The role of clathrin in mitotic spindle organisation

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Summary

Clathrin, a protein best known for its role in membrane trafficking, has been recognised for many years as localising to the spindle apparatus during mitosis, but its function at the spindle remained unclear. Recent work has better defined the role of clathrin in the function of the mitotic spindle and proposed that clathrin crosslinks the microtubules (MTs) comprising the kinetochore fibres (K-fibres) in the mitotic spindle. This mitotic function is unrelated to the role of clathrin in membrane trafficking and occurs in partnership with two other spindle proteins: transforming acidic coiled-coil protein 3 (TACC3) and colonic hepatic tumour overexpressed gene (ch-TOG; also known as cytoskeleton-associated protein 5, CKAP5). This review summarises the role of clathrin in mitotic spindle organisation with an emphasis on the recent discovery of the TACC3–ch-TOG–clathrin complex.

Key words: Clathrin, TACC3, ch-TOG (CKAP5), Mitotic spindle, Microtubule

Introduction

Membrane trafficking and mitosis are two major areas of cell biological research. Investigators in the first area are interested in questions such as how do cells internalise nutrients from the extracellular medium, how are cell surface receptors downregulated and how are lysosomes formed. Researchers in the second area would like to determine how the mitotic spindle ensures chromosomes are shared equally between the two daughter cells. At first glance these two areas appear distinct and unrelated. However, if we look more closely, we find that some proteins are involved in both processes and arguably the best understood example of such a protein is clathrin (Royle, 2011; Scita and Di Fiore, 2010).

Clathrin is best known for its role in membrane trafficking but in recent years it has emerged that clathrin has another function that occurs during mitosis (Royle et al., 2005). In non-dividing cells, clathrin forms a coat around membranes that are to be moved from one part of the cell to another (Brodsky et al., 2001). During mitosis, a proportion of clathrin becomes localised to the mitotic spindle where it has been proposed to crosslink the microtubules (MTs) comprising the kinetochore fibres (K-fibres) (Booth et al., 2011; Royle et al., 2005). This function is unrelated to its role in membrane trafficking. Recently, it has become clear that clathrin carries out its mitotic function in a complex together with transforming acidic coiled-coil protein 3 (TACC3) and colonic hepatic tumour overexpressed gene (ch-TOG; also known as cytoskeleton-associated protein 5, CKAP5) (Booth et al., 2011; Fu et al., 2010; Hubner et al., 2010; Lin et al., 2010). TACC3 and ch-TOG are two major spindle proteins with important roles in mitosis (Al-Bassam and Chang, 2011; Peset and Vernos, 2008).

The aims of this Commentary are to describe our current understanding of the role of clathrin in mitotic spindle organisation, to discuss future directions and key questions for future work, and to present the work in a way that can be understood by investigators interested in membrane trafficking or mitosis who might be unfamiliar with some concepts from the ‘other side of the fence’.

A quick guide to clathrin

Clathrin is best known for its role in membrane trafficking. Clathrin-coated vesicles (CCVs) are formed at the plasma membrane and the trans Golgi network (TGN) where they are involved in endocytosis and transport of secretory cargo, respectively (Brodsky et al., 2001). CCVs were first described in the 1960s in electron micrographs of several cellular systems, including, most famously, mosquito oocytes that uptake significant amounts of yolk protein (reviewed in Hirst and Robinson, 1998). Later, CCVs were purified from brain and their main protein constituent was identified and termed clathrin after the lattice-like (or clathrate) structures that it forms (Pearse, 1975) (Fig. 1A,B).

The assembly unit of the clathrin coat is a triskelion: a three-legged assembly of three clathrin heavy chains (CHCs), each with an associated light chain (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981) (Fig. 1C,D). The three CHCs are noncovalently trimerised at their C-termini (Nathke et al., 1992), and the light chains are bound very tightly (Winkler and Stanley, 1983). In cells, triskelia are formed upon synthesis, and monomers of clathrin heavy chain or light chain-free heavy chains are not readily observed (Brodsky, 1985; Hoffmann et al., 2010).

In humans there are four clathrin genes that encode two light (~25 kDa) and two heavy (~190 kDa) chains. The clathrin light chains a and b are encoded by the genes *CLTA* and *CLTB* on chromosome 9p13 and 5q35, respectively. The ubiquitously expressed clathrin heavy chain, referred to as CHC17 or CHC, is encoded by the gene *CLTC*, located at 17q11-qter. There is a CHC paralogue, referred to as CHC22, which is muscle-specific and is encoded by the gene *CLTCL1* at 22q11.21. However in most cells in the body, clathrin triskelia are trimers of CHC17 with a random distribution of clathrin light chain a and b. Clathrin is highly expressed in all cells with an estimated 500,000 triskelia per cell (Doxsey et al., 1987; Goud et al., 1985). Clathrin arose early in the evolution of eukaryotic life and is well conserved. For example, human CHC shows ~99% identity with CHCs from mammals and birds, ~70% homology with worms and >50% identity with CHC from yeast. In higher organisms, clathrin is an essential gene (Royle, 2006).

We have a good structural knowledge of clathrin, as summarised in Fig. 1. Several domains of clathrin have been studied by X-ray crystallography and an atomic model of a complete clathrin lattice has been built using this structural information together with data

