Never tear us apart – the importance of centrosome clustering

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Summary

The presence of more than two centrosomes (centrosome amplification) at the onset of mitosis has long been associated with multipolar spindle formation, and with the generation of genetic instability. However, in recent years, several studies have shown that a process termed 'centrosome clustering' actively contributes to bipolar division by promoting the gathering of extra centrosomes in two main poles. In this Commentary, we describe the main proteins that are involved in centriole duplication and discuss how centrosome amplification can be generated both in vitro and in vivo. We then summarize what is currently known about the processes that contribute to bipolar spindle formation when extra centrosomes are present, and which forces contribute to this process. Finally, we discuss how extra centrosomes might contribute to tumorigenesis, giving emphasis to the role of centrosome amplification in promoting genetic instability.

Key words: Bipolar spindle, Cancer, Centrosome amplification, Chromosome instability, Clustering

Introduction

Centrosomes are the main microtubule-organising centers (MTOCs) of animal cells (Bornens, 2002; Nigg and Raff, 2009). They contribute to several cellular processes that can influence cell shape and motility, and facilitate the assembly of a bipolar spindle during mitosis.

Centrosomes are formed by a pair of centrioles surrounded by amorphous material, called the pericentriolar material (PCM). Centrioles are barrel-shaped structures formed by either singlets, doublets or triplets of short and stable microtubules organized in a nine-fold symmetry (Bettencourt-Dias and Glover, 2007). In animal cells, the centriole has two main functions. During mitosis, each centriole pair recruits and organises the PCM to assemble a functional bipolar spindle that is required to equally segregate the two sister chromatids (Bornens, 2002). During interphase, or in some differentiated cells, the older of the two centrioles (the mother centriole) behaves as a basal body and serves as a template for the growth of a cilium (Kobayashi and Dynlacht, 2011).

In a normal cycling cell, centrioles duplicate once per cell cycle and only one daughter centriole is formed per mother centriole in a timely and coordinated way (Delattre and Gönczy, 2004). Perturbations of the centriole duplication cycle, cell fusion or failure in cytokinesis can result in the accumulation of more than two centrosomes per cell, also known as centrosome amplification (Boveri, 2008; Doxsey, 2002; Nigg, 2006). Extra centrosomes can, in principle, also give rise to multiple basal bodies and, therefore, to more than one cilium per cell. So far, however, little is known about the consequences of centrosome amplification during ciliogenesis (Barrera et al., 2010).

This Commentary aims to revisit the causal link between centrosome amplification and genome instability, with a particular focus on the mechanisms of centrosome clustering during mitosis. We will describe the ways in which centrosome amplification is generated, the key players that are involved in centrosome clustering, and discuss the importance of cell shape, polarity and physical constraints that are exerted by neighbouring cells within a tissue on the formation of mitotic spindles in the presence of extra centrosomes. Finally, we will discuss the consequences of centrosome amplification at the cell and organism level.

Centriole duplication and centrosome amplification

For the past 10 years, genetic screens have been fundamental in the identification of centriole duplication proteins (Bettencourt-Dias et al., 2004; Delattre et al., 2006; Delattre et al., 2004; Dobbelaere et al., 2008; Goshima et al., 2007; Kemp et al., 2004; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gönczy, 2003; O'Connell, 2002; O'Connell et al., 2000; Pelletier et al., 2006; Pelletier et al., 2004) (Table 1). Polo-like kinase 4 (PLK4) was identified as a main regulator of centriole duplication. PLK4 loss-of-function results in centriole duplication defects (Bettencourt-Dias et al., 2005; Habedanck et al., 2005), whereas overexpression of PLK4 leads to the formation of multiple daughter centrioles next to the mother centriole in a single cell cycle (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Thus, PLK4 levels must be tightly regulated in a normal cell to restrict the duplication process to occur only once during each cell cycle and to one daughter centriole per mother.

Not surprisingly, in the same way as for cyclins, the classical cell cycle proteins, the levels of centriole duplication proteins fluctuate throughout the cell cycle and are controlled by proteolysis (Table 1). Recent insights into the mechanisms that control PLK4 stability reveal that its degradation by the proteasome is regulated by an E3 ubiquitin ligase, the

Table 1. Centrosome proteins involved in the maintenance of centrosome or spindle pole integrity

