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COMMENTARY

Novel functions of endocytic player clathrin in mitosis

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Clathrin has been widely recognized as a pivotal player in endocytosis, in which several adaptors and accessory proteins are involved. Recent studies suggested that clathrin is also essential for cell division. Here this review mainly focuses on the clathrin-dependent mechanisms involved in spindle assembly and chromosome alignment. In mitosis, clathrin forms a complex with phosphorylated TACC3 to ensure spindle stability and proper chromosome alignment. The clathrin-regulated mechanism in mitosis requires the crosstalk among clathrin, spindle assembly factors (SAFs), Ran-GTP and mitotic kinases. Meanwhile, a coordinated mechanism is required for role transitions of clathrin during endocytosis and mitosis. Taken together, the findings of the multiple functions of clathrin besides endocytosis have expanded our understanding of the basic cellular activities.

Keywords**:** clathrin; endocytosis; mitosis; TACC3; spindle assembly; chromosome alignment
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Clathrin-mediated endocytosis is a vesicular transport process for a variety of cellular events including signal transduction, nutrient import and synaptic vesicles [1]. To uptake materials from environment by this process, the first step for a cell is to organize the clathrin lattice-coated vesicles [2, 3]. Clathrin lattice is assembled from triskelions, each of which is constituted from three heavy chains (CHCs) and three light chains (CLCs) [4]. Once the site for the formation of the clathrin-coated vesicles is decided, the membrane initially curves and internalizes into small vesicle pits, and around the pits, clathrin and its various binding proteins organize the clathrin coats to form the vesicles. Then, scission of the clathrin-coated vesicles from the cell membrane enables cargos to be transported to the target place [5, 6].

Mitosis is the process of cell division to maintain genomic information in eukaryotes. During mitosis, the macromolecular machine known as the spindle segregates chromosomes to two daughter cells [7, 8]. In most eukaryotic cells, the mitotic spindle is composed of microtubules, centrosomes and chromosomes. The highly dynamic microtubules form a bipolar structure regulated by a variety of motor proteins, the centrosomes function as the main microtubule sites in cells and chromosomes function in microtubule self-assembly and microtubule attachment [7]. Assembly of the mitotic spindle is orchestrated by the microtubule structure and the membranous spindle matrix together [9].

Clathrin was reported to be localized on mitotic spindles and function in mitosis a few years ago [10-14]. More recently, the role of clathrin in mitosis has been widely recognized. Several individual groups including ours have shed novel lights on the function of clathrin in mitosis at the molecular level [15-18]. These studies demonstrated that clathrin forms a complex with TACC3 to regulate spindle assembly and chromosome alignment. In this paper, we mainly discuss the progress on the regulation mechanisms of clathrin in mitotic spindle assembly and chromosome alignment and the relations between clathrin-dependent endocytosis and mitosis.

Clathrin and mitotic spindle assembly

The assembly of spindle apparatus in mitosis guarantees one cell to produce two genetically identical daughter cells. During the process of spindle assembly, the endocytic protein clathrin has been supposed to be involved [12]. It was found that clathrin is localized on spindle during cell division [10-14]. Clathrin knockdown by RNAi causes defects in spindle assembly, suggesting that

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clathrin may play a role in spindle assembly. B-Myb is a transcription factor in vertebrate cells and its knockdown by RNAi abolishes the localization of clathrin on mitotic spindles [14]. It was proposed that the B-Myb complex containing clathrin and filamin together contributes to the function of mitotic spindle and normal mitotic progression [14]. Cyclin G-associated kinase (GAK), which was previously known to regulate clathrin in endocytosis, was also found to be required for the localization of clathrin on spindles [19, 20].

Recently, several groups found that clathrin regulates spindle assembly by targeting TACC3 to the spindles (Figure 1A) [15-17]. During mitosis, Aurora A phosphorylates TACC3 and this phosphorylation ensures TACC3 to localize to the spindles in *Drosophila*, *Xenopus* and human [21-25]. The road leading to discovery of the novel function of clathrin in mitosis started from exploring how phosphorylation- catalyzed TACC3 affects mitotic spindle assembly. By mass spectrometry analysis, clathrin was initially identified as one of the specific binding partners of phosphorylated TACC3 [15-17]. Through site-directed mutagenesis studies joining with biochemical and cell biological assays, it was demonstrated that the binding of CHC and phosphorylated TACC3 is required for proper mitotic spindle assembly. In early mitosis, TACC3 is activated through the phosphorylation by Aurora A. Soon after the activation, the phosphorylated TACC3 forms a complex with CHC and together loads to spindles [15-17]. Subsequently, the CHC-TACC3 complex further recruits other molecules such as XMAP215/ch-TOG to ensure spindle stability [16, 26].

