Microtubule organization in the final stages of cytokinesis as revealed by cryo-electron tomography

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Summary

The completion of cytokinesis is dominated by the midbody, a tightly-packed microtubule (MT)-based bridge that transiently connects the two daughter cells. Assembled from condensed, spindle-MTs and numerous associated proteins, the midbody gradually narrows down until daughter cell partitioning occurs at this site. Although described many years ago, detailed understanding of the abscission process remains lacking. Applying cryo-electron tomography to purified midbodies, in combination with fluorescence microscopy, we present here new insight into MT organization within the midbody. We find that the midbody is spatially divided into a core bundle of MTs that traverses the electron-dense overlap region (continuous MTs), surrounded by MTs that terminate within the overlap region (polar MTs). Residual continuous MTs remained intact up to the verge of abscission, whereas the residual polar MTs lost their organization and retreated from the overlap region at late cytokinesis stages. A detailed localization of the microtubule-bundling protein PRC1 supports the above notion. Our study thus provides a detailed account of the abscission process and suggests that the midbody, having acquired a distinct MT architecture as compared to the preceding central spindle, actively facilitates the final stage of cytokinesis.

Key words: Cryo-electron tomography, Cytokinesis, Microtubule, Midbody

Introduction

Cytokinesis is the final stage of the cell cycle, whereby a single cell divides into two daughter cells while partitioning its cytoplasmic content (Glotzer, 2005; Eggert et al., 2006; Barr and Grueneberg, 2007). Following separation of the replicated genome in anaphase, the cell division apparatus is dominated by interpolar microtubules (ipMTs) that emanate from opposite poles and interdigitate at the midzone. The ipMTs undergo anti-parallel sliding to push the cell poles apart, while bundling progressively with neighboring MTs under the contracting force of the actomyosin-based ring. In late telophase, when the actomyosin ring has fully contracted, a unique structure of tightly packed MTs and associated proteins is formed, termed the ‘midbody’. The midbody transiently bridges the two post-mitotic daughter cells and serves as the focal point for numerous proteins and membrane vesicles (Echard et al., 2004; Skop et al., 2004), many of which have been shown to be transported along the MT tracks. Consequently, proper construction of the MT array, as well as accurate cleavage of the bridge (i.e. abscission), are of a vital importance, with failure on either front being linked to tetraploidy, a condition that can lead to the development of cancer (Fujiwara et al., 2005; Shi and King, 2005; Steigemann et al., 2009).

Midbody morphology was viewed in early electron microscopy studies by means of sectioning and heavy metal staining (Paweletz, 1967; Byers and Abramson, 1968; McIntosh et al., 1975a; Mullins and Biesele, 1977; McIntosh et al., 1979). These studies and others (e.g. McDonald et al., 1979; Euteneuer and McIntosh, 1980; Tippit et al., 1980) determined that interdigitating anti-parallel MTs, ending at the center of the telophase spindle (termed ‘polar MTs’), create the thicker overlap region (stem body). Tracking midbody MTs through serial sections confirmed this observation, and revealed that the midbody featured an additional population of MTs, which cross the overlap region (termed ‘continuous’ or ‘free’ MTs). Both ends of this latter group of MTs lie outside the dense overlap region, namely in the flanking polar regions of the midbody (McIntosh et al., 1975a; McIntosh et al., 1975b; Mastronarde et al., 1993). Notably, structural study of MT organization in late telophase mammalian spindles poses a challenge compared with visualization of spindles at earlier stages of mitosis, due to the extremely populated nature of the overlap region and its small size. This renders the midbody almost unapproachable for structural study by techniques other than electron microscopy, and limits the resolvable details in electron micrographs.

Here, we have studied the molecular organization of midbodies in mammalian cells using fluorescence microscopy and cryo-electron tomography (cryo-ET). Fluorescent tagging of MT plus ends indicated localization to four distinct foci, specifically, two inner foci flanking the overlap region, and two outer foci. Cryo-ET of frozen-hydrated midbodies enabled in-depth 3D analysis, providing a novel view of MT organization within these structures, one which correlated with our fluorescence microscopy observations. Our cryo-ET results show that, at late telophase, midbodies are dominated by a core-bundle of MTs that transverse the electron-dense region, with their plus ends found at the outer foci. The polar MTs that terminate in the overlap region surround this continuous bundle in an outer shell. A detailed localization of the MT bundling protein, protein-regulating cytokinesis 1 (PRC1), by immunocytochemistry, supports the existence of MTs that transverse the overlap region. This identified architecture persists even when MTs are severed from the midbody, almost until...
completion of division and breakage of the bridge. A marked change in architecture is, however, observed in the outer shell of late-stage midbodies, where the polar MTs lose their interdigitation and retract from the overlap region. These observations support a dynamic view of the midbody, in which the MT network is altered with respect to its organization in the central spindle prior to late telophase.