Centrosome proteins	Centrosomal function	Cell cycle degradation	Phenotypes upon gain- or loss-of-function and related diseases
[a]PLK4/SAK	Kinase involved in the regulation of centriole duplication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005).	Constitutive $SCF^{\beta TrCP}$ E3 ubiquitin ligase-dependent proteasome degradation (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Rogers et al., 2009). Constitutive trans-autophosphorylation (Guderian et al., 2010; Holland et al., 2010; Sillibourne et al., 2010). Stability in mitosis regulated by PP2A (Brownlee et al., 2011).	Centrosome amplification upon overexpression (Kleylein-Sohn et al., 2007; Habedanck et al., 2005; Bettencourt-Dias et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007a). Overexpression of PLK4 can induce de novo centriole formation in unfertilised *Drosophila* eggs (Peel et al., 2007; Rodrigues-Martins et al., 2007a). In the absence of PLK4, centrioles fail to duplicate (Habedanck et al., 2005; Bettencourt-Dias et al., 2005).
[a]SAS6	Protein required at the early stages of centriole duplication (Leidel et al., 2005). It is involved in the establishment of the 9-fold cartwheel symmetry of the procentriole (Kitagawa et al., 2011; van Breugel et al., 2011).	APC^{Cdh1} and SCF^{FBXW5} E3 ubiquitin ligase-dependent proteasome degradation (Puklowski et al., 2011; Strnad et al., 2007).	Centrosome amplification upon overexpression (Peel et al., 2007; Rodrigues-Martins et al., 2007b). Overexpression of SAS6 can induce de novo centriole formation in unfertilised *Drosophila* eggs (Peel et al., 2007; Rodrigues-Martins et al., 2007b). In the absence of SAS6, centrioles fail to duplicate (Leidel et al., 2005; Strnad et al., 2007; Strnad et al., 2007).
[a]SAS5/Ana2/ STIL	Protein required at early stages of centriole duplication (Delattre et al., 2004; Dobbelaere et al., 2008; Goshima et al., 2007). It is identified as a SAS6 (Leidel et al., 2005) and CPAP binding partner (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).	$APC^{cdc20\text{-}Cdh1}$-dependent proteasome degradation at mitotic exit (Arquint C et al., 2012).	Centrosome amplification upon overexpression in vertebrate cells and *Drosophila* spermatocytes (Tang et al., 2011; Stevens et al., 2010). Overexpression of Ana2 can drive de novo centriole formation in unfertilised *Drosophila* eggs. In the absence of STIL, centrioles fail to duplicate. Loss-of-function mutations cause primary microcephaly (MCPH7) (Kumar et al., 2009).
[a]ASL/CEP152	Centriole protein required for centriole duplication (Blachon et al., 2008) and PCM recruitment (Varmark et al., 2007) involved in the recruitment of PLK4, SAS6 and SAS4 (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010).	NK	PLK4-dependent centrosome amplification upon overexpression (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010). Overexpression of ASL can induce de novo centriole formation in unfertilised *Drosophila* eggs (Stevens et al., 2010; Dzhindzhev et al., 2010). In the absence of ASL, centrioles fail to duplicate (Blachon et al., 2008). Loss-of-function mutations cause primary microcephaly (MCPH4) (Guernsey et al., 2010).
[b]SAS4/CPAP	Centriole protein required to incorporate microtubules after procentriole assembly (Pelletier et al., 2006) originally identified in *C.elegans* (Kirkham et al., 2003; Leidel and Gönczy, 2003).	APC^{Cdh1}-dependent proteasome degradation at mitotic exit (Tang et al., 2009).	Overexpression causes abnormal lengthening of nascent procentrioles (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). In the absence of SAS4, centrioles fail to duplicate (Kirkham et al., 2003; Leidel and Gonczy, 2003; Basto et al., 2006; Kohlmaier et al., 2009; Tang et al., 2009). Loss-of-function mutations cause primary microcephaly (MCPH6) (Bond et al., 2005).
[b]CP110	Localises to the distal end of the procentriole (Chen et al., 2002; Kleylein-Sohn et al., 2007; Schmidt et al., 2009).	$SCF^{CyclinF}$-dependent proteasome degradation (D'Angiolella et al., 2010).	Involved in control of centriole length (Schmidt et al., 2009; Spektor et al., 2007).
[c]CEP63	Centriole protein. Involved in the recruitment of Cep152 to the mother centriole (Sir et al., 2011).	NK	Required to maintain centrosome cohesion (Sir et al., 2011). Loss-of-function mutations cause primary microcephaly and growth retardation (Sir et al., 2011).
[c]CNN/ CDK5Rap2	PCM protein (Megraw et al., 2001). Involved in centrosome cohesion and anchorage to mitotic spindle poles (Barr et al., 2010; Barrera et al., 2010; Buchman et al., 2010; Lucas and Raff, 2007).	NK	Mutations in genes encoding CNN or CDK5RAP2 cause loss of spindle pole integrity and, consequently, the formation of multipolar spindles (Barr et al., 2010; Barrera et al., 2010). In addition, mutations in the gene encoding CDK5RAP2 in mice also result in centriole amplification due to loss of centriole cohesion. Loss-of-function mutations cause primary microcephaly (MCPH3) (Bond et al., 2005).
[c]PCNT/PLP	PCM (Zimmerman et al., 2004) and centriole-associated protein in *Drosophila* (Martinez-Campos et al., 2004). Required to maintain the integrity of the poles of the mitotic spindle (Rauch et al., 2008).	NK	Disorganized mitotic spindles upon depletion (Zimmerman et al., 2004) and defects in primary cilia assembly (Jurczyk et al., 2004). In *Drosophila*, mutations in the gene encoding PLP affect PCM recruitment and ciliogenesis (Martinez-Campos et al., 2004). Loss-of-function mutations cause Primordial Dwarfism/MOPDII (Rauch et al., 2008).

[a]Centriolar proteins, which when overexpressed cause centrosome amplification; [b]centrosome proteins implicated in determining the length of procentrioles; [c]proteins that maintain the integrity of the mitotic spindle poles by maintaining centrosome cohesion and/or attachment to the poles. The different names of the functional homologues in different species are given for each protein. NK, not known.

Skp1–Cul1–F-box (SCF) complex $SCF^{\beta TrCP}$ (an SCF complex that contains βTrCP as the F-box protein) (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Holland et al., 2010; Rogers et al., 2009; Sillibourne et al., 2010). Interestingly, PLK4 controls its own stability by promoting the auto-phosporylation of two residues within the βTrCP phosphorylation degron (Guderian et al., 2010). Moreover, it has recently been suggested that the phosphatase $PP2A^{twins}$ ($PP2A^{PR55}$ in mammals) has an important role in counteracting the auto-phosphorylation of PLK4 within the βTrCP degron box (Brownlee et al., 2011). These results suggest that PLK4 has to be active within a short time window, which needs to be precisely regulated to ensure the accumulation of active PLK4 at the right time.

In *Drosophila melanogaster*, the analysis of overexpression of centriole duplication proteins reveals that their effect is tissue specific. Whereas SAK (the fly homologue for PLK4) and SAS6 efficiently drive centrosome amplification in embryos and brain cells (Peel et al., 2007), another centriole duplication protein, Ana2, only induces centriole amplification in male primary spermatocytes (Stevens et al., 2010).

SAS6 levels appear to depend on the activity of two distinct E3 ligases, the anaphase-promoting complex (APC) substrate APC^{Cdh1} and SCF^{FBXW5} (Puklowski et al., 2011; Strnad et al., 2007). SAS6 has an important function in the early stages of procentriole formation by assembling into oligomers to form a ring that establishes the nine-fold symmetry (Kitagawa et al., 2011; van Breugel et al., 2011). In addition, in worms, ZYG-1 – the functional PLK4 homologue – phosphorylates SAS-6, and this event is essential for procentriole stabilization (Kitagawa et al., 2009). Thus, the use of two distinct E3 ligases might just reflect how important it is to regulate SAS-6 levels for accurate control of centriole number.

Mutations in genes that control centriole duplication stability cause centrosome amplification, as shown in *Drosophila slimb* (the βTrCP homologue) and *SkpA* mutants (Murphy, 2003; Wojcik et al., 2000). However, alternative mechanisms have been suggested. For example, infection with the high-risk human papilloma virus HPV-16 was found to lead to increased levels of *PLK4* mRNA, which are sufficient to generate centrosome amplification (Korzeniewski et al., 2011).

In addition to the overexpression of centriole regulators, cells can also readily acquire extra centrosomes by a process termed cell fusion (Fig. 1). Indeed, it has been known for a long time that animal and plant cells are able to fuse to form polyploid (more than two copies of each chromosome) cells and tissues (Getsios and MacCalman, 2003; Guidotti et al., 2003; Herwig et al., 2011; Otto, 2007; Srinivas et al., 2007). The consequences of accumulating extra centrosomes in polyploid cells are not known, but it is possible that differentiated cells, such as fused muscle cells or glia (Fant et al., 2009; Tassin et al., 1985; Unhavaithaya and Orr-Weaver, 2012), have a greater tolerance for extra centrosomes compared with cycling cells (Krzywicka-Racka and Sluder, 2011).

