Controlling centrosome number: licenses and blocks
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Introduction
The centrosome is the primary microtubule-organizing center in animal cells, and it has a strong influence on microtubule-dependent processes in these cells. There are two major elements of the centrosome: centrioles, which can act as basal bodies to generate cilia and flagella, and the pericentriolar material, which nucleates and organizes microtubule arrays. Despite the near-ubiquity of the centrosome in animal cells, several of the processes with which it is usually associated, such as mitotic spindle formation, are able to occur without centrosomes. Even if centrosomes are not required for some processes, maintenance of proper centrosome number is necessary for genome stability, and hence survival [1]. Many human cancer cells show a high incidence of centrosome amplification, which may partially hence survival [1]. Many human cancer cells show a high number of centrosomes, which may partially (in this review, we will focus on how the copy number of centrosomes is controlled in the context of their duplication. The structure and composition of centrosomes and centrioles will not be discussed here, as they have been reviewed recently [3–6].

Cell-cycle-dependent centrosome duplication
Except in some specialized cell types and unusual conditions in which centrosomes can form de novo (see below), the centrosome duplication pathway requires a pre-existing centrosome. This canonical duplication cycle can be divided into ordered steps (see Figure 1). Cells in G1 have a single centrosome containing two barrel-shaped centrioles joined loosely by cohesion fibers [7,8]. At the G1/S transition new centrioles grow orthogonally from each of the two pre-existing centrioles. The initiation of centrosome duplication requires the activity of Cdk2/cyclin E or cyclin A [9–12]. The new centrioles continue to elongate until late G2 and remain in a tightly opposed orthogonal configuration during S, G2 and early M phase [13]. In early mitosis centrosome cohesion is broken, and each centrosome participates in mitotic spindle pole formation. Centriole pairs become disengaged at the end of mitosis or early G1, losing their strict orthogonal configuration [13], but are still joined by cohesion fibers formed after disengagement. The key feature of each duplication cycle is that centrosomes duplicate once and only once. In normal cells, this restriction holds even when cells are artificially arrested for long periods of time in S phase, a stage that supports centrosome duplication [14**]. However, in transformed cells or cells that lack the tumor suppressor gene p53, centrosomes can over-duplicate during prolonged S phase arrest [15,16]. It is unknown whether this over-duplication occurs via normal duplication (mother-centriole-dependent duplication), de novo centrosome assembly, or both. Thus, in normal cells, there are mechanisms that prevent centrosomes from being re-duplicated once they have duplicated. The central question is how cells achieve precise control of centrosome copy number. Recent results indicate that there are at least two controls working together to regulate centrosome number: the first prevents canonical centrosome re-duplication, and the second prevents de novo centrosome assembly. We will here discuss recent publications and some new ideas regarding the mechanism of centrosome duplication control.

‘Licensing’ duplication and ‘blocking’ re-duplication
The concepts of a ‘license’ and a ‘block’ for centrosome re-duplication derive from studies of DNA replication, where the license corresponds to the loading of MCM helicases onto DNA to unwind the DNA duplex for replication [17]. Key to this concept is that once the DNA duplex is open and primed, the licensing factors are no longer required for actual DNA replication. In DNA replication the ‘block’ to re-replication is intrinsic to
the hydrogen-bonded DNA double helix, which is not accessible for initiation of template-dependent replication without another round of licensing (unwinding). Thus each strand of the DNA duplex can serve not only as a template to be copied but also as a lock to block reduplication when licensing factors are not available.

Is there a similar control mechanism for centrosome duplication? By analogy to DNA replication, we propose the following criteria for a centrosome duplication licensing factor. First, such a factor should act at the centrosome before the G1/S transition to prime it for duplication. Second, it should be lost from centrosomes, or inactive, during other cell cycle stages so that centrosomes duplicate only once. Third, ectopic activity of the licensing factor during S or G2 phases should promote reduplication of the centrosome. As with DNA, our assumption is that a centrosome licensing factor would be distinct from centrosome assembly factors. Using these criteria, we examine the roles of two proteins that have been proposed to function as licensing factors for centrosome duplication.

