Regulation of midbody formation and function by mitotic kinases

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Abstract

Cytokinesis is the final phase of cell division and safeguards the correct distribution of genomic and cytoplasmic materials between the two nascent daughter cells. The final separation, or abscission, of the daughter cells depends on the proper assembly of an organelle at the intercellular bridge, the midbody, which acts as a platform for the recruitment and organisation of various proteins involved in both the control and execution of the abscission process. Recent studies have led to the identification of the mechanisms, signalling pathways and molecules that control the two tightly linked processes of midbody formation and abscission. Here we review our current knowledge of the role that mitotic kinases play in these processes and offer our perspectives on the potential future challenges that await researchers the field.

1. Introduction

Cell division is one of the most fundamental biological processes and is necessary for growth, development and reproduction in many organisms. It is usually divided in two major phases, karyokinesis (segregation of the nuclear material) and cytokinesis (segregation of the cytoplasmic material). Although karyokinesis is responsible for the faithful division of the genomic material, successful cytokinesis is necessary to complete cell division and to form two distinct daughter cells. Indeed, cytokinesis failure annuls all the previous cell division events, such as chromosome alignment and segregation, and causes polyploidy, which can lead to subsequent defective mitoses.

In a symmetrically dividing animal cell, cytokinesis begins with the ingression of a cleavage furrow at the cell's equatorial cortex after the sister chromatids move towards the two opposite spindle poles in anaphase. Furrow ingression is driven by the constriction of an actomyosin contractile ring that bisects the dividing cell (Fig. 1) [1]. The position of the division plane and the formation of the contractile ring are established by two populations of microtubules: astral and central spindle microtubules. The central spindle is composed by an array of interdigitating and antiparallel microtubules, whose plus ends overlap in a region known as the spindle midzone (Fig. 1). The constriction of the contractile ring progressively compacts the central spindle to form an organelle, the midbody, which provides a platform important for the recruitment and organization of many proteins that regulate the final separation, or abscission, of the two daughter cells (Fig. 1) [2]. Recent studies have also proposed that the midbody may contribute to cell fate determination [3, 4], but studies in *Drosophila* and *C. elegans* have not confirmed this role for the midbody in intact organisms [5, 6].

Most of the initial studies on the mechanics and regulation of cytokinesis have focused in the past on the mechanisms and signalling pathways that control cleavage furrow positioning and ingression. However, in recent years there has been a growing interest in the final process of abscission and the role played by the midbody in this process [2]. Like many other cell division events, midbody formation and abscission are regulated by phosphorylation and dephosphorylation. Here we review our current knowledge of the role that the

major mitotic kinases play in the formation and function of the midbody at the end of cytokinesis.

2. Midbody structure and composition

The midbody was initially described by Flemming at the end of the 19th century [7], but analysed in detail by electron microscopy (EM) more than half a century later [8-11] and more recently by cry-electron tomography [12]. This organelle is composed by tight bundles of microtubules of an initial diameter of about 1 μm, which contains at its centre an amorphous electron-dense matrix. Unfortunately, various terms over the years have been used to describe the different regions of the midbody, generating a certain degree of confusion. The major problem is that some terms refer to EM images while others to light and fluorescence microscopy images. In addition, it is now clear that the midbody changes over time and some structures become apparent only at certain stages. As a consensus has yet to be agreed by international scientific community, we have tried to summarise below the most widely used midbody terminology as illustrated in Fig. 1.

The 'midbody matrix' is universally used to indicate the amorphous electron-dense matrix observed by EM (Fig. 2A). The density of this area reflects a high concentration of proteins. The regions flanking the midbody matrix are usually referred as 'midbody arms' (Fig. 1). The midbody matrix and midbody arms together form the entire midbody. The midbody matrix corresponds to the central "bulge" of the midbody that was first observed by Flemming [7] and thus named the "Flemming body' (Fig. 1). In immunofluorescence images of cells stained for microtubules, the Flemming body appears as a non-stained "dark region" (Fig. 2B). However, overlapping microtubules can be clearly observed in this part of the midbody by both EM (Fig. 2A) and cry-electron tomography [12], indicating that this region appears 'dark' most likely because the dense cluster of proteins that accumulate around microtubules prevents the access of anti-tubulin antibodies. Some of these proteins form a ring around the Flemming body, which has led to the term 'midbody ring' (Figs.1, 2B and 2C). More recently the term 'midbody core' has also been introduced to indicate the internal region of the Flemming body (Fig. 1) [13]. However, it is important to