An alternative way to accumulate centrosomes is to inhibit cytokinesis, the last step of cell division, which will then generate a single binucleated polyploid cell instead of two diploid cells (Fujiwara et al., 2005; Storchova and Pellman, 2004) (Fig. 1). Nevertheless, at this stage, it remains to be determined whether cells can easily accumulate extra chromosomes and centrosomes at the same time. Recent data showed that repeated drug-induced-cleavage failure in transformed and non-transformed cell lines results in the rapid disappearance of binucleated cells with extra-centrosomes from the cycling cell population (Krzywicka-Racka and Sluder, 2011), suggesting that centrosome amplification is poorly tolerated under these conditions. However, evidence obtained in vivo shows that at least some tissues can handle the accumulation of extra chromosomes and centrosomes. This is the case for mammalian polyploid hepatocytes, which are generated by incomplete cytokinesis and appear post-natally in healthy livers (Faggioli et al., 2011; Guidotti et al., 2003; Margall-Ducos et al., 2007) and in *Drosophila* glia required to maintain the integrity of the blood–brain barrier (Unhavaithaya and Orr-Weaver, 2012). In a healthy organism, it is possible that only some cell types have the capacity to accumulate extra centrosomes and chromosomes, and it will be important to identify the mechanisms that allow or prevent such accumulation.

The rise and fall of supernumerary centrosomes

The first descriptions of the behaviour of extra centrosomes during mitosis were made more than 100 years ago by Theodor Boveri in his influential dispermic experiments carried out in sea urchin eggs [see the recent translation (Boveri, 2008)]. Ever since then, an almost unchallenged view persisted that there is a correlation between centrosome amplification and multipolarity of the mitotic spindle with subsequent multipolar division. More recently, however, real-time imaging of different cell types in vitro and in vivo provided evidence for a tendency of supernumerary centrosomes to cluster at the poles of mitotic spindles in order to divide in a bipolar fashion, a mechanism that appears to be more frequent than initially thought (Basto et al., 2008; Ganem et al., 2009; Kwon et al., 2008; Quintyne et al., 2005).

Centrosome clustering was first observed in the N115 mouse neuroblastoma cell line, in which up to 16 centrioles remain closely associated, not only in interphase (Brinkley et al., 1981) but also in mitosis (Ring et al., 1982). During mitosis, extra centrosomes were seen to assemble in small clusters during prometaphase, which end up forming two ring-shaped groups at the poles of a bipolar spindle in metaphase (Ring et al., 1982). A mechanism that inhibits spindle multipolarity by centrosome clustering was subsequently described in non-cancer cells, in cancer cells and in neural stem cells (neuroblasts) of flies that contained extra centrosomes (Basto et al., 2008; Ganem et al., 2009; Krzywicka-Racka and Sluder, 2011; Kwon et al., 2008; Quintyne et al., 2005; Uetake and Sluder, 2004). Although centrosome clustering appears to be a general mechanism that favours bipolar spindle assembly when extra centrosomes are present, not all cells appear to have equal capacity to cluster and, hence, divide in a bipolar way. Untransformed cell lines efficiently cluster their extra centrosomes, and the large majority of divisions are bipolar (Ganem et al., 2009; Krzywicka-Racka and Sluder, 2011; Uetake and Sluder, 2004), whereas the efficiency of clustering is quite variable in transformed cultured cells (Ganem et al., 2009; Krzywicka-Racka and Sluder, 2011; Quintyne et al., 2005; Uetake and Sluder, 2004). It will be essential to determine the reasons for such differences. Transformed cell lines are no longer diploid, and extra centrosomes and chromosomes could represent an extra burden that is difficult to carry over during subsequent cell divisions.

At the organism level, the consequences of centrosome amplification have only been studied in *Drosophila* so far. The analysis of SAK (the fly PLK4 homologue) overexpressing flies (*SakOE*) that were generated by random insertion of a transgene,