**Results**

**MT plus ends localize to four distinct foci within the midbody**

To analyze MT organization within the midbody, we first traced MT plus ends by live cell fluorescence microscopy. Transient transfection of Chinese Hamster Ovary (CHO) cells was performed using a GFP-tagged end-binding protein 1 (EB1)-encoding construct, previously shown to reflect the number and localization of MT plus end tips (Tirnauer et al., 2002; Piehl et al., 2004). In previous studies using conventional light microscopy, GFP–EB1 was localized to the midbody (Rosa et al., 2006); however, careful inspection of projections from confocal laser scanning microscopy image stacks revealed the detailed localization of the tagged EB1 to four distinct foci (Fig. 1A–C). Two inner foci flanked the center of the midbody at a distance of 460±120 nm (s.d., n=7) (Fig. 1D). These foci were shaped as parentheses enclosing the central dense region, as seen by live imaging (Fig. 1A,B). The two outer GFP–EB1 foci formed an elongated architecture at the midbody poles, >1240±210 nm away from the central region (Fig. 1A–D). Better separation between the inner and outer foci was observed in chemically fixed GFP–EB1 cells (Fig. 1C), possibly due to the improved stability of the imaged cells and fluorescent tags. However, the parenthetic shape of the inner foci was less pronounced in the fixed cells (Fig. 1C) than in the inner foci observed by live imaging (Fig. 1A,B).

Expression of both GFP–EB1 and mCherry–α-tubulin in cells confirmed the localization of MTs in the EB1 gaps (supplementary material Fig. S1). Additionally, these experiments supported the notion that MTs within the midbody form a filamentous network distinct from other cellular MT networks.

**Cryo-electron microscopy of isolated midbodies**

The surprising localization of MTs plus ends described above encouraged us to look into MT organization in midbodies in greater detail. In particular, we investigated MT organization, which is reflected by the complex EB1 fluorescent pattern seen in Fig. 1. Therefore, we performed cryo-electron microscopy (cryo-EM) on purified midbodies, enabling high-resolution analysis of midbody morphology in a close-to-native state. Isolation of midbodies was performed using established protocols (Mullins and McIntosh, 1982; Sellitto and Kuriyama, 1988) (see Materials and Methods) and provided sufficiently thin samples that were amenable to cryo-EM without the need for physical sectioning, dehydration or staining procedures. Consequently, we isolated midbodies from synchronized cultured CHO cells. Furrrow ingression and subsequent purification steps were followed by light microscopy (Fig. 2A). Frozen-hydrated midbodies were easily recognized in electron micrographs as elongated structures, 0.4–1.5 μm wide and 5–10 μm long (Fig. 2B,C). Three regions were readily defined in midbodies, namely an electron-dense region in the middle, the overlap region (Fig. 2B, OR), flanked by two more transparent regions, the polar regions (Fig. 2B, PR). Individual MTs could be easily detected within the polar regions (Fig. 2C), and a small fraction of the cell membrane was retained at the overlap region in all isolated midbodies (Fig. 2C). However, single projections were insufficient for tracing individual MTs through the midbody. Consequently, 3D analysis was required, as detailed below.

**Cryo-electron tomography uncovers MT organization in the midbody**

The motivation of this study was to gain insight into the 3D architecture of midbodies by tracing individual MTs. Therefore, we acquired tomographic tilt-series of frozen hydrated midbodies. The 3D reconstructions enabled the tracking of individual MTs (Fig. 3A,B), as well as characterization of other midbody components such as the contractile ring, the cell membrane (Fig. 4A,B) and MT-associated proteins (MAPs) (Fig. 4C–F). Fig. 3A shows an x–γ section, 3.4 nm thick, through a reconstructed volume of a midbody, where the MTs are detected as two parallel lines throughout the structure (Fig. 3A, MT). Because MTs might be slightly curved, they cannot be visualized from start to end in a single section. Nevertheless, MTs can be tracked at all midbody regions by combining sections of varying angles (Fig. 3B). Careful inspection revealed various types of MT tips, including open tips and tips in which one side was anchored to another MT (Fig. 3C, left and middle panels, respectively). Interestingly, capped tips were also detected (Fig. 3C, right panel). The capped structures highly resembled the γ-tubulin ring complex (γTuRC), as reconstructed in vitro and as seen by electron tomography of freeze-substituted and serially sectioned spindle pole bodies and centrosomes (O’Toole et al., 1999; Moritz et al., 2000; O’Toole et al., 2003). The γTuRC appears at the MT minus ends and was shown to facilitate MT nucleation. Typically, four to seven capped MTs were identified in each reconstructed midbody. This observation further supports the notion of non-centrosomal MT nucleation within mitotic spindles (Luders and Stearns, 2007).

A rendered view of all MTs detected in a representative midbody is shown in Fig. 3D,E. Isolated midbodies of all sizes adopted an oval shape in cross-section (Fig. 3G), which might have been
partially induced by the blotting of excess liquid prior to the cryo-fixation procedure. Indeed, a few MT fragments were found in the upper and lower levels of the midbodies, but were rarely observed on the sides. These MT fragments were not rendered and, therefore, are not shown. On the basis of MT continuity, origin and tip structure, we were able to identify four distinct morphological groups within the midbodies (color-coded in Fig. 3D–G). As expected, we found interdigitating polar MTs (polMTs), which presumably initiate (minus end) at the polar regions, at a distance from the midbody center and out of the tomographic range. These MTs terminated (plus end, see below) within the overlap region (Fig. 3D–G, yellow and orange), where tight connections are formed between individual MTs of opposing polar groups.