Aurora A phosphorylates TACC3 at three sites (S33/S620/S626 in *Xenopus* and S34/S552/S558 in humans) *in vitro* and phosphorylation of TACC3 enables TACC3 to target to spindle and poles for spindle assembly [23, 25, 27]. However, it was later found that only the phosphorylation of TACC3 at the latter two sites are required for the formation of the clathrin-TACC3 complex, its localization on spindle and poles and its function in mitotic spindle assembly both *in vitro* and *in vivo* [17]. It was reported that the CHC 331-542aa region, which is comprised of a linker region and the first clathrin heavy chain repeat CHCR0, directly binds with phosphorylated TACC3 to form clathrin-TACC3 complex [16]. In addition, CHC 1-479aa, especially 1-330aa, is essential for CHC to localize to spindles [12, 28]. However, as trimerization of clathrin was proposed to be required for mitotic spindle assembly [28], whether and how the TACC3-bound CHC form a triskelion requires further study. As CLC was also detected by QUBIC method in TACC3 co-precipitates [15], it may be possible that the phosphorylated TACC3 forms complexes with CHC attached by CLC, or even a clathrin triskelion. It also remains unknown whether the mitotic clathrin forms a lattice in binding with TACC3. Taken together, the functions of clathrin in mitosis to ensure spindle stability are achieved by targeting TACC3, which undergoes priming phosphorylation by Aurora A kinase, to spindle microtubules and spindle poles.

However, another recent paper by Royle and colleagues reported that, although the ablation of clathrin reduced TACC3 targeting to the spindle, the depletion of TACC3 also dramatically resulted in the reduced spindle localization of clathrin [18]. Similar to the previous report [17], they also observed that overexpression of TACC3 results in the accumulation of clathrin at the spindle [18]. Therefore, the authors proposed a distinct hypothesis that TACC3 recruits clathrin to the mitotic spindle microtubules [18]. They proposed that TACC3 and ch-TOG bind to the spindle microtubules under the regulation of Aurora A followed by the recruitment of clathrin to the microtubules through forming complex with TACC3 or TACC3/ch-TOG subcomplex. Then, the clathrin molecule in the complex may bind more than one TACC3 or TACC3/ch-TOG subcomplex between adjacent parallel microtubules to form an inter-microtubule bridge that stabilize K-fibers of the spindle by physical crosslinking the microtubules and reducing their catastrophe rates (Figure 1B) [18]. However, the previous three papers reported that knockdown of TACC3 did not reduce the spindle localization of CHC [15-17]. Treatment with Aurora kinase inhibitor also did not result in the reduction of clathrin localization on the spindle although the spindle TACC3 was abolished [15]. Moreover, the facts that the CHC fragment 331-1639aa, which contains the TACC3 interaction domain, failed to localize to the spindle [16, 28] and that CHC 1-330aa, which lacks the TACC3 interaction domain, was able to target to the spindle [12, 16] do not support the hypothesis that TACC3 recruits clathrin to the spindle. Therefore, the order of the recruitment of clathrin and TACC3 to the mitotic spindle remains controversial and needs to be further clarified. Given that the conservation of the protein sequences of clathrin, Aurora A and TACC3 in vertebrates, it is also very likely conserved that clathrin and TACC3 form a complex to carry out their roles in mitosis.

Clathrin regulates mitotic chromosome alignment

Depletion of TACC3 by RNAi leads to chromosome misalignment and reduced localization of kinetochore proteins in mitosis, suggesting that TACC3 is required for microtubule-kinetochore interaction [26, 29]. Meanwhile, clathrin was also found to be required for chromo-