note that midbody ring and midbody core cannot be distinguished in EM images, as they both form the midbody matrix (Fig. 2A). In addition, while some antigens localize specifically to the midbody ring, others localize to both the midbody core and midbody ring (Fig. 2). Furthermore, midbody core and midbody ring proteins co-localize during the last stages of cytokinesis in a single structure that often persists as a midbody remnant after abscission (Figs 1 and 2B).

The midbody is not a static structure, but it undergoes a series of morphological changes during the late stages of cytokinesis. When furrow ingression is complete, two symmetric constrictions form at both sides of the midbody ring, making the midbody look similar to a 'bow tie' (Fig. 2B). At this stage the chromosomes are almost completely de-condensed. Subsequently, the microtubule bundles become progressively thinner, an event most likely mediated by microtubule de-polymerizing factors such as katanin and spastin [14, 15], and ultimately a distinct abscission site appears usually first at one side of the midbody ring (Fig. 2B). The midbody remnant is therefore typically inherited by one of the two daughter cells and slowly eliminated by autophagy [16]. However, the abscission event can vary in different cell types and the formation of two abscission sites at both sides of the Flemming body has also been described [17].

Both contractile ring and central spindle proteins contribute to midbody formation. Actomyosin filaments, however, disappear soon after completion of furrow ingression. Different midbody components display distinct spatial and temporal localization patterns [18]. Some contractile ring proteins, such as Anillin and Citron kinase (CIT-K), localize mostly to the midbody ring (Fig 2C). Most central spindle proteins, such as the kinesin KIF4A and the MKLP1/KIF23 kinesin component of the centralspindlin complex accumulate throughout the Flemming body (Fig. 2B). Many central spindle and contractile ring proteins persist until the final stages of abscission when, as mentioned above, they merge into a single midbody structure (Figs 1 and 2B) that becomes the midbody remnant after abscission. By contrast, the kinesin MKLP2/KIF20A and the Chromosomal Passenger Complex (CPC) accumulates at the midbody arms and disappear after the 'bow tie' stage (Fig. 2C). Whether these different spatio-temporal localization patterns reflect distinct functions is not fully

understood yet and it is a very interesting and active area of research. What is becoming clear, however, is that kinases - including the major mitotic kinases of the Aurora, Polo, and Cyclin-dependent families - and their counteracting phosphatases play a key role in the organization and function of the midbody and in the abscission process.

3. Aurora B and the Chromosomal Passenger Complex

Aurora B is a member of the Aurora kinase family [19] and the enzymatic component of the CPC, which comprises also Borealin, Survivin and the scaffolding subunit Inner Centromeric Protein (INCENP) [20]. The name of the complex reflects its dynamic distribution during mitosis. It localizes to centromeres from prophase until metaphase, relocates to the central spindle after anaphase onset and then accumulates at the midbody arms after furrow ingression. The translocation from centromeres to the central spindle depends on the interaction of the INCENP's coiled-coiled domain with microtubules and requires the kinesin MKLP2/KIF20A [21, 22]. Consistent with this dynamic distribution, the CPC controls various events throughout cell division, from chromosome condensation in prophase to the final separation or abscission of the two daughter cells. The best-known and most studied role of the CPC is undoubtedly the correction of improper kinetochore-microtubule attachments in prometaphase, but recent evidence have highlighted essential roles for the CPC also during cytokinesis, including midbody assembly and abscission.

