Cytokinesis is a fundamental step of cell proliferation, and its high-fidelity completion is crucial for stable maintenance of the genome (Ganem et al., 2007; Lacroix and Maddox, 2012). Anti-parallel microtubule bundle structures such as the central spindle and the midbody play various important roles throughout cytokinesis, from positioning of cleavage furrow to final separation of the two daughter cells (Barr and Gruneberg, 2007; Glotzer, 2009; Fededa and Gerlich, 2012). Although a large number of different factors that are important for abscission, including membrane trafficking machinery, localise to the midzone during cytokinesis (Caballe and Martin-Serrano, 2011; Guizetti and Gerlich, 2010; Neto and Gould, 2011), here we focus on the current understanding of protein–protein interactions between microtubule organisers and their regulators that form cytokinetic microtubule structures.

During anaphase, a barrel-shaped array of bundles of interpolar microtubules appears between the segregating chromosomes (Barr and Gruneberg, 2007; Glotzer, 2009). Although this structure has been referred to in different ways, such as ‘midzone microtubule bundles’, here we call it the ‘central spindle’ (Douglas and Mishima, 2010). It is made of two sets of microtubules that come from each half of the mitotic spindle with their plus ends at the centre and form interdigitating anti-parallel overlaps referred to as the ‘stem body’ (see Poster). In some cell types, especially in larger cells such as blastomeres of sea urchin embryos, growth of non-spindle microtubules that emanate from spindle poles (astral microtubules) towards the cell cortex is also promoted after the onset of anaphase (von Dassow et al., 2009). When astral microtubules from opposite poles meet at the equatorial region, they also form anti-parallel bundles. Irrespective of their origins, both of these anti-parallel microtubule bundle structures play important roles in specifying the cleavage furrow by recruiting cytokinesis effectors, such as activator(s) of Rho GTPase, the
master regulator of contractile ring formation (Piekarz et al., 2005).

The ingressing cleavage furrow gathers both the aster-derived equatorial microtubule bundles and the central spindle into a single compact microtubule bundle, which, upon completion of furrowing, tightly contacts the surrounding plasma membrane in the intercellular bridge (Buck and Tisdale, 1962) (see Poster). Often, a distinct disk-like expansion of the intercellular bridge is observed, which corresponds to the central zone, where anti-parallel microtubules overlap. Again, there has been a history of ambiguity in terminology. Here we use ‘midbody’ to refer the entire microtubule bundle structure that is retained at the intercellular bridge after furrow completion and call the central distinct structure the ‘Flemming body’ (Mollinari et al., 2002; Paweletz, 1967). The intercellular bridge is maintained for up to several hours until the final separation of sister cells (abscission) occurs and is believed to serve as the platform for the membrane trafficking and fusion events that are essential for abscission.

Using traditional electron microscopy (EM), overlapping anti-parallel microtubules in the stem body and the Flemming body were shown to be embedded in highly electron-dense matrices (‘stem body matrix’ and ‘midbody matrix’, respectively) (McIntosh and Landis, 1971). Dense matrix material also fills the space between the microtubule bundle and the plasma membrane (Buck and Tisdale, 1962). A recent cryo-electron tomography study confirmed that this dense region is not just caused by staining with heavy metals, but actually reflects the deposition of high-density matrix material (Elad et al., 2011). Indeed, owing to this crowding effect, antibodies cannot easily access the inside of the Flemming body, resulting in a dark gap in immunofluorescence staining of tubulin by epitope masking (Saxton and McIntosh, 1987). This means that it is essential to use a combination of immunofluorescence and GFP tagging to determine the localisation of proteins to the Flemming body (Arnaud et al., 1998). It remains unclear whether the midbody matrix is a uniform amorphous aggregation of the matrix substances, or whether it has a regular substructure.

A number of molecules have been identified at the central anti-parallel overlap zone of the central spindle and the midbody. These can be classified into three categories. The first class includes the core microtubule organisers, such as protein regulator of cytokinesis 1 (PRC1) and centralspindlin. The second category is made up of their upstream regulators, represented by mitotic kinases, such as Aurora B kinase, which is a component of the chromosomal passenger complex (CPC) (Ruschau et al., 2007), and polo-like kinase 1 (PLK1) (Archambault and Glover, 2009). The third class comprises the downstream effectors, including epithelial cell-transforming sequence 2 oncogene (ECT2), which is the major activator of Rho GTPase, and centrosomal protein of 55 kDa (CEP55), which is a key to recruitment of abscission factors to the midbody. However, this classification is not always obvious because the relationship of these factors to each other is not a simple cascade, but instead comprises a complex network with multiple feedback loops as described below.