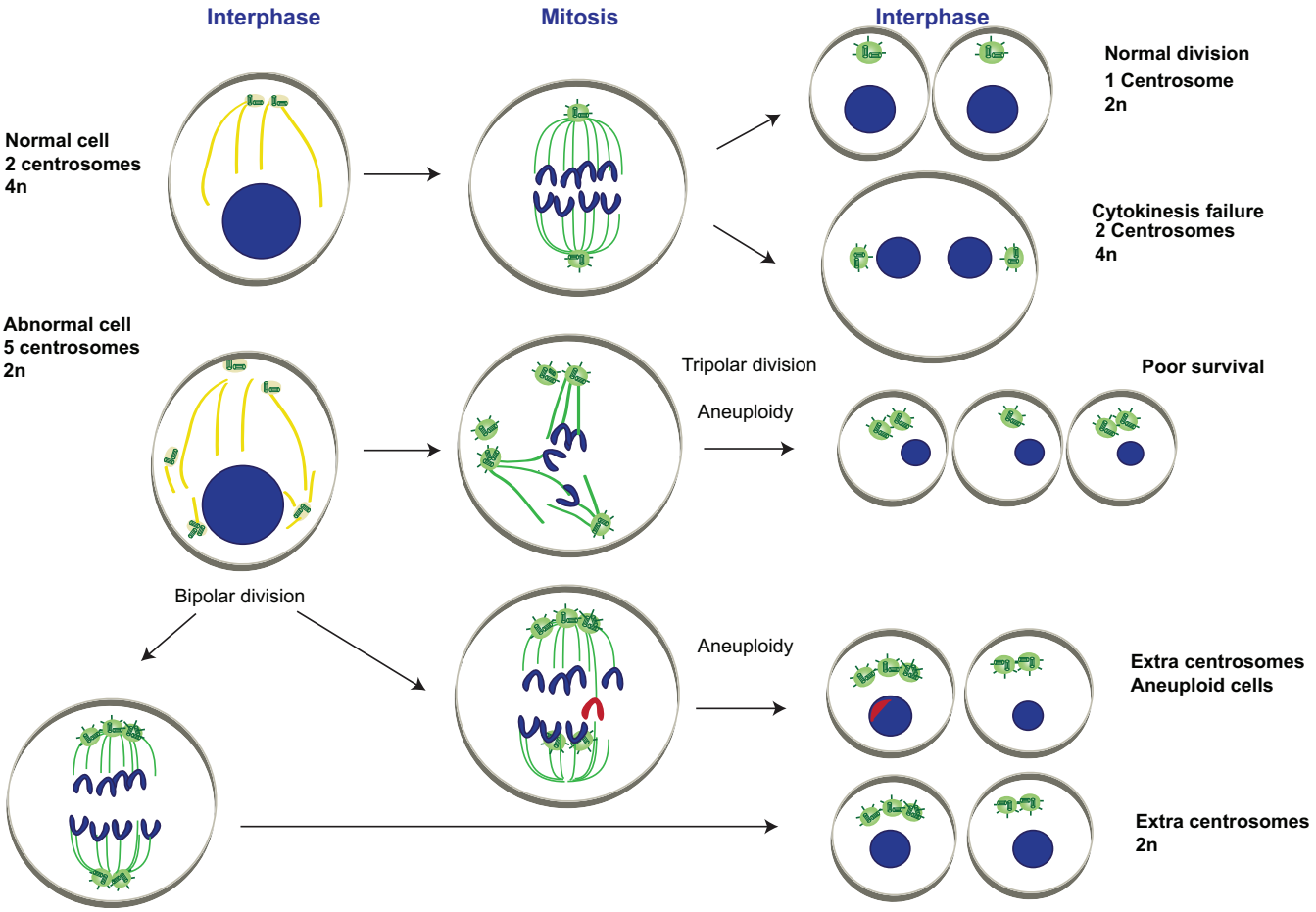

Fig. 1. Causes and consequences of centrosome amplification. A normal cell in interphase (top) contains a set of duplicated chromosomes (4n) and two centrosomes, as indicated by the yellow circles with two centrioles (green barrels) each. After cytokinesis, two diploid (2n) daughter cells with one centrosome each are generated. Cytokinesis failure generates a single daughter cell with two nuclei and two centrosomes. In the next cell cycle, centriole duplication will cause centrosome amplification. In a cell that contains extra centrosomes owing to the overexpression of centriole duplication proteins (bottom), two important scenarios have been described. Supernumerary centrosomes fail to cluster and form multipolar spindles that divide in a multipolar manner. This type of division is thought to have a poor outcome for cell survival. In cells where the extra centrosomes cluster to form a bipolar spindle, merotelic attachments (indicated by the red chromosome) can result in lagging chromosomes during anaphase, generating aneuploid cells. If all the chromosomes are correctly attached and bi-oriented at the metaphase plate, two daughter cells of equal genetic content (but with extra centrosomes) can be generated.

in which the SAK cDNA is under the control of a moderate promoter, reveals that the presence of extra centrosomes is poorly tolerated at early embryonic stages, as ~60% of *SakOE* embryos die during embryogenesis (Basto et al., 2008). The remaining 40%, however, continue to develop until adult stages without any obvious morphological defects (Basto et al., 2008).

Live imaging analysis of *SakOE* neuroblasts with extra centrosomes reveals the presence of a robust mechanism that promotes centrosome clustering (Fig. 2). Neuroblasts are large cells with a diameter of around 10 μm that display prominent rounding in preparation for mitosis. Their centrosome cycle is unusual in that the two centrosomes spend most of the time separated from each other (Rebollo et al., 2007; Rusan and Rogers, 2009), whereas in vertebrate cultured cells, centrosomes remain together until the beginning of mitosis (Kuriyama and Borisy, 1981; Splinter et al., 2010). In *SakOE* neuroblasts, several centrosomes spread throughout the cytoplasm can be detected before nuclear envelope breakdown (NEB). As mitosis proceeds, most centrosomes gather together into two main foci to form a bipolar spindle. Frequently, one or more centrosomes fail to cluster or lose their 'clustered status' during prometaphase and metaphase. Un-clustered centrosomes can be found at different random locations, such as near the spindle, near chromosomes, in the cytoplasm or close to the cortex. Regardless of their location, these 'wandering' centrosomes remain in an inactive (or silenced) state. They do not maintain microtubule nucleation activity (or only in a very reduced manner) and do not participate in spindle formation. Interestingly, both events, clustering and centrosome inactivation, take place exclusively during mitosis, when the activity of cyclin-dependent kinase 1 (Cdk1)-cyclinB complex is high. As cells enter anaphase, rapid centrosome de-clustering can be observed and several centrosomes re-populate the cytoplasm. In addition, the previously inactive centrosomes transiently regain the capacity to nucleate microtubules (Fig. 2). Importantly, centrosome inactivation has been only described in *Drosophila* neuroblasts and it remains to be investigated whether such a mechanism can also contribute to spindle bipolarity in cancer cells when extra centrosomes are present.