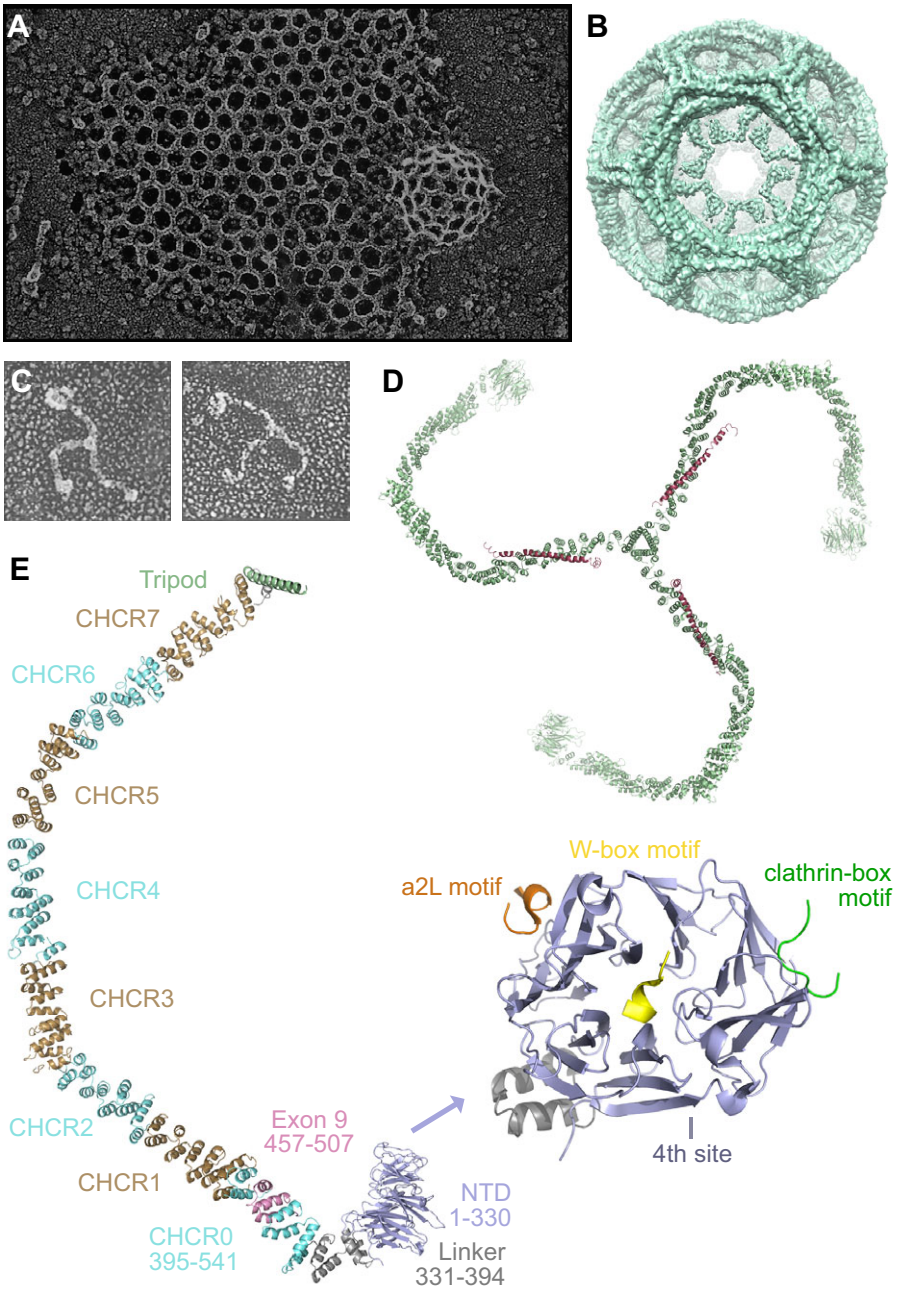

Fig. 1. The structure of clathrin. (**A**) A clathrin lattice and budding vesicle at the plasma membrane as seen by deep-etch electron microscopy. © 1987 Rockefeller University Press. Originally published in *J. Cell Biol*. **105**, 1999-2009 (Heuser et al., 1987). (**B**) Cryoelectron microscopy map of a clathrin-coated vesicle. The image is generated from EMDataBank ID EMD-5119 using the UCSF Chimera program. (**C**) Two examples of isolated clathrin triskelia viewed using electron microscopy. ©1988 Rockefeller University Press. Originally published in *J. Cell Biol*. **107**, 877-886 (Heuser and Keen, 1988). (**D**) Molecular model of a clathrin triskelion. CHCs are green, the central portion of the clathrin light chain is shown in dark orange. The image is generated from PDB files 1XI4 and 3LVG using Pymol (Fotin et al., 2004; Wilbur et al., 2010). (**E**) Structural features of a single CHC. The NTD (residues 1–330, blue), linker (grey), CHCR0–CHCR7 (alternating blue and brown), hairpin (grey) and tripod helix (green) are shown. Note the region encoded by exon 9 of CHC (residues 457–507, pink) is a subregion of the TACC3-binding region. The enlarged structure is a view of the 'foot' region of CHC (residues 1–363) with the approximate positions of the various interacting motifs shown: clathrin-box motifs LΦXΦ[DE] (green), W-box motifs PWXXW (yellow), arrestin-2L site [LI][LI]GXL (dark orange). The position of the fourth interaction site, for which the motif is unknown, is also indicated. The image is generated from PDB files 1UTC, 1C9I and 3GD1 using Pymol.

from electron microscopy of purified CCVs (Fotin et al., 2004). The globular N-terminal domain (NTD) of CHC comprises a seven-bladed β-propeller (ter Haar et al., 1998). Four interaction sites on the NTD that are important for endocytic function have also been described (Kang et al., 2009; Miele et al., 2004; ter Haar et al., 2000; Willox and Royle, 2011) and are important for interactions with adaptor proteins. Just C-terminal of the NTD, there is a short linker followed by eight CHC repeats (CHCRs) numbered CHCR0–CHCR7. Next, there is a short linker followed by the trimerisation domain, but the final C-terminal residues are unresolved (Fotin et al., 2004). The triskelion is often described as a leg, with the NTD being the foot. Interactions between CHCRs on adjacent clathrin triskelia regulate lattice formation. The light chain binds to the heavy chain at the region most proximal to the trimerisation domain (Fotin et al., 2004), and recent evidence indicates that the light chains could influence triskelion structure (Wilbur et al., 2010).

A key concept in the function of clathrin in membrane trafficking is its inability to bind membranes or cargo directly (Unanue et al., 1981). Instead, clathrin binds to adaptor proteins, which in turn can bind to membranes or to proteins destined for trafficking (Reider and Wendland, 2011). In interphase cells, assembled clathrin can be visualised in coated pits and vesicles at the plasma membrane, in the TGN and free in the cytoplasm (Fig. 2A). Some of these clathrin spots colocalise with adaptors, such as AP-2, that are involved in endocytosis at the plasma membrane, whereas another subset of clathrin colocalises with AP-1 at the TGN (Robinson, 2004). In addition, there are many other adaptors and accessory proteins involved in clathrin-mediated membrane traffic that form an extensive network of interactions, with clathrin as a hub interacting with many of these proteins (Traub, 2011). It is worth noting that accessory proteins for membrane trafficking are sometimes referred to as CLASPs (for clathrin-associated sorting

