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COMMENTARY

Citron kinase – renaissance of a neglected mitotic kinase

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ABSTRACT

Cell division controls the faithful segregation of genomic and cytoplasmic materials between the two nascent daughter cells. Members of the Aurora, Polo and cyclin-dependent (Cdk) kinase families are known to regulate multiple events throughout cell division, whereas another kinase, citron kinase (CIT-K), for a long time has been considered to function solely during cytokinesis, the last phase of cell division. CIT-K was originally proposed to regulate the ingression of the cleavage furrow that forms at the equatorial cortex of the dividing cell after chromosome segregation. However, studies in the last decade have clarified that this kinase is, instead, required for the organization of the midbody in late cytokinesis, and also revealed novel functions of CIT-K earlier in mitosis and in DNA damage control. Moreover, CIT-K mutations have recently been linked to the development of human microcephaly, and CIT-K has been identified as a potential target in cancer therapy. In this Commentary, I describe and re-evaluate the functions and regulation of CIT-K during cell division and its involvement in human disease. Finally, I offer my perspectives on the open questions and future challenges that are necessary to address, in order to fully understand this important and yet unjustly neglected mitotic kinase.

KEY WORDS: Citron kinase, Cell division, Spindle orientation, Polyploidy, Microcephaly, Cancer

Introduction

The fascinating process of cell division is essential for growth, development and reproduction in many organisms. Cell division controls the correct segregation of genome and cytoplasm, including organelles, into daughter cells. In addition, the orientation of the plane of division is crucial for determining cell fate and tissue organization. It is, therefore, not surprising that errors in this process have been implicated in many human diseases, including chromosomal syndromes, microcephaly, sterility and cancer. Complex control and surveillance mechanisms have evolved to ensure the fidelity and robustness of mitotic and meiotic processes. As the chromatin is highly compacted during cell division, these mechanisms rely in large part on post-translational modifications (PTMs) of proteins involved in the assembly and orientation of the mitotic spindle (Prosser and Pelletier, 2017), the attachment and segregation of chromosomes (Musacchio, 2015; Musacchio and Desai, 2017), and the formation and ingression of a cleavage furrow that separates the two daughter cells (Fig. 1) (D'Avino et al., 2015). Two types of PTM are mainly used to regulate cell division, ubiquitylation and phosphorylation/de-phosphorylation. Ubiquitin-mediated protein degradation helps to ensure accurate, timely and unidirectional progression through different stages of mitosis and meiosis, whereas phosphorylation/de-phosphorylation controls the activity, localization and interactions of a wide variety of mitotic factors. Members of the Aurora, Polo and cyclin-dependent (Cdk) kinase families regulate many mitotic events by phosphorylating a plethora of proteins (Archambault and Glover, 2009; Carmena et al., 2012; Malumbres, 2014). Other kinases have, instead, been described to have a more 'restricted' role and to act only during specific phases of cell division. One of these kinases is Citron kinase (CIT-K; gene name *CIT*) that, for a long time, has been considered to function only during the last phase of cell division, i.e. during cytokinesis. CIT-K was originally identified as a binding partner of active forms of the small GTPases Rho and Rac (Di Cunto et al., 1998; Madaule et al., 1995). It was later proposed to promote the constriction of the actomyosin contractile ring that drives cleavage furrow ingression during cytokinesis by phosphorylating the myosin regulatory light chain (MRLC) (Madaule et al., 1998, 2000; Yamashiro et al., 2003). Subsequent studies, however, have revealed that, instead, CIT-K has an evolutionarily conserved role in later stages of cytokinesis, after completion of furrow ingression (Bassi et al., 2011, 2013; D'Avino et al., 2004; Echard et al., 2004; Gai et al., 2011; Gruneberg et al., 2006; McKenzie et al., 2016; Naim et al., 2004; Shandala et al., 2004; Watanabe et al., 2013), and recent evidence indicates that CIT-K is also important in early mitosis (Gai et al., 2016). Furthermore, mutations in CIT-K have been recently found to cause human primary microcephaly, and this kinase has been proposed as a target in anti-cancer therapy (Basit et al., 2016; Fu et al., 2011; Harding et al., 2016; Li et al., 2016; McKenzie and D'Avino, 2016; Shaheen et al., 2016). Finally, there is growing evidence that CIT-K is subject to multiple levels of regulation by both intra- and extra-cellular mechanisms (Jungas et al., 2016; McKenzie et al., 2016). These studies clearly indicate that CIT-K has a much more important role in cell division and human disease than previously thought, suggesting this kinase has been unjustly neglected and misunderstood for a long time. To rectify this, in this Commentary, I review what is known about the functions of CIT-K in cell division, how it is regulated by other signalling molecules, as well as its implication in human disease, before offering my views on the unanswered questions and future challenges that need yet to be tackled in order to fully understand this multifaceted and important mitotic kinase.