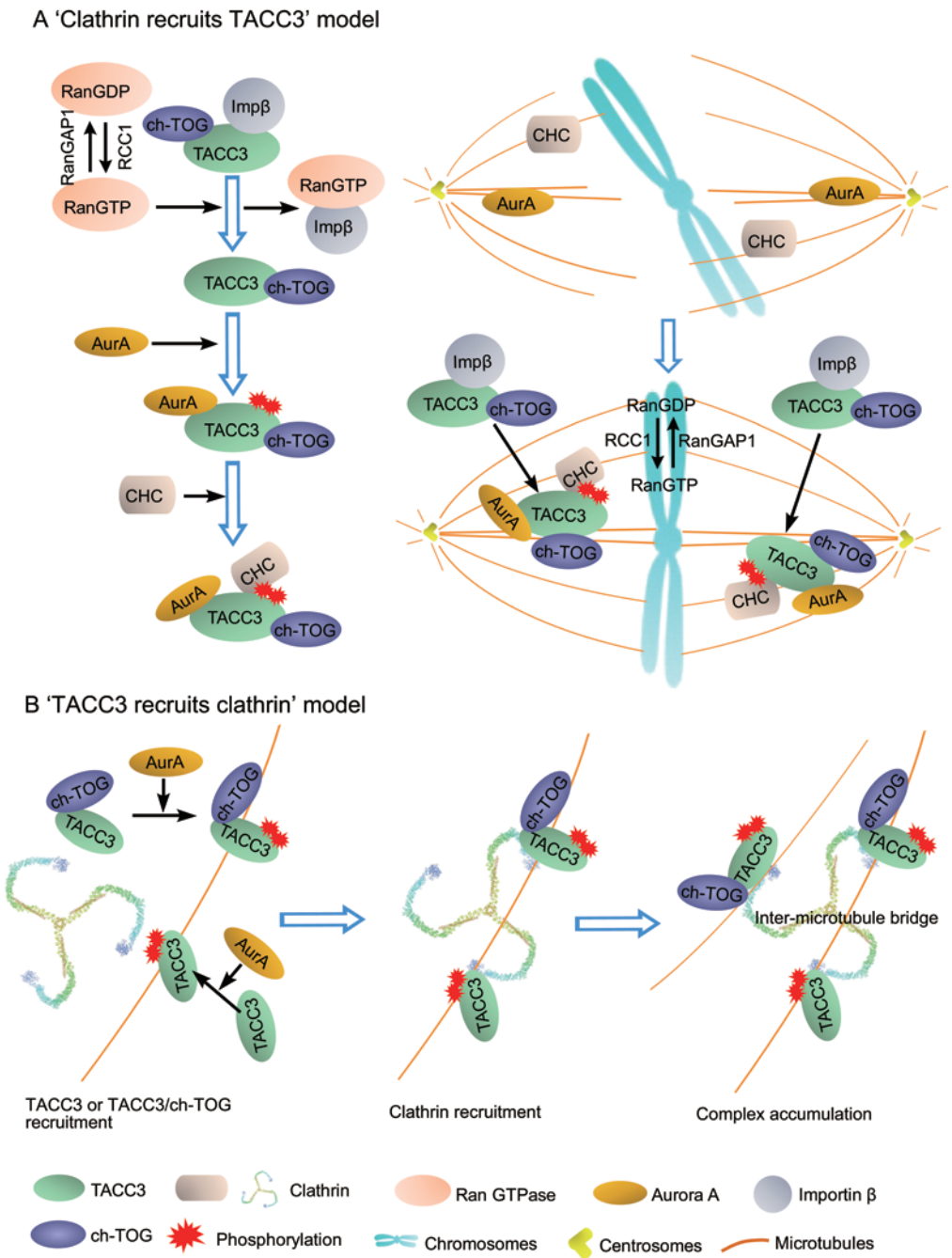

Figure 1 Clathrin-TACC3 complex regulates mitotic spindle assembly. Two models describing the function of clathrin-TACC3 complex in mitotic spindle assembly**. (A)** The 'clathrin recruits TACC3′ model. The conversion between GTP-bound and GDP-bound states of Ran GTPase is ensured by guanine nucleotide exchange factor RCC1 and Ran GTPase activating protein RanGAP1. When the cell enters mitosis, RanGTP is generated by RCC1 around the chromosomes. Through specific binding of RanGTP with importin-β, TACC3, one of the spindle assembly factors (SAFs), is released from the inhibitory binding with importin β. Then, Aurora A kinase activates the released TACC3 by phosphorylating it at two sites (Ser620 and Ser626 in *Xenopus*) and enables it to bind with clathrin to form the clathrin-TACC3 complex. Finally, the clathrin-associated TACC3 is targeted to spindle poles and spindle microtubules for proper spindle assembly. Along with TACC3, ch-TOG also targets to spindles to regulate spindle stability. **(B)** The 'TACC3 recruits clathrin model'. First, TACC3 alone or complexed with ch-TOG is recruited to the spindle microtubules in a phosphorylation-dependent manner regulated by Aurora A. Then, clathrin is recruited to the spindle by the spindle microtubule-localized TACC3 or TACC3/ch-TOG. Finally, a clathrin molecule forms an inter-microtubule bridge by interacting with additional TACC3 or TACC3/ch-TOG on adjacent microtubules to stabilize the spindle microtubules.