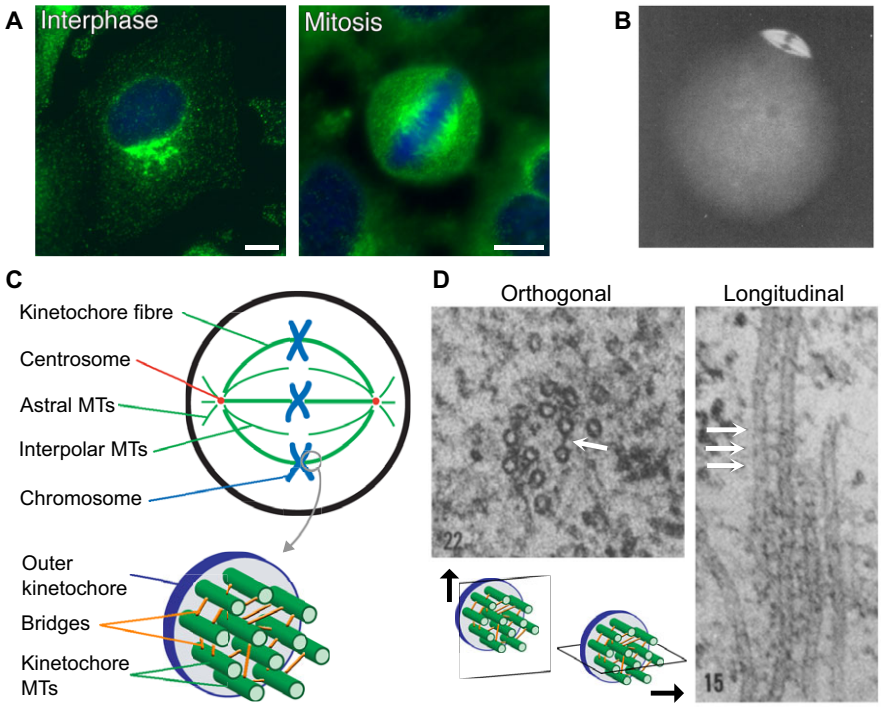

Fig. 2. Clathrin localisation during mitosis. (**A**) The subcellular distribution of clathrin in HeLa cells in interphase and mitosis. Clathrin was detected using the monoclonal antibody X22 (green) and DNA was stained with DAPI (blue). Scale bars: 10 µm. (**B**) Micrograph of clathrin as detected by indirect immunofluorescence on the second metaphase spindle in an unfertilised mouse egg. Reproduced with permission ©1985 Company of Biologists (Maro et al., 1985). (**C**) Schematic diagram of the MT organisation of the mitotic spindle at metaphase. The kinetochore (K)-fibre is a bundle of parallel MTs that extend from the spindle pole and terminate with their plus end at the kinetochore. Only 11 MTs are shown for clarity, bridges are represented as orange sticks. (**D**) Electron micrographs of inter-MT bridges in the mitotic spindle. Two views are seen: on the left, a fibre is sectioned orthogonally so that microtubules appear as circles; on the right, a fibre is sectioned in parallel with the spindle axis so that MTs are long stripes. Bridges are the electron-dense connections between microtubules marked by white arrows. A schematic diagram (inset) shows the sectioning orientation. ©1970 Rockefeller University Press. Originally published in *J. Cell Biol.* **54**, 438-444 (Hepler et al., 1970).

proteins) (Reider and Wendland, 2011), whereas in cell division research, CLASP refers to a family of MT-binding proteins [cytoplasmic linker protein (CLIP)-associated proteins] (Al-Bassam and Chang, 2011).

The mitotic function of clathrin: background

The first clues for a mitotic function for clathrin can be traced back to studies performed more than 30 years ago that examined the subcellular distribution of clathrin using immunofluorescence. Maro et al. found that clathrin is located on the second metaphase spindle in unfertilised mouse eggs (Maro et al., 1985) (Fig. 2B). Much later, the subcellular localisation of clathrin during mitosis was studied using several different antibodies and various cell lines (Okamoto et al., 2000). In addition, clathrin was identified as a spindle protein by two untargeted methods. First, a gene trap intended to find nuclear proteins revealed that clathrin can be localised to spindles (Sutherland et al., 2001). Second, clathrin was identified as being a component of purified mitotic spindles by mass spectrometry (Mack and Compton, 2001). For technical reasons, that study only found a subset of the total spindle proteome (Sauer et al., 2005), but, interestingly, clathrin and the factors that recruit it to the spindle were present in this subset (see below). With hindsight, we can see clearly that clathrin is a bona fide spindle protein; however, at the time there were several reasons why these results were not pursued further. Uncertainty over antibody specificity was a worry with the original study (Maro et al., 1985). In addition, the presence of clathrin at the spindle could have been a contaminant – especially as it is a large protein that frequently appears erroneously in mass spectrometry studies (Trinkle-Mulcahy et al., 2008).

With no known role at the mitotic spindle, the simplest conclusion was that the presence of clathrin at the spindle reflected a large store of coated vesicles, a pervasive idea in the field. Seminal studies of mitotic cell morphology did report coated vesicles among microtubules of the spindle but did not quantify whether they were enriched at this location (Robbins and Jentzsch, 1969). Attempts to label membranes in the spindle generally show that, whereas membranes might be present, they are not enriched at the spindle (Waterman-Storer et al., 1993), although this is somewhat dependent on the cell line. Electron microscopy studies have confirmed a relative lack of endoplasmic reticulum and/or nuclear envelope (Puhka et al., 2007), Golgi-derived membranes (Lucocq et al., 1989) and coated vesicles (Tooze and Hollinshead, 1992) within the spindle itself. The spindle apparatus, however, is surrounded by membranes, and it has been proposed that these membranes are important for spindle function (Zheng, 2010). If a large store of CCVs were present, it would be expected that adaptors would be found together with clathrin, but they are not (Royle et al., 2005). Finally, immunogold labelling of clathrin shows that clathrin is not associated with membranes but rather with MTs (Booth et al., 2011; Royle et al., 2005). It is therefore unlikely that the clathrin that is located at the spindle is associated with membranes.

The subcellular localisation of clathrin during mitosis that was first observed with antibodies was revisited recently using GFP-tagged clathrins in parallel with antibody staining (Royle et al., 2005). That study confirmed that clathrin is colocalised with the MTs of the spindle apparatus early in mitosis. Clathrin is localised to the K-fibres of the spindle and there is no obvious localisation to astral, interpolar or midzone MTs as the cell goes through mitosis. The association with MTs becomes less obvious in telophase and cytokinesis (Royle et al., 2005). Evidence for a functional role of clathrin at the spindle came from studies using RNA interference (RNAi) against CHC, which caused a delay in mitosis as a result of defects in chromosome congression at the metaphase plate (Royle et al., 2005). The congression defects stem from a destabilisation of the K-fibres of the mitotic spindle (Royle et al., 2005). In the next section, the mechanism by which clathrin stabilises K-fibres will be considered.

