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EML PROTEINS IN MICROTUBULE REGULATION AND HUMAN DISEASE

Andrew M. Fry[1], Laura O'Regan[1], Jessica Montgomery[1], Rozita Adib[1] and Richard Bayliss[2]

[1]Cancer Research UK Leicester Centre, University of Leicester, Department of Molecular and Cell Biology, Leicester LE1 9HN, U.K.

[2]Cancer Research UK Leeds Centre, Faculty of Biological Sciences, Astbury Building, University of Leeds, Leeds LS2 9JT, U.K.

Correspondence:

Andrew Fry (amf5@le.ac.uk)

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Introduction: EMLs, a novel class of MAPs

The EMLs are a conserved family of microtubule-associated proteins (MAPs). The founding member was discovered in sea urchins as a 77 kDa polypeptide that co-purified with microtubules. This protein, termed EMAP for echinoderm MAP, was the major non-tubulin component present in purified mitotic spindle preparations made from unfertilised sea urchin eggs (Suprenant et al., 1993; Suprenant and Marsh, 1987). Orthologues of EMAP were subsequently identified in other echinoderms, such as starfish and sand dollar, and then in more distant eukaryotes, including flies, worms and vertebrates, where the name of ELP or EML (both for EMAP-like proteins) has been adopted (Hueston et al., 2008; Suprenant et al., 2000). The common property of these proteins is their ability to decorate microtubules. However, whether they associate with particular microtubule populations or exercise specific functions in different microtubule-dependent processes remains unknown. Furthermore, although there is limited evidence that they regulate microtubule dynamics, the biochemical mechanisms of their molecular activity have yet to be explored. Nevertheless, interest in these proteins has grown substantially since the identification of EML mutations in neuronal disorders and oncogenic fusions in human cancers. Here, we summarise our current knowledge of the expression, localization and structure of what is proving to be an interesting and important class of MAPs. We also speculate about their function in microtubule regulation and highlight how studies of EMLs in human diseases may open up novel avenues for patient therapy.

Expression, localization and structure of EMLs

Mammals express six EML proteins that have been reported under different names but which we refer to here as EML1 to EML6 (Figure 1). EML1, 2, 3 and 4 share a similar protein organization with an N-terminal region of approximately 175-200 residues that appears largely unstructured apart from a short coiled-coil, and a C-terminal structured domain of approximately 650 residues consisting of multiple WD (tryptophan-aspartate) repeats. EML5 and 6 are distinct in that they lack the N-terminal region and have three contiguous repeats of the C-terminal WD repeat domain. Expression analyses reveal that EML1 and EML4 are highly expressed in early mouse embryos, but exhibit a lower and more restricted expression pattern in late embryos and adults with the most common site of expression being the nervous system, including the hippocampus, cortex, cerebellum, eyes and olfactory bulb (Houtman et al., 2007; Kielar et al., 2014). EML5 is also primarily expressed in the nervous system of the adult mouse (O'Connor et al., 2004), although EML3 was detected not only in the brain but also in the liver and kidney (Huttlin et al., 2010; Villen et al., 2007). Human studies suggests widespread expression of at least EML1 and EML2, although for all the EMLs

there is clear evidence of differential splicing (Lepley et al., 1999). *C. elegans* ELP-1 is also expressed in a variety of adult tissues, including the body wall, muscles and intestine (Hueston et al., 2008).

Localization studies, mostly in cultured cells, together with in vitro biochemical studies provide overwhelming evidence that EMLs are microtubule-binding proteins. EMAP was originally purified using a biochemical approach from spindles prepared from unfertilized sea urchin eggs. Antibodies raised against EMAP detected a similar sized protein by Western blot in microtubule preparations from a variety of echinoderm species, while it decorated microtubules in sea urchin embryos and adult coelomocytes by immunofluorescence microscopy (Suprenant et al., 1993). Expression of recombinant proteins confirmed even association of mammalian EML1, 2, 3 and 4 with the microtubule lattice (Eichenmuller et al., 2002; Houtman et al., 2007; Pollmann et al., 2006; Richards et al., 2015; Tegha-Dunghu et al., 2008). However, analysis of endogenous EML localization has, at least until recently, been hampered by the lack of good, commercially available antibodies. Peptide-specific antibodies raised to EML3 and EML4 have though confirmed localization of these proteins to microtubules in cultured cells (Chen et al., 2015; Tegha-Dunghu et al., 2008). Taken together, the expression and localization studies are indicative of a family of cytoplasmic microtubule-binding proteins with widespread expression but which may have particular roles in neuronal tissues.