some alignment. Knockdown of CHC by RNAi resulted in kinetochore fiber destabilization and defective congression of chromosomes to the metaphase plate [12]. More recently, it was found that TACC3 regulates chromosome alignment dependent on its phosphorylation by Aurora A and that CHC specifically recruits phosphorylated TACC3 to the mitotic spindle and poles, linking the roles of clathrin and TACC3 together in proper mitotic spindle assembly and chromosome alignment [15-17]. Although the order of the recruitment of clathrin and TACC3 remains controversial [18], it seems possible that clathrin and TACC3 function together in mitosis. Moreover, the chromosome alignment defects caused by RNAi knockdown of TACC3 can be rescued by expressing exogenous phospho-mimic TACC3, but not its non-phosphorylatable mutants. Considering that CHC targets phosphorylated TACC3 to the mitotic spindle, it may be suggested that CHC regulates mitotic chromosome alignment also through forming clathrin-TACC3 complex.

Knockdown of TACC3 by RNAi leads to reduced localization of structural and checkpoint proteins at kinetochores, mitotic index increase, prolonged mitosis and apoptosis [30, 31]. Knockdown of clathrin by RNAi leads to persistent activation of the spindle checkpoint as well as destabilization of kinetochore fibers [12]. The traditional view is that the defects of kinetochore-microtubule attachment and tension can lead to activation of the spindle checkpoint [30, 31]. However, as the CLC A interacts with the mitotic arrest deficient protein MAD2B during mitosis [32], the previous research could not rule out the possibility that clathrin-TACC3 complex can directly regulate spindle checkpoint. Therefore, the clathrin-TACC3 complex probably regulates chromosome alignment by ensuring both proper kinetochore-microtubule attachment and spindle assembly checkpoint [15-17]. Moreover, a complex of GAK and B-Myb, which functions in spindle assembly upstream of clathrin, is also involved in chromosome congression [14, 19, 20]. However, it is not clear whether the failure of chromosome alignment in clathrin or TACC3 knockdown studies is caused by the defects of the spindle assembly. Of course, the mechanisms may be much more complicated. Thus, understanding the role of clathrin could be pivotal to elucidate the real relationships between spindle assembly and chromosome alignment.

Crosstalk among clathrin, SAFs, Ran and mitotic kinases in mitosis

The spindle assembly is a very complicated process, which requires coordination of a variety of spindle assembly factors (SAFs), mitotic kinases, Ran GTPase and Ran-binding proteins [33]. The Ran GTPase cycle is regulated by RanGAP, the GTPase activity stimulator, and RCC1, the GDP-GTP exchange factor for Ran. Ran, with its regulators and effectors, was first recognized to regulate the nuclear transport between the nuclear envelope. The Ran system was then found to regulate the mitotic spindle assembly and the nuclear envelope assembly. RanGTP binds to nuclear transport factors and controls their association with cargos. When the cell enters mitosis from interphase, RanGTP regulates nuclear pore complex disassembly and nuclear envelope breakdown. The SAFs, which usually contain nuclear localization sequences, are targeted into nucleus in interphase by importin-α/β dimer, two Ran system factors that were firstly recognized as nuclear import regulators. In mitosis, the binding of SAFs with importin proteins would be inhibitory and the inhibition must be lifted for proper mitotic spindle assembly. Ran in its GTP-bound form could specifically bind with importin-β and release the SAFs. So far a number of SAFs, such as TPX2, NuMA, Rae1 and TACC3, have been identified to be released from importins by RanGTP for mitotic spindle assembly [33].

Clathrin was previously reported to be required for the function of mitotic spindle and proposed to act as a brace between two or three microtubules within a kinetochore fiber to increase fiber stability [12, 28]. The recent finding that clathrin specifically binds and recruits phosphorylated TACC3 to spindle and poles for proper spindle assembly and chromosome alignment is an important progress in the field [15-17]. It may suggest a general mechanism for spindle assembly as well as chromosome alignment by SAFs, Ran and mitotic kinases (Figure 1). When the cell enters mitosis from G2, the inhibitory binding of SAFs with importins, here at least TACC3, will be released by RanGTP and immediately modified by mitotic kinases. The SAFs will then be recruited to the mitotic spindle and poles by additional regulator(s), here the clathrin protein triskelion. Once recruited, the SAFs alone or complexed with other factors will locally perform their mitotic functions. Therefore, the coordinated regulation among SAFs, clathrin, Ran and mitotic kinases will be a general mechanism for the mitotic spindle assembly and the proper chromosome alignment (Figure 1).

