain WBC6 genomic DNA using either PfuTurbo Hotstart PCR Master Mix or Easy-A High Fidelity PCR Master Mix (Agilent) and were cloned into the ThermoFisher Scientific Gateway entry vectors pENTR/D-TOPO (blunt-end, directional TOPO cloning) or pCR8/GW/TOPO (for efficient TOPO TA cloning), respectively. Inserts were sequenced to confirm gene identity and correct orientation. To generate DAP-GFP fusions, entry vectors were recombined with the *E. coli*/*Giardia* Gateway destination vector pcGFP1F.pac (Dawson and House, 2010) via LR recombination using LR Clonase II Plus (ThermoFisher Scientific). Clones were screened by AscI digest and Qiagen's Plasmid Plus Midi Kit was used to prepare bulk plasmid DNA. To create *Giardia* strains expressing GFP-tagged candidate DAPs, 20 µg plasmid DNA was introduced into 10^7 *G. lamblia* strain WBC6 (ATCC 50803) trophozoites by electroporation using a Bio-Rad GenePulserXL as previously described (Dawson et al., 2007). Transfectants were initially selected with 10 µg /ml puromycin; the antibiotic concentration was increased to 50 µg /ml once cultures reached 50% confluence

(typically 7 to 10 days). Cultures were maintained with selection for at least two weeks prior to the preparation of frozen stocks.

Biochemical fractionation of the Giardia cytoskeleton

Detergent extraction of *Giardia's* microtubule cytoskeleton was done as previously described (Hagen et al., 2011). Medium was decanted from one confluent culture tube and trophozoites were washed *in situ* three times with 5 mls of warmed 1X HEPES Buffered Saline (HBS). To demembranate cytoskeletons, cells were iced for 15 minutes in the last 1X HBS wash, pelleted by centrifugation (1000 × g, 5 min), and resuspended in 1 ml of 1X PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 1 mM MgCl2, pH 7.4) containing 1% Triton X-100 and 1M KCl. 1X HALT protease inhibitor cocktail (Roche) was added to prevent proteolysis. This solution was transferred to an Eppendorf tube and vortexed continuously for 30 min at medium speed. Ventral disc cytoskeletons were then pelleted as above and washed twice in 1X PHEM lacking both Triton X-100 and KCl. Sufficient extraction of cytoskeletons was confirmed by wet mount using DIC microscopy.

Cytoskeletons were fractionated as previously described (Crossley and Holberton, 1983a; Crossley and Holberton, 1983b; Crossley and Holberton, 1985) and Figure 2A). Following detergent extraction, an aliquot of demembranated cytoskeletons in PHEM was retained as 'P1.' Cytoskeletons were then pelleted as described above, washed, and resuspended in CB buffer (10mM Tris, 1mM EDTA, pH 7.7) for 48 hours to dissolve the crossbridges. Leftover cytoskeletal complexes were pelleted at 1000 x g for 5 min and the supernatant was retained as 'S2.' Complexes were next washed and resuspended in MR buffer (10mM HEPES, 5mM EDTA,

pH 8.7) for 48 hours to dissolve microribbons. The remaining tubulin complexes were pelleted, and the supernatant was retained as 'S3.' The pelleted tubulin complexes were resuspended in 1X PHEM and retained as 'P3.'

SDS-PAGE and Western Blotting

A total of 20 µg of total cell lysate in RIPA buffer was run on SDS/PAGE using Precise Tris·HEPES protein gels (ThermoFisher), 4–20%. Gels were transferred onto 0.22 micron nitrocellulose and blocked for 1 h at room temperature in 5% (wt/ vol) milk in PBS with 0.05% Tween. Antibodies were diluted in 5% (wt/vol) milk in PBS with 0.05% Tween as follows: anti-HA 1:5,000, (Sigma) 1:5,000, goat antimouse IgG HRP (Bio-Rad) 1:10000. ECL substrate (Millipore) was used for detection and development. For SDS-PAGE of fractionated proteins, 20 ul of total undiluted fraction samples were run as for western blots. Gels were stained in True Blue for 1 hour then rinsed with MilliQ water and incubated with MilliQ water overnight at room temperature to help destain.

Proteomic analyses of fractions and mass spectrometry

All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version X! Tandem Alanine (2017.2.1.4)). X! Tandem was used to search the uniprotgiardiaintestinalis_Craprev database (14528 entries) assuming the digestion enzyme trypsin and with a fragment ion mass tolerance of 20 PPM and a parent ion tolerance of 20 PPM. X! Tandem variable modifications used were Glu->pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, gln->pyro-Glu of

the N-terminus, deamidated of asparagine and glutamine, oxidation of methionine and tryptophan and dioxidation of methionine and tryptophan. Scaffold (version Scaffold_4.8.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Protein identifications were accepted if they contained at least 5 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Live imaging of GFP-tagged DAP strains

Strains were thawed from frozen stocks and cultured for 24 to 48 hours prior to live imaging. Trophozoites were iced 15 min, harvested by centrifugation at 1000 x g, resuspended in warm medium, and incubated in 96-well black glass bottom imaging plates (Cellvis, Mountain View, CA) for up to 2 hours at 37C in a nitrogen-enriched atmosphere to promote attachment. The medium was replaced with 1X HBS, and the trophozoites were incubated under the same conditions for 30 min. Additional warm HBS washes were performed as needed during imaging to remove detached cells. Imaging was performed using differential interference contrast (DIC) and epifluorescence with a Leica DMI6000B wide-field inverted fluorescence microscope (PlanApo 100X, NA 1.40 oil immersion objective). Optical sections were acquired at 0.2-µm intervals using Metamorph (MDS Technologies). Images were processed using Fiji, and two-dimensional maximum intensity projections were created from the three-dimensional data sets if required.

Structured Illumination Microscopy

3D stacks were collected at 0.125 um intervals on the Nikon N-SIM Structured Illumination Super-resolution Microscope with a 100x/NA 1.49 objective, 100 EX V-R diffraction grating, and an Andor iXon3 DU-897E EMCCD. Images were recollected and reconstructed in the "2D-SIM" mode (no out of focus light removal; reconstruction used three diffraction grating angles each with three translations).

Transmission Electron Microscopy

Cytoskeleton preps of wild type and knockdown cells were prepared as above and applied to 400 mesh formvar/carbon coated glow-discharged grids. Negative staining was performed by applying 1% phosphotungstic acid, pH 5.4, and grids were dried by blotting without washes. For thin sections, *Giardia* trophozoites that were pelleted or attached to ACLAR discs (Electron Microscopy Sciences) were fixed for 10 minutes in 4% paraformaldehyde and secondarily fixed for 1 hour in 1% osmium tetroxide. Cells were washed three times with cold ddH2O to remove fixative, dehydrated through ascending concentrations of ethanol (30%, 50%), and incubated for 1 hour in 2% uranyl acetate in 50% ethanol. Dehydration continued through 70% and 3 x 95% ethanol, and was completed with three changes in 100% ethanol for a minimum of 10 minutes each change. Cell were embedded in 1:1 epoxy resin:acetone overnight at room temperature. The next day the resin was removed and replaced with 100% resin twice for two hours each. The ACLAR discs were placed at the bottom of a flat bottom beam capsule with the cells facing up and the capsule was filled with fresh resin. The blocks were polymerized at 70C overnight. The blocks were trimmed, and thin sections were cut with a Leica UCT

ultramicrotome (Leica Ultracut UCT, Leica, Vienna, Austria) and stained with 2% uranyl acetate in 70% ethanol and lead citrate before viewing in the Talos L120C electron microscope (FEI/TheromoScientific Company, Hillsboro, OR., U.S.A. made in Eindhoven, The Netherlands) at 100KV. Images were acquired using the fully integrated Ceta CMOS camera.