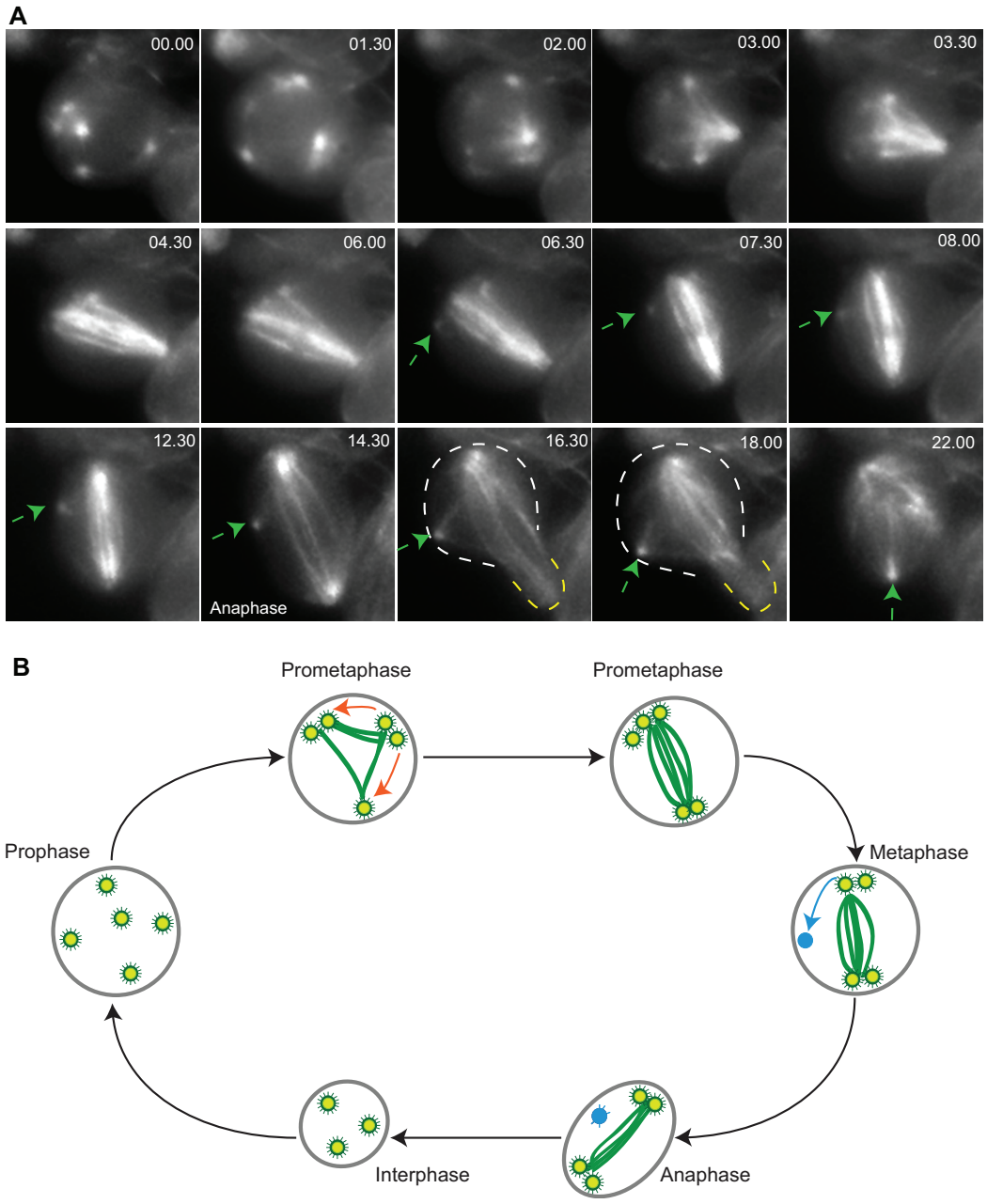

Fig. 2. Dynamics of extra centrosomes during mitosis. (**A**) This panel shows a time-lapse series of a *Drosophila SakOE* neuroblast expressing a GFP fusion to microtubule-associated protein Jupiter (Jupiter–GFP) (Morin et al., 2001). Before NEB, five MTOCs can be identified. As the cell progress into mitosis, two centrosomes cluster to form a focused spindle pole (03.00). The remaining centrosomes cluster afterwards to form a broader pole opposite to the first one (04.30). As this second pole becomes focused (top pole) one centrosome loses its spindle pole localisation (06.30, green arrow). This centrosome is now inactivated, as it is maintained in the cytoplasm throughout mitosis but does not nucleate microtubules. As the cell goes into anaphase, this centrosome regains its microtubule nucleating capacity. The white dash line outlines the neuroblast, and the yellow line outlines the small GMC. Time is shown in minutes. (**B**) A schematic illustration of centrosome clustering and inactivation events observed in *SakOE* neuroblasts. Orange arrows represent the movements of extra centrosomes towards the spindle poles (similar to the events occurring at times 03.00, 04.30 and 07.30 in Fig. 2A), whereas the blue arrow indicates the inactive centrosome (similar to the events occurring at times 06.30 and 07.30).

Even if little is known with regard to the mechanisms that promote centrosome inactivation, the mechanisms and proteins required for centrosome clustering have now begun to be identified (Kwon et al., 2008; Leber et al., 2010). The first genome wide screen performed with the aim of identifying proteins required for centrosome clustering was performed in *Drosophila* S2 cells. This screen identified three main classes of proteins, regulators of acto-myosin contractility, components of the spindle assembly checkpoint (SAC) and microtubule associated proteins (MAPs) (Kwon et al., 2008). Identification of components of the actin cytoskeleton in this screen suggests that both individual cell shape and forces exerted by

neighbouring cells might contribute to clustering, and this aspect will be discussed in more detail below.

In a cell with two centrosomes, a high number of kinetochore–microtubule interactions are needed before these become stabilized, which then satisfy the SAC for further progression through mitosis (Musacchio and Salmon, 2007). Therefore, it is not surprising that a cell with multiple poles (even if just transiently at the beginning of mitosis) requires more time to perform stable (but not necessarily accurate, see below) kinetochore–microtubule attachments. This lengthening of mitosis is consequently used to favour clustering of extra centrosomes. Reducing the time spent in mitosis leads to a failure in centrosome clustering and, consequently, to multipolar division (Basto et al., 2008; Kwon et al., 2008; Sussan et al., 2008).

Microtubule motors, including the minus-end-directed motor dynein and the spindle pole associated protein nuclear mitotic apparatus protein (NuMA), were originally proposed to have important roles in the clustering process (Quintyne et al., 2005). In addition, the minus-end-directed motor, Non-claret disjunctional (NCD, a member of the kinesin-14 family, also known as HSET in vertebrate cells) was identified in the S2 screen described above (Kwon et al., 2008). This kinesin is particularly promising in the context of therapeutic purposes, because it is not essential for cell division in normal cells (Endow and Komma, 1998), but it is required for the efficient clustering of spindle poles in cancer cells with extra centrosomes (Kwon et al., 2008) or in *SakOE* neuroblasts (Basto et al., 2008).

A new group of proteins has been identified in another genome-wide screen. This screen aimed to identify proteins that promote clustering, specifically in cancer cells. Several components of the chromosome passenger complex, proteins involved in kinetochore microtubule attachment, sister chromatid cohesion components and members of the augmin complex (required for spindle assembly) were identified (Leber et al., 2010). This study provides evidence that kinetochore and spindle proteins might help to maintain extra centrosomes in a clustered state at the poles of the spindle by contributing to the establishment and maintenance of tension across the spindle apparatus. Taken together, it appears that a combination of forces provided by, and exerted on, the spindle poles influence the efficiency of centrosome clustering and the maintenance of the clustering status.

Further validation of the proposed hits obtained from these two screens should include the comparison of both microtubule- and actin-driven forces in a three-dimensional environment to preserve cell–cell contacts and cell–extracellular matrix (ECM) interactions. In addition, such validation should take into account cell type diversity and, in particular, it should be considered that different machineries might be activated according to the cell type.

Forcing bipolarity

Bipolar spindle formation can be considered as a self-assembly process that results from forces exerted by mitotic motor proteins and MAPs on microtubules that are nucleated from both centrosomes and chromosomes (Burbank et al., 2007; Loughlin et al., 2010). The robustness of bipolar spindle assembly is very likely to rely on the formation of bipolar anti-parallel microtubule bundles, which is a process that can be reconstituted in vitro with a limited set of components (Fache et al., 2010; Gaillard et al., 2008; Janson et al., 2007; Subramanian et al., 2010).