3.1 The role of Aurora B in central spindle and midbody assembly

Midbody formation depends of course on the proper assembly of the contractile ring and central spindle. Recent studies have implicated the CPC in the regulation of two protein complexes that are essential for the proper assembly and dynamics of central spindle microtubules: centralspindlin and the KIF4A/PRC1 complex [23, 24]. The centralspindlin complex is a heterotetramer composed by two subunits of the kinesin 6 family member MKLP1/KIF23 and two molecules of RacGAP1/MgcRacGAP/Cyk4 [25]. Centralspindlin is required for central spindle and midbody formation (hence its name) in many organisms,

from nematodes to humans [25-28], but is also known to perform many other crucial roles during cytokinesis, such as RhoA activation and consequent contractile ring assembly and constriction [29-32]. Centralspindlin activity is important for bundling central spindle microtubules and to exert this function it needs to form high-order oligomers [33]. This clustering of centralspindlin complexes is promoted by Aurora B phosphorylation of a serine, S708, located in the MKLP1 C-terminal tail. This phosphorylation prevents the association of MKLP1 to 14-3-3 protein, which inhibits centralspindlin clustering [34]. Thus, Aurora B promotes the assembly of the central spindle via phosphorylation of the kinesin component of the centralspindlin complex.

The kinesin KIF4A and the microtubule-associated protein PRC1 form another complex important for central spindle assembly. PRC1 is able to cross-link and bundle microtubules and is transported to the spindle midzone by KIF4A [35-39]. The formation of the PRC1/KIF4A complex is prevented by cyclin-dependent kinase 1 (Cdk1) phosphorylation in metaphase [36], but after anaphase onset this interaction is instead promoted through Aurora B phosphorylation of KIF4A [40]. This phosphorylation also stimulates the microtubule-dependent ATPase activity of KIF4A, which suppresses microtubule dynamics and limits the length of the central spindle and midbody [40]. Quite interestingly, KIF4A is also responsible for maintaining phosphatase PP2A-B56 at the central spindle, thereby creating a spatially restricted negative feedback loop counteracting Aurora B in cytokinesis [41].

3.2 Regulation of abscission by the CPC

The CPC has been proposed to prevent abscission in the presence of DNA at the cleavage site, thereby avoiding the formation of genetically abnormal daughter cells [42]. In this study it was reported that, if lagging chromatin lingered at the cleavage site, Aurora B remained active and stabilized the intercellular bridge. The Aurora B target(s) in abscission, however, have remained elusive until a few years ago when two studies simultaneously showed that one of such targets is the Snf7 component of the Endosomal Sorting Complex Required for Transport III (ESCRT-III) [43, 44]. ESCRT proteins are highly conserved and best known for catalyzing membrane fission

events both in virus budding and in the sorting of receptors into vesicles that bud off into the lumen of the endosome, creating multivesicular bodies (MVBs) [45]. Four distinct ESCRTs, known as ESCRT-0, -I, -II and -III are sequentially recruited to endosomes, and the final complex in the pathway, ESCRT-III, provides the core machinery that mediates membrane deformation and fission events during MVB biogenesis [46]. Cytokinesis is topologically similar to virus budding and MVB biogenesis, and thus it is not surprising that recent studies have indicated that the ESCRT machinery could also catalyze membrane fission during abscission. ESCRT-III Snf7 components (known as CHMP4 proteins in humans) have been observed to form spiral filaments that appear to remodel and constrict the membrane in order to create the abscission site (Fig.1) [17, 47]. In human cells, ESCRT proteins are initially recruited to the midbody ring through direct interaction of Cep55 with the ESCRT-I component TSG101 and another MVB player, ALIX, which in turn recruits CHMP4 proteins [48, 49]. Cep55, however, is not present in lower eukaryotes such as *Drosophila*, and therefore this recruitment mechanism probably evolved in higher eukaryotes.

The CPC has been proposed to regulate abscission timing through direct interaction with the ESCRT-III Snf7 components in both *Drosophila* and humans [43, 44]. In human cells, Borealin directly interacts with all three CHMP4 proteins, CHMP4A, CHMP4B and CHMP4C, and Aurora B phosphorylates the C-terminal tail of CHMP4C. Two different models have been proposed to explain the regulation of CHMP4 proteins by the CPC. Carlton et al. (2012) proposed that Aurora B phosphorylation promotes CHMP4C translocation to the midbody ring, where this ESCRT-III component inhibits abscission. By contrast, we proposed that CPC controls abscission through inhibition of CHMP4 polymerization and membrane association using two concurrent mechanisms: interaction of its Borealin component with all three CHMP4 proteins and phosphorylation of CHMP4C by Aurora B [43]. These two concomitant events could preclude the formation of the ESCRT-III filaments essential for the formation of the constriction that physically separate the two daughter cells. In this model, CHMP4 proteins could assemble into spiral filaments only after CPC removal from the midbody. Overall, the CPC-mediated regulation of ESCRT-III

has been suggested to act as a surveillance mechanism that prevents abscission in the presence of DNA at the cleavage site [42-44] (Fig.2).