Structural studies on human EML1, 2 and 4 reveal interesting and unexpected features of this family of proteins (Richards et al., 2014; Richards et al., 2015). The crystal structure of the C-terminal WD domain from human EML1, comprising residues 167-815, was determined to a resolution of 2.6 Å (Figure 2A). The WD repeats form 13 individual beta-sheet structures that form the blades of two beta-propeller domains that are closely and rigidly connected. The first beta-propeller is formed from 7 WD repeats. Unexpectedly, the second propeller is assembled from 6 WD repeats and an additional sub-domain that is formed from separate regions of the primary sequence of EML1. This domain appears to be a ubiquitous and unique feature of EML family proteins, and was termed the TAPE (tandem atypical propeller in EML) domain. The tandem propeller arrangement creates a relatively planar structure with a concave and convex surface and, sequence comparison amongst the human EMLs, suggests stronger conservation of the concave surface. Crystal structures of the coiled-coil regions of human EML2 and EML4 were determined to resolutions of 2.1 Å and 2.9 Å, respectively (Figure 2B). In both structures, three molecules of the EML protein come together through a core of hydrophobic interactions stabilized by salt-bridges to form a trimerization domain (Figure 2C). The conservation of primary sequences indicates that the coiled coil domains of human

EML1-4 are all trimeric, although coprecipitation experiments indicate that these proteins have the potential to assemble heterotrimers, as well as homotrimers (Richards et al., 2015).

Microtubule binding and regulation of EMLs

The mechanism of microtubule binding of EMLs is intriguing. Studies on sea urchin EMAP revealed that microtubule binding was conferred through a region towards the N-terminus of the protein (Eichenmuller et al., 2001). Indeed, it was originally suggested that a highly conserved 'HELP' (hydrophobic ELP) motif of approximately 40 residues within this N-terminus was responsible for microtubule binding (Eichenmuller et al., 2002; Suprenant et al., 2000). However, chimeras that specifically fused the HELP motif to EGFP did not localize to microtubules (Pollmann et al., 2006), and structural studies have since revealed that the HELP motif is an integral part of the TAPE domain and contributes to protein folding rather than microtubule binding (Richards et al., 2014). An alternative feature of the N-terminus that could be responsible for microtubule association is its basic nature that is conserved across metazoans. A number of MAPs interact with microtubules through electrostatic interactions between basic regions of the MAP and the negative surface of microtubules created by the exposed C-terminal tails of α/β-tubulin that are rich in acidic residues, particularly glutamate (Janke and Bulinski, 2011). The one letter code of glutamate, E, has led to these tails being referred to as 'E-hooks' with various MAPs, including dynein and kinesin motors, binding via the E-hooks. Limited proteolysis with subtilisin can cleave the E-hooks from polymerized microtubules in vitro and this has been exploited to test whether binding of MAPs is dependent on E-hooks. Subtilisin digestion of microtubules did not though prevent association of sea urchin EMAP arguing against binding occurring via electrostatic forces (Hamill et al., 1998). However, we have recently found that binding of the basic N-terminal fragment of human EML1 to microtubules is disrupted by subtilisin digestion, arguing in favour of an electrostatic mechanism of interaction for this protein (Montgomery *et al., in preparation*).

The N-terminal regions of EML1-4 have a coiled-coil that promotes trimerization (Richards et al., 2015). Deletion of the coiled-coil reduces microtubule binding of EML1, and this domain might contribute to microtubule association either by direct binding or via promoting oligomerization. EML5 and 6 lack the coiled-coil but do have three repeats of the TAPE domain encoded within a single polypeptide. Hence, they may adopt a similar overall tertiary structure that favours microtubule binding, although whether EML5 and 6 associate with microtubules remains to be tested. Intriguingly, sea urchin EMAP does not have a coiled-coil and has only one copy of the TAPE