**Cdk2/cyclin E and A**

As in DNA replication, Cdk2, paired with either cyclin E or cyclin A (Cdk2/E/A), has been shown to be required for centrosome duplication in several systems [9–12] and proposed to be a licensing factor for centrosome duplication. The evidence came from in vitro experiments using S-phase-arrested Xenopus egg extract [9]. The authors visualized the repeated doubling of microtubule asters using polarized light microscopy, suggesting that centrosomes were continuously duplicating in the extract. Inactivation of Cdk2/E did not affect the first round of aster doubling, but blocked subsequent doubling, indicating that Cdk2/E is required for repeated centrosome duplication, consistent with Cdk2/E being a licensing factor for centrosome duplication [9]. However, two critical pieces of information are missing in this experiment: first, the number of centrioles in each microtubule aster is not known; second, the licensing and cohesion status of the donor centrosomes (from demembranated sperm nuclei) is unclear. Without this information it is possible that the first aster doubling could be simply separation of the two sperm centrioles. If this is the case, the role of Cdk2/E as a licensing factor is in doubt, because it might only be required for centriole growth, preventing subsequent aster doubling. Consistent with this interpretation, the first aster doubling time was much shorter than the second and third doubling times [9], suggesting that it is mechanistically different from later rounds. In addition, we note that when normal somatic cells are arrested in S phase, where Cdk2/E/A activity is high and all the machinery for centrosome duplication is available, centrosomes still only duplicate once [14**]. We therefore suggest that Cdk2/E/A is an
essential centrosome duplication factor, but is not sufficient to license centrosomes for re-duplication.

Nucleophosmin

Nucleophosmin is a multifunctional protein localized to the nucleolus and the centrosome, and is a substrate of Cdk2/E. Okuda et al. 2000 [18] showed that during G1/S, nucleophosmin dissociates from the centrosome after phosphorylation by Cdk2/E and that this dissociation is required for centriole duplication. Duplicated centrosomes remain free of nucleophosmin until mitosis, when nucleophosmin reassociates with the centrosomes. On the basis of the localization pattern and its inhibitory effect on centrosome duplication, the authors proposed that nucleophosmin could be a licensing factor that limits centrosome duplication to once per cell cycle [18]. Consistent with it having an inhibitory role in centrosome duplication, Wang et al. further showed that the centrosomal association of nucleophosmin relies on the Ran–Crm1 complex [19*], a nuclear-cytoplasmic shuttle system that, when abolished, results in centrosome amplification, similar to the phenotype caused by nucleophosmin depletion by RNAi [19*]. However, if absence of nucleophosmin at centrosomes licenses them for duplication, this model cannot explain why centrosomes do not normally re-duplicate during S and G2 when nucleophosmin is not present at centrosomes. Therefore, we suggest that nucleophosmin is a downstream effector of Cdk2/E and is involved in centrosome assembly, but probably not directly in licensing centrosome duplication. That nucleophosmin, a substrate of Cdk2/E, is not a licensing factor is consistent with the above argument that Cdk2/E is not a licensing factor.

The role of centriole configuration

If Cdk2/E and nucleophosmin are not licensing factors for centrosome duplication, is there any evidence that such a factor really exists? As for DNA replication, if there is a block to the process, there must be a licensing event to relieve that block. Strong evidence for such a block to centrosome duplication comes from cell fusion experiments, which show that there is a centrosome-intrinsic block to re-duplication during a single cell cycle [14**]. The authors fuse combinations of G1, S and G2 cells and show that centrosome duplication is observed only when the fusion products contain previously unduplicated centrosomes. After ruling out a counting mechanism that compares the number of centrosomes to the number of nuclei, they conclude that there must be an intrinsic difference between unduplicated G1 centrosomes and duplicated G2 centrosomes such that centrosomes that have already duplicated are not competent to duplicate again within a single cell cycle [14**].