CIT-K functions in cell division: cytokinesis and beyond

CIT-K is a large – >2000 amino acids long – multifunctional protein with a complex structure. It contains an N-terminal kinase domain; two central coiled-coil domains (CC1 and CC2), with the latter harbouring a Rho/Rac binding domain (RBD); a cysteine-rich (C1) motif that is adjacent to a pleckstrin homology (PH) domain, and a Citron–Nik1 homology (CNH) domain in the C-terminus (Fig. 2A). Two different CIT isoforms, CIT-K and CIT-N, have been described in mammals, with CIT-N being shorter and lacking the kinase domain (Di Cunto et al., 1998; Madaule et al., 1998). CIT-K is expressed throughout the cell cycle. It is uniformly

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Journal of Cell Science (2017) 130, 1701-1708 doi:10.1242/jcs.200253

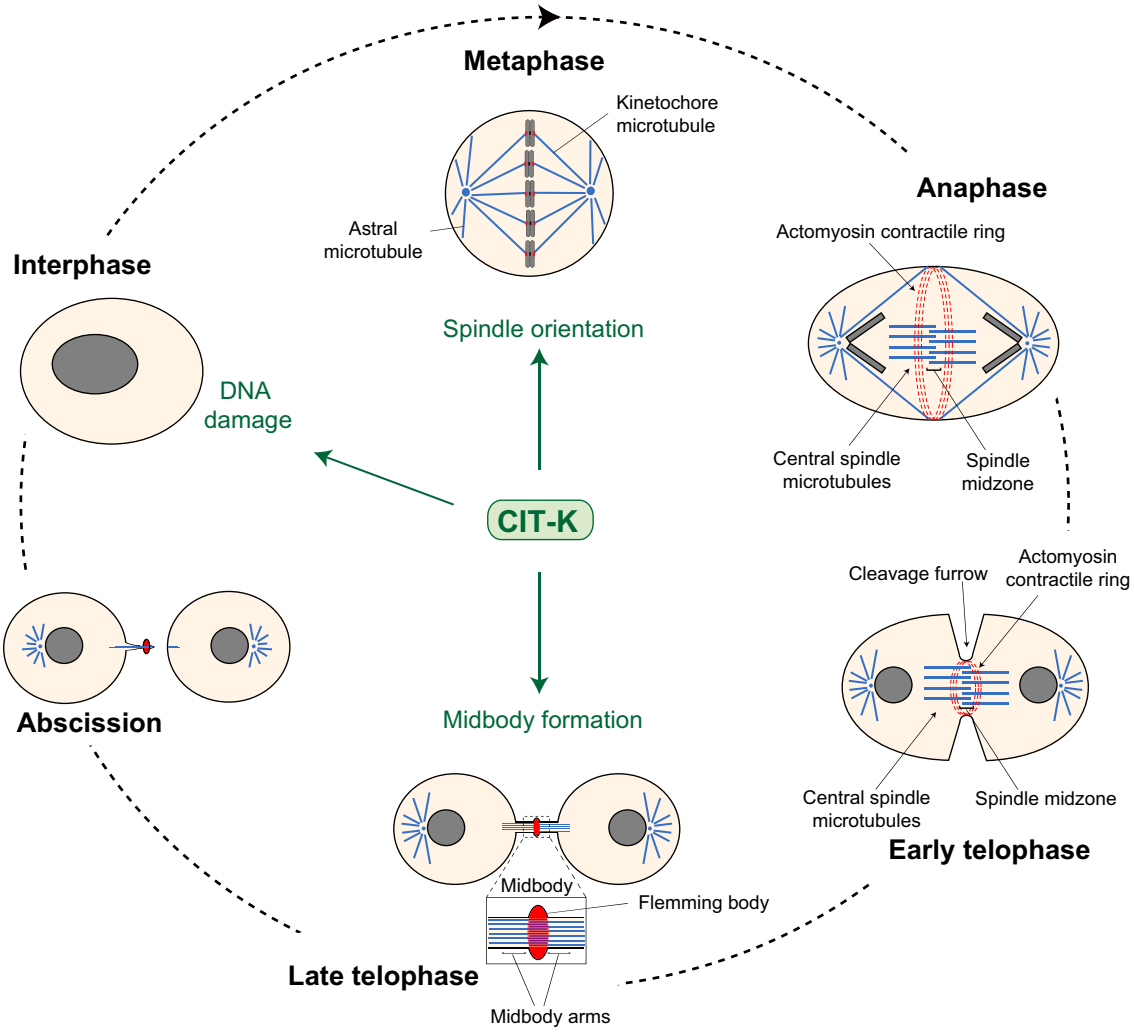

Fig. 1. Schematic representation of the cell cycle with indicated roles of CIT-K. Microtubules are depicted in blue, DNA/chromosomes in grey and kinetochores, actomyosin contractile ring and midbody in red. The different populations of microtubules are also indicated. A magnification of the midbody is shown during late telophase to illustrate its different regions. For a complete description of the midbody, please see D'Avino and Capalbo, 2016.

distributed within the cytoplasm during interphase (Eda et al., 2001) but, during cell division, is enriched at the spindle poles during metaphase (Gai et al., 2016) before accumulation increases substantially at the cleavage furrow after onset of anaphase (Eda et al., 2001; Madaule et al., 1998). After completion of furrowing, CIT-K forms a ring at the midbody, an organelle located at the

