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Centrosome duplication: of rules and licenses

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Most microtubule arrays in animal cells, including the bipolar spindle required for cell division, are organized by centrosomes. Thus, strict control of centrosome numbers is crucial for accurate chromosome segregation. Each centrosome comprises two centrioles, which need to be duplicated exactly once in every cell cycle. Recent work has begun to illuminate the mechanisms that regulate centriole duplication. First, genetic and structural studies concur to delineate a centriole assembly pathway in *Caenorhabditis elegans*. Second, the protease Separase, previously known to trigger sister chromatid separation, has been implicated in a licensing mechanism that restricts centrosome duplication to a single occurrence per cell cycle. Finally, Plk4 (also called Sak), a member of the Polo kinase family, has been identified as a novel positive regulator of centriole formation.

Introduction

Upon fertilization, the sperm of most animal species contributes not only one complement of chromosomes but also, depending on the species, one or two tiny barrel-shaped bodies, the centrioles, which combine with proteins stored in the egg to reconstitute the centrosome [1]. After formation of the first centrosome, this organelle needs to be duplicated and segregated during each cell division cycle in synchrony with the genome [2]. The main function of the centrosome is to organize dynamic arrays of microtubules (MTs) [3,4]. During interphase of the cell cycle, MTs determine cell shape, polarity and motility, whereas during M phase, they form the bipolar spindle required for chromosome segregation. As illustrated in Figure 1, the single centrosome present in a G1-phase cell comprises two centrioles embedded in a protein matrix known as pericentriolar material (PCM). Before division, this whole structure needs to be duplicated once, so that a G2-phase cell harbours two centrosomes, each comprising two closely linked centrioles (Figure 1). Centrioles are tiny, barrel-shaped structures that are structurally related to (and often interconvertible with) basal bodies, which, in turn, are essential for the formation of cilia and flagella (Box 1). In vertebrates, centrioles are composed of nine triplet microtubules, whereas in *Drosophila* and *Caenorhabditis elegans* they mostly comprise doublet and singlet microtubules, respectively [1]. The PCM surrounding the centrioles has been visualized as a fibrous lattice [5] and, in a human centrosome, contains over 100 different proteins [6]. These include components required for microtubule nucleation, notably γ-tubulin, and associated proteins, which are also conserved in fungal spindle pole bodies (the functional equivalents of centrosomes) [7]. Other PCM components are less well conserved although many harbour predicted coiled-coil domains [6], suggesting that they perform scaffolding functions, notably for the recruitment of cell cycle regulatory proteins [8–12].

In organisms or cells that lack centrosomes (e.g. higher plants and the eggs of many animal species), bipolar spindles can form through centrosome-independent mechanisms [13]. However, when they are present, centrosomes exert a strong influence on the number of spindle poles formed [14]. Therefore, the cell cycle regulatory machinery must control not only a chromosome cycle but also a centrosome–centriole cycle; the integration between the two cycles is crucial for genome stability [2]. Any deviation from normal centrosome numbers can result in the formation of mono- or multipolar spindles, with dire consequences for the accuracy of chromosome segregation. Accordingly, centrosome abnormalities have long been related to aneuploidy and proposed to contribute to the development of cancer [15–17]. As summarized in Box 2 and expertly reviewed elsewhere [11,18–20], the centrosome cycle can be subdivided into distinct steps, with centriole duplication occurring during S phase and centrosome segregation during M phase.

Recent studies have begun to shed some light on the fundamental process of centriole duplication. Here, I focus mainly on three recent developments that bear on both the mechanism of centriole formation and the cell cycle regulation of centrosome duplication. Specifically, I discuss elegant studies describing the assembly of centrioles in the nematode *C. elegans*, the unexpected role of Separase (a protease previously implicated in sister chromatid separation) in centriole disengagement, and the identification of a protein kinase, Plk4 (also called Sak), as a positive regulator of centriole formation. Furthermore, I argue that, conceptually, the maintenance of correct centrosome and centriole numbers during successive cell cycles depends on two distinct rules: one imposing cell cycle control, the other limiting centriole copy number (Box 3). Hopefully, these concepts and new findings will contribute to a better understanding of centriole formation and the mechanisms controlling centrosome numbers during cell cycle progression.