**Conserved midzone motors and microtubule-associated proteins (MAPs)**

Generally speaking, the organisation of a microtubule-based structure depends on the combination of various elementary steps involved in microtubule dynamics, such as nucleation, elongation, end-capping, depolymerisation, bundling, sliding, etc. The microtubules in the spindle midzone are stabilised after anaphase onset (Murthy and Wadsworth, 2008), although the underlying molecular mechanism is not clear. It remains to be determined whether a stabilisation of interpolymer microtubules that pre-exist from metaphase is sufficient for the formation of the central spindle or whether de novo microtubule polymerisation (Uehara and Goshima, 2010) is also required. The molecular details of the anchoring of the microtubule minus ends or their stabilisation without a specific anchoring structure are also unclear, although γ-tubulin (Julian et al., 1993; Shu et al., 1995) and its associated proteins, such as augmin (Uehara et al., 2009) and minus-end-directed motor kinesin-14 (Cai et al., 2010), as well as the recently identified microtubule minus-end stabiliser patronin (Goodwin and Vale, 2010) have been suggested to be involved. By contrast, the roles of motors and microtubule-associated proteins (MAPs) that localise to the central anti-parallel overlaps have been better characterised as described below. The localisation of these factors has been studied using different methods and in different cell types (as discussed below and summarised on the Poster), and has recently been confirmed in a comparative survey in HeLa cells (Hu et al., 2012).

**PRC1**

PRC1 (Jiang et al., 1998; Verbrugge and White, 2004; Vermi et al., 2004) is a microtubule bundling protein, which belongs to a highly conserved family of MAPs that includes plant MAP65 (Hamada, 2007; Sasabe and Machida, 2006) and yeast Ase1p (Schuyler et al., 2003). Crossbridges of ~35 nm length are observed between anti-parallel microtubules that are bundled by PRC1 (Mollinari et al., 2002; Subramanian et al., 2010). Although the C-terminal portion of PRC1 is predicted to be unfolded, the remainder of the molecule is predicted to form a rod-like dimer consisting of repeated triple helix bundles similar to an actin bundling protein, α-actinin (Li et al., 2007). The microtubule-binding region of PRC1 has been mapped to the C-terminal half of the molecule, which covers the unfolded tail and the adjacent part of the α-helical region (Mollinari et al., 2002; Subramanian et al., 2010). The triple helix bundle structure in the microtubule-binding domain of human PRC1 has been confirmed by X-ray crystallography and cryo-EM helical reconstruction (Subramanian et al., 2010).

PRC1 is essential for the formation of the central spindle in vivo (Mollinari et al., 2002; Verbrugge and White, 2004; Vermi et al., 2004). Before the onset of anaphase, PRC1 is diffusely localised throughout the cytoplasm and only a minor population associates weakly with the entire spindle. At anaphase onset, PRC1 rapidly accumulates to the spindle midzone, forming the central spindle. Phosphorylation by cyclin-dependent kinase 1 (CDK1) has a crucial role in this temporal regulation (Jiang et al., 1998; Mollinari et al., 2002; Zhu and Jiang, 2005). Although PRC1 is enriched in the stem body and the Flemming body, its localisation is not as restricted as that of centralspindlin, another key cytokinetic factor described below (Elad et al., 2011). In vitro experiments at the single molecule level reveal that both PRC1 and Ase1p move diffusively along a single microtubule and have a preference for anti-parallel bundling compared with parallel bundling (Bieling et al., 2010; Kapitein et al., 2008; Subramanian et al., 2010). PRC1 interacts with various motors and MAPs as discussed below.