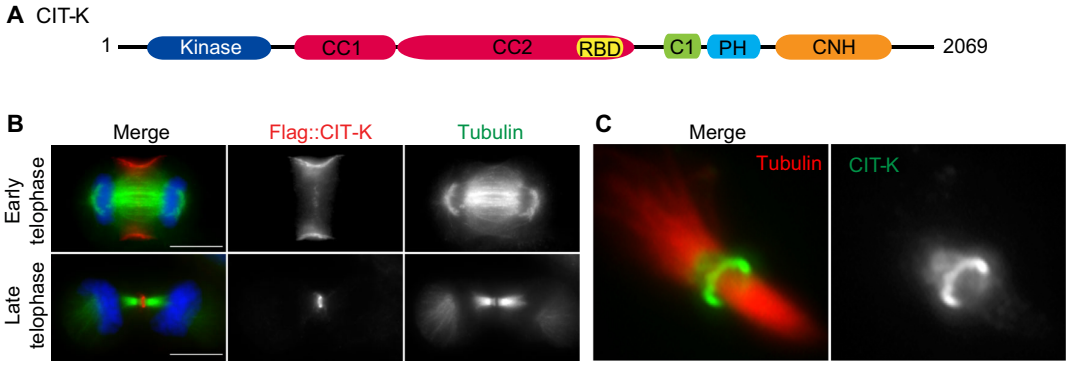

Fig. 2. Structure and localization of CIT-K. (A) Schematic diagram of CIT-K protein domains: Kinase, kinase domain; CC1, coiled-coil region 1; CC2 coiled-coil region 2; RBD, Rho-binding domain; C1, cysteine-rich motif; PH, pleckstrin-homology domain; CNH, Citron–Nik1 homology domain. (B) Human HeLa cells stably expressing a Flag-tagged CIT-K transgene were fixed and stained to detect Flag (red), tubulin (green) and DNA (blue). Scale bars: 10 µm. Adapted with permission from McKenzie et al., 2016, where it was published under a CC-BY licence (https://creativecommons.org/licenses/by/4.0/). (C) Midbodies were purified from HeLa cells and fixed and stained, to detect CIT-K (green) and tubulin (red); notice the ring-like distribution of CIT-K.

centre of the intercellular bridge that acts as a platform for the recruitment of proteins required for the final separation – i.e. abscission – of the two daughter cells (Fig. 2B and C) (D'Avino and Capalbo, 2016; Eda et al., 2001; Gai et al., 2011; Hu et al., 2012; McKenzie et al., 2016; Watanabe et al., 2013). A pool of CIT-K also accumulates at the central spindle, an array of antiparallel and interdigitating microtubules that assemble between the segregating chromosomes during anaphase (Fig. 2B) (Eda et al., 2001; McKenzie et al., 2016; Paramasivam et al., 2007). An identical localization to the cleavage furrow and to the midbody has also been reported for the *Drosophila* CIT-K ortholog Sticky (Sti) (Bassi et al., 2013; D'Avino et al., 2004; Naim et al., 2004; Shandala et al., 2004). However, it is not yet known whether Sti accumulates at the spindle poles during metaphase, despite the fact that the functions of CIT-K during metaphase appear to be conserved in both human and fly cells (Gai et al., 2016). Initial reports more than a decade ago indicated that CIT-K has a role in the contraction of the actomyosin ring by phosphorylating MRLC (Madaule et al., 1998, 2000; Yamashiro et al., 2003). However, these studies were solely on the basis of CIT-K overexpression and *in vitro* experiments, and were challenged by the findings that, in *Drosophila*, depletion or mutations of the *sti* gene caused failure of cytokinesis at a late stage, i.e. after completion of furrow ingression (D'Avino et al., 2004; Echard et al., 2004; Naim et al., 2004; Shandala et al., 2004). Later studies confirmed a similar function for CIT-K in human cells and highlighted its role in the organization of the midbody and in abscission (Bassi et al., 2011, 2013; Gai et al., 2011; McKenzie et al., 2016; Watanabe et al., 2013).

The midbody is composed of several proteins localized to the preceding contractile ring and central spindle that show a very precise and stereotypic distribution (Hu et al., 2012; Mierzwa and Gerlich, 2014). Proteomics-based approaches, as well *in vivo* and *in vitro* binding assays, have indicated that CIT-K has an evolutionarily conserved role in midbody organization by linking components of the contractile ring and central spindle, including anillin, myosin, RhoA, KIF23/MKLP1, KIF14 and the chromosomal passenger complex (CPC) – of which Aurora B is the kinase component – and by maintaining the correct arrangement of midbody proteins (Bassi et al., 2011, 2013; Gai et al., 2011; Gruneberg et al., 2006; McKenzie et al., 2016). Thus, a current model is that CIT-K does not promote furrow ingression through MRLC phosphorylation but that – owing to its modular structure – it, instead, acts as an organizing centre for the midbody (Fig. 3). In the absence of CIT-K, the highly ordered arrangement of midbody proteins and the connection between the cortex and the central spindle microtubules are lost, which in turn leads to abscission failure (McKenzie et al., 2016). Another additional mechanism by which CIT-K can contribute to the stabilization of midbody microtubules is by indirectly altering the phosphorylation of tubulin beta III (TUBB3) (Sgrò et al., 2016). CIT-K is necessary for the recruitment of casein kinase 2 alpha (CK2α) at the midbody, which in turn phosphorylates TUBB3 to stabilize microtubules.