Two main innovative approaches have been fundamental to the assembly of spindle-like structures in vitro, the combination of chromatin-coated beads with *Xenopus* extracts (Heald et al., 1996) and the engineering of molecular motors with multiple heads (Nédélec et al., 1997; Surrey et al., 2001). These approaches helped to identify the molecular components and the physical principles leading to spontaneous assembly of such complex structures. They also lead to the emergence of an important concept, which is that bipolar spindle-like structures appear to form robustly in various contexts, even in the absence of centrosomes. The assembly of mitotic spindles from purified components, combined with realistic simulation approaches (Burbank et al., 2007; Loughlin et al., 2010; Loughlin et al., 2011), will help to draw a general ‘phase diagram’ of spindle assembly to identify the key parameters that define the boundaries existent between the assembly of robust bipolar spindles and transient (or stable) multipolar spindles.

Multipolar spindles have been observed under several conditions, even if only transiently, during the assembly of acentriolar spindles (Cullen and Ohkura, 2001; Schuh and Ellenberg, 2007), or when extra centrosomes are present (Basto et al., 2008; Ganem et al., 2009; Kwon et al., 2008; Silkworth et al., 2009). The destabilizing effect of centrosome amplification on spindle bipolarity is based on their capacity to both nucleate microtubules and to anchor their minus-ends. The two mechanisms, clustering and inactivation, that are involved in promoting bipolar spindle formation in cells with extra centrosomes amount to the same result, that is, to concentrate microtubule-nucleating and minus-end-anchoring activities at the two main poles of the spindle.

In a cell that contains extra centrosomes, three main forces might contribute to centrosome clustering. The first type of force can be exerted by centrosomes on each other at the beginning of mitosis to facilitate centrosome clustering. The second type of force is generated by anti-parallel microtubule-bundling complexes in regions of overlapping microtubules. These forces depend on an intrinsic property of microtubules, their stiffness, which promotes the alignment of centrosome microtubules towards the metaphase plate. Finally, forces exerted by the cell cortex on astral microtubules that are in close proximity are likely to contribute to clustering by facilitating the movement of centrosomes towards each other (Fig. 3).

In interphase, clustering of microtubule asters depends on the activity of cytoplasmic dynein complexes (Rodionov and Borisy, 1997), and dynein is also required to facilitate clustering during mitosis in some cell types (Quintyne et al., 2005). However, at the beginning of mitosis, it remains to be explained how this motor can regulate both processes. Mechanisms that drive centrosome separation in G2 or prophase involve the recruitment of dynein to the nuclear envelope through two adaptors, bicaudal D homolog 2 (BICD2) and centromeric protein-F CENPF (Bolhy et al., 2011; Splinter et al., 2010). Another motor, Kinesin 5 (Eg5, also known as KIF11), which pushes anti-parallel microtubules that emanate from both centrosomes apart, is also required for centrosome separation (Tanenbaum et al., 2009). Indeed, when KIF11 is active, the dynein pathway is not essential. In cells that overexpress the motor protein KIF15, which can form bipolar spindles in the absence of KIF11, the dynein pathway becomes essential for