Centriole assembly pathways

It is well established that some cells, notably ciliated epithelial cells and male gametes of lower plants, are able

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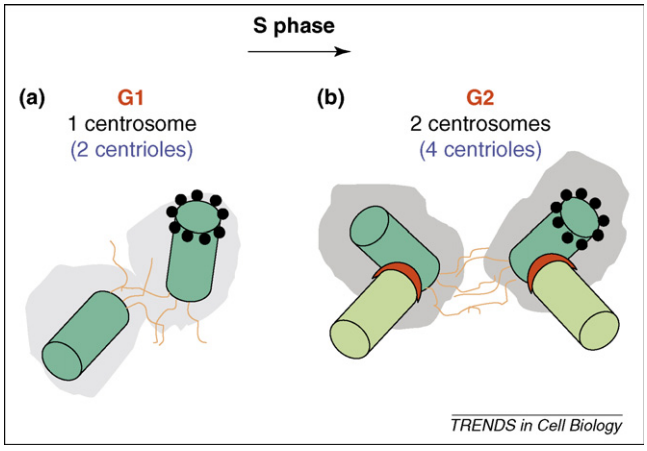

Figure 1. Centrosomes in G1 and G2 phase cells. **(a)** A cell in G1 phase of the cell cycle harbours a single centrosome comprising two centrioles (green). The two centrioles are structurally distinct, reflecting their different ages: the older one (a 'parent' in the preceding cell cycle) carries distal and subdistal appendages (indicated in black), structures implicated in both MT anchoring [3] and ciliogenesis [60], whereas the younger one (a 'progeny' formed during the preceding cell cycle) lacks appendages. Both centrioles are embedded in PCM (grey). During G1, the two centrioles are tethered to each other only loosely, apparently through entangling fibers associated with their proximal ends ('base-to-base' association; brown dashed lines) [61,62]. **(b)** In the following S phase both centrioles then give rise to progeny, so that by G2 phase the cell harbours two centrosomes, each made up of two tightly associated centrioles. Within each centrosome, parent and progeny centriole display a close orthogonal association ('base-to-side'; red disks); according to a recent model, this association (termed 'engagement') prevents reduplication in the same cell cycle [20]. Note that the PCM undergoes a phosphorylation-dependent 'maturation' event in late G2 (indicated by dark grey) [63,64]. This enhances the recruitment of γ-tubulin ring complexes and allows the increased MT nucleation activity required for spindle formation.

to generate *de novo* large numbers of centrioles/basal bodies (Box 1). This *de novo* assembly pathway has been described morphologically but is not well understood at a mechanistic level [21]. Until recently, the acentriolar pathway for the biogenesis of centrioles/basal bodies was thought to be restricted to specialized cell types, whereas most somatic cells were believed to require pre-existing centrioles for the production of new centrioles through a poorly defined templating mechanism. Recently, however, the distinction between *de novo* and templated centriole formation has been blurred, thanks to a series of ingenuous experiments performed in cell culture. Noticeably, the *de novo* formation of centrioles was shown to be inducible, provided that resident centrioles were first removed by laser ablation or microsurgery [22–24]. The available evidence thus suggests that pre-existing centrioles are not strictly required for centriole assembly but that centrioles, when present, restrict the numbers of new centrioles (procentrioles) to one per template [25]. How this control of the copy number is implemented is not presently understood, but the observed restriction on centriole assembly imposed by pre-existing centrioles might hint at a process in which procentriole assembly in close proximity to a pre-existing centriole is kinetically favoured over *de novo* assembly in the cytoplasm (similar kinetic arguments could also be invoked to rationalize the formation of only one procentriole next to each parental centriole; see later). Alternatively, it is possible that two distinct assembly pathways coexist but that templated pathways normally suppress *de novo* pathways [25]. Interestingly, the formation of centriole triplets has been observed following inactivation of the mitotic kinase Cdk1 in *Drosophila* [26], indicating that a parental centriole carries more than one potential site for the assembly of procentrioles.