There had been no evidence for a role of CIT-K in other mitotic events besides cytokinesis, until a recent study reported that it is also required for the orientation of the mitotic spindle during metaphase (Gai et al., 2016); this work used as a starting point the previous finding that CIT-K interacts with the abnormal spindle-like microcephaly-associated (ASPM) protein, which is the ortholog of the *Drosophila* abnormal spindle protein (Asp) (Paramasivam et al., 2007). Asp/ASPM associates with the minus ends of microtubules and localizes to the spindle poles during metaphase, and to the central spindle after anaphase onset; thus, pointing to an important role in the organization and polymerization of spindle microtubules throughout cell division (do Carmo Avides and Glover, 1999; Higgins et al., 2010; Saunders et al., 1997; Wakefield et al., 2001). Gai et al. now found that CIT-K accumulates at the spindle poles during metaphase and is necessary for the orientation of the mitotic spindle in human, mouse and fly cells (Gai et al., 2016). Both events depend on the interaction of CIT-K with ASPM, and the latter also requires its kinase activity. Furthermore, the authors also showed that CIT-K can regulate the nucleation, length and stability of astral microtubules (Gai et al., 2016).

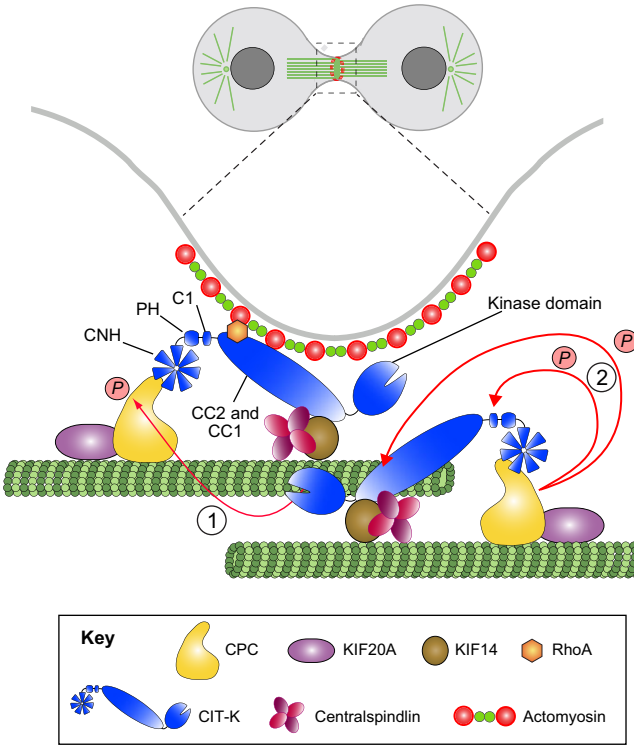

Fig. 3. Cartoon illustrating the interactions of CIT-K at the midbody. The centralspindlin complex is a heterotetramer composed of two subunits of MKLP1 and two subunits of RacGAP1. The chromosomal passenger complex (CPC) is composed of four subunits: the kinase Aurora B, the scaffold subunit INCENP, the cell division cycle-associated protein Borealin and the apoptosis inhibitor protein Survivin (also known as BIRC5). Red arrows indicate (1) phosphorylation of the INCENP TSS motif within the CPC by CIT-K and (2) phosphorylation of the CIT-K CC1 and C1 domains of by the CPC component Aurora B. The figure has been adapted from McKenzie et al., 2016 where it was published under a CC-BY licence (https://creativecommons.org/licenses/by/4.0/).

Although the exact molecular mechanisms by which CIT-K regulates spindle orientation and microtubule dynamics still need to be elucidated, these recent findings clearly demonstrate that the roles of CIT-K in cell division have been underestimated and overlooked. Moreover, as discussed in the next paragraph, there is growing evidence that CIT-K is subject to multiple levels of regulation, which suggests that the activity and function of this kinase is tightly controlled, thus, further underscoring its importance in cell division.

CIT-K regulation by Rho GTPases, mitotic kinases and ephrin/ephrin receptor signalling

As mentioned above, CIT-K was initially identified as a protein interacting with active, GTP-bound forms of Rho and Rac GTPases (Madaule et al., 1995). This led to the obvious hypothesis that

CIT-K is a Rho/Rac effector and, subsequently, the proposition that RhoA activates CIT-K during cytokinesis to promote the constriction of the actomyosin ring by mediating MRLC phosphorylation, similarly to the role of the closely related Rho kinase (ROCK) (Madaule et al., 2000). However, clear evidence that Rho GTPases can, indeed, activate CIT-K is still lacking, mainly owing to the challenge in designing an assay, either *in vitro* or *in vivo*, to test the possible 'activation' of this large and complex kinase. Furthermore, the paucity of demonstrated CIT-K substrates also makes it difficult to test whether its kinase activity is possibly promoted by Rho GTPases. Indeed, only a single *bona fide* substrate of CIT-K has been identified thus far, the inner centromere protein (INCENP) component of the CPC; but, unfortunately, the site in INCENP that is phosphorylated by CIT-K is also targeted by the CPC component Aurora B, further adding to the difficulty in assessing CIT-K activity *in vivo* (McKenzie et al., 2016). Moreover, the RBD domain does not appear to be evolutionarily conserved because the *Drosophila* RhoA homologue Rho1 interacts with the CNH domain of Sti (Bassi et al., 2011; Shandala et al., 2004). It was initially reported that RhoA is necessary for the recruitment of CIT-K to the cleavage furrow in human cells (Eda et al., 2001). However, this is likely to be an indirect effect because of evidence in *Drosophila* that Sti is recruited to the cleavage furrow through its association with actomyosin filaments (Bassi et al., 2011). Finally, CIT-K is required for correct RhoA localization at the cleavage site during late cytokinesis in both *Drosophila* and human cells, indicating that CIT-K behaves more like a RhoA regulator than an effector (Bassi et al., 2011; Gai et al., 2011; Watanabe et al., 2013). Therefore, the relationship between RhoA and CIT-K appears less straightforward than initially anticipated.