4. Cyclin B/Cyclin-dependent kinase 1

The Cyclin B/Cyclin-dependent kinase 1 (CycB/Cdk1) complex is well known to promote cell division by phosphorylating a large number of proteins required for multiple mitotic events. In parallel, Cdk1 phosphorylation also inhibits the activity of cytokinesis proteins until anaphase onset, when CycB is targeted for destruction by the Anaphase Promoting Complex (APC). For example, as mentioned in section 3.1, Cdk1 prevents the formation of the KIF4/PRC1 complex [36]. Similarly, Cdk1 also phosphorylates the motor domain of MKLP1/KIF23 to prevent the association of centralspindlin with microtubules [50]. These inhibitory phosphorylations prevent the formation of the central spindle until the metaphase/anaphase transition, when CycB is degraded and counteracting phosphatases de-phosphorylate and activate these central spindle-promoting factors. However, in contrast to this inhibitory function, a recent study reported that a small population of CycB persisted at the midbody in both *Drosophila* ovaries and mammalian cells where CycB/Cdk1 activity promoted abscission [51]. The molecular mechanisms and Cdk1 substrates involved in this process are, however, unknown. Furthermore, these authors also showed that CycB/Cdk1 abscission-promoting activity is inhibited by Aurora B phosphorylation of CycB. Thus, the CPC uses at least two redundant pathways, through parallel regulation of CycB/Cdk1 and ESCRT-III, to control abscission timing.

5. Citron kinase

5.1 Citron kinase: A unique midbody organiser

Citron kinase (CIT-K) is a multifunctional protein that, in addition to its N-terminal kinase domain, contains a long central coiled coil region and a C-terminal Citron-Nik1 homology (CNH) domain that mediate the interaction with contractile ring proteins and central spindle components [52-55] (McKenzie, C.,

Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). CIT-K accumulates predominantly to the cleavage furrow [56, 57], but also, to a lesser extent, to the spindle midzone [58] (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). This kinase was initially identified as a RhoA effector and proposed to promote contractile ring constriction by phosphorylating the myosin regulatory light chain (MRLC) [59, 60]. However, CIT-K and its *Drosophila* ortholog, Sticky (Sti), are not required for furrowing [54, 56, 61-63] and evidence in both *Drosophila* and human cells has shown that CIT-K is required for proper RhoA localization at the cleavage site during late cytokinesis, thus behaving more like a RhoA regulator than an effector [53, 54]. Moreover, Sti is not required for MRLC phosphorylation [53, 64]. Together, these findings challenge the original model that CIT-K could, in parallel to Rho kinase (ROK), promote contractile ring constriction during cytokinesis through MRLC phosphorylation [57, 59, 60]. In contrast, a growing body of evidence indicates that CIT-K plays a major role in establishing the proper organization and architecture of the midbody. In the absence of CIT-K, the midbody matrix becomes scarce, fragmented, detached from the cortex, and mis-positioned towards one of the two daughter cells in both *Drosophila* and human cells [52] (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). Moreover, as mentioned previously, CIT-K interacts with several components of the actomyosin ring - actin, myosin, Anillin, and RhoA – and of the central spindle - KIF14, MKLP1/KIF23, and the CPC - thereby providing a crucial link between these two structures [52-55] (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). Consistent with these molecular interactions, CIT-K is required for the recruitment of KIF14 to the cleavage site and proper distribution of actin, myosin, RhoA, MKLP1/KIF23, PRC1 and the CPC [52-55] (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). Strikingly, the orderly arrangement of some midbody proteins, including MKLP1/KIF23 and the CPC, is lost after CIT-K depletion (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). As no other midbody protein has so far been described to have a similar role, this data highlights the

unique role that CIT-K plays in shaping and maintaining the architecture of the midbody.