The mitotic function of clathrin is apparently distinct from its role in membrane trafficking. This might seem obvious given that the clathrin at the spindle is not associated with membranes and

that the mitotic function of clathrin occurs when clathrin-mediated endocytosis is inhibited (Warren, 1993). However, the function of clathrin in membrane trafficking is so well established that this point needed to be thoroughly investigated. The best evidence for the distinction between the membrane trafficking and mitotic function of clathrin came from experiments using CHC mutants that were found to be capable of fulfilling only one function but not the other (Blixt and Royle, 2011; Hood and Royle, 2009; Royle and Lagnado, 2006).

Mitotic spindle structure: role for non-motor proteins in fibre stability

In mammalian cells, the spindle apparatus is composed of three classes of MT: astral, interpolar and kinetochore (Fig. 2C). Astral MTs radiate from the spindle pole to the cell cortex, whereas interpolar MTs run along most of the way from the spindle pole to the opposing pole (Mastronarde et al., 1993; McDonald et al., 1992). Kinetochore MTs connect the spindle pole with the kinetochore and are responsible for chromosome movement (McDonald et al., 1992; Rieder, 2005). Numerous kinetochore MTs are bundled together to form a K-fibre. The number of MTs in such a fibre depends on the size of the kinetochore, but not necessarily on the size of the chromosome it has to move (Rieder, 1982), and range from 20–40 MTs in humans to only a few in yeast (Ding et al., 1993).

In many cellular systems, bundles of MTs are crosslinked by electron-dense inter-MT bridges (Stephens and Edds, 1976) that are composed of motor proteins or non-motor microtubule-associated proteins (MAPs). A good example is in dendrites, in which the parallel MT bundles are crosslinked by single MAP2 molecules, conferring an MT spacing of ~62 nm, and those in the axon which are crosslinked by tau resulting in a shorter inter-MT distance of ~20 nm (Chen et al., 1992; Kim et al., 1979). In the mitotic spindle, MTs of K-fibres are similarly crosslinked by bridges (Hepler et al., 1970; Mastronarde et al., 1993; McDonald et al., 1992; Witt et al., 1981), which are probably also formed by non-motor structural proteins (Manning and Compton, 2008; Peterman and Scholey, 2009) (see Fig. 2D for examples). However, unlike the inter-MT bridges in neuronal cells, which are formed by single MAPs, the bridges in K-fibres appear to be of various lengths, suggesting that a number of different proteins mediate the crosslinking (Booth et al., 2011).

The ‘bridge hypothesis’ of clathrin function

As described above, depletion of clathrin, a protein found on K-fibres, results in K-fibre destabilisation (Royle et al., 2005). So, what is the mechanism by which clathrin stabilises K-fibres? There are two regions of clathrin that are important for its localisation at the spindle. The first is in the NTD (Royle et al., 2005; Royle and Lagnado, 2006) and the second is a stretch of 50 amino acids in the CHCR0 or ‘ankle’ region (Hood and Royle, 2009) (Fig. 1E). As these regions, which are closely located on one CHC, are spaced far away from each other in the clathrin triskelion, it has been proposed that clathrin could stabilise kinetochore fibres by physically cross-bracing adjacent MTs (Royle et al., 2005). This idea, the ‘bridge hypothesis’, is supported by experiments with CHC mutants in the context of cells depleted of endogenous CHC (Royle and Lagnado, 2006); CHC mutants that are unable to either trimerise or to bind to the spindle (i.e. unable to act as a bridge) do not rescue the mitotic defects caused by RNAi against CHC. Moreover, trimeric or dimeric CHCs that contain the spindle-interaction sites on the NTD and the ankle but lack most of the leg region are capable of functional rescue (Blixt and Royle, 2011; Royle and Lagnado, 2006). However, although these experiments provide support for the bridge hypothesis, they are not a direct test.

Clearly, if clathrin acts as an inter-MT bridge in K-fibres, upon its depletion, the bridges should disappear. Using electron microscopy, it has been found that ~40% of inter-MT bridges are missing from clathrin-depleted K-fibres compared with those from control RNAi experiments (Booth et al., 2011). Importantly, it was found that inter-MT bridges in K-fibres exist in several populations of different lengths. The ~40% decrease in bridges in clathrin-depleted K-fibres can be almost entirely explained by the loss of the shortest inter-MT bridges (Fig. 2E). A second test of whether clathrin is indeed an inter-MT bridge is whether they are labelled with anti-clathrin antibodies; Booth et al. observed anti-clathrin immunogold labelling of inter-MT bridges, which could be clearly distinguished from tubulin labelling (Booth et al., 2011), providing strong evidence that clathrin indeed acts as an inter-MT bridge.

The simplest model for the function of inter-MT bridges, such as clathrin, is that they stabilise K-fibres by physically cross-bracing them (Rieder, 1982). However, clathrin-depleted K-fibres also exhibit MT loss, and, as a result, the fibres are thinner and there is more space between MTs (Booth et al., 2011). Therefore, destabilisation of K-fibres could be the result of the loss of bridges and/or of MTs. It has been suggested that bridge loss precedes MT loss and that bridges might protect from MT catastrophe (Booth et al., 2011). Further work is needed to determine the relative contribution of physical crosslinking and prevention of MT catastrophe to K-fibre stabilisation.

Clathrin is different from the other non-motor MAPs that have been shown to crosslink MTs. Besides the trimeric structure of clathrin, rather than the bipolar MAP structure, most MAPs, such as protein regulator of cytokinesis 1 (PRC1) are able to bind to MTs directly (Peterman and Scholey, 2009). Clathrin has no known microtubule-binding domains, and it does not bind to MTs that have been assembled in vitro unless other mitotic proteins are added (Booth et al., 2011), suggesting that other factor(s) are required to recruit clathrin to the mitotic spindle. This is a familiar concept of clathrin function. As mentioned above, during interphase, clathrin requires adaptor proteins to mediate membrane or cargo interactions for membrane trafficking, and it appears that, during mitosis, clathrin also needs adaptor protein(s) to be able to bind to kinetochore MTs.