domain, yet localizes to microtubules via its N-terminus. Moreover, *Drosophila* ELP-1, also called DCX-EMAP, contains within its N-terminus a specific sequence that bears homology to a domain in doublecortin (DCX) that directly contributes to microtubule binding (Bechstedt et al., 2010). Together with the difference in subtilisin response of EMAP, this may suggest that EMAP, ELP-1 and the human EML proteins have different mechanisms of microtubule binding. On the other hand, it may simply reflect differences in the relative contributions of electrostatic and conformational factors within the N-terminal regions that contribute to microtubule binding. Furthermore, studies on sea urchin EMAP suggest a second, weaker microtubule binding site in the C-terminal TAPE domain of the protein that could contribute to its overall microtubule affinity (Eichenmuller et al., 2001). The isolated TAPE domain of human EML1 did not localize to microtubules in cultured cells, but did associate tightly with soluble α/β-tubulin heterodimers via interactions with conserved residues on its concave surface (Richards et al., 2014; Richards et al., 2015). Whether this reflects a distinct role in binding soluble tubulin, or rather a second microtubule binding site that is only effective in the context of a trimer will be important to examine.

To date, most published studies have reported no significant differences in the association of EMLs with microtubules in interphase and mitotic cells. Indeed, sea urchin EMAP, as well as mammalian EML1-4, have all been reported to localize to spindle microtubules in mitosis, as well as the microtubule cytoskeleton in interphase (Eichenmuller et al., 2002; Kielar et al., 2014; Pollmann et al., 2006; Suprenant et al., 1993; Tegha-Dunghu et al., 2008). However, we recently found that there may well be important differences in the relative affinity of EMLs for microtubules in interphase and mitosis, with EML3 and EML4 exhibiting reduced localization to microtubules in mitosis as compared to interphase (Montgomery *et al., in preparation*). This is significant as there is a dramatic difference in the dynamic properties of microtubules between these two phases of the cell cycle, and the altered localization of EMLs could reflect a functional contribution to these changes. Moreover, they raise the question of how the affinity of EMLs for microtubules is regulated through the cell cycle. A strong candidate is phosphorylation with hyperphosphorylation of EML4 reported in mitotic cells, and evidence that sea urchin EMAP is not only phosphorylated in mitosis but can interact with the mitotic kinase, CDK1 (Brisch et al., 1996; Pollmann et al., 2006). EMLs have also been identified in interactome studies with the mitotic NEK6 kinase (Ewing et al., 2007). Our preliminary studies support a functional relationship with NEK6 phosphorylation reducing the affinity of EML3 for microtubules in mitotic cells (Montgomery *et al., in preparation*). Phosphoproteome data reveal a concentration of phosphorylation sites within the N-terminal basic regions of human EMLs

consistent with the hypothesis that phosphorylation could directly regulate microtubule affinity by altering electrostatic interactions.

EML functions in differentiated and proliferating cells

Biochemical studies on the sea urchin EMAP first revealed the potential of these proteins to alter microtubule dynamics. Purified EMAP caused an increase in microtubule dynamics with suppression of rescue events in vitro that is consistent with overall destabilization (Hamill et al., 1998). The limited studies that have since been undertaken on the human EMLs suggests that they might represent an unusual class of MAPs in which some members promote MT stabilization while others promote destabilization. This notion is based on the fact that overexpression and depletion studies argue that EML4 is a microtubule stabilizing protein (Houtman et al., 2007; Pollmann et al., 2006), whereas EML2 can act as a microtubule destabilizer reducing growth rates and promoting catastrophe (Eichenmuller et al., 2002).