5.2 Cross-regulation between CIT-K and Aurora B during cytokinesis

We have recently identified a cross-regulatory mechanism between Aurora B and CIT-K that is important for proper midbody formation (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). The CPC and CIT-K depends on each other for proper midbody localization and CIT-K interacts directly with three CPC subunits: Aurora B, Borealin and INCENP. Furthermore, Aurora B phosphorylates several CIT-K residues, including the serine S699 in a region of the coiled coli domain that binds to KIF14 and MKLP1/KIF23. This phosphorylation is important for correct CIT-K localization and interaction with its central spindle partners. Finally, CIT-K stimulates Aurora B activity at the midbody via phosphorylation of the TSS residues in the INCENP IN-box, which is know to be responsible for Aurora B binding and activation [65-68] (McKenzie, C., Bassi, Z.I., Debski, J., Gottardo, M., Callaini, G., Dadlez, M. and D'Avino P.P., manuscript submitted). This mechanism establishes a feedback regulatory loop by which CIT-K reinforces its own localization and consequently the correct architecture of the entire midbody.

6. Polo-like kinase 1

Polo-like kinase 1 (Plk1) is a member of the Polo-like kinase family, which in mammals includes also Plk2, Plk3 and Plk4. These kinases regulates multiple events during cell divisions and Plk1 has been involved in mitotic entry, centrosome maturation, chromosome alignment, and cytokinesis [69]. Plk1 accumulates at centrosomes and kinetochores in early mitosis but after anaphase onset translocates to the spindle midzone thanks to its interaction with PRC1 [70, 71]. Plk1 is known to be important for RhoA activation in early cytokinesis [72-74] but has also been described to regulate the orderly recruitment of ESCRT components at the abscission site through phosphorylation of Cep55 [75]. As mentioned in section 3.2, ESCRT proteins

are recruited at the midbody by Cep55, which is in turn brought to the cleavage site by its interaction with MKLP1/KIF23. Plk1 phosphorylates Cep55 to prevent its binding to MKLP1/KIF23 during furrow ingression and the two proteins can only interact when Plk1 is degraded after completion of furrow ingression [75]. This mechanisms controls abscission timing by ensuring that Cep55, and in turn ESCRT proteins, accumulates at the midbody ring at a very late stage in cytokinesis. It is noteworthy, however, that a Cep55 orthologue is not present in lower eukaryotes, such as *Drosophila*, and therefore this abscission timing mechanism must have appeared late during evolution.

A recent study reported that the localization of the *Drosophila* Plk1 homologue Polo is regulated by Aurora B during cytokinesis [76]. Aurora B was already known to promote Polo kinase activity by phosphorylating its activation loop in mitosis [77]. Kachaner et al. [76] have shown that the same phosphorylation also induces the dissociation of Polo from the microtubule-associated protein Map205 during cytokinesis, allowing Polo to accumulate at the spindle midzone. Failure in this process induced cytokinesis defects [76]. Whether this mechanism is conserved in higher eukaryotes, however, is not yet known.

7. Concluding remarks and future perspectives

It is becoming evident that midbody formation and abscission are finely orchestrated and tightly regulated processes that have been initially underestimated and neglected by the cell division field. In retrospect, this was clearly an oversight because, while cleavage furrow ingression is very rapid - usually about 20-30 minutes depending on the cell type - abscission is much longer and can last several hours. This should have hinted the existence of robust and sophisticated surveillance mechanisms that ensure that abscission occurs at the right time and without harmful consequences for the two daughter cells. It is also not surprising that mitotic kinases have been found to regulate this process, although the discovery of different and complex levels of cross-regulation and redundancy amongst these kinases is, in some way, unexpected (Fig. 3). Aurora B appears to play a central and leading role as it is implicated in every step of midbody formation and function and regulates all the other

kinases, but we have probably just scratched the surface and it is likely that other mechanisms and players are involved. Consistent with this, there is already evidence that other, non-mitotic kinases, are involved in the regulation of abscission [78] and we have very little information about the identity and functions of the phosphatases that counteract the activity of these kinases. Finally, all these phosphorylation and dephosphorylation signalling pathways must be integrated with the orderly and targeted degradation of midbody proteins, an aspect that has yet to begin to be addressed.