Interestingly, we identified an additional group of MTs that persisted through the overlap region and both polar regions (Fig. 3D–G, red). The ends of these continuous MTs (conMTs) were shifted considerably from the overlap region (see next paragraph; supplementary material Fig. S2). The conMTs localized around the core of the midbody, forming an inner cylinder that exhibited half of the diameter of the midbody at the overlap region. These MTs comprised about one third of the midbody MT pool (supplementary material Table S1). We could not determine the polarity of the conMTs because both of their ends lay beyond the boundaries of the tomogram, and the resolution of the data was insufficient to reveal subunit polarity. By contrast, the polMTs were assumed to have their plus end at the overlap region and minus end at one of the midbody poles (Euteneuer and McIntosh, 1980). Notably, previous studies observed that anaphase and telophase spindle bundles are composed of anti-parallel MTs.

Fig. 2. Fluorescence and cryo-EM of midbodies. (A) Synchronized CHO cell at late telophase and an isolated midbody (inset) immunolabeled with anti-α-tubulin antibodies and visualized by fluorescence microscopy. (B) Cryo-electron micrograph of an isolated, frozen-hydrated midbody. Three regions are identified: the overlap region (OR), which is an electron-dense region at the middle of the midbody, and two polar regions (PR), which are lighter regions flanking the overlap region. (C) Single microtubules (MT) and some attached membrane (mem) are seen in a single projection of a higher magnification view of the overlap region shown in B. Scale bars: 5 μm (A), 0.5 μm (B,C).

Fig. 3. MT organization at the overlap region as revealed by cryo-ET. (A) Typical 3.4-nm thick x–y section through a tomogram centered at the midbody overlap region. The reconstructed area seen in the tomogram is indicated by the blue square in the inset and comprises the overlap region and small parts of both polar regions. Individual MTs can be seen in the tomographic sections as parallel double lines that can be tracked through the midbody. Other midbody components can be identified, including the contractile ring (CR), remnants of the cell membrane (mem) and MAPs (better seen in B). (B) Despite the high protein density in the midbody overlap region, individual MTs can be tracked (red arrows). The panel represents three 3.4-nm sections at angles that correspond to the MT curvature. Sections are not orthogonal to the tomogram boundaries but are in arbitrary angles in order to optimize the visualization of a particular MT. The electron-dense region is indicated. Scale bars: 200 nm (A,B). (C) MT tips observed in the midbody exhibit an open end (left panel), an open end anchored to a neighboring MT (middle panel) and a capped end (right panel). All MTs are decorated with elongated densities. Scale bar: 20 nm. (D–G) Rendered views of a midbody from a top (D) and side view (E). Horizontal section and vertical cross-section are shown in F and G, respectively. Tracked MTs are segmented as follow: red, continuous MTs; yellow, polar MTs originating (minus end) from the side of one daughter cell; orange, polar MTs originating (minus end) from the side of the other daughter cell; purple, minus-end capped MTs. Four minus-end capped MTs were identified in the current reconstruction (G).
with polMT-end positions found in our cryo-ET analysis. The localization of GFP–EB1 into the two outer foci (Fig. 1A–C) resulted in the formation of an elongated architecture at the midbody poles. Our cryo-ET analysis indicated that most midbody MTs emanated from the polar regions and extended to the overlap region or beyond, to the opposite polar region, with very few MTs being confined to a polar region (Fig. 3D–F; supplementary material Fig. S2). We furthermore established that the inner EB1 foci comprised the plus end tips of the polMTs. These MTs emanated from each of the midbody polar regions and terminated at the opposing face of the overlap region, shortly after the middle of the structure. We can, therefore, attribute the outer EB1 foci to the conMTs. These MTs also emanated from each of the midbody polar region, spanned the overlap region and terminated at the polar regions of the opposing daughter cell. Fig. 1A–C indicates a lower abundance of MT plus ends between the inner and outer EB1 foci. Similarly, our cryo-ET analysis showed symmetric gaps containing no (or very few) MT-end-tips at the outer edges of the overlap region (Fig. 5). This can be seen in Fig. 5 as a reduced density of end-points at distances larger than ±800 nm from the center.