The key question with regards to the biochemical activity of EMLs is how they influence the dynamic properties of microtubules at the molecular level. Structure-function studies with both sea urchin EMAP and mammalian EMLs have revealed the presence of two distinct domains, one that binds to polymerized microtubules and one that binds to soluble α/β-tubulin heterodimers (Richards et al., 2014; Richards et al., 2015). In this respect, they resemble the chTOG/XMAP215 family of microtubule polymerases that associate with microtubules via a disordered basic region, whilst binding to tubulin heterodimers via multiple TOG domains (Akhmanova and Steinmetz, 2015). The combination of these two properties within a single protein, together with their preference for plus ends, allows them to stimulate microtubule growth and prevent catastrophes by processively tracking plus ends and catalysing incorporation of tubulin dimers. The presence of multiple TOG domains increases the local concentration of tubulin heterodimers also promoting growth and it is intriguing that the human EMLs, through trimerization or having multiple WD domains in a single polypeptide, could have the same effect. Hence, it is attractive to speculate that this could explain how EML4 acts to stabilize microtubules. However, there is no evidence to date that EML4 concentrates at plus ends. Equally, it would not explain how EML2 promotes microtubule destabilization unless this reflects a subtle change in equilibrium between a conformation that promotes assembly and one that promotes disassembly. It is also undoubtably true that in the 'busy' confines of the microtubule cytoskeleton, some of the consequences of EML proteins in regulating

microtubule dynamics will arise from competition with other microtubule stabilization and destabilization factors.

Although the molecular details remain elusive, there is sufficient evidence that EMLs contribute to microtubule organization to expect them to have roles in both differentiated and proliferating cells (Figure 3). First of all, the pronounced expression of EMLs in the nervous system suggests a functional significance in microtubule dependent processes in neuronal cells. They may be important for maintaining the particular architecture of these cells or they may have specific roles in mechanical or sensory signal transduction. In *C. elegans,* expression of ELP-1 in mechanoreceptor and ciliated neurons suggests a role in mechanotransmission and this is backed-up by observing reduced touch sensitivity in cells lacking ELP-1 (Hueston et al., 2008). Meanwhile, in muscle cells, ELP-1 may promote force generation by enabling attachment of adhesion complexes on the cell surface to the underlying microtubule network (Hueston et al., 2008). In flies, the ELP-1 (DCX-EMAP) protein localizes to ciliated neurons in the auditory organ of the fly and insertional mutants have mechanosensation defects, including deafness and uncoordinated movement (Bechstedt et al., 2010). Hence, in both these organisms, there is evidence for mechanosensory roles, including in ciliated cells, although the mechanisms remain far from clear. Most differentiated epithelial cells in vertebrates possess a primary cilium that contributes to detection of various external stimuli, including chemicals, movement and light (Ishikawa and Marshall, 2011). However, there is no evidence to date implicating the mammalian EMLs in cilia; for example, they are not present in ciliary proteomes or transcriptomes regulated by ciliary-specific transcription regulators, such as RFX or FoxJ1 (Choksi et al., 2014). There is also no evidence for localization of EMLs to ciliary, or centriolar, microtubules, both of which exhibit increased stability and post-translational modifications such as acetylation and glutamylation (Janke and Bulinski, 2011). This lack of localization to ciliary or centriolar microtubules might suggest that the mammalian EMLs are not required for assembly of highly stabilized microtubule structures nor for intraflagellar trafficking. On the other hand, they may have specific mechanosensory roles in restricted tissue types that have yet to be identified.

There is good evidence that EMLs also play a role in proliferating cells and particularly during cell division. During mitosis the microtubule cytoskeleton undergoes a dramatic reorganization with microtubules becoming short and highly dynamic in contrast to the long, relatively stable microtubules characteristic of interphase cells (Heald and Khodjakov, 2015). This change is regulated

at least in part via differential binding of MAPs. EML3 colocalises with the mitotic spindle and midbody microtubules in HeLa cells and is important for metaphase chromosome alignment (Tegha-Dunghu et al., 2008). As well as being hyperphosphorylated in mitosis, EML4 is localized to the mitotic spindle and its depletion inhibits cell proliferation (Brisch et al., 1996; Pollmann et al., 2006). EML4 is required for organization of the mitotic spindle and specifically for the proper attachment of spindle microtubules to kinetochores in metaphase. This function seems to involve the recruitment of the nuclear distribution gene C (NuDC) protein to the mitotic spindle (Chen et al., 2015). Study of EML1 function using HeCo mice, a spontaneous model of a neurodevelopment disorder, suggests that EML1 may contribute to spindle orientation (Kielar et al., 2014). Unlike in wild-type brains, cells at the ventricular lining of mutant animals exhibited a reduced frequency of vertically oriented spindles. The authors proposed that the consequence of this was the inappropriate release of cells from the ventricular lining that retained progenitor markers; the presence of these ectopic cells in the white matter are the cause of neuronal dysfunction. The question though of how EMLs contribute to spindle orientation, as well as spindle dynamics and chromosome capture, remains to be addressed.