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Figure Legends

Fig. 1. Schematic diagram illustrating the different stages of cytokinesis in animal cells. Microtubules are depicted in blue, the actomyosin contractile ring and various midbody regions in red, and the ESCRT-III (endosomal sorting complex required for transport-III) spiral filaments in green.

Fig. 2. Midbody structure in late cytokinesis. (A) Electron microscopy image of a HeLa cell in cytokinesis. A magnification of the midbody is shown on the right. MM, midbody matrix; MT, microtubules. Bars, 1 μm. (B) Midbody stages in late cytokinesis. Human HeLa cells were fixed and stained to detect MKLP1/KIF23, tubulin and DNA. Insets show a 2X magnification of the midbody. The arrowheads indicate the midbody rings while the arrows marks the dark zone. Bars, 10 μm. (C) Human HeLa cells were fixed and stained to detect CIT-K, Aurora B, tubulin and DNA. The bottom panel shows a 3X magnification of the midbody.

Fig. 3. Schematic diagram showing the role of mitotic kinases in midbody formation and abscission. For details see text.

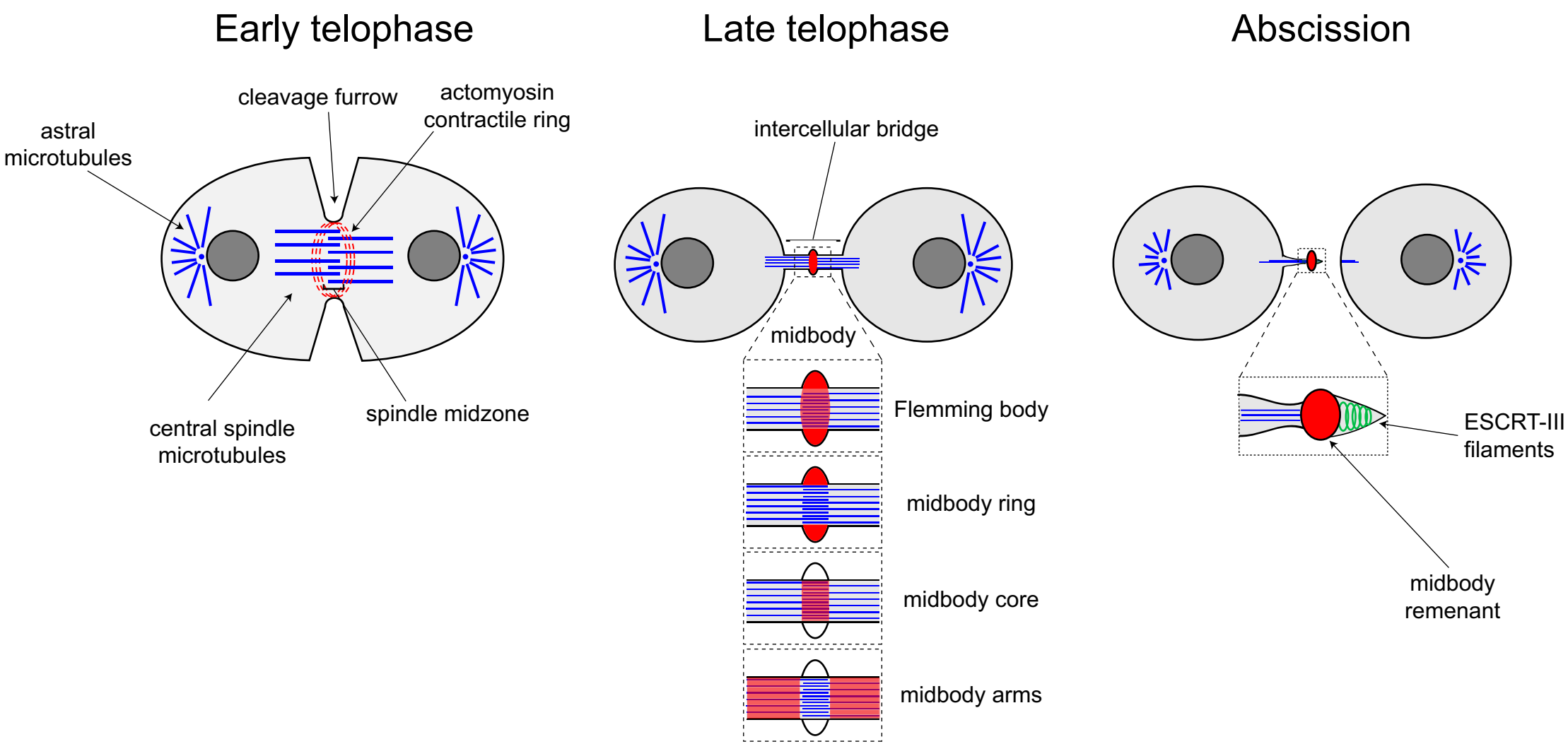

Figure 1
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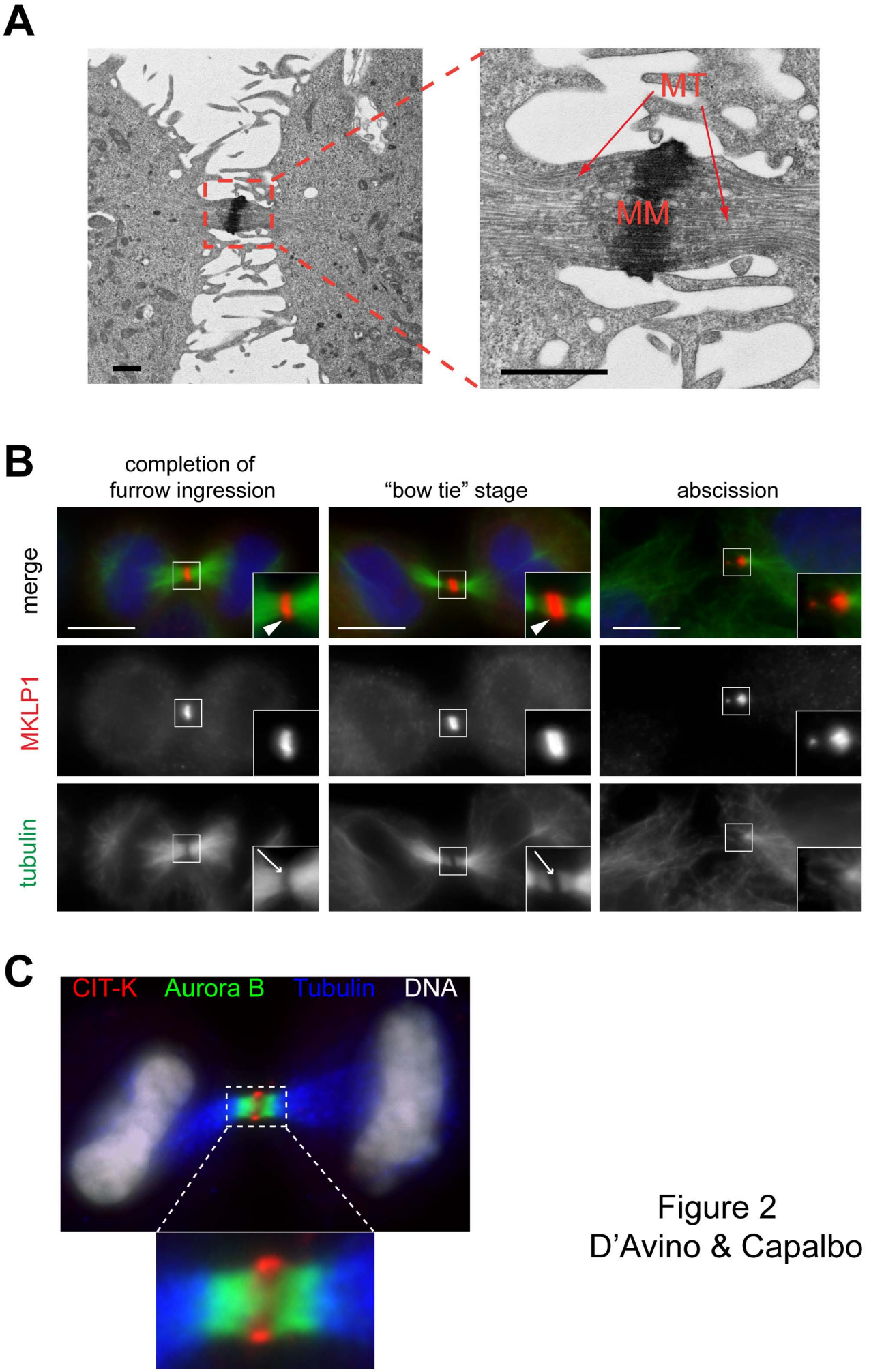