The mechanism of the centrosome-intrinsic block to re-duplication and the nature of the license that relieves that block are unknown. However, examination of the morphology of centrioles during their duplication cycle by electron microscopy [13] suggests a possible model. Centrioles in S and G2, which are blocked for re-duplication, display a strict orthogonal configuration between mother and daughter centrioles. We shall refer to this configuration as ‘engaged centrioles’. Might this engaged configuration represent the block to centrosome re-duplication? Centrioles usually grow from the side of pre-existing centrioles; although the significance of this is not known, the engaged state might block a site for nucleating new centriole growth. Centrioles remain engaged through S, G2 and early M phases (Figure 1), only becoming disengaged at the end of mitosis or early G1 [13]. It is not known what triggers centriole disengagement, but transition from metaphase to anaphase is required [20]. This cell-cycle-specific pattern of centriole configurations exhibits some similarities to the ‘license and block’ model for DNA replication. We therefore propose that the activity that disengages centrioles at the end of mitosis licenses the centrosome for duplication at the subsequent G1/S transition, and that the engaged state of centrioles after duplication blocks re-duplication.

We note that the previously described ability of Cdk2/E to separate centriole doublets in an in vitro assay [10] was probably revealing this protein’s role in separating previously disengaged centrioles, as the experiments largely made use of G1 centrosomes, which are disengaged but remain joined by cohesion fibers. It is still unknown how engaged centrioles become disengaged at the end of M phase. We believe that characterization of this disengagement activity will be required to identify the licensing factor for centrosome re-duplication.

De novo centrosome assembly

Although in most cells centrosome duplication requires a pre-existing centrosome, de novo centrosome formation does occur naturally in some biological systems. Studies of multiciliated epithelial cells [21] and early mouse embryos [22] clearly show that centrioles and centrosomes, respectively, can be assembled de novo. Under special experimental conditions, other biological systems can also support de novo centrosome assembly, although canonical centrosome duplication is the dominant pathway. These systems include Chlamydomonas [23], surf clam oocyte extract [24], and parthenogenetically activated Drosophila embryos [25]. Strikingly, de novo assembly can also occur in vertebrate somatic cells when the endogenous centrosomes are destroyed or removed [26,27**]; however, the number of centrosomes formed through the de novo pathway is variable. The spontaneous formation of centrosomes therefore poses a grave risk for dividing cells, which require strict control over centrosome number to maintain genomic stability.

A critical question then is how the de novo assembly pathway is suppressed so that only the tightly controlled canonical pathway is used. Experiments in Chlamydomonas
show that the de novo assembly pathway becomes active only when endogenous centrioles are absent, and forms new centrioles at ~50% of the rate of templated assembly [23]. This result suggests that in Chlamydomonas the de novo pathway is present and significant but is somehow inhibited by the presence of a pre-existing centriole [23]. Suppression of the de novo assembly pathway by pre-existing centrioles has also been observed in HeLa cells [27*]. The authors found that de novo formation of centrioles occurred in HeLa cells when all endogenous centrioles were destroyed by laser ablation, but that the presence of even a single endogenous centriole inhibited the assembly of additional centrioles [27*]. These results indicate that although centrioles are able to form de novo, this pathway is inhibited by a pre-existing centriole, such that only the canonical mechanism depending on that centriole can occur. Because the canonical pathway has a strict dependence on a pre-existing centriole, this pathway is inhibited by a pre-existing centriole [23].

Conclusions
The mechanism that limits centrosome duplication to once per cell cycle is still poorly understood. We have discussed how the proteins known to be associated with centrosome duplication do not fulfill the requirements of a licensing factor, and have proposed that a specific process, the disengagement of duplicated centrioles, might constitute the licensing event. Similarly, the engaged state of centrioles after duplication might block re-duplication until this blocking is relieved at the end of mitosis by the disengagement activity. Recent genetic screens and direct proteomic analysis of centrosomes in several biological systems have begun to reveal many of the components involved in centriole and centrosome assembly [28–31]. The combination of these with specific assays will allow us to unravel the mechanisms of biogenesis of this remarkable organelle.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

15. Using cell fusion experiments, the authors provide evidence for an intrinsic blocking mechanism in centrosome duplication that is independent of the canonical pathway. Inhibition of centrosome duplication by a pre-existing centriole is thought to be mediated by the canonical pathway. Inhibition of centrosome duplication by a pre-existing centriole is thought to be mediated by the canonical pathway. Inhibition of centrosome duplication by a pre-existing centriole is thought to be mediated by the canonical pathway.


The authors provide a detailed account of de novo centrosome assembly in cycling somatic cells. They also show that the presence of a single centriole is sufficient to inhibit the assembly of additional centrioles, indicating that centrioles have an activity that suppresses the de novo pathway.