While interaction of EMLs with mitotic kinases may reflect regulation of EML function by phosphorylation, it is possible that EML proteins may act as scaffolds to localize mitotic kinases to microtubules. Indeed, besides direct regulation of microtubule dynamics by the EMLs themselves, it is worth more generally considering the potential role of EMLs in recruiting other regulators of microtubule dynamics to the microtubule cytoskeleton in both interphase and mitosis.

EMLs in human disease

Genetic defects involving EMLs have been associated with neuronal disorders and cancer. However, the first suggestion of a disease link came when the EML1 gene was mapped to a locus on chromosome 14 that was responsible for Usher syndrome type 1, in which patients suffer from deafness and blindness (Eudy et al., 1997). These symptoms are typical of syndromic ciliopathies, inherited disorders that result from defects in primary cilia (Badano et al., 2006). Primary cilia, as indicated above, are microtubule-based organelles raising the possibility that EML1 may represent a ciliopathy disease gene. However, causative mutations in EML1 responsible for Usher syndrome type 1 have yet to be identified and there is no evidence for localization of EML1 to either primary or motile cilia. Mutations in EML1 on the other hand have more recently been shown to lead to a developmental brain disorder in both humans and mice (Kielar et al., 2014). Specifically, point

mutations in EML1 that most likely disrupt folding of the TAPE domain cause a null phenotype that manifests as neuronal heterotopia. This condition involves the presence of misplaced neural progenitor cells in the white matter of the brain that potentially arise as a result of defects in spindle orientation in the neocortex. Epilepsy is one symptom of neuronal heterotopia, and as it happens unusually high expression of EML5 is detected in the anterior temporal neocortex of patients with intractable epilepsy (Sun et al., 2015). In this latter case though it remains to be seen whether EML5 expression contributes to the disease phenotype.

Particularly exciting is the finding that EMLs are present in oncogenic fusion proteins in human cancer. EML1-ABL1 fusions were first identified in T-cell acute lymphoblastic leukaemia, while EML4-ALK fusions were subsequently identified in lung, breast and colorectal cancers (De Keersmaecker et al., 2005; Lin et al., 2009; Rikova et al., 2007; Soda et al., 2007). The fusions have the catalytic domain of the ABL or ALK tyrosine kinases at the C-terminal end of the protein joined to variable amounts of the N-terminal region of the EML protein (Figure 1). All fusions have at least the coiled-coil motif of the EML and there is good reason to believe that this promotes constitutive activation of the tyrosine kinase through oligomerization and trans-autophosphorylation. However, in the case of non-small cell lung cancer patients, multiple different EML4-ALK variants have been identified in which the breakpoint can lead to substantially different amounts of the EML4 protein being present. At first sight this may not seem particularly important. However, there is emerging evidence that the position of the breakpoint can be crucial both in terms of progression of disease and response to particular treatments (Bayliss et al., 2016).

Future perspectives

Despite identification of the EMLs as abundant and conserved MAPs three decades ago, our journey to understand their mechanisms of action and biological roles has only just begun. Yet what we have learnt to date reinforces the essential roles that these proteins are likely to have in cell and tissue organization. Clearly, there is a need for detailed biochemical studies in purified systems to get to grips with how EML proteins regulate MT dynamics. Equally, it is imperative to know how they interact and cooperate with other MAPs, and how they respond to the complex tubulin 'code' created by tubulin isotype expression and post-translational modification (Janke and Bulinski, 2011). In terms of studying their role in interphase and mitotic events, interpreting the data will be complicated by the fact that the human proteins can exhibit hetero-oligomerization (Richards et al., 2015), and there is the potential for redundancy and adaptation by alternative splicing within the