**Centralspindlin**

Centralspindlin is another microtubule-bundling protein that is critical for the
formation of the central spindle. It is a stable 2:2 heterotetrameric complex of a kinesin-6 orthologous to mammalian mitotic kinesin-like protein 1 (MKLP1) and a Rho-family GTPase-activating protein (RhoGAP) orthologous to Caenorhabditis elegans CYK-4 (CYK4) (Mishima et al., 2002; Somers and Saint, 2003). In mitotic cells, the majority of MKLP1 and CYK4 are in the centralspindlin complex and there is no clear evidence for the presence of free components. The motor domain of MKLP1 possesses the microtubule plus-end-directed motor activity (Hizlan et al., 2006; Mishima et al., 2004; Nislow et al., 1992), which is essential for proper microtubule bundle formation and deposition of the midbody matrix (Matulienė and Kuriyama, 2002). The neck region of MKLP1, which links the motor domain to a relatively short coiled-coil stalk domain, is unusually long and contains the binding site for CYK4 (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007; Somers and Saint, 2003). Both subunits are vital for microtubule bundling in vitro and central spindle formation in vivo. The atomic structure of centralspindlin is not known, except for the GAP domain of CYK4, whose target Rho-family GTPase is under debate (Canman et al., 2008; Jantsch-Plunger et al., 2000; Miller and Bement, 2009; Yamada et al., 2006; Zavortink et al., 2005). Similar to PRC1, the interaction of centralspindlin with microtubules is suppressed by CDK1 phosphorylation before anaphase onset (Goshima and Vale, 2005; Mishima et al., 2004). The heterotetrameric centralspindlin forms higher-order clusters in a manner that is regulated by Aurora B kinase and 14-3-3 proteins (Douglas et al., 2010; Hutterer et al., 2009). This clustering activity is essential for its processive motility in vitro and for its distinct sharp accumulation to the centre of the central spindle in vivo (Hutterer et al., 2009). Centralspindlin also interacts with a number of downstream cytokinesis regulators and effectors as well as with PRC1 (see below).

KIF4

Another midzone motor is the kinesin-4 family member KIF4, which has an N-terminal motor domain followed by a long coiled-coil tail (~100 nm and longer than that of the conventional kinesin) that is responsible for its dimerisation (Sekine et al., 1994). It moves along the microtubules towards their plus ends and upon its accumulation at the plus end, reduces microtubule polymerisation and depolymerisation dynamics (Bieling et al., 2010; Bringmann et al., 2004). KIF4 was the first kinesin found to associate with mitotic chromosomes and was thus termed chromokinesin (Wang and Adler, 1995). After anaphase onset, a subpopulation of KIF4 accumulates at the central spindle and the midbody (Wang and Adler, 1995). Reflecting this localisation pattern, KIF4 is involved in multiple steps of cell division, including the accurate formation of the central spindle (D’Avino et al., 2007; Kurasawa et al., 2004; Mazumdar et al., 2006; Mazumdar et al., 2004; Williams et al., 1995; Zhu and Jiang, 2005). KIF4 depletion causes abnormal elongation of the central spindle with unfocused microtubule overlaps (Hu et al., 2011). Its interaction with PRC1 has been proposed to be important for cytokinesis (see below).

MKLP2

Mitotic kinesin-like protein 2 (MKLP2) is a less conserved member of the kinesin-6 family, which, in contrast to MKLP1, has not been reported to form a stable complex with other polypeptides. MKLP2 is crucial for the relocation of the CPC from the centromere to the central spindle (Cesario et al., 2006; Gruneberg et al., 2004; Hümmel and Mayer, 2009) and for the proper localisation of PLK1 to the spindle midzone (Cesario et al., 2006; Neef et al., 2003). In contrast to centralspindlin, MKLP2 localises to regions adjacent to the Flemming body during late telophase. Although C. elegans does not have the MKLP2 orthologue, vertebrates have a related molecule MPP1, which is also localised to the spindle midzone and required for proper cytokinesis (Abaza et al., 2003).

CLASP

CLASP is a microtubule plus-end tracking protein that contains repeats of TOG and TOG-like domains, and is orthologous to mammalian cytoplasmic linker-associated protein 1 and 2 (CLASP1 and CLASP2) (Akhmanova and Steinmetz, 2008; Slep, 2009). In addition to its function in the regulation of interphase microtubule dynamics, CLASP also controls the architecture of the bipolar mitotic spindle (Walczak, 2005) and the central spindle (Inoue et al., 2000; Inoue et al., 2004). The central spindle localisation of CLASP depends upon its interaction with PRC1 (Liu et al., 2009), similarly to its Schizosaccharomyces pombe orthologue cls1p (also called peg1p) (Bratman and Chang, 2007). Depletion of one of the three CLASPs in C. elegans causes a synthetic cytokinesis failure in PRC1 mutant embryos, which show only mild cytokinesis failure in the first cell division (Bringmann et al., 2007).

Other MAPs and kinesins

ASPM, the mammalian homologue of a Drosophila centrosomal MAP abnormal spindle (Paramasivam et al., 2007), and other kinesin-like motors, including those belonging to the kinesin-3 class [KIF14, KIF13A and GAKIN (also known as KIF13B)] (Gruneberg et al., 2006; Sagona et al., 2010; Unno et al., 2008), the kinesin-7 class (CENP-E, also known as KIF10) (Brown et al., 1994) and the kinesin-8 class (Klp67A) (Savoian et al., 2004), have also been reported to localise to the centre of the central spindle and the midbody. Their roles in organisation of the midzone microtubule bundles are still unclear.