**All groups of MTs persist during midbody breakdown**

At late telophase, the midbody narrows gradually by severing its MTs in a process that is not yet fully understood. As such, we studied the organization of MTs during midbody breakdown. Fig. 6A–C shows horizontal slices through tomograms of representative midbodies at three distinct stages of breakdown. Notably, a single preparation of synchronized cells contained midbodies of varying diameters. The synchronization was not sufficiently accurate to produce a population of equally sized midbodies at the spatial resolution presented here. Therefore, the sequential order, in which the tomographic sections are presented (Fig. 6A–C), was based on the well-documented process of gradual, time-dependant midbody narrowing, and on our live-imaging analysis (see below; Fig. 6F). The three presented midbodies had average diameters of 0.95, 0.7 and 0.45 μm, at their overlap regions (Fig. 6A–C, respectively). The widest and medium-sized midbodies (Fig. 6A,B) were well-preserved and contained well-defined, intact MTs. By contrast, the

**The inner EB1 foci comprise the plus ends of the polMTs, and the outer EB1 foci comprise the plus ends of the conMTs**

To compare the information obtained from fluorescence and cryo-ET experiments, we marked the end-points of the polMTs from several midbody cryo-tomograms, projected them, and superimposed the coordinates onto a single cryo-image of a midbody (Fig. 5). This analysis revealed that polMT end-points taken from the tomograms fell into a pattern similar to that obtained in the fluorescence microscopy analysis, and were 350±250 nm (s.d.) distant from the center of the midbody. Therefore, we conclude that the distance and shape of the inner EB1 foci coincide

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Midbody before cytokinesis completion in GFP–EB1-expressing CHO cells. The midbody in Fig. 1A,B is displayed here 14 minutes later. It can be seen that the parentheses-like shape of the inner foci is lost. Scale bar: 2 µm.

Fig. 6. MT groups persist during midbody breakdown. (A–C) x–y slices from tomograms of representative midbodies at three different stages of abscission. The average diameters of the midbodies are (A) 0.95 µm, (B) 0.7 µm and (C) 0.45 µm. Scale bar: 200 nm. (D,E) Rendered views of the medium midbody displayed in B. Shown are a top view (D) and a horizontal slice at the same orientation (E). Although the total number of MTs decreased, compared with those seen in the midbody shown in Fig. 3, the four MT groups including conMTs (red), opposing polMTs (orange, yellow) and minus-end-capped MTs (purple) remained.

(F) Midbody before cytokinesis completion in GFP–EB1-expressing CHO cells. The midbody in Fig. 1A,B is displayed here 14 minutes later. It can be seen that the parentheses-like shape of the inner foci is lost. Scale bar: 2 µm.

A rendered view of the midbody shown in Fig. 6A was presented earlier (Fig. 3D–G) and can be compared to the rendered view of the midbody shown in Fig. 6B (Fig. 6D,E). In the latter rendering, we found and tracked 74 MTs, whereas 193 MTs were detected in the midbody shown in Fig. 3D–G. Nevertheless, the general architecture remained, with all MT groups being detected. The medium midbody contained well-defined conMTs stretching through the overlap region at its central axis, with polMTs wrapped around them (Fig. 6D, E). Notably, MTs with minus-end-capping were recognized in midbodies of both sizes and presented similar characteristics (Fig. 3C,D,G and Fig. 6D). Differences between midbodies existed, however, in the architecture of the polMTs. The polMTs in the medium midbody retracted from the overlap region and barely interdigitated with opposite polMTs. As can be seen in Fig. 6D,E, polMTs of the medium midbody formed bundles with neighboring MTs of the same daughter cell, rather than with MTs of the opposite daughter cell. In general, the overlap region appeared more distorted than the overlap region of the large midbody (Fig. 3D–G). This data was confirmed by examining fluorescence images of GFP–EB1 (Fig. 6F). Late cytokinesis midbodies with a diameter smaller than ~0.8 µm did not show a well-defined MT organization, as presented in Fig. 1A–C. Moreover, the midbody seen in Fig. 1A,B, which represents an early stage in midbody development, was followed by live cell imaging and is shown in Fig. 6F at 14 minutes after it was imaged in Fig. 1A,B (see supplementary material Fig. S3 for another example). The overlap region of the late-stage midbody was more diffuse than in the earlier stage midbody, as can be judged from the fluorescence microscopy analysis. The parentheses-like foci detected in early stages (Fig. 1A,B) were indistinguishable, and a continuum between the outer and inner foci was noted.

Distribution of PRC1 within midbodies supports MT rearrangement

Next, we probed the distribution of two key MAPs, PRC1 and MKlp1. These MAPs, which localize to the midbody, are essential for central spindle assembly and cytokinesis progression (Glotzer, 2009). PRC1 is the main MT-bundling factor in cytokinesis and is highly conserved (Jiang et al., 1998; Mollinari et al., 2002). PRC1 was, moreover, shown to crosslink anti-parallel MTs in the midzone during the period spanning anaphase to late telophase.