family. The discovery of EML1 mutations in a neurodevelopmental disease makes one suspect that EML mutations are going to underlie other diseases, including not only neuronal conditions but also perhaps ciliopathies. In this regard, studies of knockout animals should be highly informative in relation to roles in developmental and tissue-specific processes. Finally, there is the exciting opportunity to use our growing knowledge of the biology of EMLs to consider novel therapeutic approaches for cancers driven by EML fusion proteins. The appreciation through structural analysis of the TAPE domain that oncogenic fusions that disrupt ordered domains are heavily dependent upon chaperones for stability (Richards et al., 2014), has justified testing chaperone inhibitors not only against EML4-ALK positive tumours but other cancers driven by inherently unstable fusion proteins. Moreover, the demonstration that the fusion breakpoint can influence stability and therapeutic response strengthens the argument for stratified approaches to cancer treatment. Furthermore, the involvement not only of EMLs but also other MAPs in oncogenic fusions, such as FGFR3-TACC3 (Williams et al., 2013), will lead to new rationales for more targeted use of the well-proven microtubule poisons in future cancer treatment.

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FIGURE LEGENDS

Figure 1. EML protein organization

A schematic cartoon of the sea urchin EMAP, the six human EML proteins and known oncogenic EML fusion proteins (two variants for EML4-ALK) is shown. A number of different splice variants exist for the human EMLs with the longest known variant shown here; synonyms of the human proteins are also indicated. Asterisks on EML1 indicate the position of mutations identified in two families with neuronal heterotopia; one family had heterozygous mutations of R138C and T243A, and the other had a homozygous mutation of W225R. The position of the breakpoints in the oncogenic fusion proteins is indicated with an arrowhead. Domains shown include coiled-coil (purple), TAPE (green), SH3 (orange), SH2 (blue) and kinase domain (red). aa, amino acids.

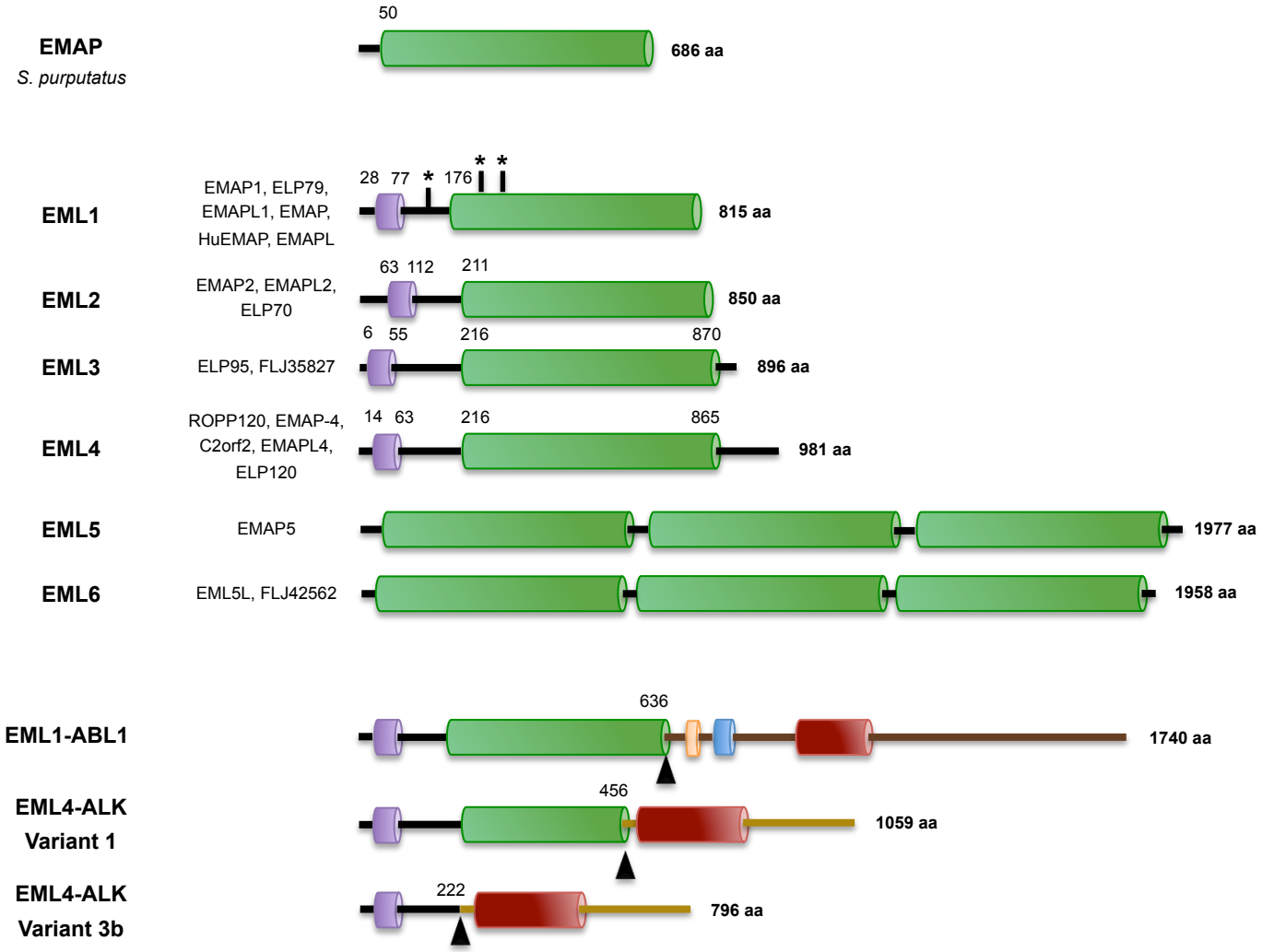
Figure 2. Structured domains of the EML protein family