Protein–protein interactions between spindle midzone proteins

As discussed above, the assembly of the central spindle and the midbody depends on multiple microtubule modulators and their regulators. Although the biophysical details of the effects of individual factors on microtubule dynamics or organisation remain to be clarified, current research efforts are shifting towards investigating how these factors cooperate with each other. However, this is a challenging goal because they are not merely interacting with each other in a simple cascade, but instead form a complex network with multiple feedback loops. The fact that many of these factors also directly interact with microtubules as well as with each other adds further complexity. An important test for the functional significance of an interaction between midzone proteins is to determine how the localisation of a protein is affected by depletion of another protein (localisation epistasis). However, this analysis faces serious limitations when the roles of hub factors, such as PRC1 or centralspindlin, which interact with many different proteins, are to be dissected. Ultimately, we need to devise a tool to specifically disrupt the interaction between two proteins without affecting any other functions, such as the generation of
‘separation-of-function’ mutants, ideally in a temporally controlled manner. Our current knowledge on the interactions and localisation epistasis between the midzone proteins is discussed below and summarised on the Poster. A more complete survey that also includes additional midzone proteins not discussed here can be found in supplementary material Tables S1 and S2.

Interactions between midzone organisers and their upstream and downstream factors

Interactions between core midzone organisers and their upstream regulators, such as mitotic kinases, or downstream factors, such as Rho pathway regulators, are relatively well established. Aurora B kinase, which as a CPC component controls various aspects of mitotic progression, is recruited to the central spindle by MKLP2 after anaphase onset, although it is unclear which of the CPC subunits directly interacts with MKLP2 (Gruneberg et al., 2004; Hümer and Mayer, 2009). It also remains to be determined how the CPC is recruited to the central spindle in organisms that lack MKLP2, such as C. elegans, although a direct interaction between AIR-2 (its Aurora B orthologue) and ZEN-4 (its MKLP1 orthologue) has been reported (Severson et al., 2000). PLK1 is recruited to the central spindle through the binding sites that PLK1 itself produces on PRC1 (Neef et al., 2007) and MKLP2 (Neef et al., 2003) by phosphorylation.

The recruitment of downstream effectors for cleavage furrow induction and abscission also depend on their direct interactions with the midzone motors. For example, ECT2, a major activator of Rho GTPase during cytokinesis, directly interacts with CYK4, and this interaction plays a crucial role in the establishment of the equatorial zone of active Rho, which specifies the division plane (Chalamalasetty et al., 2006; Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yüce et al., 2005; Zhao and Fang, 2005). CYK4 also interacts with CYK4, and this interaction is even more challenging. A relatively well-characterised interaction among the microtubule modulators is the one between PRC1 and CYK4. This interaction can be detected both by co-immunoprecipitation and yeast two-hybrid assays and is thus likely to be direct (Kurasawa et al., 2004). CDK1 negatively regulates this interaction by phosphorylating PRC1 (Zhu and Jiang, 2005; Zhu et al., 2006). Although the central spindle localisation of CYK4 is lost when PRC1 is depleted, PRC1 still localises to the central spindle and the midbody in the absence of CYK4, albeit in a broadened pattern (Zhu and Jiang, 2005). This observation could reflect the role of the PRC1–CYK4 interaction in transporting PRC1 to the plus ends of microtubules (Zhu and Jiang, 2005). However, aberrant PRC1 localisation in the absence of CYK4 can also be explained by an increase in the length of the microtubule overlap (Hu et al., 2011), which is consistent with the in vitro observation that PRC1 and CYK4 have a combinatorial effect in regulating the length of the anti-parallel overlap (Bieling et al., 2010). Further study is needed to clarify the in vivo importance of their direct interaction because a CYK4 construct that lacks the C-terminal tail and thus does not bind PRC1 can still tightly focus itself and PRC1 to the centre of the central spindle (Hu et al., 2011).