Interaction of clathrin with TACC3 and ch-TOG

The question of which protein(s) clathrin associates with at the spindle has been answered independently by four groups (Booth et al., 2011; Fu et al., 2010; Hubner et al., 2010; Lin et al., 2010). Using a variety of approaches and systems, these groups found that clathrin is in a complex with TACC3 and ch-TOG at the mitotic spindle. These two proteins are well-characterised spindle components with established roles in MT growth and stabilisation (Barr and Gergely, 2007). Schematic diagrams of TACC3 and ch-TOG are shown in Fig. 3A, and molecular details of their homologues is presented in Table 1. Previously, ch-TOG has been described to be important for MT outgrowth from the centrosome, and TACC proteins were thought to be important for loading ch-TOG onto spindle MTs (Barr and Gergely, 2008; Gergely et al., 2003; Kinoshita et al., 2005; Lee et al., 2001). Below, I will discuss that, although there is consensus regarding the composition of the TACC3–ch-TOG–clathrin complex and the importance of TACC3

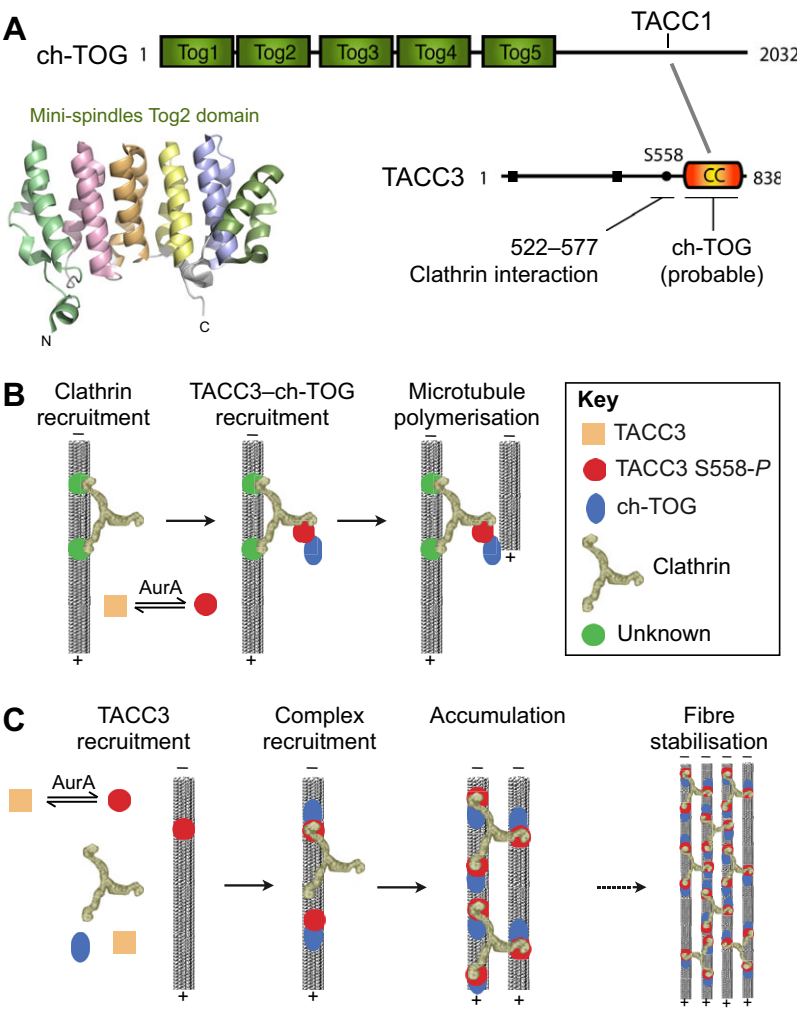

Fig. 3. Models for the interaction of clathrin with TACC3 and ch-TOG at the spindle. (**A**) Schematic diagrams of ch-TOG and TACC3. Human ch-TOG has five TOG domains (green) that bind to soluble tubulin dimers. The structure of the second TOG domain from Mini-spindles (Msps), the *Drosophila* ch-TOG homologue, is shown (PDB code 2QK2). TACC3 has several Aurora-A phosphorylation sites (black boxes) and a C-terminal coiled-coil domain (CC, orange). The interaction between TACC3 and ch-TOG probably occurs between the CC domain of TACC3 and ch-TOG C-terminal domain, the site of TACC1 interaction (Peset and Vernos, 2008). (**B**) The 'clathrin recruits TACC3' model. In this model, clathrin is initially recruited to microtubules by an unknown protein. Phosphorylated TACC3 binds clathrin and is therefore recruited to the spindle. TACC3 also binds ch-TOG, and the MT polymerisation activity of ch-TOG enhances spindle stability (Fu et al., 2010; Hubner et al., 2010; Lin et al., 2010). (**C**) The inter-MT bridge model. In this model, Aurora-A (AurA)-phosphorylated TACC3 is the initial recruitment factor. Clathrin can bind to TACC3–ch-TOG complexes, which might be located on adjacent microtubules allowing clathrin to crosslink microtubules. The resulting TACC3–ch-TOG–clathrin complex is more stable owing to multiple interactions and, therefore, accumulates on the spindle (Booth et al., 2011). Microtubules, triskelia and the distances between them are shown to scale (based on electron microscopy experiments).

phosphorylation by the kinase Aurora-A in regulation of the complex, there is some disagreement over the mechanism by which the complex binds to MTs and the role phosphorylation might have in this event (Fu et al., 2011; Hood and Royle, 2011).

The TACC3–ch-TOG–clathrin complex

Using quantitative proteomics, clathrin was identified as a binding protein for TACC3 (Hubner et al., 2010), and ch-TOG was found to be associated with clathrin and TACC3 (Hubner et al., 2010). Lin et al. showed that clathrin can interact with a minimal 50-residue fragment of TACC3 (Lin et al., 2010). The corresponding region on clathrin for the interaction with TACC3 in vitro was found to be residues 331–542 (Fig. 1E), a region that contains the previously identified site required for recruitment of clathrin to the spindle (Hood and Royle, 2009). The interaction between clathrin and the *Xenopus* TACC3 homologue, maskin, demonstrates that this functional complex is conserved in frogs (Fu et al., 2010). This study also showed that the complex is under the regulation of importin-β and RanGTP. Booth et al. have also isolated the native clathrin complex from purified mitotic spindles from human cells and found it contains a complex of TACC3, ch-TOG and clathrin (Booth et al., 2011).