Figure 2
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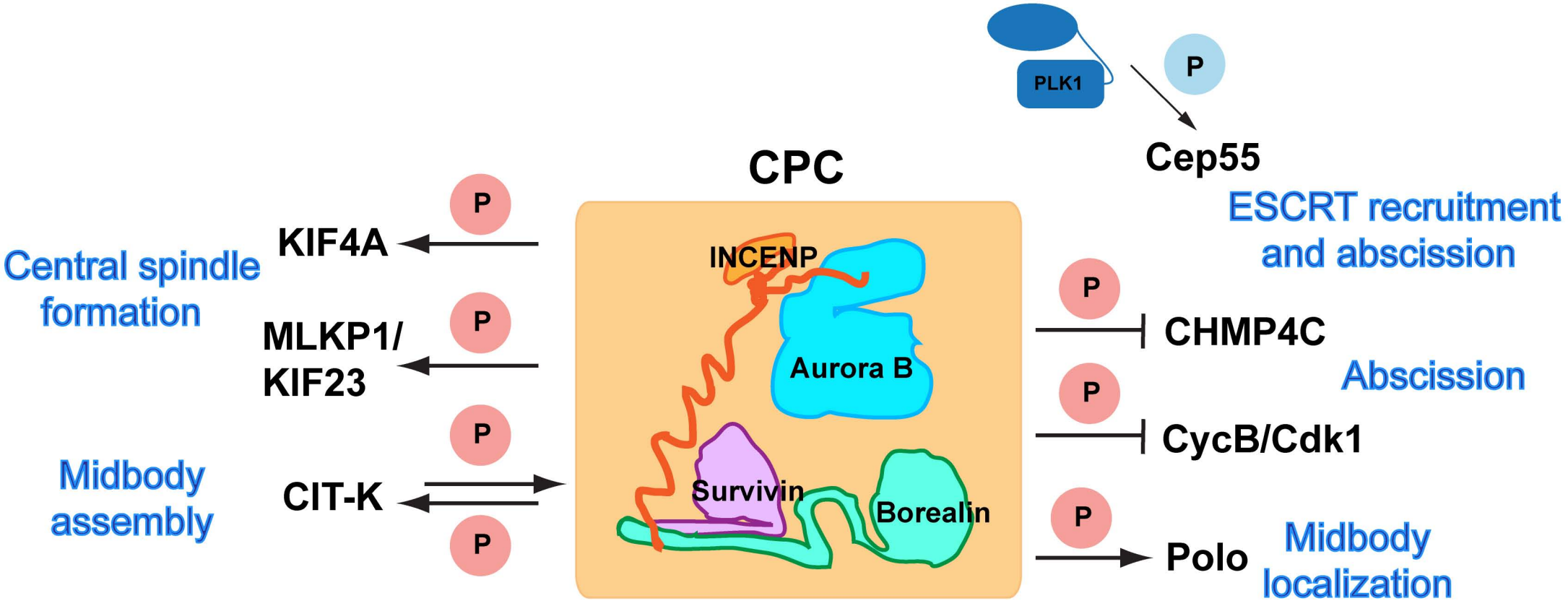

Figure 3
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