There is also clear evidence emerging that CIT-K is subject to complex phosphorylation-mediated regulation by both intra- and extra-cellular signalling pathways. Various studies, based on either large-scale or CIT-K-focused phospho-proteomics analyses, have identified several phosphorylation sites distributed across the entire protein (Jungas et al., 2016; McKenzie et al., 2016; McKenzie and D'Avino, 2016) (see also the PhosphoSitePlus database, http://www.phosphosite.org/, for a full list of phosphorylated CIT-K residues). Many phosphorylated residues match the consensus sequence of other mitotic serine/threonine kinases, including Aurora B, Cdk1 and Plk1, and my group has recently confirmed that CIT-K is a direct substrate of both Aurora B and Cdk1 (McKenzie et al., 2016; and Zuni Irma Bassi and P.P. D'A. unpublished data). Although the effect of Cdk1-mediated CIT-K phosphorylation is still unclear, we have found that Aurora B and CIT-K crossregulate each other, and that this regulatory mechanism is important for the organization of the midbody (McKenzie et al., 2016). CIT-K is required for the correct localization of the CPC at the midbody and directly binds to at least three CPC components, Aurora B, Borealin (also known as CDCA8) and INCENP. In addition, CIT-K phosphorylates INCENP at a threonine–serine–serine (TSS) motif that is required for Aurora B activation, thereby promoting full activation of Aurora B. Reciprocally, Aurora B also controls CIT-K localization and phosphorylates CIT-K within its CC1 and C1 domains (see Fig. 2A) (McKenzie et al., 2016). The function of the C1 domain of CIT-K has not been characterized but it is likely to mediate its association with membrane phospholipids, similarly to the role of the C1 domain of RacGAP1 (also known as MgcRacGAP), a subunit of the centralspindlin complex (Lekomtsev et al., 2012). This suggests that Aurora B regulates the interaction of CIT-K with the plasma membrane but further studies are needed to define such a regulative mechanism. In our recent work, we have established that Aurora B-mediated phosphorylation of the CC1 domain prevents accumulation of CIT-K at the spindle midzone in early cytokinesis by inhibiting its interaction with the central spindle protein MKLP1 (also known as KIF23) – the motor component of the centralspindlin complex – and the CPC itself (McKenzie et al., 2016). We have proposed that this acts as a mechanism to temporally regulate the transition from central spindle to midbody (McKenzie et al., 2016). In the early stages of cytokinesis, when Aurora B, CIT-K and MKLP1 colocalize to the spindle midzone, Aurora-B-mediated phosphorylation of CIT-K would inhibit a strong association between CIT-K and MKLP1 during furrow ingression in order to not interfere with the clustering of centralspindlin, which is also promoted by Aurora B and necessary for the bundling of central spindle microtubules (Fig. 4A) (Douglas et al., 2010). However, after furrow completion, Aurora B accumulates at the midbody arms, parting from CIT-K and MKLP1 that, instead, localize to the Flemming body (Fig. 4B) (D'Avino and Capalbo, 2016; McKenzie et al., 2016). Because of this separation, Aurora B is likely to have less effect on CIT-K, allowing it to strongly bind to MKLP1 and stabilize the midbody (Fig. 4B).

It is less straightforward to speculate on the possible role of CIT-K-mediated activation of Aurora B through phosphorylation of INCENP. One simple hypothesis is that this is a regulatory feedback loop to both reinforce Aurora B activity and prevent CIT-K accumulation at the spindle midzone during furrow ingression (Fig. 4). However, the evidence that CIT-K and the CPC are both required for midbody organization and for the orderly arrangement of midbody proteins (McKenzie et al., 2016) suggests that CIT-K and CPC also cooperate during late cytokinesis, and that this crossregulatory mechanism is important to establish different 'identities' in distinct regions of the midbody.

CIT-K has also recently been found to be regulated by extracellular signals (Jungas et al., 2016), as activation of the Ephrin/ephrin receptor (Eph) signalling pathway, which is known to regulate cell adhesion, causes persistent midbody and cytokinesis failure. Jungas and colleagues reported that CIT-K failed to form a distinct ring and showed diffuse distribution at the midbody after activation of Ephrin/Eph signalling through the EphB2 receptor. The authors also showed that CIT-K is directly phosphorylated by Src kinase in EphB2-activated cells and that Src-mediated phosphorylation of the RBD of CIT-K promotes its association with active RhoA (Jungas et al., 2016). On the basis of these data, the authors speculated that Ephrin/EphB2 forward-signalling interferes with cytokinesis by promoting the association of CIT-K with active RhoA. This association might prevent RhoA inactivation during late cytokinesis, an event necessary for the disassembly of the contractile ring and subsequent abscission. However, this mechanism does not fully explain the abnormal localization of CIT-K in EphB2-activated cells and why a CIT-K fragment containing only the C1 domain – which is not phosphorylated by Src, can rescue the EphB2-induced cytokinesis defects (Jungas et al., 2016). Consistent with this, Src kinase was shown to be able to phosphorylate several CIT-K residues outside the RBD *in vitro* (Jungas et al., 2016). Thus, further analysis is necessary to fully understand the regulation of CIT-K by Ephrin/EphB2 signalling. The exact scope of such Ephrin/EphB2-mediated regulation of CIT-K in the context of whole tissues is not completely clear. Loss of Ephrin/Eph signalling in mouse neural progenitors results in a reduction of polyploid neurons in the neocortex, which was proposed to be achieved by affecting the function of CIT-K during cytokinesis (Jungas et al., 2016). However, as the role of Ephrin/Eph signalling is established in