Results

***Biochemical fractionation readily extracts disc and axoneme proteins for shotgun proteomic analysis and mass spectrometry*.**

Using cryo-ET of detergent extracted ventral discs, Brown et al. (Brown et al., 2016) recently defined specific regional variations in the ventral disc architecture, such as the ventral groove, disc margin and overlap zone areas. To find additional proteins that comprise these structurally distinct regions of the disc, we developed a novel biochemical fractionation protocol to disrupt the disc and used proteomic analysis and GFP tagging to identify candidate proteins.

Detergent extraction of *Giardia* trophozoites with Triton X-100 removes membrane and cytosol from the microtubule cytoskeleton and yields intact ventral discs that are stable in PHEM buffer for weeks (Crossley and Holberton, 1983a; Crossley and Holberton, 1983b; Crossley and Holberton, 1985; Holberton and Ward, 1981). Here we extracted trophozoites with both 1% Triton X-100 and 1M KCl (Figure 2A, P1 fraction). Microtubules and other disc proteins (Figure 2B, C) were retained following the high salt extraction, confirming the overall stability of the disc and cytoskeleton. Negative staining of the P1 fraction (Figure 2D) showed the loss of the

nuclei, and retention of the disc, flagella, and the funis. Subsequent extractions with TRIS-EDTA and HEPES buffers were used to destabilize and disrupt the disc, resulting in pellet or supernatant fractions (S2, S3, P2, and P3). Using a strain with fluorescently tagged delta-giardin (Figure 2C, D), we determined that these additional treatments caused significant changes to the ventral disc ultrastructure (S3 fraction), including a relaxation of the normally domed and closed ventral disc spiral, and separation of microtubule/microribbon pairs from crossbridges (Figure 2E, F). Flagellar components are removed from the disc fractions in the procedure (Figure 2E, F). SDS-PAGE of each fraction resolved proteins enriched in each pellet (P1, P3) and supernatant (S2, S3) fraction.

Proteomics and mass spectrometry of ventral disc fractions

Following mass spectrometry of each fraction (P1, P3 and S2, S3) and compared the overlap between proteins in various fractions. One hundred and five proteins were identified with at least five hits in any fraction after mass spectrometry (Table 1). Highly abundant known disc-associated proteins were identified in every fraction sampled (tubulin, MBP, delta giardin, etc). Other proteins had significant overlap between all fractions, yet sixty-two proteins were found to be exclusively enriched in S2 as compared to S3.

A revised inventory of disc-associated proteins

In a prior proteomic analysis of detergent-extracted, isolated ventral discs, we identified over twenty new DAPs that specifically localize to regions of the ventral disc or lateral crest (Hagen et al., 2011). To determine the cellular localization of candidate DAPs identified here, we again used C-terminal GFP tagging of candidate DAPs under the control of native promoters (Figure 3, Figure 4 and Supplemental Figures 1-4). The disc-specific localization of new 54 DAPs was confirmed with this method, bringing the total number of verified DAPs to 87 (Table 1). Many of the newly identified DAPs also localize to one or several other cytoskeletal structures (Figure 3A), such as the membrane bound or cytoplasmic regions of the eight axonemes or the eight basal bodies (31 total DAPs), the median body (23 total DAPs), or the lateral crest structure surrounding the disc (4 total DAPs). Forty-two of the 87 known DAPs only to specific regions of the ventral disc, and not to other cytoskeletal structure (Figure 3).

DAP compositional variation in distinct regions of the ventral disc

To classify the 54 new DAPs, we divided them into categories based on their GFP localization to six distinct regions of the disc: the disc body (body), the disc margin (DM), the overlap zone (OZ), the ventral groove (VG), the dense bands (DB) and the lateral crest (LC) (Figure 4 and Table 1)**.** Some DAPs localized to more than one disc region, and some also localized to other cytoskeletal structures. Overall, the majority of DAPs localize to more than one region of the disc (Figure 4 and Table 1). Thrity-six DAPs localize to only one of the six disc regions: six DAPS to the disc body only; three to overlap zone; twenty-two to the disc margin; four to the dense bands, and only one DAP is associated specifically with the ventral groove. Most DAPs localize

to the disc body, overlap zone, or disc margin regions, which highlights the overall structural complexity of these areas of the disc relative to other regions such as the dense bands that have five DAPS.

The majority of DAPs lack known MT-binding motifs

Of the total confirmed DAPs, only two are related to known MT-binding proteins (Figure 5). Specifically, one DAP has a CAP-GLY domain protein (DAP5374) (Weisbrich et al., 2007), and one kinesin, kinesin-6a (DAP102455), localizes to the ventral disc in the disc margin region. Twenty-six of the eight-seven DAPs lack homology to known proteins in other eukaryotes. An additional twenty-nine DAPs simply contain ankyrin repeat domains (Figure 3C and Figure 4). A handful of the identified DAPs are members of conserved protein families, including: three members of the striated fiber (SF)–assemblins (beta-giardin, delta-giardin, and SALP-1 (Palm et al., 2003)); four annexin family members (e.g., alpha-giardins (Bauer et al., 1999; Peattie, 1990; Weiland et al., 2005; Weiland et al., 2003)), and thirteen NEK kinases (Figure 5 and Table 1). Nine of the thirteen NEKs lack conserved catalytic residues or are pseudokinases which may, however, still retain kinase activity (Hagen et al., 2011). Other DAPs include those with a WD40 repeat domain (DAP15218), a DUF1126 domain (DAP41512), a Mlf1p domain (DAP16424), an ENTH domain (DAP3256), or SHIPPO repeat domains (DAP103164 and DAP9148).

Ventral disc composition and integrity is insensitive to microtubule drugs and high salt extraction.

To evaluate the effect of MT stabilizing or depolymerizing drugs such as Taxol or nocodazole on disc structure, we treated cells with each of these drugs separately and quantified the area and overall structure of the disc body, overlap zone and central bare area (Nosala et al., 2018). Treatment with either MT stabilizing or depolymerizing drugs had no effect on disc area or conformation (Figure 6A). In contrast, other cytoskeletal elements such as the flagella or median body are dynamic and sensitive to these MT drugs (Figure 6B).

We also determined that the detergent extraction of ventral disc cytoskeletons with up to 2M KCl had little effect on the recovery of disc-associated proteins as seen by SDS-PAGE (Figure 6C). Furthermore, the GFP-tagged disc protein DAP16343 (or MBP) remains associated with the disc even after extraction with 1M KCl, and both alpha tubulin and delta-giardin, a component of the microribbons, remain associated with the disc following salt extraction (Figure 6D).