Several years ago, the apparent self-replication of centrioles prompted a search for a centrosome-associated nucleic acid. However, no conclusive evidence was found to support this possibility. At this time, it seems safe to exclude the existence of centriolar DNA [27], but the idea that RNA might have a role in centrosome duplication has recently seen a revival. Indeed, following the identification of specific RNA species associated with centrosomes of surf clam oocytes [28], the proposal that specific RNAs might participate in centriole structure and/or function has resurfaced. However, to substantiate or refute a physiological involvement of the purified clam RNA in centrosome biology, it will be important to clarify its source. Because viruses often assemble in proximity to centrosomes [29], it will be crucial to exclude a viral origin. In the meantime, a protein-based (rather than nucleic acid-based) mechanism appears most likely to control centriole biogenesis. Centriole/basal body assembly has long been studied, both by electron microscopy and genetic analyses, in ciliated protozoan organisms and green algae. Recent studies carried out in *Tetrahymena* have confirmed a requirement for

Box 1. Centrioles, basal bodies and human disease

Centrioles are structurally related to (and often interconvertible with) basal bodies [65,66], the organelles required for the formation of cilia and flagella. This can readily be seen, for instance, in the unicellular green alga *Chlamydomonas*, where the exact same organelles function as flagellar basal bodies during interphase and as centrioles at spindle poles during cell division [65]. Interestingly, basal bodies do not always form next to pre-existing organelles: in ciliated epithelia for instance, two pathways for the biogenesis of basal bodies and centrioles can be distinguished, a centriolar pathway and an acentriolar pathway [21]. In the centriolar pathway, new centrioles form adjacently to pre-existing ones, but in the acentriolar pathway, centrioles/basal bodies assemble *de novo* without apparent contact with pre-existing centrioles. In this latter case, centrioles/basal bodies are generated around fibrous aggregates termed deuterosomes (probably comparable to the spherical electron dense masses known as blepharoplasts in lower plants) [21]. To what extent the acentriolar and centriolar pathways use common regulatory mechanisms is an important unresolved question. Likewise, it will be interesting to determine the nature of the controls that activate the acentriolar pathway in the appropriate cells and/or suppress it in all others.

Growing out of basal bodies, cilia and flagella are microtubule-based cell surface protrusions. Motile cilia and flagella have long been known to be important for cell locomotion and/or transport of material over cellular surfaces. By contrast, the functional relevance of non-motile cilia has only recently been fully recognized. In particular, the so-called primary cilium, a structure that forms on the surface of most quiescent vertebrate cells, is now thought to constitute the cell's 'antenna', with both sensory and signalling functions [67,68]. Hence, by performing various mechano- and chemosensory functions, cilia are crucial for normal development and health, including the determination of left–right asymmetry, kidney function, photoreception and brain development. Underscoring this conclusion, recent proteomic and genetic studies have identified several novel proteins associated with centrioles/basal bodies and the ciliary apparatus, whose dysfunction leads to human disease syndromes, including Bardet–Biedl syndrome, polycystic kidney disease, oral–facial–digital syndrome and primary microcephaly [68–71].

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Box 2. The centrosome cycle in a nutshell

The centrosome–centriole duplication–segregation cycle is traditionally subdivided into discrete steps, notably centriole disengagement, centriole duplication, centrosome maturation and centrosome separation (Figure I). In a metaphase cell (Figure Ia), each of the two spindle poles is characterized by the presence of one centrosome comprising two centrioles (green). These two centrioles represent a parent–progeny pair originating from the previous cell cycle; they are tightly associated with each other (red disks) and usually have an orthogonal arrangement. The tight link between the two centrioles is lost upon exit from M phase (or during early G1), in a process now referred to as 'disengagement' (formerly 'disorientation') [47,72]. Importantly, this disengagement is proposed to license the two centrioles for a new round of duplication [20]. During G1 (Figure Ib), a different, highly dynamic linker structure (thin brown lines) is established between the two disengaged (duplication-competent) centrioles (yellow). According to recent evidence, this 'base-to-base' connection involves the tethering of filaments associated with the proximal ends of the two centrioles [61,62]. During S phase (Figure Ic), one new centriole (procentriole) begins to grow at an orthogonal angle next to each licensed centriole, again establishing tight 'base-to-side' connections between parental and progeny centrioles. Duplication requires the activity of Cdks, Plk4 (ZYG-1 in *C. elegans*) and several centrosomal proteins predicted to have structural roles (see the main text). The two procentrioles then elongate until they reach full length in G2 and, in late G2 (Figure Id), the younger of the two parental centrioles acquires appendages (black bars), thereby reaching full maturity. At about the same time, the loose tether between the two parental centrioles is severed, apparently in response to phosphorylation of linker proteins, such as C-Nap1 [73] and Rootletin [61,62], to enable centrosome separation and spindle formation. Note that three different generations of centrioles coexist throughout S and G2 phase of the cell cycle. Furthermore, the completion of centriole biogenesis (full maturation and acquisition of appendages) requires more than one full cell cycle. (During M phase, one centriole at each spindle pole is competent for appendage formation but appendages escape detection by electron microscopy; they are therefore indicated by open bars.)