Journal of Cell Science

Journal of Cell Science (2017) 130, 1701-1708 doi:10.1242/jcs.200253

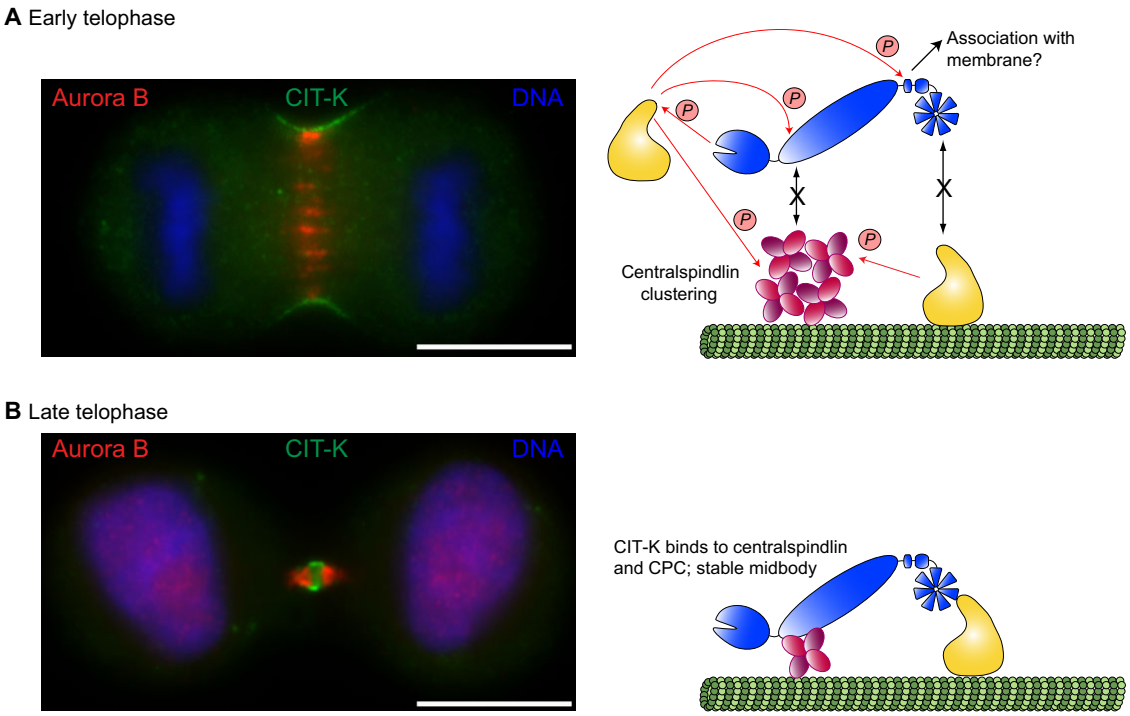

Fig. 4. Model for crossregulation between CIT-K and the chromosomal passenger complex (CPC) during cytokinesis. (A,B) The images on the left show HeLa cells stained to detect the CPC kinase component Aurora B (red), CIT-K (green) and DNA (blue). CIT-K marks the cleavage furrow in A and the midbody ring in B. Note that, although not evident in the image shown in A, Aurora B and CIT-K are also known to colocalise at the cleavage furrow (McKenzie et al., 2016). Scale bars: 10 µm. (A). During early telophase, Aurora B-mediated phosphorylation of the CC1 domain of CIT-K prevents its interaction with centralspindlin and its accumulation of at the spindle midzone. This allows the clustering of centralspindlin, which is promoted by phosphorylation of Aurora B. At the same time, Aurora B-mediated phosphorylation of the CC1 domain within CIT-K could promote its association with the plasma membrane. (B) During late telophase, Aurora B accumulates at the midbody arms, whereas CIT-K and MKLP1 localise to the Flemming body. This different localization would reduce the influence of Aurora B on both CIT-K and MKLP1, so the two proteins can strongly interact to stabilize the midbody. Red arrows indicate phosphorylation.

cell adhesion, it is also tempting to speculate that EphB2-mediated regulation of CIT-K contributes to control the adhesion of dividing cells to their neighbours during the dramatic cell shape changes that occur during cytokinesis. Finally, because Src kinase is involved in many signalling pathways that are activated by plasma membrane receptors, multiple pathways might converge to regulate CIT-K.