Discussion

Paradigms of microtubule function, dynamics, and nucleation have classically been defined by studies of the mitotic spindle and cilium in model systems. Yet for nearly 300 years, diverse and unique interphase cytoskeletal organelles have also been described in microbial eukaryotes, or protists (Dawson and Paredez, 2013). In these organisms, microtubules are often arranged into higher-order assemblies that have forms and functions that are quite different from those of model organisms (Chaaban and Brouhard, 2017). The complex architecture and understudied functional abilities of non-canonical microtubule organelles like the ventral disc challenge our conceptions of the capabilities of cytoskeletal polymers.

The ventral disc is critical for parasite attachment to the host epithelium, and *Giardia* trophozoites are believed to attach to surfaces via hydrodynamic suction-based forces that result in a pressure differential underneath the disc relative to the outside medium (Woessner and Dawson, 2012). Overall conformational changes of regions of the disc may create or modulate these attachment forces in the absence of canonical MT dynamics (Woessner and Dawson, 2012). Defining the molecular mechanisms that underlie disc functions in attachment requires a more thorough understanding of the novel proteins and complexes that decorate almost all protofilaments of the disc MTs, and vary in composition and conformation along the length of the microtubules (Brown et al., 2016).

A dramatically expanded inventory of disc-associated proteins

Given the relatively small number of proteins known to regulate microtubule dynamics and assembly, how do diverse eukaryotic cells assemble and regulate elaborate microtubule organelles like the ventral disc? Diverse protistan cytoskeletal structures are often composed of proteins that lack homology to proteins in other eukaryotes (Hagen et al., 2011; Hu et al., 2006; Preisner et al., 2016), and thus may be an untapped reservoir of non-canonical MT-binding proteins governing MT assembly, nucleation, or dynamics (Hampl et al., 2009). Some DAPs may represent novel MT binding proteins with unique functions, such as facilitating the curved MT array of the disc. Only one DAP has a bonafide MT binding motif – DAP5374, a CAP-Gly protein (Weisbrich et al., 2007). One other MT-associated protein– kinesin-6a (DAP102455) – localizes to the ventral disc, in the disc margin region. Fifty-four of the total 87 DAPs localize exclusively to the disc, and the remainder also localize to other microtubule structures such as the flagella,

basal bodies, or median body which may indicate their more general MT-binding properties. Thus, while DAPs are primarily uncharacterized with respect to their microtubule binding or biochemical properties, many still may directly influence ventral disc MT nucleation, MT + end binding, or MT stability. DAPs may also contribute to novel aspects of the disc structure, likely generating and stabilizing the curved spiral array of microtubules (Brown et al., 2016; Schwartz et al., 2012).

Using a new method for disc biochemical fractionation followed by shotgun proteomic analyses, we identified and confirmed 54 new disc-associated proteins (DAPs), adding to the 33 previously described DAPs (Nosala et al., 2018). Many of the 87 known DAPs are likely structural components of the disc architectural elements (e.g., microribbons, crossbridges, disc margin). Overall, the ventral disc is composed of novel, non-homologous proteins and ankyrin repeat proteins (Figure 5), which are known to mediate protein-protein interactions, protein folding, and protein stability (Li et al., 2006). Eighty-five DAPS lack homology to known MT-binding proteins, and more than a third are novel, lacking homology to proteins outside of *Giardia* species – including those of other closely-related diplomonads like *Spironucleus* (Andersson et al., 2007). Among this group may be proteins involved in regulating the assembly or disassembly of the intricate disc architecture.

Close to 30 DAPs simply contain ankyrin repeat domains. Ankyrins can also interact directly with tubulin *in vitro* (Bennett and Davis, 1981; Davis and Bennett, 1984). The 30 disc-associated ankyrin repeat proteins may contribute to disc assembly or architecture, as ankyrin repeat proteins have been associated with the microtubule cytoskeleton in diverse eukaryotes. For

example, in the apicomplexan protist *Toxoplasma gondii*, the MT based apical complex acts as an invasion machine that is necessary for infection of host cells and parasite replication, and is constructed from canonical tubulins, non-canonical tubulin polymers, and novel proteins (Hu et al., 2006; Morrissette, 2015). Like the ventral disc, the conoid is largely composed of ankyrin-repeat containing proteins that are integral to conoid structure and functioning (Long et al., 2017). Ankyrin proteins stabilize subsets of microtubules in human erythrocytes, muscles, and neurons [42]. Thus, the adaptability of ankyrin repeat domains may facilitate the stability of microtubule organelles in *Giardia* and other eukaryotes.

Giardia has an expanded repertoire of over 70 NEK kinases (Manning et al., 2011), and 13 NEK kinases are associated with the disc. NEKs in the disc could act as structural or regulatory proteins involved in disc biogenesis or disassembly during cell division. Five NEK kinase DAPs also have ankyrin repeat domains. Nine of the twelve disc-associated NEKs lack conserved catalytic residues or are pseudokinases, yet may still retain kinase activity (Hagen et al., 2011).

Regional variation in disc composition is correlated to similar variations in disc architecture

The strikingly distinct localization patterns of the 54 disc and median body associated DAPs that delineate the disc (Figure 4 and Supplemental 1-4) are consistent with the structural variations in the disc defined by cryo-ET (Brown et al., 2016). Due to regional variations in the disc architecture, a single microtubule can be coated with different DAPs in different regions, beginning at the “dense band” nucleation zone and terminating at the disc margin (Brown et al., 2016). The majority of disc microtubules terminate with their plus ends either at the disc

margin or in the overlap zone, with a small subset observed to terminate within the disc body itself (Brown et al., 2016).

Of the 54 disc and median-body localizing DAPs, six localize specifically to the disc body, with 22 DAPs the disc margin and two the lateral crest, three to the overlap zone, four to the dense bands, and one to the ventral groove region (Figure 4). The function and mechanism by which regional variation in DAP localization is generated is unknown, yet the localization of DAPs to these specific regions (e.g., disc margin, dense bands, etc.) implies unique molecular properties associated with these regions.

Do DAPs confer "hyperstability" to the ventral disc?

Emerging microbial eukaryotic model systems like *Giardia* offer a wealth of unexplored cytoskeletal organelles with unique properties and functions (Russell et al., 2017). The insensitivity of the disc to MT drugs and its integrity following extraction in high salt, or "hyperstability," could be explained by the high number of DAPs and protein complexes coating the disc microtubules (Brown et al., 2016). The extensive microribbon and crossbridge complexes may also contribute to disc stability, as Holberton and Ward previously observed that detergent-extracted discs unwind and lose cohesion as crossbridge connections dissociate over time (Holberton and Ward, 1981). This was particularly evident in the ventral groove region (Holberton and Ward, 1981). Despite the loss of crossbridges, disc microtubules remained curved, implying that disc curvature is governed by other structures such as the microribbons (Holberton, 1981; Holberton and Ward, 1981). We observed the loss of

crossbridges in the S3 fraction, yet curvature of the microtubule/microribbon structure remained intact (Figure 2E, F). Holberton argued that mild dissolution of crossbridges results in a flat disc that is more energetically stable with fully extended CBs, and that outer ventral disc structures may help to hold the lattice in the spiral conformation (Crossley and Holberton, 1983b). Our lab has observed that the ability of the ventral disc to form a dome is crucial for functional attachment of *Giardia* to surfaces (Woessner and Dawson, 2012). Therefore, the crossbridges may be critical for both the structure and function of the ventral disc.