It has been previously noticed that PRC1 localizes to the midbody polar regions in late telophase, whereas its localization is restricted to the midzone in earlier stages of cytokinesis (Kurasawa et al., 2004). This observation is particularly intriguing in the context of our model for MT organization in the midbody. The elongation of conMTs upon midbody assembly implies that new stretches of anti-parallel MTs would form in the polar regions, creating new binding sites for PRC1 outside the overlap region. Additionally, we observed cross-bridges in the polar region (Fig. 4E,F), similar to those seen in vitro in PRC1–MT complexes (Mollinari et al., 2002; Subramanian et al., 2010). We localized PRC1 before and after midbody formation using immunofluorescent and immunocytochemical electron microscopy. As previously observed, PRC1 localized to the midzone during early telophase, whereas after midbody assembly, fluorescent labeling also appeared in the polar regions (Kurasawa et al., 2004) (Fig. 7A,B). Detailed examination of PRC1 within the midbody revealed that its localization resembled that of EB1 (Fig. 1), namely appearing as two parentheses-shaped inner foci and two elongated outer foci (Fig. 7B). However, it is noteworthy that antibody
micrographs were scaled to the calculated average midbody diameter (1.4 μm). The number of gold labels was normalized to the calculated average midbody diameter (1.4 μm in the overlap region). Error bars represent s.d. from the long axis (Fig. 7E, normalized as above). Additionally, previous studies have indicated that some telophase MTs transverse the overlap region (McIntosh et al., 1975a; McIntosh et al., 1975b; Mastronarde et al., 1993). Nonetheless, we now present direct evidence for differential localization along the midbody vertical axis, as well as separation between the plus ends of two MT groups along the midbody horizontal axis (Fig. 8). The midbody features a central bundle of conMTs surrounded by an outer shell of polMTs. The plus ends of these polMTs are restricted to parentheses-shaped foci at the boundaries of the overlap region, whereas the plus ends of the conMTs extend >1.2 μm away from the center of the midbody. Notably, the structure described here is short lived and occurs under normal circumstances for only several minutes, typically at late telophase. The midbody is constantly being disassembled and loses some of its distinctive features (such as interdigitation of the polMTs) when it narrows. Careful live imaging of cells undergoing cytokinesis, as performed here, was crucial for detecting the described structures.

Our novel spatial differentiation in MT organization is in agreement with published structural studies reporting on midbody morphology. First, our data indicate that the continuous bundle contains about one third of the midbody MTs, a value in accordance with serial section EM studies performed on cultured mammalian cell strains, including HeLa, PtK and CHO cells (Brinkley and Cartwright, 1971; McIntosh and Landis, 1971; McIntosh et al., 1975b; Mastronarde et al., 1993). Containing polMTs alone, this ratio should be 2:1. However, because a third of the MTs are continuous from one polar region to the other, the ratio between the number of MTs in the overlap region to either polar regions is 1.5:1, as also observed by these earlier studies. Second, as indicated, the conMTs were identified by tracking individual MTs within stained serial sections (McIntosh et al., 1975a; McIntosh et al., 1975b; Mastronarde et al., 1975a; McIntosh et al., 1975b; Mastronarde et al., 1993). Indeed, hardly any gold labels were detected in the MKlp1-labeled polar regions, showing an average of 0.5±0.9 gold labels (Fig. 7D; supplementary material Fig. S4A). As a control, we immunogold-labeled tyrosinated tubulin, which showed a considerably greater number of gold labels in the polar region (66.1±29.2) (Fig. 7D; supplementary material Fig. S4B). In the overlap region, on the other hand, a similar number of gold labels were detected for all protein targets (Fig. 7D). The distribution of gold labels across the overlap region was similar for both PRC1 and MKlp1 (Fig. 7E; supplementary material Fig. S4A). It is noteworthy that the localization of PRC1 in the gold labeling experiment showed no noticeable foci, as detected using fluorescence labeling. Additionally, in the gold labeling experiment, the overlap region showed tenfold more label than did the polar region (Fig. 7D), an effect that was not observed as different levels of intensity in the fluorescence experiment (Fig. 7B). Thus, the labeling of PRC1 in the midbody polar region agrees with the existence of conMTs with opposite polarity, as seen by cryo-ET. However, the low occurrence of MKlp1 detected at the polar region supports the notion that conMTs emergence does not involve anti-parallel sliding.

**Discussion**

Using cryo-ET in combination with fluorescence microscopy to examine isolated midbodies from mammalian cells, we present novel insight into MT architecture during late telophase. Our findings are summarized in the model presented in Fig. 8. It is well-established that the plus ends of MTs nucleating from opposing daughter cells interdigitate at the midbody overlap region to construct the tightly-packed array found in this structure. Additionally, previous studies have indicated that some telophase MTs transverse the overlap region (McIntosh et al., 1975a; McIntosh et al., 1975b; Mastronarde et al., 1993). Nonetheless, we now present direct evidence for differential localization along the midbody vertical axis, as well as separation between the plus ends of two MT groups along the midbody horizontal axis (Fig. 8). The midbody features a central bundle of conMTs surrounded by an outer shell of polMTs. The plus ends of these polMTs are restricted to parentheses-shaped foci at the boundaries of the overlap region, whereas the plus ends of the conMTs extend >1.2 μm away from the center of the midbody. Notably, the structure described here is short lived and occurs under normal circumstances for only several minutes, typically at late telophase. The midbody is constantly being disassembled and loses some of its distinctive features (such as interdigitation of the polMTs) when it narrows. Careful live imaging of cells undergoing cytokinesis, as performed here, was crucial for detecting the described structures.