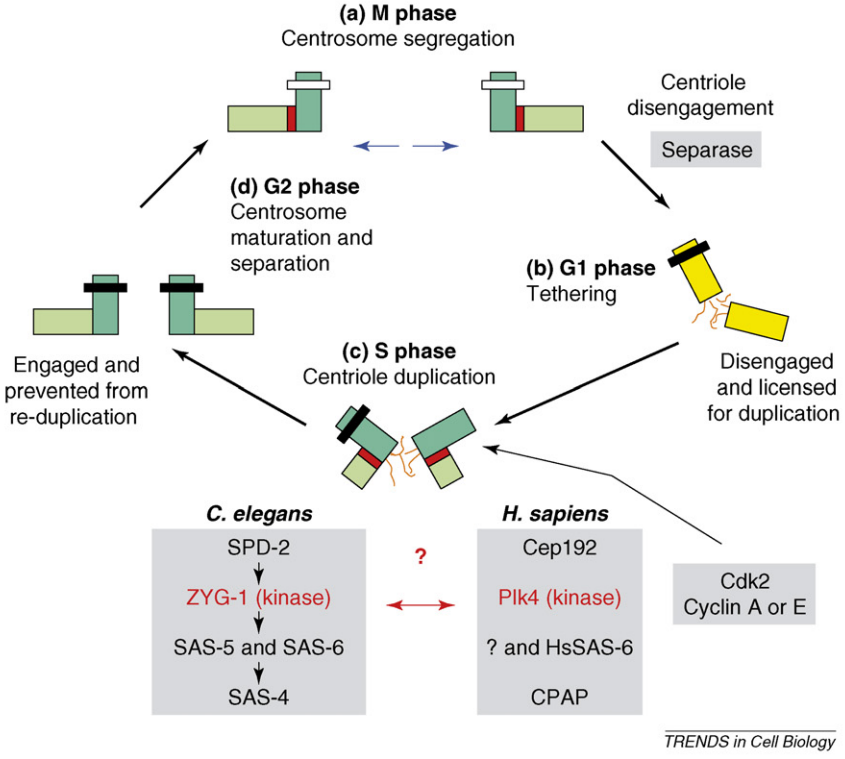

Figure I. The centrosome cycle. Major steps in the centrosome duplication–segregation cycle **(a–d)** are depicted, along with major components implicated in the regulation of this cycle (in grey boxes). In (b), the two centrioles are in yellow to emphasize their disengagement, which results in a unique competence for duplication. In (c), note that no human homologue of *C. elegans* SAS-5 has yet been identified (indicated by?). To what extent the various gene products identified in *C. elegans* and *H. sapiens* function in analogous pathways remains to be established (double arrow). Also, whereas the *C. elegans* gene products have been placed into a pathway [39,40] (indicated by arrows), no comparable information has yet been obtained for *H. sapiens*.

centrins (small calcium-binding proteins) in basal body duplication, extending earlier work from other species [30]. Moreover, morphological studies have long emphasized a distinct, symmetrical structure, termed cartwheel, that appears early during (pro)centriole formation in many organisms. Interest in this putative scaffolding structure has been rekindled by the recent identification of a cartwheel-associated coiled-coil protein, Bld10p, that clearly has a crucial role in centriole/basal body assembly in *Chlamydomonas* [31]. It would obviously be interesting to identify functional homologues of Bld10p in other species.

Centriole assembly in *C. elegans*

Genetic studies and RNA interference screens have identified a total of five centrosome/centriole-associated proteins as being essential for centrosome duplication in *C. elegans*: ZYG-1, a protein kinase, as well as SAS-4, SAS-5, SAS-6 and SPD-2, which all display coiled-coil domains [32–38]. Careful molecular epistasis experiments indicate that the five proteins act sequentially during centriole biogenesis [39]. After fertilization of the *C. elegans* egg, SPD-2 is initially recruited to paternal centrioles where it is required for the centriolar localization of the other four proteins: ZYG-1 comes next and is in turn required for the

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Box 3. Controlling centriole numbers: of birth cycles and litter sizes