To conclude, it is evident that many signalling events are able to affect CIT-K, suggesting that the activity, localization and functions of this kinase need to be precisely regulated. Therefore, it did not come as a total surprise that, as discussed below, deregulation and loss of CIT-K were recently found to be involved in human diseases (Basit et al., 2016; Harding et al., 2016; Li et al., 2016; Shaheen et al., 2016).

CIT-K and disease

Early findings that CIT-K-knockout mice show growth delay, reduced life span, ataxia and defective neurogenesis (Di Cunto et al., 2000), and that a *CIT* mutation in rat affects brain development (Sarkisian et al., 2002) led to the hypothesis that CIT-K has a particularly important role in the development of the central nervous system (CNS). Indeed, four recent studies have reported that mutations in *CIT* are responsible for some cases of primary microcephaly in humans (Basit et al., 2016; Harding et al., 2016; Li et al., 2016; Shaheen et al., 2016), thereby, further supporting this hypothesis and, for the first time, linking CIT-K to human disease. In both rodents and humans, the defects in CNS development were associated with failure of cytokinesis and severe apoptosis. Mutations of *CIT* that were found to be associated with human microcephaly mapped to different locations within the gene, and included point mutations in conserved amino acids and splice sites, as well as deletions (Basit et al., 2016; Harding et al., 2016; Li et al., 2016; Shaheen et al., 2016). Some of the mutations identified in the kinase domain abolish the kinase activity of CIT-K *in vitro* (Li et al., 2016), clearly indicating that the kinase activity is essential for brain development and cytokinesis. The CIT-K-binding partner ASPM is one of the most frequently mutated genes in autosomal-recessive human microcephaly and one of the main determinants of human brain size (Nicholas et al., 2009), suggesting that a common cellular mechanism underlies the cause of primary microcephaly. The presence of multinucleated neurons in patients carrying *CIT* mutations (Harding et al., 2016; Li et al., 2016) and the established roles for both CIT-K and ASPM in cytokinesis (Higgins et al., 2010; Paramasivam et al., 2007) suggest that cytokinesis failure and, consequently, apoptosis is one such possible mechanism. However, as both proteins also cooperate in spindle orientation (Gai et al., 2016), defects during this step of mitosis cannot be discounted as a possible cause.

Chromosomal instability (CIN) is a hallmark of many cancers (Hanahan and Weinberg, 2011) and is characterized by recurrent chromosomal changes that contribute to tumorigenesis by altering the balance of critical growth and death pathways. CIN has been implicated in cancer evolution, diversification and heterogeneity, drug resistance, as well as in the development of metastases (Heng et al., 2013; McGranahan et al., 2012). CIN often originates from cell division defects and, therefore, a potential anti-cancer strategy could be to increase aneuploidy and polyploidy above the thresholds that are compatible with cell viability by interfering with cell division in cancer cells (McGranahan et al., 2012). Furthermore,

such a strategy would specifically target highly proliferating CIN cancer cells and, thus, be potentially less toxic for normal tissues. Following this line of thought and because CIT-K had been indicated to be a potential target in hepatocellular carcinoma (Fu et al., 2011), my lab recently investigated the depletion of CIT-K as a a strategy to promote cell death in cancer cells. Indeed, we found that CIT-K depletion caused failure of cytokinesis and dramatically decreased cell proliferation in a large panel of breast, cervical and colorectal cancer cell lines. This proliferation decrease directly correlated with an increase in apoptosis via both p53-dependent and -independent pathways. Finally, we have also shown that actively dividing and polyploid cancer cells are more susceptible to CIT-K depletion, suggesting that targeting CIT-K is a promising anti-cancer therapeutic approach for a wide range of cancers, especially those that are characterized by rapid cell proliferation and polyploidy (McKenzie and D'Avino, 2016). However, it is important to note that CIT-K loss can cause CIN owing to its involvement in DNA damage control and independently of its role in cytokinesis (Bianchi et al., 2017). This raises the possibility that the effects on cancer cells upon CIT-K depletion result from a combination of cytokinesis failure and increased DNA damage.

Taken together, these findings clearly indicate a crucial role for CIT-K in mammalian CNS development and point to its potential targeting for anticancer therapy. A detailed knowledge of the mechanisms of action of this kinase is, therefore, likely to lead to the identification of new molecules and pathways that could be used for the diagnosis and treatment of human diseases.