Lastly, the disc is a "hyperstable" structure in that drugs that normally affect MT dynamic instability have no effect on ventral disc microtubules (Dawson et al., 2007). No turnover of any disc-associated protein has been reported (Hagen et al., 2011). The large number of unique microtubule-associated proteins and protein complexes decorating the disc spiral, the disc margin, and the dense bands may also contribute to the observed hyperstability of the plus and minus ends of the disc MTs.

Evolution of a complex microtubule organelle for attachment

The ubiquity and diversity of unique cytoskeletal organelles in microbial eukaryotes underscores the fact that cytoskeletal variation is the norm rather than the exception. The complex ventral disc spiral MT array and associated structures (e.g., lateral crest) have evolved only in *Giardia* species, by the cooption of existing proteins or structures or through the invention of novel MT-binding proteins. Because several components of the disc are MT-associated proteins (e.g., those with CAP-Gly or kinesin domains) or are related to flagellar root structures (SF-assemblins (Weber et al., 1993)), portions of the ventral disc may be derived from

ancestral flagellar structures. However, the sheer number of non-homologous and ankyrin repeat domain proteins in the disc suggests that much of the complexity of the ventral disc may have evolved through the invention of novel cytoskeletal proteins rather than the elaboration and modification of an existing cytoskeletal structure like an axoneme.

Given a high-resolution structure and a growing inventory of DAPs, the ongoing development of molecular genetic and biochemical tools in *Giardia* (Carpenter and Cande, 2009; Dawson et al., 2007; Touz et al., 2005) will be central toward understanding the roles of specific DAPs in the assembly and functions of the complex disc architecture as well as their roles in modulating disc conformational dynamics that promote attachment to the host.

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Figure legends

Figure 1. Ventral disc substructures support the microtubule array. A schematic of the ventral disc indicating the primary structure elements is shown in panel A: disc body, oz: overlap zone, lc: lateral crest, vg: ventral groove region, db: dense bands, dm: disc margin (A), MT: microtubule, MR: microribbon, CB: crossbridge (B,C). In B, a negative-stained cytoskeletal preparation of the ventral disc highlights the primary structural elements as well as the eight cytoplasmic axonemes (CF = caudal flagella; VF = ventral flagella; PF = posteriolateral flagella; AF = anterior flagella) and basal bodies (BB). In C, transmission electron microscopy of thin sections of whole embedded trophozoites show both microribbons (MR) associated with each MT of the spiral array and the associated lateral crest (LC). Negative staining of the disc (D) highlights the microribbons (MR) linked with regularly spaced crossbridges (CB). In E, the overlap zone (OZ) of the MT spiral array, along with the MR-CB complexes are shown in cross section (N = nucleus).

Figure 2: Biochemical fractionation readily extracts disc and axoneme proteins for shotgun proteomic analysis and mass spectrometry. Extraction of *Giardia* trophozoites with detergent and high salt (A) removed membrane and cytosol from the microtubule cytoskeleton (P1). Subsequent extractions with TRIS-EDTA and HEPES to further disrupted the disc resulting in the S2, P2, S3 and P3 fractions. Immunostaining of P1 fraction (B, C) retained the disc (green, anti-delta-giardin) and flagella (purple, anti-alpha-tubulin). Negative staining of the P1 fraction (D) confirms the loss of the nuclei (N) but retention of the disc (body, vg, oz, lc), flagella (AF, PF)

and the funis (fn); however, greater dissociation of the disc from the axonemes is evident in the S3 fraction as shown by delta-giardin immunostaining (E, F). SDS-PAGE resolves proteins that are enriched in each pellet (P1, P3) and supernatant (S2, S3) fraction. Following mass spectrometry of each fraction (P1, P3 and S2, S3), the Venn Diagram comparison indicates some overlap between protein the various fractions (H).

Figure 3. Many disc-associated proteins (DAPs) also localize to other cytoskeletal structures. Subcellular localizations of 87 DAP-GFP fusions identified by fractionation and mass spectrometry (Figure 2) were categorized into 42 that associated exclusively to the disc (disc only, B) or localizations of both the disc and other cytoskeletal structures including the flagella (N=31), the median body (N=12), and lateral crest (N=4). The Venn diagram indicates DAP-GFP fusions with overlapping localization to the disc, flagella, median body, and lateral crest (A). Representative localizations are shown for the 12 DAP-fusions localizing to the disc and median body (mb, C); the 11 localizing to the disc, median body and flagella (mb and fl, D); and the 18 localizing to the disc and flagella only (N=18, E). Two DAPs localized to the lateral crest and disc (F) and two DAPs localized to the lateral crest and flagella (2). The flagella category includes localizations to either the basal bodies and /or to the cytoplasmic or membrane bound regions of the flagella.

Figure 4. Disc-associated proteins localize to specific structurally distinct regions of the ventral disc. A Venn Diagram (A) shows categorization of the 54 new DAPs by their localization

to distinct regions of the disc including the MT spiral array (body), disc margin (DM), ventral groove (VG), dense bands (DB), and overlap zone (OZ). In B, representative DAPs defining each localization category are shown with their functional annotations.

Figure 5. The majority of disc associated proteins (DAPs) lack known microtubule binding domains

PFAM domains are summarized and ranked by abundance for the 87 confirmed *Giardia* DAPs. CAP_GLY and kinesin motor domains are the only known MT-binding motifs. The five DAPs with Nek kinase domains that also possess ankyrin repeat domains are not included in the final summary.

Figure 6. The overall composition and structural integrity of the ventral disc is insensitive to microtubule drugs and high salt extraction. The area and overall structure of the disc body, overlap zone (oz) and central bare area (ba) are not affected by treatment with either MT stabilizing or depolymerizing drugs such as Taxol or nocodazole (A). In contrast, other cytoskeletal elements, including the flagellar, are dynamic and sensitive to MT drugs (B). SDS-PAGE of fractions (pellet, P, and supernatant S) from 0 to 2M KCl extractions shows that salt also has little effect on extracted proteins (C). Furthermore, the GFP-tagged disc protein MBP remains associated with the disc even after extraction with 1M KCl (images in D green), as do α-tubulin and the microribbon protein δ-giardin as seen via Western blot (D).