Depletion of TACC3 results in strikingly similar mitotic defects (Gergely et al., 2003) to those observed for clathrin depletion (Royle et al., 2005). Lin et al. nicely demonstrated that the mitotic phenotypes observed with RNAi against either clathrin or TACC3 are equivalent to the depletion of both proteins (Lin et al., 2010). Using *Xenopus* egg extracts, Fu et al. have shown that depletion of either TACC3 or clathrin results in defective spindle assembly (Fu et al., 2010). These results suggest that both proteins work together in the same pathway and that independent mitotic functions for TACC3 and clathrin are unlikely (Hood and Royle, 2011). This finding is underscored by the observations that TACC3-depleted K-fibres have fewer inter-MT bridges and that the extent of bridge loss is very similar to that seen in CHC-depleted K-fibres (Booth et al., 2011). Depletion of ch-TOG leads to mitotic defects that are much more severe compared with the effects of RNAi against TACC3 or clathrin (Booth et al., 2011; Gergely et al., 2003), suggesting that, although ch-TOG is part of the TACC3–ch-TOG–clathrin complex in spindles, it has additional independent functions.

Regulation by Aurora-A kinase

TACC3 is a well-characterised substrate for Aurora-A kinase (Barr and Gergely, 2007). Several TACC3 serine and threonine residues outside of its coiled-coil region are phosphorylated during mitosis, but, of these, only S25, T30, S402 and S558 are sensitive to inhibition of Aurora-A kinase (Kettenbach et al., 2011). The phosphorylation of TACC3 on residue S558 by Aurora-A, in particular, is thought to be crucial for localisation of TACC3 to the

Table 1. Details of members of the TACC3–ch-TOG–clathrin complex

Member	Composition	Human proteins	Homologues	Notes	Reference
TACC3	2 × 90 kDa subunits (monomer runs at 150 kDa on SDS-PAGE gels)	TACC3 (ERIC1) (838)	TACC3 (AINT) (mm, 630); TACC3 (Maskin) (xl, 931); TACC (dm, 1226); C7G031 (dd, 616); TAC-1 (2P40) (ce, 260); ALP7 (Mia1) (sp, 474)	TACC1 and TACC2 are expressed in mammals; there is no clear homologue of TACC3 in plants or *S. cerevisiae*, the best *S. cerevisiae* candidates are SLK19 (sc, 821) with weak homology, or Spc72 (sc, 622) owing to interaction with STU2; the TACC domain is best conserved and the NTD is variable	(Still et al., 2004)
ch-TOG	1 × 225 kDa subunit	CKAP5 (ch-TOG) (2032)	CKAP5 (mm, 2032); XMAP215 (xl, 2065), Msps (dm, 2050); MOR1 (at, 1978), CP224 (dd, 2013); Zyg-9 (ce, 1415); Alp14 (Dis1) (sp, 809); STU2 (sc, 888)	Higher eukaryotes have five TOG domains; Zyg-9 has three TOG domains and yeast homologues have two TOG domains	(Al-Bassam and Chang, 2011)
Clathrin	3 × (190 + 27 kDa) subunits	CLTC (CHC) (1675); CLTCL1 (CHC22) (1640)	CLTC (mm, 1675); cltc (xl, 1675); Chc (dm, 1678); AT3G11130 (at, 1705); chcA (dd, 1694); chc-1 (ce, 1681); chc1 (sp, 1666); CHC1 (sc, 1653)	The domain organisation of CHC is completely conserved down to yeast; homologues of CHC22 are present in mammals (a pseudogene in mice), birds, amphibians and fish	(Wakeham et al., 2005)
		CLTA (LCa) (248) CLTB (LCb) (229)	CLTA (mm, 216); Clta (xl, 203); clc (dd, 194); F4J5M9 (at, 258); Clc (dm, 219); clic-1 (ce, 226); clc1 (sp, 229); CLC1 (sc, 233)	Homologues of both LCa and LCb in mammals, birds, amphibians and fish; only LCa homologues are shown here; there is only a single light chain in flies, worms, slime mould and fungi; there are three light-chain-encoding genes in *Arabidopsis*	(Wakeham et al., 2005)

Any synonyms are given in parentheses, whereas numbers in parentheses refer to the number of amino acids of the longest isoform. Abbreviations: mm, mouse; xl, frog; dm, fruitfly; at, plant; dd, slime mould; ce, nematode; sp, fission yeast; sc, budding yeast.

spindle (Booth et al., 2011; Kinoshita et al., 2005; LeRoy et al., 2007; Lin et al., 2010); mutation of S558 to an alanine residue results in a TACC3 variant that is unable to localise properly to spindles. Similarly, treatment of cells with the selective Aurora-A kinase inhibitors MLN8054 or MLN8237 leads to the loss of TACC3 from spindles (Booth et al., 2011; Cheeseman et al., 2011; Hubner et al., 2010; LeRoy et al., 2007).

Several lines of evidence indicate that the phosphorylation of TACC3, probably at S558, is required for clathrin–TACC3 binding. First, clathrin does not interact with a TACC3 mutant where alanine residues replace S558 and two other serine residues (Hubner et al., 2010). Second, Aurora-A-mediated phosphorylation of the 50-residue TACC3 fragment, containing S558, is necessary in order to detect an interaction with clathrin (Lin et al., 2010). Finally, the interaction between clathrin and maskin is enhanced by Aurora-A kinase activity, with the most N-terminal Aurora-A phosphorylation site not required for the interaction (Fu et al., 2010).

Two models for binding of TACC3–ch-TOG–clathrin to MTs

Depletion of clathrin by RNAi reduces the amount of TACC3 on the spindle (Booth et al., 2011; Fu et al., 2010; Hubner et al., 2010; Lin et al., 2010), and this observation, together with the phosphorylation data, suggests a model in which clathrin recruits TACC3 to the mitotic spindle (Fig. 3B). Here, TACC3 phosphorylation is needed for TACC3 to be able to bind to clathrin. Spindle stability is then achieved by TACC3 recruiting ch-TOG to promote MT assembly and therefore stability.

The ‘clathrin recruits TACC3’ model explains some of the results, but not all. First, the majority (>80%) of clathrin is not at the spindle, whereas TACC3 is localised almost exclusively at the spindle. Thus, as a targeting protein, clathrin would not be very efficient and, in fact, on the basis of localisation, it would make more sense if TACC3 recruited clathrin to the spindle. Secondly, trimerisation-deficient CHCs cannot function in mitosis (Royle and Lagnado, 2006), but if clathrin simply recruited TACC3 to achieve spindle stability, we would not expect any mitotic defects with these mutants CHCs. It is also difficult to reconcile this model with the finding that TACC3–ch-TOG–clathrin complexes form inter-MT bridges. Third, as described above, clathrin cannot bind MTs, but TACC3 (or maskin) and ch-TOG are able to do so (Charrasse et al., 1998; O’Brien et al., 2005; Peset et al., 2005). Therefore the question arises why these proteins would require clathrin for their recruitment to MTs, and, if this is the case, which protein is responsible for recruiting clathrin to the spindle?