Interaction between PRC1 and KIF4

Dissecting the roles of protein–protein interactions between midzone organisers is even more challenging. A relatively well-characterised interaction among the microtubule modulators is the one between PRC1 and KIF4. This interaction can be detected both by co-immunoprecipitation and yeast two-hybrid assays and is thus likely to be direct (Kurasawa et al., 2004). CDK1 negatively regulates this interaction by phosphorylating PRC1 (Zhu and Jiang, 2005; Zhu et al., 2006). Although the central spindle localisation of KIF4 is lost when PRC1 is depleted, PRC1 still localises to the central spindle and the midbody in the absence of KIF4, albeit in a broadened pattern (Zhu and Jiang, 2005). This observation could reflect the role of the PRC1–KIF4 interaction in transporting PRC1 to the plus ends of microtubules (Zhu and Jiang, 2005). However, aberrant PRC1 localisation in the absence of KIF4 can also be explained by an increase in the length of the microtubule overlap (Hu et al., 2011), which is consistent with the in vitro observation that PRC1 and KIF4 have a combinatorial effect in regulating the length of the anti-parallel overlap (Bieling et al., 2010). Further study is needed to clarify the in vivo importance of their direct interaction because a KIF4 construct that lacks the C-terminal tail and thus does not bind PRC1 can still tightly focus itself and PRC1 to the centre of the central spindle (Hu et al., 2011).

Interaction between PRC1 and centralspindlin

PRC1 has also been reported to interact with centralspindlin on the basis of co-immunoprecipitation experiments in HeLa cell lysates (Ban et al., 2004; Gruneberg et al., 2006; Kurasawa et al., 2004). PRC1 was also found to interact with the CYK4 subunit of centralspindlin in a yeast two-hybrid assay (Ban et al., 2004). It is interesting to note that in fission yeast, which does not have centralspindlin, ase1p interacts with the kinesin-6 klp9p, which itself is more closely related to MKLP2 than MKLP1 (Fu et al., 2009). Observation of animal cells that are depleted of PRC1 or of centralspindlin components indicates that their respective localisation to the central spindle is interdependent (Verbrugghe and White, 2004; Verni et al., 2004). However, the significance of the interaction between PRC1 and centralspindlin for their recruitment to the spindle midzone and the formation of the central spindle is unclear because defects in either factor disrupt the central spindle as described in the previous sections. To answer the question of why both of these microtubule-bundling factors are needed for the formation of the central spindle, it will be crucial to generate and study ‘separation-of-function’ mutants of PRC1 and CYK4, which specifically disrupt their interaction without affecting their other functions, such as microtubule bundling.

Conclusion and future perspectives

How exactly multiple microtubule organisers cooperate to form the central spindle and the midbody remains an important and challenging question from both theoretical and technical perspectives. In vitro reconstitution assays and in silico models need to be developed and combined with live cell observation using tools to perturb the protein–protein interactions in a temporally and spatially regulated manner. These approaches will give us greater insight into the mechanisms underlying the complex network of protein machineries that drives precise cytokinesis and ensures genome stability.

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We thank D. Gerlich, A. Piekny and G. Correia for helpful critical comments. We apologise for unintended omissions from the interaction and localisation tables.

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References


**Supplementary Table 1 Interaction matrix of midzone proteins**

Interaction matrix of midzone proteins. Data obtained by co-immunoprecipitation (IP, orange), yeast two-hybrid (Y2H, blue) and in vitro binding with recombinant proteins (IV, green) were summarised. References with colours indicate positive interaction. References without any colour indicate negative interaction. Interactions not tested were left empty.

### Interaction Matrix

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<th>MKLP1</th>
<th>CYK4</th>
<th>KIF4</th>
<th>INCENP</th>
<th>Aurora B</th>
<th>Survivin</th>
<th>Dasra/Borealin</th>
<th>MKLP2</th>
<th>CENPE</th>
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<th>CLASP</th>
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**Central spindle**

**Chromosomal Passenger Complex**

References with colours indicate positive interaction. References without any colour indicate negative interaction. Interactions not tested were left empty.
### Supplementary Table 2: Localisation interdependency matrix of midzone proteins in mammalian cells

Localisation interdependency matrix of midzone proteins in mammalian cells. The effect of the depletion of the proteins listed in rows on the localisation of the proteins in columns is illustrated by colour with additional comments. Dark pink, severe effect; pale pink, weak effect; pale blue, no clear effect. CS: central spindle, FB: Flemming body, CF: cleavage furrow.

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<th>Effects on the localisation of</th>
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<th>MKLP1</th>
<th>CYK4</th>
<th>KIF4</th>
<th>INCENP</th>
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<th>Survivin</th>
<th>Dasra/ Borealin</th>
<th>MKLP2</th>
<th>CENPE</th>
<th>KIF14</th>
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References for Supplementary Tables