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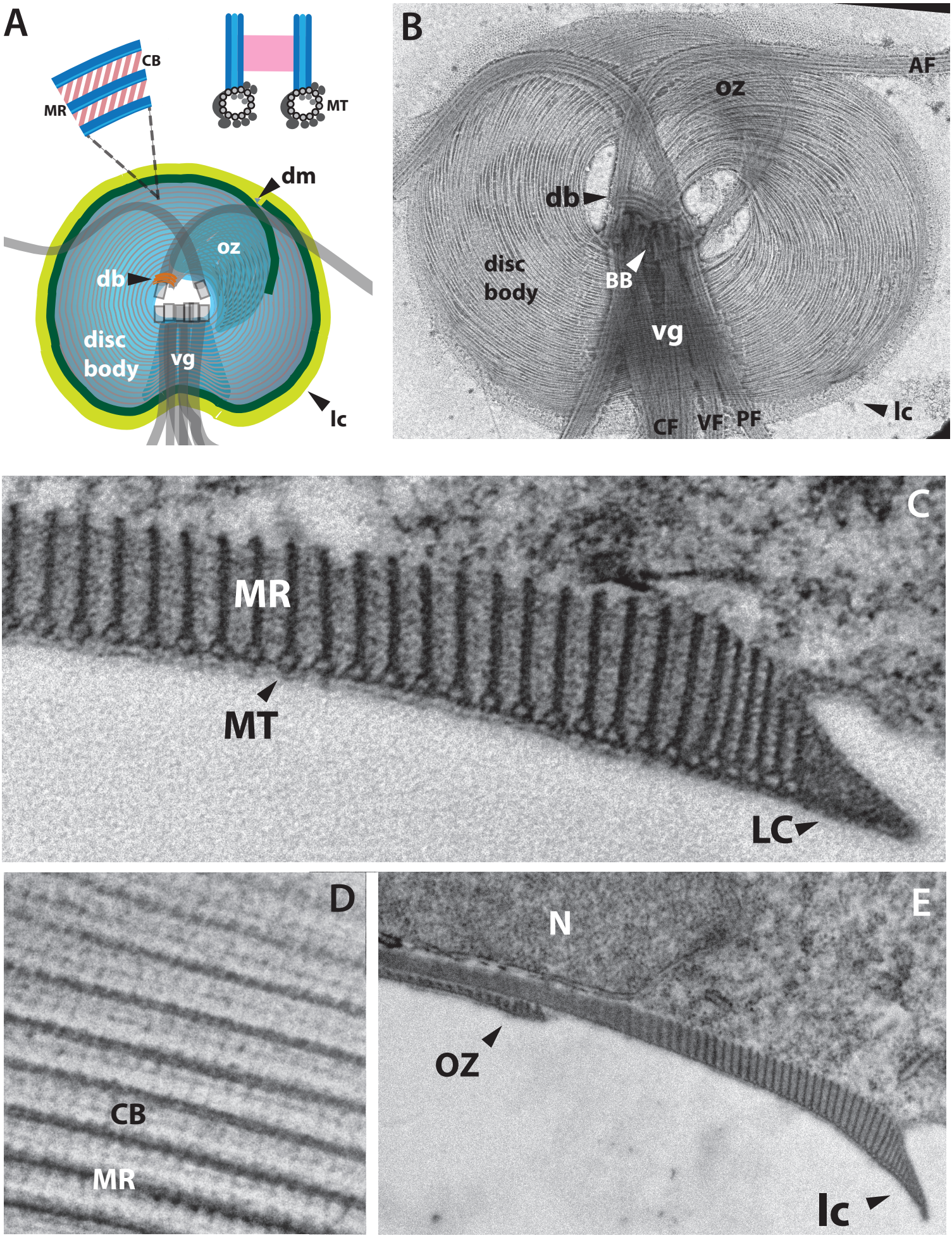
A
CB
MR
MT
dm
oz
db
disc
body
vg
lc
B
AF
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body