The native clathrin complex found on spindles contains only TACC3, ch-TOG and clathrin (Booth et al., 2011). Therefore, unless an unknown protein dissociated from the complex during purification, the factor recruiting the complex to MTs is one of these three proteins. Although depletion of clathrin reduces the amount of TACC3 and ch-TOG at the spindle (Booth et al., 2011; Lin et al., 2010), depletion of TACC3 (and to a lesser extent ch-TOG) also decreases the recruitment of the other two complex members (Booth et al., 2011). If the converse experiment is performed (i.e. TACC3 is overexpressed in mitotic cells), increased amounts of ch-TOG and clathrin are recruited to the spindle (Booth et al., 2011; Fu et al., 2010). However, overexpression of ch-TOG or clathrin has no effect on the distribution of the other complex members, and, furthermore, overexpression of clathrin quickly saturates its binding sites on the spindle and fills the cytoplasm (Booth et al., 2011). Thus, TACC3 appears to be the limiting factor

in the recruitment of the complex to MTs, suggesting that TACC3 recruits clathrin to the spindle, and not the other way around.

However, as depletion of any member of the TACC3–ch-TOG–clathrin complex affects the localisation of the other two, a simple 'single-step' serial recruitment model, whereby either clathrin recruits TACC3 or TACC3 recruits clathrin (Fu et al., 2011) is not the full story. Booth et al. therefore have proposed a two-step model in which the complex is recruited to MTs, where, after initial recruitment, further complexes accumulate (Fig. 3C). Such an accumulation is made possible by the triskelion structure of clathrin, which allows it to bind multiple TACC3 and ch-TOG molecules. If subcomplexes of TACC3 and ch-TOG are bound by different MTs, clathrin will be able to bridge between them. This model consolidates the known structures of the proteins involved, the ultrastructural data showing that TACC3–ch-TOG–clathrin complexes are inter-MT bridges and the observations from RNAi and overexpression studies. The model is, however, not without its problems and requires further refinement.

It has been proposed that phosphorylation of TACC3 by Aurora-A kinase is required for TACC3 to bind to MTs before the recruitment of ch-TOG and clathrin (Fig. 3C). There are several observations that suggest this is indeed the case. First, inhibition of Aurora-A kinase activity using Alisertib (MLN8237) after the spindle has assembled results in loss of TACC3 and clathrin from spindles that displays similar kinetics (Booth et al., 2011) and also initiates a loss of inter-MT bridges from K-fibres, which causes their subsequent destabilisation (Cheeseman et al., 2011). Second, non-phosphorylatable TACC3 mutants do not localise to spindles and, in cells expressing these mutants, clathrin is also not found on the spindle (Booth et al., 2011). An alternative view is that phosphorylation of TACC3 is only required for binding to clathrin and not for the interaction with MTs. For example, TACC3 phosphorylated at S558 was originally thought to be restricted to the centrosome (Kinoshita et al., 2005). However, recent work shows that the phosphorylated form of TACC3 is found along the length of the spindle MTs and that the original antibody erroneously detected an unrelated protein on centrosomes (Lin et al., 2010). It was also reported that chronic inhibition of Aurora-A kinase (16 hours of treatment with MLN8054) displaces TACC3 but not clathrin from the spindle (Hubner et al., 2010). However, the normal spindle morphology and intact clathrin–TACC3 binding under these conditions point to an incomplete inhibition of the kinase. There are some additional concerns about the phosphorylation-dependent recruitment of TACC3 to MTs that remain valid. First, there is evidence that maskin can bind to MTs independently of its phosphorylation (Fu et al., 2010; Kinoshita et al., 2005). Second, the interaction between clathrin and TACC3 requires, or at least is enhanced by, phosphorylation of TACC3 at S558 (Fu et al., 2010; Hubner et al., 2010; Lin et al., 2010). S558 cannot simultaneously act as both a MT-binding site and a clathrin-binding site. One possible solution to this apparent paradox is that the interaction between phosphorylated S558 of TACC3 and clathrin creates the MT-binding site.

Despite the many pieces of evidence described here, more work is needed to determine the precise conformation of the TACC3–ch-TOG–clathrin complex on MTs. The binding sites that govern the interactions between these proteins are currently poorly defined and many questions remain. For example, does the NTD of CHC bind to TACC3 or ch-TOG? Further insight into the function of the TACC3–ch-TOG–clathrin complex will come from detailing the interactions between complex members at high resolution. Furthermore, understanding the role of phosphorylation of TACC3 at S558 in the interactions with complex members and MTs is crucial to refining the current models.

Other proteins involved in mitotic function of clathrin

Clathrin-interacting proteins

Before the identification of the TACC3–ch-TOG–clathrin complex, two other proteins had been implicated as having a role in the function of clathrin during mitosis. First, the transcription factor Myb-related protein B (B-myb, also known as Mybl2) was found to be in a complex with clathrin and filamin in mitotic cells, and depletion of B-myb resulted in a loss of clathrin at the spindle (Yamauchi et al., 2008). However, neither B-myb nor filamin are enriched on the mitotic spindle, so they are not involved directly in recruiting clathrin to the spindle. Interestingly, B-myb is important for the transcription of a number of cell-cycle-regulated genes including Aurora-A kinase (Knight et al., 2009) and it is possible that the observed effect on clathrin was an indirect result of the reduced expression of one of these target genes. The second protein implicated in clathrin function is cyclin-G-associated kinase (GAK), a protein involved in uncoating CCVs (Greener et al., 2000). Depletion of GAK results in an accumulation of cells at prometaphase indicating a problem in spindle function (Shimizu et al., 2009; Tanenbaum et al., 2010). RNAi against GAK also reduces the amount of clathrin on the mitotic spindle. Again, like B-myb, GAK is not enriched at the spindle and, therefore, might only have an indirect effect on clathrin localisation. For example, a reduction in the amount of free clathrin that is able to bind the spindle might be the result of the inhibition of vesicle uncoating in GAK-depleted cells (Hirst et al., 2008; Tanenbaum et al., 2010). The mitotic defects in GAK-depleted cells are more severe than those observed in cells depleted of clathrin or TACC3, which suggests that GAK has a mitotic function that is independent of clathrin.