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Fig. 8. Model for MT organization and remodeling within the midbody. (A) Newly-formed midbody in late telophase contains a core bundle of conMTs (red), surrounded by opposing polMTs (yellow, orange) that interdigitate at the overlap region, and a minority of minus-end-capped MTs (purple). The midbody is gradually broken down by a severing of its MTs, although all MT overlap region, and a minority of minus-end-capped MTs (purple). The midbody is gradually broken down by a severing of its MTs, although all MTs have to be extended significantly from early to late telophase, which might extend through the spindle midzone to create stretches of overlap during early telophase. Such extended bundles would then form the midbody core in late telophase. However, a more plausible explanation for the formation of the core bundle is that the midbody overlap region is initially formed from early telophase bundles with varying overlap lengths. Then, in late telophase, MT bundles localizing in close proximity to the core region would polymerize and elongate, whereas the outer, surrounding MTs would be restricted. In support of this hypothesis, Rosa and colleagues (Rosa et al., 2006) observed considerable MT dynamics at the midbody poles, a result corroborated by live imaging in our study (data not shown). Additional support was gained upon following the distribution of PRC1 and MKlp1 in the midbody. Recently, it was shown that PRC1 can scan MTs by one-dimensional diffusion so as to identify locations of anti-parallel MTs, where it binds (Subramanian et al., 2010). Thus, when conMTs organize, stretches of anti-parallel MTs are formed in the polar regions, providing binding locations for PRC1. Indeed, PRC1 localization during cytokinesis is partially controlled by the kinesin motor protein Kif4 (Kurasawa et al., 2004). However, PRC1 localizes to the midzone even in Kif4-deficient cells (albeit to a broader region), and Kif4 does not seem to localize to the midbody polar regions (Kurasawa et al., 2004). The lack of an accompanying motor protein might explain the relatively low concentration of PRC1 in the polar region compared with the level of the protein seen in the overlap region. In contrast to PRC1, MKlp1 (which is involved in anti-parallel sliding in MT plus ends) remains restricted to the overlap region, indicating that the mechanism of conMT elongation does not involve anti-parallel sliding.

Architecture of the MT groups during midbody breakdown
Our findings suggest that the central bundle persists until the very last stages of midbody breakdown. Although reduction in the number of the conMTs from early to late-stage midbodies occurs, MT architecture is retained as in earlier stages. This further implies that the observed organization supports midbody function to the verge of completion of cell division. Interestingly, we distinguished between (at least) two structurally distinct states of midbodies during late telophase, mainly on the basis of polMT morphology (Fig. 8). When the midbody diameter is between 1.5 μm and ~0.8 μm, opposing polMTs interdigitate and end in clear parentheses-like structures enclosing the overlap region. However, when midbody diameter is less than ~0.8 μm, the polMTs are no longer sustained in the above organization. Instead, bundles of polMTs from the same, rather than opposing, daughter cells are formed, with the polMTs plus ends retracting from their original foci at the edges of the overlap region. Thus, we can establish that midbody contraction affects core MTs as well as those found in the outer shell, although disassembly of the overlap region seemed to be more pronounced in the outer shell, especially towards abscission. It remains unknown whether MT elimination occurs simultaneously at all midbody shells or whether conMTs found at the outer rim of the core bundle are first shortened to become polMTs and are only then severed. However, the persistence of the conMTs to the verge of abscission, combined with the dynamic nature of the MTs at the midbody ends (Rosa et al., 2006), suggests their involvement in the elongation of the bridge occasionally seen in motile cells before cell division (Byers and Abramson, 1968).

Midbody function in cell division
Our analysis of the midbody structure from the time of assembly to the late stages of contraction offers a dynamic and functional view of MT organization within the midbody. There is ever-growing evidence for the existence of molecular control governing midbody formation and abscission (Eggert et al., 2006; Barr and...
Grunenberg, 2007; Steigemann and Gerlich, 2009). The finding of a continuous MT core bundle that transverses the electron-dense region, together with the active nature of these MTs, transforms the current view of the midbody as only a remnant of the mitotic spindle, as well as the manner by which cells discard the central spindle. The spindle network, initially designed for separating the duplicated chromosome and, later, for pushing the poles of the mitotic cell apart, is apparently redesigned upon formation of the midbody and adapts to its new role in daughter cell partitioning. The midbody network is, therefore, actively modified to better facilitate its role in cell division. This is supported by observations that breakage of the bridge might be delayed for very long periods (Byers and Abramson, 1968; Mullins and Bieseke, 1977; Cimini et al., 2003; Bottcher et al., 2009; Steigemann et al., 2009).

Finally, our approach of combining confocal-based fluorescence microscopy and cryo-ET holds high potential for structural characterization of the midbody. Cryo-ET enables 3D visualization of large cellular assemblies without using heavy metal staining and dehydration, while fluorescence microscopy enables easy tagging of specific proteins and a broader view of the specimen. Detailed analysis of the midbody is therefore possible despite its small size and dense content. Consequently, direct observation of MT network organization is now possible, as is direct observation of other midbody components, such as the actomyosin-based ring, MAPs and the γTuRC (Fig. 4A,B,C–F and Fig. 3C, respectively). This study thus serves as a platform for high-resolution investigation of the final phase of cell division.