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vg
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PF
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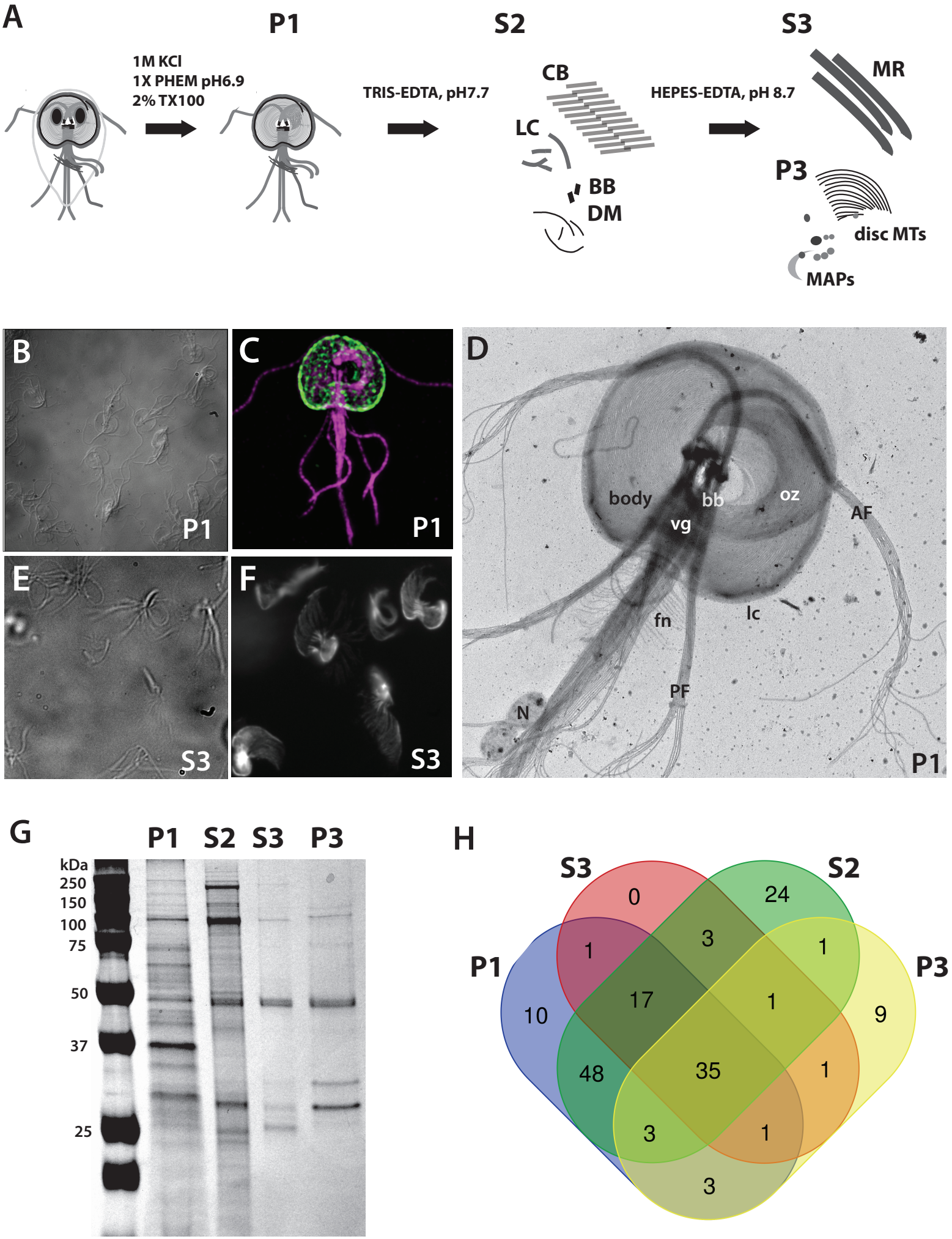