Finally, the quantitative proteomics study that identified the TACC3–clathrin interaction also reported that G2- and S-phase-expressed protein 1 (GTSE1) strongly interacts with TACC3 and clathrin (Hubner et al., 2010). GTSE1 is a cell-cycle-regulated protein that is strongly phosphorylated during mitosis. It localises to the mitotic spindle, which suggests that it has a role in spindle function. Curiously, the interaction profile of GTSE1 has been reported to be identical to that of clathrin (Hubner et al., 2010), including binding to coated vesicle components. However, it needs to be clarified whether or not GTSE1 is truly a cofactor of clathrin. Although it is unclear whether any other proteins are directly involved in the function of the clathrin–TACC3–ch-TOG complex, it is apparent that this complex is not exclusively responsible for all inter-MT bridges in K-fibres and that other factors might help to stabilise K-fibres.

Inter-MT bridges in K-fibres

As mentioned above, there are four apparent populations of inter-MT bridges in K-fibres with lengths that range from 15 to 53 nm, with the shortest bridge type corresponding to the TACC3–ch-TOG–clathrin complex, but the identity of the remaining bridges is unclear (Booth et al., 2011). Several proteins have been proposed to act as MT crosslinkers in spindles (Manning and Compton, 2008; Peterman and Scholey, 2009), but only a few of these are found on K-fibres. Good candidates for the longer inter-MT bridges are HSET (also known as KIFC1) and hepatoma upregulated protein (HURP; also known as DLGP5, DAP-5 and DLGAP5).

HSET is a kinesin-related protein that preferentially localises between parallel MTs in K-fibres (Mountain et al., 1999), and overexpression of HSET causes bundling of spindle MTs (Cai et al., 2009). HURP is a MT-associated protein that localises to the mitotic spindle in a gradient with the highest concentration nearest to the chromosomes. Depletion of HURP causes problems in K-fibre attachment (Wong and Fang, 2006). Ultrastructural studies will be important to confirm whether or not these proteins are indeed involved in inter-MT bridges.

For many years, research into spindle function has focused on MT motors, and only recently have non-motor proteins begun to receive attention (Manning and Compton, 2008). As an example, a recent study into the biophysics of the spindle highlighted the importance of proteins that crosslink spindle MTs (Shimamoto et al., 2011). Identifying and characterising these proteins is definitely an area that should be prioritised. Once we have a good understanding of the proteins that can crosslink parallel MTs in K-fibres, it will be interesting to determine how these bridge complexes differ from those formed by proteins known to crosslink other MT arrays [e.g. MAP2, nuclear mitotic apparatus protein 1 (NuMA) and PRC1]. Another outstanding question is how K-fibre bridge complexes select parallel rather than anti-parallel MTs. Analysis of MT dynamics in the mitotic spindle shows that the MTs in K-fibres are turned over more slowly than non-K-fibre MTs (McIntosh et al., 2002). This is likely to be due to the stabilisation conferred by attachment to the kinetochore and also by crosslinking MTs along their length. It will be important to understand the contribution of inter-MT bridges to K-fibre stabilisation and to determine whether all inter-MT bridge complexes stabilise MTs in similar ways.

Perspectives

Over the past couple of years our understanding of the role of clathrin in mitosis has accelerated considerably. We have progressed from the curious observation of clathrin on the spindle, through validation of clathrin as a genuine spindle protein with a role in K-fibre stabilisation, to recent work showing that clathrin works in concert with two core spindle proteins. Despite this progress, many questions remain and some of these are discussed below.

How conserved is the function of clathrin in mitosis? Equivalent observations to those in human and rat cells (Royle et al., 2005) have been made in mouse (Han et al., 2010; Yamauchi et al., 2008), pig (Holzenspies et al., 2010), frog (Fu et al., 2010) and plants (Tahara et al., 2007). Removal of clathrin is lethal in chicken DT40 cells, but an apoptosis-resistant strain was isolated (Wettey et al., 2002) that only exhibited mild mitotic defects after removal of CHC (Borlido et al., 2008). It remains to be seen whether the function of clathrin in mitosis is not conserved in birds or if these results were specific for the strain used in the study. Similarly, it is unclear whether or not the mitotic function of clathrin is conserved in lower organisms. As discussed above, the conservation of the clathrin protein is excellent in eukaryotes, but TACC3 is less well conserved and there is no clear homologue in *Saccharomyces cerevisiae* (Table 1). *Schizosaccharomyces pombe* has the TACC homologue Alp7 (also known as Mia1p), and this protein works together with the ch-TOG homologue Alp14 in spindle assembly (Sato and Toda, 2007). Interestingly, *S. cerevisiae* have a single MT that acts as a 'K-fibre', obviously not requiring inter-MT bridges; whereas *S. pombe* has four MTs that do have inter-MT bridges (Ding et al., 1993). Alp7 has been shown to be able to crosslink MTs in *S. pombe* suggesting that it has a 'minimalistic' version of inter-MT bridges (Thadani et al., 2009).

A related question is why clathrin needs three legs to fulfil its bridging function in mitosis given that bipolar MT cross-linkers would probably be the most logical configuration (Peterman and Scholey, 2009). A trimeric crosslinker would allow for bridging between three MTs simultaneously and, in theory, would result in less parallel movement during the crosslinking of two MTs compared with that performed by a bipolar linker. However, the answer could also be that it might not be optimal to have a tripolar MT crosslinker, but that clathrin acquired this function later in evolution. The fact that clathrin is a triskelion is owing to its role in membrane trafficking, a process that arose earlier in evolution than either open mitosis or K-fibres that contain more than one MT and thus require crosslinking. As the structural resolution of clathrin inter-MT bridges is lagging behind that of proteins such as PRC1 that crosslink anti-parallel MTs (Subramanian et al., 2010), a full answer to this question will require analysis of the fine structure of these bridges.

Finally, it is also uncertain whether clathrin forms a multimeric lattice at the spindle; the shape of electron-dense inter-MT bridges observed in single electron microscopy sections suggests that clathrin is present as individual triskelia and CHC mutants that are unlikely to be able to form lattices are still able to localise and function at the spindle, arguing that lattices are not necessary for mitotic function (Blixt and Royle, 2011; Royle and Lagnado, 2006). To answer this question will also require a more detailed visualisation of the clathrin inter-MT bridges.

We are just beginning to understand at the molecular level how K-fibres are stabilised by inter-MT bridges. The fibres must be strong enough to perform their function but not become 'overstabilised' to the point that MT dynamics are affected. Because the expression of many non-motor spindle proteins, such as TACC3 and ch-TOG, is altered in many cancers (Manning and Compton, 2008), determining how K-fibre stabilisation is optimised might therefore be important for uncovering new targets for anti-cancer therapeutics in the future.

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Journal of Cell Science

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