Materials and Methods

Cell culture

CHO cells were cultured in Ham’s F12 medium supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin and L-glutamine (Biological Industries, Beit Haemek, Israel) at 37°C and 5% CO2.

Cell synchronization and isolation of midbodies

For synchronization of mitotic cells, 4 mM thymidine (Sigma-Aldrich) was applied to cells growing at 60–70% confluency for 16 hours. The cells were washed and cultured for 4 hours in fresh medium, followed by a 4-hour incubation with 0.1 μg/ml nocodazole (Sigma-Aldrich). Rounded mitotic cells were gently shaken off the plate, harvested, washed twice in fresh medium and incubated at 37°C. Phase microscopy was used to monitor furrow ingression and the formation of midbodies 50–60 minutes after the fresh medium wash. After the incubation period, midbodies of varying diameters were obtained, from ~2 μm to less than 0.5 μm, as measured in electron micrographs. Appropriate samples were either prepared for immunofluorescence or subjected to the isolation procedure described below.

A midbody isolation protocol was adapted from Mullins and Mcintosh (Mullins and McIntosh, 1982) and from Sellitto and Kuriyama (Sellitto and Kuriyama, 1988). After 50–60 minutes into cytokinesis, 40 μg/ml taxol (LC Laboratories) was added to cells growing at 60–70% confluency for 16 hours. The cells were disrupted by vortexing and the midbodies sedimented at 600 g/ml taxol (LC Laboratories) was added to cells growing at 60–70% confluency for 16 hours. The cells were disrupted by vortexing and the midbodies sedimented at 600 g for 10 minutes. The pellet was resuspended in 50 mM cold 2-(N-morpholino)ethane sulfonic acid (MES), pH 6.3, and maintained at 4°C. Prior to cryo-fixation, gentle centrifugation at 70 g for 2 minutes at 4°C was performed to remove large aggregates.

Cryo-electron tomography

A 3 μl drop of midbody sample, along with 3 μl of BSA-coated 15 nm colloidal gold in PBS, was applied onto a glow-discharged 200-mesh carbon-coated copper grid (Quantifoil, Jena, Germany), and plunge-frozen into liquid nitrogen-cooled ethane. Tomographic tilt-series (typically −66 to +60°, 2° increment) were taken with a 300 kV FEI (FEI Polara) equipped with a postcolumn GIF 2002 energy filter and a 2K CCD camera (Gatan). The defocus value ranged from −10 mm to −14 mm and the pixel size was 0.82 nm at the specimen level. Projection images were aligned to a common origin using fiducial gold markers, after which sampling was reduced to 1.65 mpx. 3D reconstruction was done by weighted back-projection, as implemented by the TAL software suite (Niell et al., 2005). For visualization, contrast enhancement was performed using an anisotropic-diffusion denoising algorithm (Frangakis and Hegerl, 2001). Image segmentation, MT tracking and isosurface rendering were performed using the Amira 4.1 software package. Because isolated midbodies adopted an oval shape in cross-section (see Fig. 3G), their diameter was calculated as the average of their horizontal and vertical diameters.

Immunofluorescence

Cells on coverslips were fixed for 15 minutes in 3% paraformaldehyde (PFA), washed three times in PBS and incubated for 4 minutes with 0.5% Triton X-100 (Sigma-Aldrich) in PBS. Fixed cells were incubated with a primary antibody (monoclonal anti-α-tubulin antibodies (Sigma-Aldrich)) for 1 hour, washed three times with PBS and incubated with a secondary antibody (goat anti-mouse-Cy3 antibodies from Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Images were taken on a Nikon TE2000-S inverted microscope using a 100× objective. For immunofluorescence of isolated midbodies, a concentrated sample of isolated midbodies was applied to a glass slide, labeled with the above antibodies and imaged using an Olympus Fluoview FV1000 confocal microscope.

Transient transfections and live imaging

For transfection, cells were plated onto 35-mm glass-bottomed dishes. GFP-EB1- and mCherry-α-tubulin-encoding plasmids (kind gifts from Alexander Bershadsky, Weizmann Institute of Science, Rehovot, Israel) were introduced using Lipofectamine 2000, according to the protocol provided by the manufacturer (Invitrogen). At 8 hours after transfection, the cells were transferred to fresh Ham’s F12 medium supplemented with 10% FCS and L-glutamine, without antibiotics) for 24 hours. Fixed cells were then washed in ice-cold methanol and imaged or taken for live imaging. Imaging was performed using an Olympus Fluoview FV1000 confocal microscope. Live imaging was performed in Ham’s F12 medium (supplemented with 10% FCS and L-glutamine, without antibiotics) using the same confocal microscope equipped with a channel (Live Imaging Services, Reinach, Switzerland), which provided a constant temperature of 37°C and a humidified atmosphere of 5% CO2.