The importance of the kinase activity of CIT-K

There has been some debate over the last years with regard to the role and physiological relevance of the kinase activity of CIT-K. Initial studies indicated that the isolated kinase domain of CIT-K is active *in vitro* (Di Cunto et al., 1998), and the first clue that its kinase activity might be a physiologically relevant role came from the analysis of the mouse gene knockout (Di Cunto et al., 2000; Di Cunto et al., 2002). As mentioned earlier, the mouse *CIT* gene encodes two different proteins, CIT-K and CIT-N, with the latter lacking the kinase domain (Di Cunto et al., 1998; Madaule et al., 1998). Already some time ago, Di Cunto et al. generated a knockout mouse mutant carrying an insertion that specifically affected CIT-K, without altering the expression of CIT-N (Di Cunto et al., 2000). CIT-K$^{-/-}$ mice show abnormal CNS development and defects in spermatogenesis (Di Cunto et al., 2000; Di Cunto et al., 2002), supporting an *in vivo* function for the kinase activity of CIT-K. However, because CIT-K and CIT-N do not show identical expression patterns (Di Cunto et al., 2000), it was impossible to extrapolate the exact role of the kinase activity of CIT-K from these experiments. Further support for a physiological role of the kinase activity of CIT-K came from observations that a kinase-dead mutant cannot rescue the defects caused by depletion of endogenous CIT-K, such as cytokinesis failure, midbody organisation and spindle orientation, in both *Drosophila* and human cells (Bassi et al., 2011; Gai et al., 2016; McKenzie et al., 2016). By contrast, other groups reported, instead, that the kinase activity of CIT-K is not necessary for cytokinesis in *Drosophila* and human cells (El Amine et al., 2013; Watanabe et al., 2013). However, in their study, Watanabe et al. reported that a kinase-dead CIT-K mutant only partially rescues the multinucleate phenotype caused by depletion of endogenous CIT-K (Watanabe et al., 2013), indicating that the kinase activity of CIT-K is necessary for some functions in cytokinesis. Although these contradictory reports had cast some doubts on the physiological relevance of the kinase activity of CIT-K, the more recent findings, implying impaired kinase activity in microcephaly and cytokinesis failure in humans (Li et al., 2016), convincingly demonstrate the *in vivo* importance of this particular function of CIT-K.

Thus, because the vast majority of data strongly support a physiological role for the kinase activity of CIT-K, any discrepancies amongst studies might simply reflect differences in techniques and/or methodologies used.

Conclusions and future perspectives

The story of CIT-K exemplifies and emphasises how important it is in science to have an open mind and to think 'outside the box'. The initial but, later found to be incorrect, model positing a role for CIT-K as a Rho effector that promotes the constriction of the actomyosin ring during cytokinesis (Madaule et al., 1998, 2000; Yamashiro et al., 2003), might have hindered efforts to further characterise the multiple roles and mechanisms of action of this important kinase (Fig. 1) because the cellular function of CIT-K was assumed to have been established, with it only playing an ancillary role in cytokinesis. I hope that this Commentary will serve to 'rehabilitate' and re-evaluate this important kinase, and also eliminate the misconception that CIT-K is required for the constriction of the actomyosin ring – which is still present in the scientific community. Undoubtedly, the recent studies are likely to stimulate the interest of the scientific community in CIT-K. Indeed, there are a number of questions that remain to be answered. First and foremost, we need to identify the substrates and partners of CIT-K at different stages of the cell cycle to fully understand its mechanisms of action. Only a single *bona fide* CIT-K substrate has been identified thus far and the CIT-K interactome has only been characterised during telophase (McKenzie et al., 2016). Second, it will be important to establish tools, such as small-molecule inhibitors and analogue-sensitive mutants, to address the role of its kinase activity *in vivo*. Third, we do not know why CIT-K is necessary in every tissue in fly (D'Avino et al., 2004; Naim et al., 2004; Shandala et al., 2004), whereas it appears to be more important for the normal development and function of the CNS in mammals (Basit et al., 2016; Di Cunto et al., 2000, 2002; Harding et al., 2016; Li et al., 2016; Shaheen et al., 2016), despite the fact that its depletion causes cytokinesis failure in different types of human cultured cell (McKenzie and D'Avino, 2016). This discrepancy between *in vivo* an *ex vivo* model systems might simply reflect a difference in the requirement of CIT-K and midbody formation in cultured cells versus whole tissues. Another possibility is that some tissues are more sensitive than others to the loss of CIT-K. For instance, Sgrò et al. proposed recently that the expression of the tissue-specific tubulin variant TUBB3 sensitizes cultured neuronal progenitors to CIT-K loss (Sgrò et al., 2016); however, we could not find a correlation between TUBB3 expression and CIT-K depletion in various cancer cell lines (McKenzie and D'Avino, 2016). It is also important to point out that CIT-K is involved in both spindle orientation and midbody formation, two events that have been implicated in establishing cell fate and polarity (Bergstralh and St Johnston, 2014; Dionne et al., 2015). Therefore, it would be interesting to know whether CIT-K functions in yet more biological processes, such as stemness, differentiation, and tissue organization and remodelling. Last, there is evidence that CIT-K has other roles besides in cell division. In addition to its recently described role in DNA damage (Bianchi et al., 2017), CIT-K has also been proposed to function in virus budding, organization of the Golgi

complex and epigenetic gene silencing (Camera et al., 2003; Ding et al., 2016; Loomis et al., 2006; Sweeney et al., 2008) but, as yet, there are no major insights into its mechanisms of action in these processes.

There is clearly still much work to do before we fully understand this complex and multifunctional kinase but, hopefully, the discussion in this Commentary contributes to raising the profile of this fascinating but understudied kinase, and sparks interest in studying its roles more extensively in the future.

Acknowledgements

I thank all the people that have contributed to our studies of CIT-K and, in particular, two of my former graduate students, Zuni Bassi and Callum McKenzie, who enthusiastically embarked on a treacherous CIT-K journey against all the odds. I am grateful to F. Di Cunto and L. Capalbo for their critical reading of the manuscript and apologise to all those colleagues, whose work could not be discussed and/or cited due to length limitations. Finally, I thank the Cambridge Cancer Centre for a PhD studentship to investigate the role of CIT-K in cancer.

Competing interests

The authors declare no competing or financial interests.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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