As reviewed, the interplay among clathrin, Aurora A and TACC3 presents a crosstalk between clathrin and Aurora A kinase. Moreover, other studies also suggested the potential cooperation between mitotic kinases and clathrin. The endocytic protein Numb, which directly associates with clathrin-associated adaptor complex AP-2 [34], is phosphorylated by AAK1 to promote clathrin-coated pits maturation [35]; while in asymmetric cell

division, Numb is regulated by Aurora and Polo kinase: Aurora A phosphorylates Par6 for the release of Numb from posterior cell cortex and Polo phosphorylates Pon for the localization of Numb at the anterior cell cortex [36-38]. With no doubt, in the cell, the crosstalk among clathrin, Ran-GTP and mitotic kinases could be much more complicated than expected and more regulators need to be found.

Role transitions of clathrin between endocytosis and mitosis

The functions of clathrin in interphase and mitosis have simply taken endocytosis and mitosis into the same pool; however, the relationships and regulations between them must be complicated. A lot of previous work suggested that clathrin-mediated endocytosis is arrested during mitosis as other endocytic steps [39, 40]. Moreover, inhibition of clathrin-mediated endocytosis does not disrupt mitosis, suggesting that endocytosis and spindle assembly are two distinct processes [12]. However, it was found recently that clathrin-dependent internalization of materials on the cell plasma membrane takes place throughout the cell cycle, while the endosomal recycling is ceased in mitosis and enhanced in cytokinesis [41, 42]. Moreover, several other clathrin related membrane vesicle proteins were found recently to function in mitosis and in endocytosis. The clathrin adaptor protein AP2 was found to interact with the mitotic checkpoint kinase BubR1 [43]. Another endocytic clathrin-associated adaptor protein autosomal recessive hypercholesterolemia (ARH) functions to sort members of the LDL receptor superfamily (LDLR, megalin, LRP) and participates in centrosomal and mitotic dynamics by interacting with centrosomal proteins. Cyclin G-associated kinase (GAK) is known to be essential for clathrin-mediated membrane trafficking; while in mitosis, cells lacking GAK show strongly reduced levels of clathrin on the mitotic spindle. GAK and clathrin function cooperatively in centrosome maturation, chromosome congression, spindle assembly and microtubule outgrowth from kinetochores/chromosomes [19, 20]. Moreover, the major function of GAK at spindles is probably performed by recycling clathrin from endocytic vesicles at the onset of mitosis [20]. Taken together, these findings suggest that some endocytic players including clathrin switch to function in spindle assembly when the cell enters mitosis.

Recently, the functions of epsin in mitosis may provide a new insight into the role transition between spindle assembly and endocytosis. Epsin directly modifies membrane curvature in binding to the membrane lipid PtdIns(4,5)P2 in conjunction with clathrin polymerization and limits clathrin coat assembly to the size of newly formed vesicles [44, 45]. The membrane deformation function of epsin contributes to spindle organization during mitosis [46].As spindle matrix proteomics has revealed several endocytosis proteins [47, 48], we believe that endocytic players, which were previously thought to be outside the spindles, may also switch to function in microtubule organization inside the spindles in a very complicated way [46]. Furthermore, quantitative phosphorproteomics suggests that some endocytic partners including clathrin are regulated by phosphorylation throughout the cell cycle [49, 50], implicating that both kinases and phosphatases may coordinate the switch between endocytosis and mitosis through regulating phosphorylation state of endocytic regulators.

Conclusions and prospects

Besides playing a pivotal role in endocytosis, clathrin also regulates spindle assembly and chromosome alignment. However, there is still a long way to go to fully understand the roles of clathrin during the cell cycle at the molecular, structural and evolutionary levels. Except for the well documented roles of clathrin in the coated membrane vesicle formation in endocytosis, how clathrin play roles in other processes in the cell remains mysterious. Moreover, when the cell is in the different stages of the cell cycle, how the functions of clathrin are coordinated is also a big question. The last but not the least, the function of clathrin in tumorigenesis needs to be further elucidated [51].

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