I would like to argue that, conceptually, the maintenance of correct centrosome and centriole numbers during successive cell cycles depends on the observance of two distinct rules, one imposing cell cycle control, the other limiting copy numbers of centrioles (Figure 2). To use a metaphor, the first rule dictates how often a parental centriole gives 'birth' during the cell cycle ('birth cycle'), whereas the second limits the number of centrioles produced at each birth ('litter size'). Clearly, these two rules are reminiscent of those that govern the chromosome duplication cycle. In the case of DNA replication, cell cycle control is implemented through distinct requirements (notably with regard to levels of Cdk activity) for the assembly of pre-replication complexes and the firing of replication origins, respectively, whereas copy number control follows logically from the semi-conservative replication of the DNA double helix [74,75]. In the case of the centrosome cycle, the recently proposed licensing model provides an attractive explanation for cell cycle control [20,47]. The mechanism(s) ensuring copy number control remain largely enigmatic, but the ability of excess Plk4 activity to trigger extra procentriole formation might finally offer a handle to solve this mystery [56].

recruitment of SAS-5 and SAS-6, followed by SAS-4. Thus, SPD-2 clearly has a key role in the initiation of centrosome assembly, possibly acting as a scaffold for the recruitment of the ZYG-1 kinase and its substrates [39]. This assembly pathway has been extended to a structural level by the use of electron tomography [40]. In addition to confirming that SPD-2 and ZYG-1 act upstream in the process, this remarkable study revealed that centriole assembly involves the formation of a central tube that required the SAS-5 and SAS-6 proteins (as either structural or regulatory components), followed by an SAS-4-dependent assembly of MTs onto the periphery of the tube. These important observations set a precedent for future studies on centriole biogenesis in other species, notably vertebrates. SPD-2, SAS-4 and SAS-6 all have homologues in human cells [19], termed Cep192 [6], CPAP [41] and HsSAS-6 [37], respectively. There are no obvious homologues of ZYG-1 in the human genome, but in view of the properties of Plk4 described below, it is tempting to speculate that Plk4 might have a role functionally analogous to that of ZYG-1. In the future, it will be important to determine whether centriole biogenesis in vertebrates also involves the formation of a central tube as well as to clarify the relationship between the central tube described in *C. elegans* and the cartwheel observed in other organisms.

What controls determine centriole numbers?

The question of how cells keep centriole numbers constant over successive cell divisions continues to represent one of the most mysterious problems in contemporary cell biology. When considering the centrosome cycle from a purely conceptual perspective (Box 3), one can discern two distinct rules (Figure 2). The first rule stipulates that centrosomes duplicate once and only once in every cell cycle (cell cycle control), whereas the second enforces the formation of only one progeny centriole next to each parental centriole (copy number control). Although conceptually distinct, these two modes of control are expected to be coordinated at the molecular level. Importantly, adherence