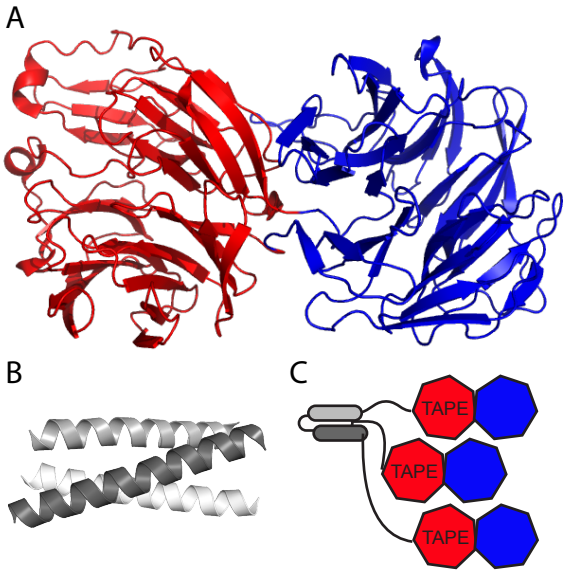
A. Crystal structure of the C-terminal, TAPE domain of EML1 (PDB code 4CI8). **B**. Crystal structure of the N-terminal, coiled coil, trimerisation domain (TD) of EML4 (PDB code 4CGC). **C**. Cartoon of the molecular architecture of human EML1-4 to show how the TD brings three copies of the TAPE domain into close proximity.