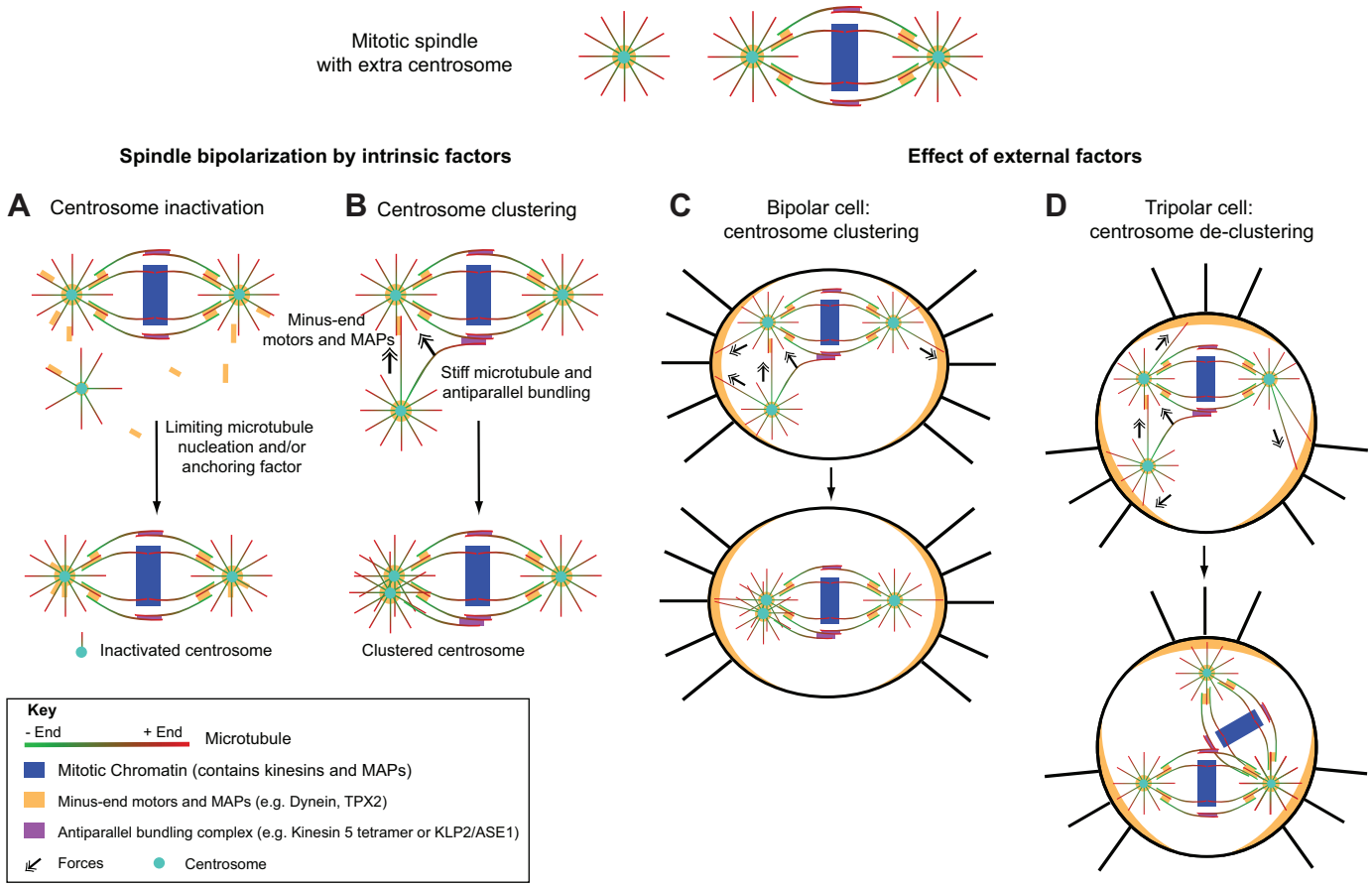

Fig. 3. Forces involved in bipolar spindle formation. The fate of supernumerary centrosomes during mitosis is determined by factors that are intrinsic to the spindle assembly machinery (**A**,**B**) and by external factors, such as the interaction of the cell cortex with astral microtubules (**C**,**D**). Intrinsic factors include centrosome inactivation through depletion of microtubule anchoring and nucleating proteins (A). Centrosomes that are attached to poles of the spindle have more connected minus ends and tend to win the competition for limiting factors, whereas isolated centrosomes would gradually lose their microtubule-anchoring and nucleating capacity. If a centrosome is sufficiently close to a spindle pole, it can also be captured and clustered (B) through interactions between its astral microtubules and microtubules that emanate from the spindle pole microtubules. In this process, the activity of minus-end directed motors such as NCD (HSET) and dynein, or of certain MAPs, such as TPX2 and NuMA, is essential. External factors are exemplified here by the effect of the asymmetric distribution of forces at the cell cortex that pull on astral microtubules. In this example, the asymmetry is owing to the geometry of cell adhesion (Théry et al., 2005). When the cell cortex is bipolar (C), forces that act on astral microtubules will favour bipolar spindle formation, whereas when the cortex is multipolar (e.g. tripolar, as shown in D), the formation of a multipolar spindle is favoured, because forces acting on the astral microtubules tend to pull each pole towards one of the cell cortex pulling regions.

centrosome separation (Tanenbaum et al., 2008; Tanenbaum et al., 2009; Tanenbaum and Medema, 2010). Thus, KIF15 (together with the microtubule minus-end-binding protein TPX2) provides an alternative pathway to KIF11 to promote bipolar spindle formation (Tanenbaum et al., 2009). Taken together, these results provide evidence that at least two robust pathways exist that define the two main MTOCs at the beginning of mitosis. In addition, in cells with extra centrosomes, these pathways might also help to define the sites at which supernumerary centrosomes could form clusters.

A different means of maintaining bipolarity in the presence of supernumerary centrosomes is through centrosome inactivation where un-clustered centrosomes loose microtubule nucleation activity. Although not much is known with regard to centrosome inactivation (Basto et al., 2008), a simple hypothesis is that the extra centrosomes compete for limited amount of PCM components. Such a mechanism has been proposed in budding yeast to explain the asymmetry between the two spindle pole bodies (SPBs) (Grava et al., 2006). It is possible that either a minus-end-anchoring factor or a microtubule-nucleating factor is actively recruited by microtubules and transported to the minus-ends, generating a feedback loop. Such a positive feedback mechanism should then lead to the concentration of this factor at the centre of the main MTOC(s). If this factor was present in limited amounts, it would be depleted from other less ‘important’ MTOCs, which would, therefore, have reduced microtubule nucleating or anchoring capacity. The two microtubule minus-end-containing poles of the bipolar spindle would then rapidly become the dominant MTOCs of the cell (Fig. 3).

All the above considerations can be applied to the mitotic spindle when it is perceived as an isolated structure in space. However, within a cell, external factors act on the spindle and on the centrosomes, and these factors can either destabilize or reinforce spindle bipolarity (Fig. 3). Indeed, cell shape and polarity might have such a role, as the use of disc-shaped adhesive micropatterns to plate cells with extra centrosomes

results in the formation of bipolar spindles (Kwon et al., 2008). The proportion of cells with bipolar spindles was found to be even higher in cells that are plated on a bar-shaped adhesive pattern. However, if Y-shaped micropatterns are used, an increased amount of multipolar spindles is observed (Kwon et al., 2008; Théry et al., 2007; Théry et al., 2005). Bar-shaped adhesive patterns induce a distinct accumulation of actin at the two poles of the cell, whereas a disk-shape pattern induces the formation of a rotating actin cloud (Fink et al., 2011). Therefore, it is likely that Y-shaped patterns lead to three main sites of actin accumulation, which could then result in three main foci that pull on astral microtubules and, thus, attract centrosomes. The Y pattern corresponds to a 'tripolar' cortex and favours a tripolar spindle. This suggests that, within a tissue, the microenvironment of a cell aggravates the effect of an abnormal number of centrosomes and induces multipolar spindle formation, or by contrast, in polarized tissues, contributes to the survival of these cells by promoting centrosome clustering and bipolar spindle formation.

The shape of a cell influences the distribution of forces that act on the spindle, and it is thought to have an important role in the orientation of the spindle (Minc et al., 2011). Accordingly, defects in the shape of a mitotic cell, such as those induced by the depletion of moesin, a protein linking the actin cortex to the plasma membrane, result in the assembly of abnormal spindles (Carreno et al., 2008; Kunda et al., 2008). Interestingly, these defects can be rescued by coating cells with the lectin Concanavalin A, which cross-links sugars outside the cell, thus, providing a rigid shell to restore cell shape but not cortical integrity (Kunda et al., 2008). Recent studies have shown that the 'doughnut-like' spatial organisation of chromosomes in prometaphase, called the 'Rabl star' after the 19th century cell biologist who first described this arrangement, is important for proper kinetochore attachment (Kitajima et al., 2011; Magidson et al., 2011). This further suggests that defects in cell shape can impact on spindle assembly by preventing the proper formation of the 'Rabl star'. The majority of cells become rounded for mitosis, and it is possible that rounding and cortical stiffening protects mitotic cells from possible deformations that might be caused by neighbouring cells. Therefore, it is tempting to speculate that the round mitotic cell shape can be regarded as a means to securing a free space, which would then favour robust bipolar spindle assembly.

Consequences of centrosome amplification

The initial observations made by Boveri suggested that the main consequence of centrosome amplification is the generation of multipolar spindles and abnormal cell division. Centrosome amplification is a common feature of solid and hematopoietic tumours, and is often correlated with chromosome instability (CIN). As a result, a link between centrosome amplification and cancer can be easily established (Doxsey, 2002; Guo et al., 2007; Lingle and Salisbury, 1999; Lingle and Salisbury, 2000; Nigg, 2006; Zyss and Gergely, 2009). Importantly, however, we should also take into consideration the fact that centrosome amplification is not a unique feature of cancer cells. Certain cells in our body contain extra centrosomes, either transiently or permanently (Faggioli et al., 2011; Margall-Ducos et al., 2007), suggesting that in a healthy organism, centrosome amplification is tolerated and even required to promote genetic variability (Duncan et al., 2010; Kingsbury et al., 2005; Yang et al., 2003).