Immunogold electron microscopy

Synchronized cells were centrifuged and fixed with freshly prepared solution of 4% PFA, 0.1 M sucrose in 0.1 M cacodylate buffer containing 5 mM CaCl2 for 1 hour at 24°C. Fixed cell pellets were incubated in 10% gelatin at 37°C for 30 minutes and centrifuged. Excess gelatin was removed at 37°C, followed by post-fixation in the same solution at 40°C for 24 hours. Fixed cell pellets were air-dried and resin-embedded after overnight infiltration with 2.3 M sucrose in cacodylate buffer and then frozen by injection into liquid nitrogen. Ultrathin (75 nm) frozen sections were cut with a Diatome diamond knife at −120°C using a Leica UC6 ultramicrotome. The sections were transferred to formvar-coated 200 mesh nickel grids (EMS, Hatfield, PA). Sections were treated with conditional medium (0.5% BSA, 3% NGS, 0.1% glycine and 1% Tween 20 in PBS) for 5 minutes to block nonspecific binding, followed by a 2-hour incubation with antibodies. Rabbit anti-PRC1, rabbit anti-MKlp1 (kind gift of Francis A. Barr, Cancer Research Centre, University of Liverpool, UK), and rat anti-tyrosinated α-tubulin (YLI/2; Abcam) antibodies were used. After extensive washing in PBS containing 0.1% glycine, the primary antibody was detected by goat anti-rabbit or anti-rat 15 nm colloidal gold conjugate (EMS, Hatfield, PA). The grids were then washed in PBS-glucine, stained for 5 minutes with neutral uranyl acetate oxide followed by 10 minutes staining with 2% uranyl acetate in H2O2, and embedded in 2% methyl cellulose/uranyl acetate, as previously described (Tokuyasu, 1980). Grids were analyzed in a CM100 FEI electron microscope equipped with a Eagle 2K CCD camera (FEI, Eindhoven, Netherlands).

We thank J. Richard McIntosh for critical discussion and invaluable comments on the manuscript. We thank Benjamin Geiger, Larisa Gheber and Jerry Eichler for critical review, Reinhard Fässler and Alexander Bershadsky for providing plasmids and fruitful discussion, Francis Barr for providing the PRC1 and MKlp1 antibodies, Uri Abdu for providing antibodies, and Alon Monseonge and Shay Abutbul for access to the confocal microscope and assistance with the live imaging. This study was supported by grants from the German-Israeli Cooperation Project (DIP, H.2.2) and by an ERC Starting Grant to O.M. (23407 INCEL).

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/2/207/DC1

References


Figure S1

Localization of MTs and MT plus ends within the midbody.

Fluorescent microscopy images of cells expressing both GFP-EB1 and mCherry-α-tubulin showing four plus end foci, with respect to MT organization. Bar, 2 μm.
Reconstruction of the midbody Polar Region.

The reconstructed volume in (A-C) includes ~0.2 μm of the Overlap Region (A and B, left) and ~1.5 μm of the Polar Region (A and B, right), as illustrated in the inset in panel (A). Shown are a top view of the entire rendered tomogram (A), a horizontal slice (B), and two vertical cross sections at the locations in (A) indicated by the arrows. Since only part of the Overlap Region is evident in the reconstruction, MTs could not be tracked through the Overlap Region. Assignment to MT groups was, therefore, made based on comparison to other reconstructed midbodies, such as the one shown in Fig. 3. MTs around the central axis found within a diameter equal to half the diameter of the midbody are assigned to the continuous group (colored red). MTs located in the outer shell of the midbody, including the upper and lower rims, are assigned to one of the polar groups (yellow). MTs that terminated within a range of 0.7 μm from the Overlap Region side of the tomogram (left) were assigned to the opposite polar group (orange). MTs which
exhibited minus-end-capping (Fig. 3C, right panel) are colored purple. MTs that did not enter the Overlap Region and did not exhibit minus-end-capping are colored grey.

(D) MTs located around the central axis of the midbody (red) are represented by horizontal bars and arranged according to their lengths. 60% of the MTs within the core bundle of the midbody were continuous through the reconstructed Polar Region (lower part of the graph), while 40% terminated gradually, starting from a distance of 0.3 μm from the Overlap Region (upper part of the graph).
MT plus ends within a late midbody.

A midbody before cytokinesis completion is visualized by transient transfection of CHO cells to express GFP-EB1. This midbody has a diameter of ~0.8 μm and, accordingly, does not feature well-defined MT organization, as presented in Fig. 1. Bar, 2 μm.
Localization of MKlp1 and tyrosinated tubulin in the midbody.

Electron micrographs of a chemically-fixed and stained sections, immuno-labeled with anti-MKlp1 (A) and anti-tyrosinated tubulin (B) antibodies. Bar, 500 nm.
Table S1

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<th>Mid. 1</th>
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<td>% conMTs</td>
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Distribution of MTs to the continuous and polar groups in three 'early' midbodies.

Only midbodies in which the Overlap Region was well-shaped, as seen in Fig. 3, are included.