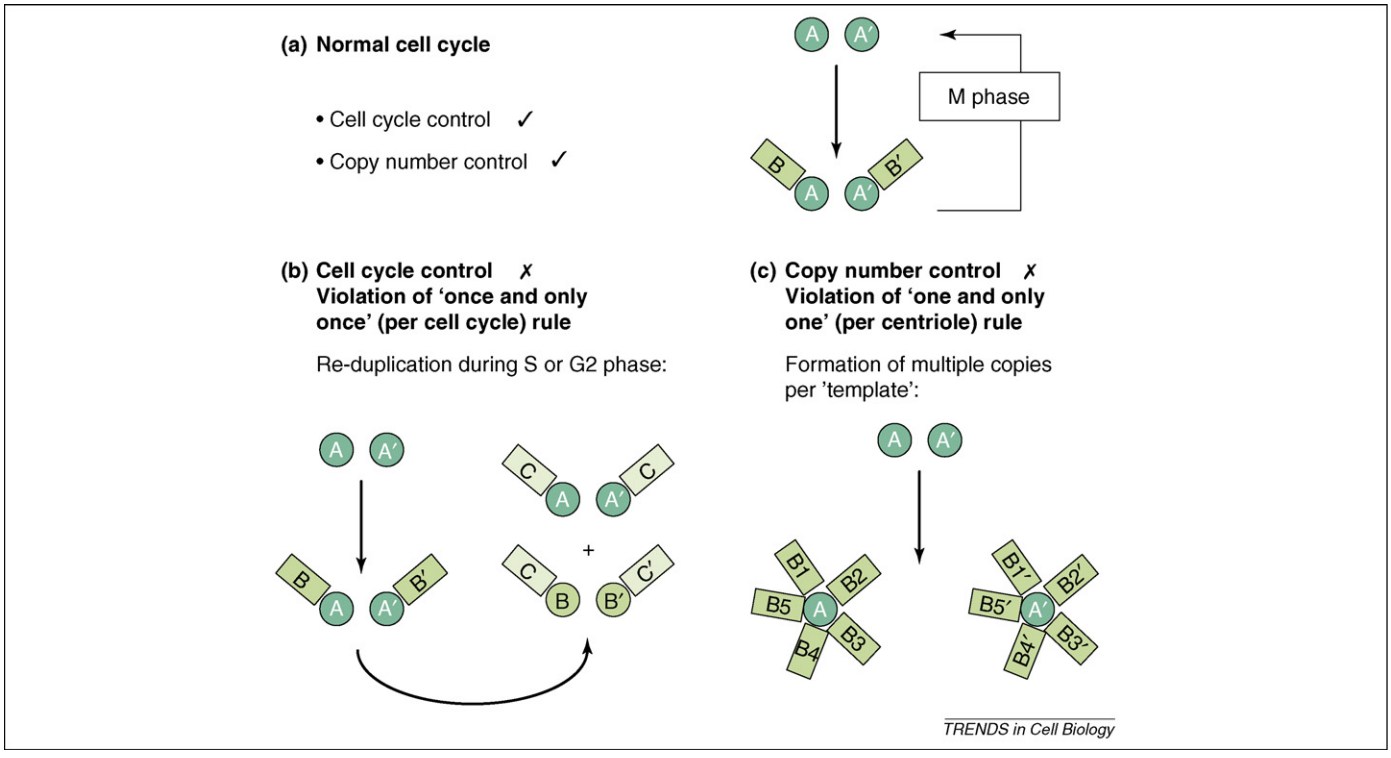

Figure 2. Two rules governing the centrosome cycle. **(a)** Centriole duplication in a normal cell cycle involves two centrioles (A and A′) giving rise to progeny (B and B′). This process is proposed to be controlled by two mechanisms (Box 3). **(b)** The first mechanism imposes cell cycle control and ensures that a new round of duplication can occur only after passage through M phase. Violation of this 'once and only once' per cell cycle rule results in reduplication during S or G2 phase, leading to extra centrioles (C and C′). **(c)** The second mechanism imposes copy number control at each duplication event and limits the formation of procentrioles to one per pre-existing centriole. Violation of this 'one and only one' per centriole rule results in the formation of multiple (pro)centrioles (B1–B5 and B1′–B5′) per template. Deregulation of either cell cycle control or copy number control has the potential to produce excessive numbers of centrioles, a phenotype commonly observed in cancer cells [16,17].

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to both rules is crucial for the maintenance of constant centrosome/centriole numbers over successive cell generations. Conversely, deregulation of either cell cycle control or copy number control is expected to give rise to aberrant centriole numbers and, consequently, genome instability [17] (Figure 2). In the next two sections I discuss recent findings that begin to clarify the mechanisms underlying these two proposed types of controls.

Cell cycle control: Separase and the licensing model

Through the adaptation of classical cell fusion studies [42], strong evidence has been obtained for a centrosome-intrinsic block to centriole reduplication during S and G2 phase of the cell cycle [43]. This observation, combined with detailed electron microscopic descriptions of the centriole duplication cycle [44–46], has led to the proposal of an attractive licensing model [20]. According to this model, the engagement of newly duplicated centrioles (i.e. their tight orthogonal association) blocks further duplication until disengagement at the end of mitosis licenses the two centrioles for a subsequent round of duplication. By simultaneously studying centriole disengagement and centriole growth after addition of purified centrosomes to *Xenopus* egg extracts, Tsou and Stearns obtained strong experimental support for the above model [47]. Specifically, their data suggest that Separase, a protease already well-known for its role in sister chromatid separation [48], is also required for centriole disengagement and that this event is in turn crucial to the subsequent growth of new centrioles. This study thus supports the view that centriole engagement, established during centriole duplication in S phase, prevents further duplication throughout the remainder of the same cell cycle until passage through M phase (and concomitant disengagement) issues a license for a new round of duplication [20].