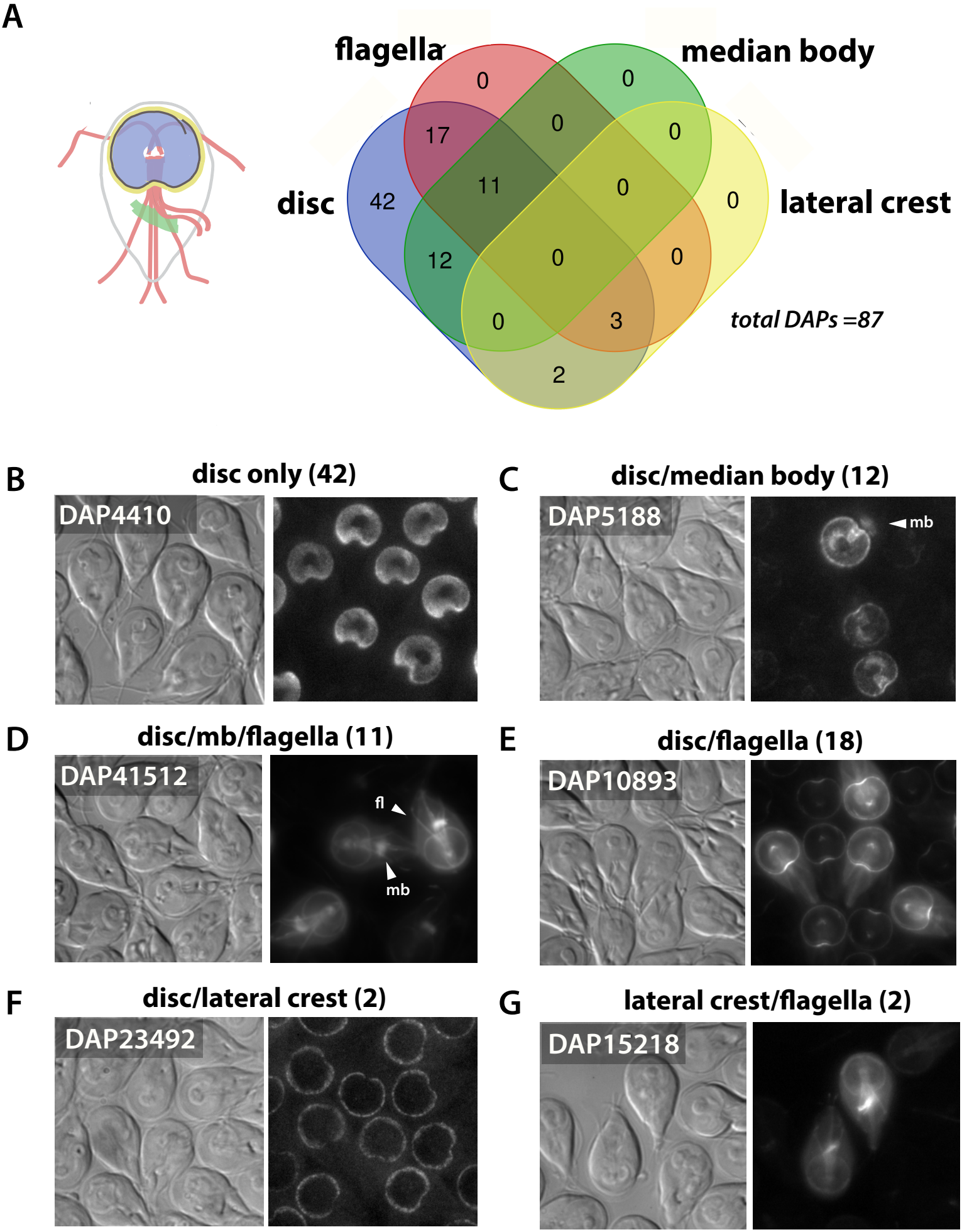
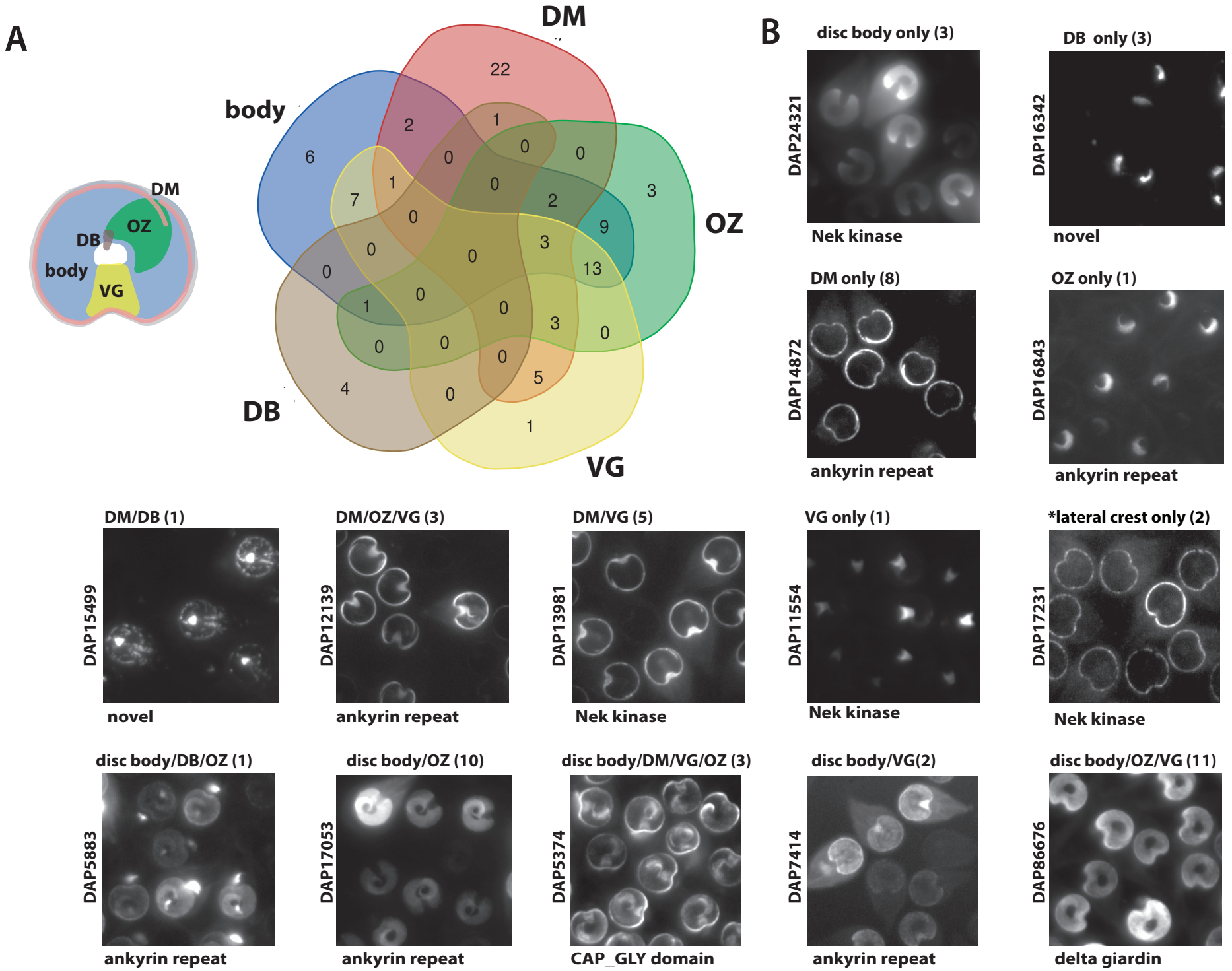
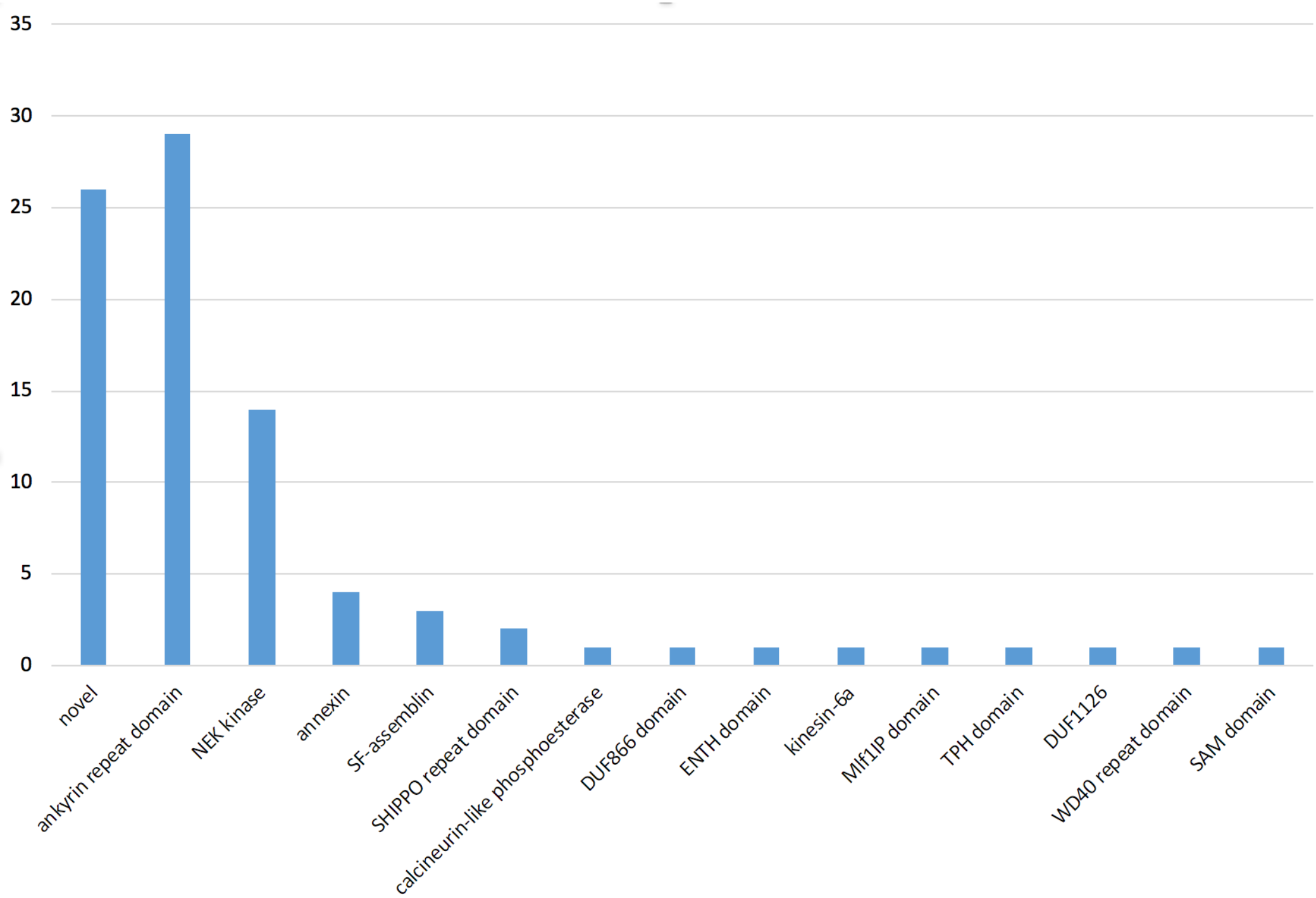
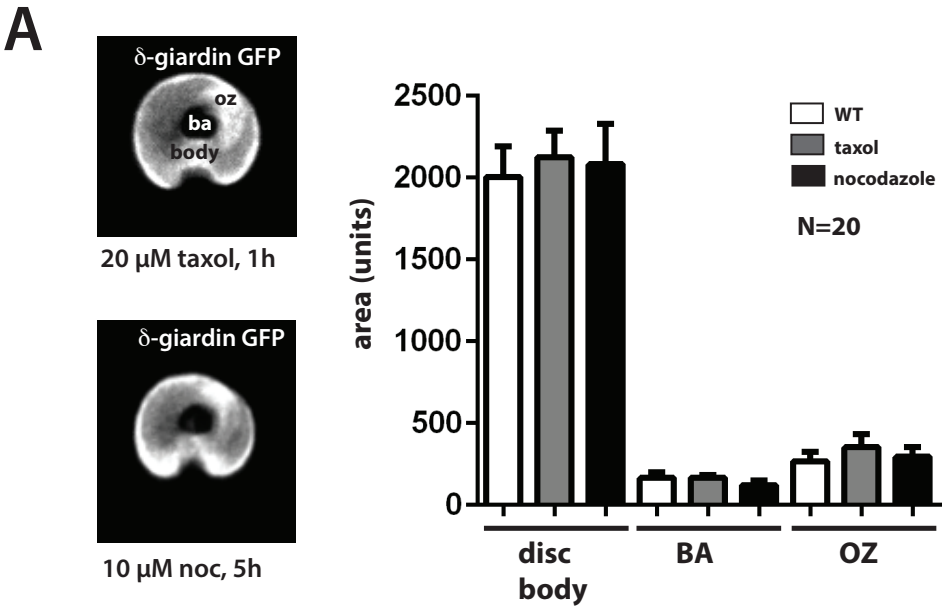