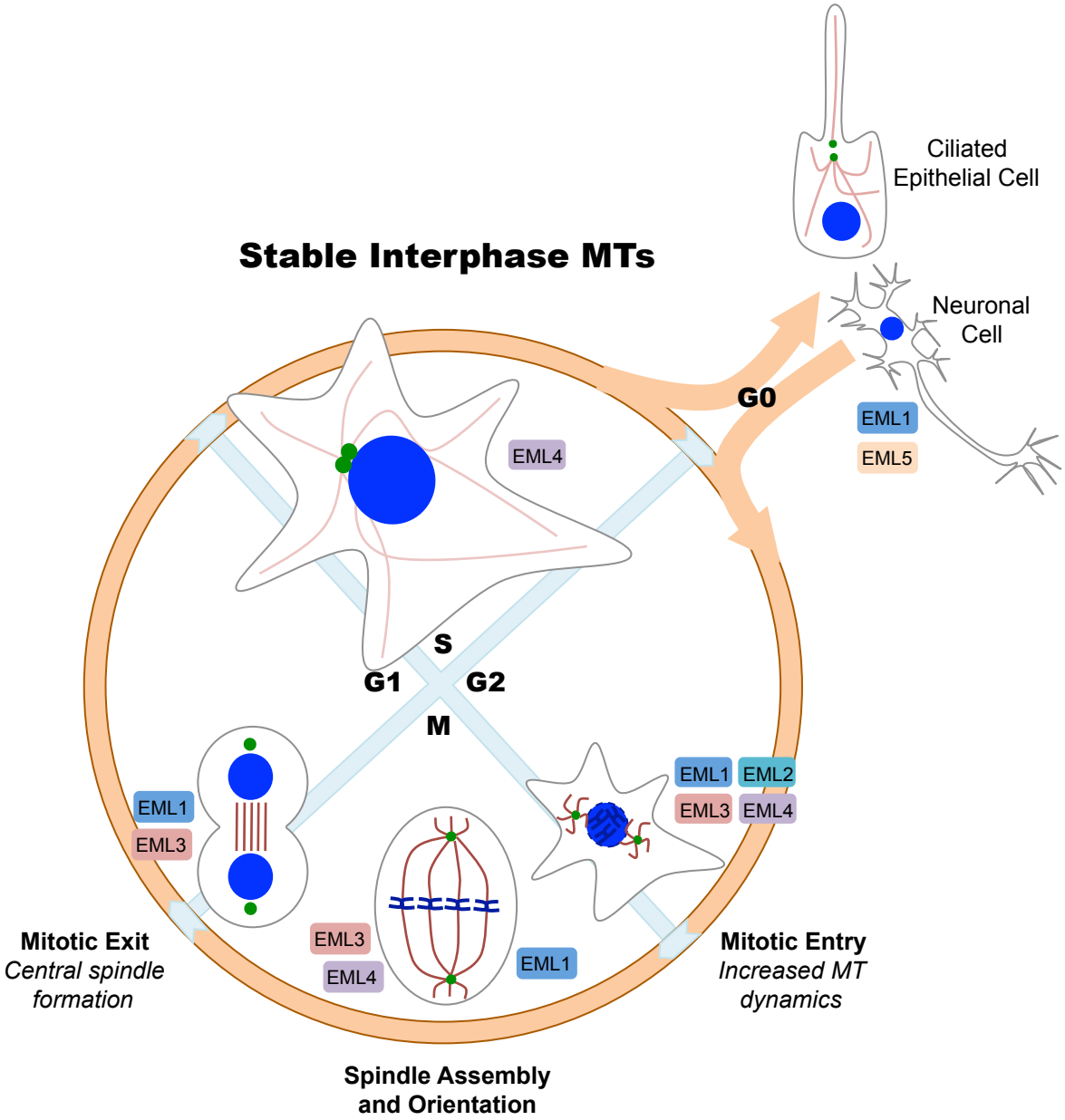
Figure 3. Potential functions of EML proteins in cell cycle progression

Schematic representation of the cell cycle indicating microtubule-dependent processes in which EML proteins are thought to participate. Although specific functions remain unclear, EML1-4 may well regulate the changes in microtubule (MT) dynamics required for the dramatic reorganization of the cytoskeleton that occurs as cells progress from interphase into mitosis. Evidence from localization and functional analyses suggest specific roles for EMLs 1, 3 and 4 in mitotic spindle organization and mitotic exit, while there may be mechanosensory functions yet to be defined in differentiated neuronal and ciliated epithelial cells.

Figure 1


EMAP
S. purputatus
50
686 aa
EML1
EMAP1, ELP79, EMAPL1, EMAP, HuEMAP, EMAPL
28 77 176
815 aa
EML2
EMAP2, EMAPL2, ELP70
63 112 211
850 aa
EML3
ELP95, FLJ35827
6 55 216 870
896 aa
EML4
ROPP120, EMAP-4, C2orf2, EMAPL4, ELP120
14 63 216 865
981 aa
EML5
EMAP5
1977 aa
EML6
EML5L, FLJ42562
1958 aa
EML1-ABL1
636
1740 aa
EML4-ALK Variant 1
456
1059 aa
EML4-ALK Variant 3b
222
796 aa


A
B
C
TAPE
TAPE
TAPE


Ciliated
Epithelial Cell
Neuronal
Cell
EML1
EML5
Stable Interphase MTs
G0
EML4
S
G1
G2
M
EML1
EML3
EML1
EML2
EML3
EML4
EML3
EML4
EML1
Mitotic Exit
Central spindle
formation
Mitotic Entry
Increased MT
dynamics
Spindle Assembly
and Orientation
Dynamic Mitotic MTs