The proposed licensing model holds considerable appeal because it might explain how centrosome duplication is limited to once per cell cycle. Several important questions remain nonetheless to be addressed. In particular, presently, the evidence implicating Separase remains indirect. Therefore, it will be important to design experiments that directly address the role of this protease in centriole disengagement and to examine carefully the centriole duplication cycle in Separase-deficient cells. It will also be interesting to clarify the mechanism that leads, in the apparent absence of disengagement, to centriole reduplication in *Drosophila* wing discs depleted of Cdk1 [26], and to consider the implications of the licensing model for the centrosome overduplication observed in some S and/or G2 arrested cells [49–51]. Conversely, it will be interesting to explore how certain experimental conditions can induce centrosome splitting [52,53] even though they activate the spindle assembly checkpoint that is expected to prevent global activation of Separase. Finally, it remains to be determined whether Separase acts directly or indirectly on centrosomes. If the action is direct the next challenge will be to identify the centrosomal proteins that are cleaved by this protease. Alternatively, if Separase acts indirectly, for instance through regulation of kinase or phosphatase activities, it will be important to delineate the pathway that ultimately triggers centriole disengagement. Perhaps arguing in support of an indirect mechanism, centriole disengagement appears to occur considerably later than the metaphase-to-anaphase transition, the time when Separase is first activated.

Copy number control: Plk4 (and a counteracting phosphatase?)

In addition to the *C. elegans* ZYG-1 kinase discussed above, several vertebrate kinases have also been implicated in the regulation of centrosome duplication [18]. The most definitive evidence supports a role for Cdk2–Cyclin A and/or –Cyclin E [47,54,55]. However, a detailed mechanistic understanding of the Cdk requirement for centrosome duplication has not yet emerged. Thus, until a direct action of Cdk2–Cyclin A and/or –Cyclin E at the centrosome can be demonstrated, it remains possible that Cdk activity is required primarily to advance cells to a cell cycle stage that is permissive for centrosome duplication. In view of these unresolved issues, it is of considerable interest that a member of the Polo kinase family, Plk4 (also known as Sak), has unequivocally been identified as a positive regulator of centriole duplication in both human cells and *Drosophila* [56,57]. The activity of Plk4 is required at the centrosome [56], reminiscent of data obtained for *C. elegans* ZYG-1 [19,33]. Thus, although Plk4 shows no clear-cut sequence similarity to ZYG-1, it is tempting to conclude that these two kinases might carry out analogous functions. In the absence of Plk4 activity, both vertebrate and invertebrate cells progressively lose centrioles through impaired duplication, leading to severe anomalies in spindle formation [56,57]. Moreover, the spermatids of *Drosophila plk4* mutants lack basal bodies and are therefore unable to form flagella [57]. Plk4 had previously been shown to be essential for embryogenesis in mice [58] and, interestingly, $Plk4^{+/-}$ mice are prone to develop tumors [59]. Clearly, it will be interesting to determine whether these phenotypes reflect the centrosomal function of Plk4 or, alternatively, hint at other functions for this kinase [56,59]. It is of particular interest that the overexpression of *PLK4* in human cells results in the production of multiple centriole precursors surrounding a single parental centriole [56]. This remarkable effect is strictly dependent on the association of Plk4 with centrioles, suggesting that procentriole formation might critically depend on the phosphorylation of one or more proteins at the procentriole assembly site. For instance, one could envision a scenario in which the localized phosphorylation of a particular protein triggers the formation of a 'seed' (e.g. through protein stabilization or recruitment) that initiates the rapid growth of a single procentriole in close proximity to the pre-existing centriole, at the expense of procentriole formation elsewhere. If so, cellular Plk4 activity would be expected to be closely balanced by phosphatases to prevent the simultaneous formation of multiple centrioles.

Conclusions and prospects

Encouraging progress has been made towards understanding centrosome duplication. Major recent achievements concern not only the description of the assembly process *per se*, but also insight into the controls that limit

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the frequency of duplication to once per cell cycle, and those that determine the numbers of centrioles produced on each occasion. Major challenges for the future include a more detailed definition of the pathway that leads to centriole disengagement, the functional characterization of the direct substrates of Plk4 (and ZYG-1) and the identification of the phosphatase(s) that is expected to counteract these kinases. Furthermore, the continued analysis of centriole biogenesis through the templated versus *de novo* pathways will hopefully clarify the mechanisms that normally restrict procentriole formation to one per template. One attractive possibility is that the templated mechanism involves kinetic principles akin to those that govern crystal formation. Finally, time has come to ask how cells ensure the correct size of their centrioles or, in other words, what 'rulers' they use to prevent centriolar MTs from growing indefinitely into space.

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