A
P1
S2
S3
1M KCl
1X PHEM pH6.9
2% TX100
TRIS-EDTA, pH7.7
HEPES-EDTA, pH 8.7
CB
LC
BB
DM
MR
P3
disc MTs
MAPs
B
P1
C
P1
D
body
bb
oz
vg
AF
fn
lc
PF
N
P1
E
S3
F
S3
G
P1 S2 S3 P3
kDa
250
150
100
75
50
37
25
H
S3
S2
P1
P3
0
24
1
3
1
10
17
1
9
48
35
1
3
1
3

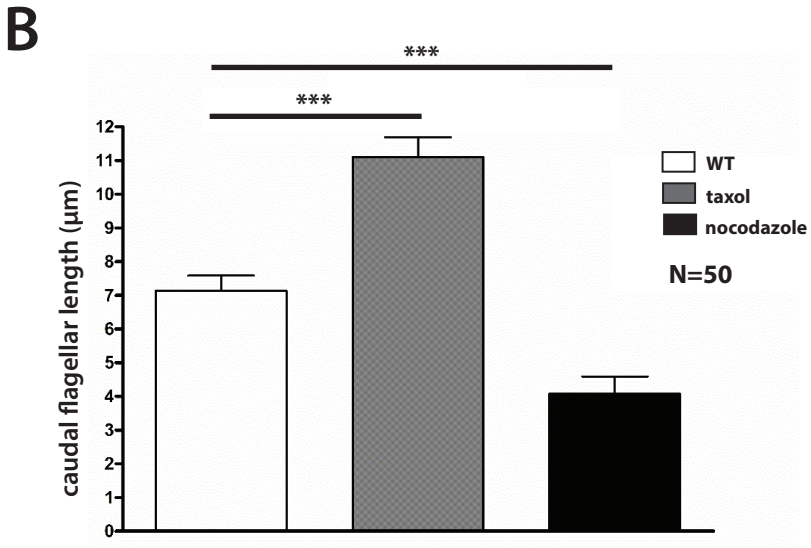

A
flagella
median body
disc
lateral crest
0
0
17
0
0
42
11
0
0
12
0
0
0
3
2
total DAPs =87
B
disc only (42)
DAP4410
C
disc/median body (12)
DAP5188
mb
D
disc/mb/flagella (11)
DAP41512
fl
mb
E
disc/flagella (18)
DAP10893
F
disc/lateral crest (2)
DAP23492
G
lateral crest/flagella (2)
DAP15218

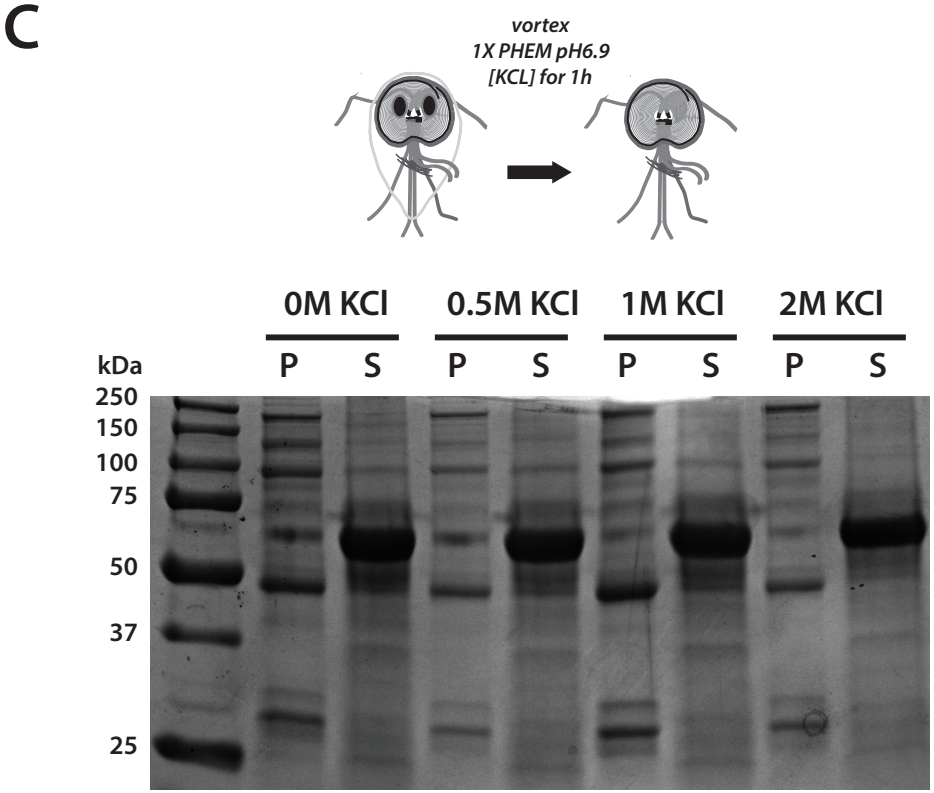


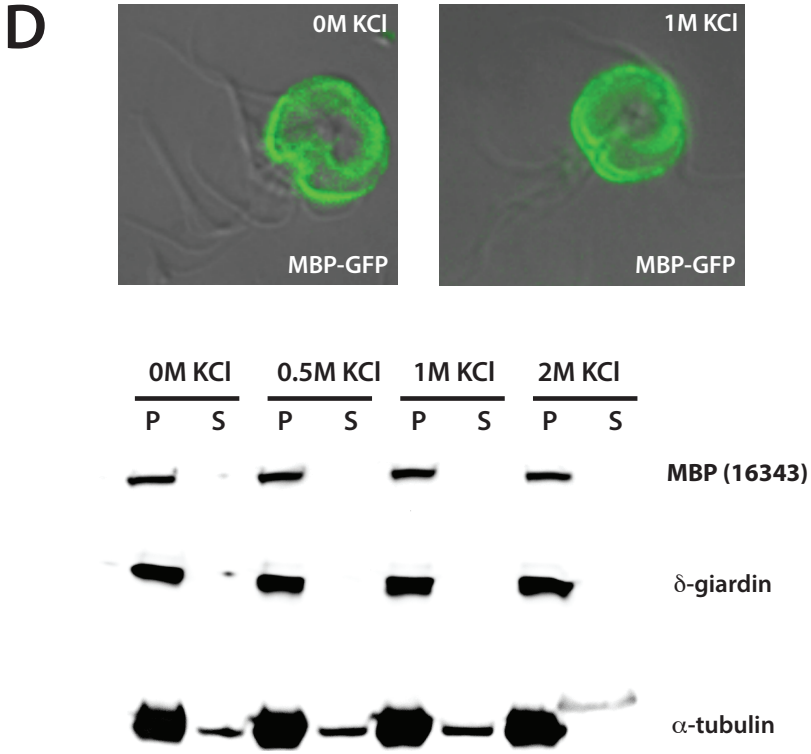
***no image disc body/DM (2)*


35
30
25
20
15
10
5
0
novel
ankyrin repeat domain
NEK kinase
annexin
SF-assemblin
SHIPPO repeat domain
calcineurin-like phosphoesterase
DUF866 domain
ENTH domain
kinesin-6a
Mlf1IP domain
TPH domain
DUF1126
WD40 repeat domain
SAM domain


A
δ-giardin GFP
oz
ba
body
20 μM taxol, 1h
δ-giardin GFP
10 μM noc, 5h
area (units)
2500
2000
1500
1000
500
0
WT
taxol
nocodazole
N=20
disc body
BA
OZ


B
***
***
caudal flagellar length (μm)
12
11
10
9
8
7
6
5
4
3
2
1
0
WT
taxol
nocodazole
N=50


C
vortex
1X PHEM pH6.9
[KCL] for 1h
0M KCl
0.5M KCl
1M KCl
2M KCl
P
S
kDa
250
150
100
75
50
37
25


D
0M KCl
MBP-GFP
1M KCl
MBP-GFP
0M KCl
0.5M KCl
1M KCl
2M KCl
P
S
MBP (16343)
δ-giardin
α-tubulin