It is important, however, to understand whether there is a correlation between the presence of extra centrosomes and CIN found in the majority of human tumours. Multipolar spindles are often observed in cancer cell lines, but the progeny of multipolar division is not consistently viable, as seen in vertebrate cells in culture (Ganem et al., 2009; Krzywicka-Racka and Sluder, 2011; Quintyne et al., 2005) and in *Drosophila* embryos, in which extra centrosomes fail to cluster (Basto et al., 2008; Peel et al., 2007). Hence, how can CIN be generated in cells with extra centrosomes if the majority of the spindles are bipolar? This important question was recently addressed, and it has been proposed that the transition from multipolarity to bipolarity (at the beginning of mitosis) favours the establishment of merotelic attachments, in which a single kinetochore is attached to both spindle poles (Ganem et al., 2009; Silkworth et al., 2009). Merotelic attachments are not always corrected, because sensing any kind of microtubule attachment, even if incorrect, can satisfy the SAC (Cimini et al., 2001). In principle, uncorrected merotelic attachments generate lagging chromosomes during anaphase, and consequently, aneuploidy. However, recent experiments suggest that it is not that simple, because the large majority of lagging chromosomes segregate to the correct daughter cell, at least in the cell line that was analysed in this study (Thompson and Compton, 2011). Thus, it is possible that extra centrosomes generate CIN by more than one mechanism when cells divide in a bipolar fashion, and that alternative mechanisms remain to be described.

Aneuploidy, the gain or loss of whole chromosomes, has long been associated with malignancy and tumour progression (Boveri, 2008; Brinkley and Goepfert, 1998; Nigg, 2006). Yet, it is very difficult to determine the exact role of aneuploidy in tumorigenesis. An emerging view suggests that the consequences of gaining a chromosome depend on a combination of factors, among which the identity of the gained chromosome is of the uttermost importance. For example, experiments performed in four different aneuploid mouse embryonic fibroblast (MEF) cell lines, each containing a different additional chromosome, show that they have different capacities to proliferate, metabolize glucose and become immortalized (Williams et al., 2008). In addition, single chromosome mis-segregation, which can be generated in the presence of extra centrosomes (see above), normally compromises the proliferation of diploid cells (Thompson and Compton, 2008), a process that seems to be dependent on the p53 pathway (Thompson and Compton, 2010).

It is also possible that not all tissues respond in a similar way to aneuploidy. For instance, random aneuploidy that is caused by overexpressing the spindle assembly checkpoint protein MAD2 or by reducing the levels of the mitotic kinesin CENPE, generates tumours in some tissues, but not in others (Sotillo et al., 2007; Weaver et al., 2007). Moreover, the exposure of heterozygous *Cenpe*$^{+/-}$ aneuploid mice to carcinogens does not result in tumour formation, whereas wild-type mice treated under the same conditions develop tumours (Weaver et al., 2007). In agreement with these observations, the incidence of intestinal tumours, which normally develop in the APC$^{Min+/-}$ mouse model, decreases in the Ts65Dn background, a trisomic mouse model for human Down syndrome (Sussan et al., 2008). Together, these results suggest that aneuploidy either promotes or inhibits tumour formation depending on the cellular and tissue context combined with the identity of the gained chromosome (Weaver and Cleveland, 2009).

Aneuploidy also influences the capacity of a tumour to recur. For example, in K-Ras-driven lung tumours (caused by the constitutive overexpression of the *K-Ras*-encoding gene), transient overexpression of *Mad2* to induce aneuploidy causes tumour relapse, even after removal of K-Ras (Sotillo et al., 2010).

In flies that have extra centrosomes, there is neither a substantial increase in the level of chromosome instability, nor are merotelic or lagging chromosomes detectable (Basto et al., 2008). However, importantly, flies only have four chromosomes, which might facilitate the correction of possible attachment errors. Accordingly, *Drosophila* mutants lacking *mad2* are viable, as the decrease in the length of mitosis is sufficient to allow the attachment and congression of all four chromosomes (Buffin et al., 2007).

Although *Drosophila SakOE* brains do not show increased levels of aneuploidy, defects in asymmetric cell division of neural stem cells (neuroblasts) are noticable (Basto et al., 2008). Neuroblasts divide in an asymmetric manner to self-renew and to generate a ganglion mother cell (GMC) that will divide once more before undergoing differentiation. A complex machinery ensures the differential localisation of stem cell proteins to the apical cortex, and of proteins that are required for differentiation to the basal side (Knoblich, 2008). Asymmetry is generated by the precise position of the mitotic spindle along the polarity axis, further demonstrating that spindle positioning is a key factor in determining the outcome of stem cell division (Gonzalez, 2007; Knoblich, 2008; Morin and Bellaïche, 2011). During asymmetric cell division of *SakOE* neuroblasts, defects in astral microtubule nucleation can be detected, which cause defects in spindle position and lead to an increase in the neuroblast population (Basto et al., 2008). This increase in the stem cell population proves to be tumorigenic when *SakOE* brains are transplanted into the abdomen of a healthy host. *SakOE* brain tissue is able to overproliferate, which causes the death of the host in less than two weeks (Basto et al., 2008).

In flies, mutations that deregulate the balance between stem cell renewal and differentiation are known to cause tumours in transplantation assays (Caussinus and Gonzalez, 2005), whereas mutations that cause chromosome instability do not (Castellanos et al., 2008). It is, therefore, possible that in flies aneuploidy alone cannot initiate tumorigenesis because the gain or loss of one of the four chromosomes would have dramatic consequences for cell survival. Importantly, the results in flies also suggest that there is more than one route that leads to tumour formation when extra centrosomes are present.

Ideally, a cell should be able to sense the presence of supernumerary centrosomes, and hazardous cells with abnormal centrosome content should be effectively disposed of. Unfortunately, such a mechanism does not appear to operate, and instead, robust pathways ensure that bipolar spindles are formed in such cases, even at the expense of generating low levels of aneuploidy (Ganem et al., 2009; Silkworth et al., 2009). Consequently, cells with extra centrosomes can continue to proliferate. Affecting bipolar spindle formation, and thus, directing cells towards apoptosis, is a very promising approach to kill tumour cells that contain extra centrosomes, and explains why, in recent years, much effort has been geared towards developing drugs that affect KIF11 (see above). In principle, the use of NCD (or HSET) inhibitors should be an even more promising approach, as normal cells do not require this kinesin for mitosis, whereas cancer cells rely on it to survive (Kwon et al., 2008). Nevertheless, perturbing spindle bipolarity might not be so easy, as cells can find alternative pathways to ensure bipolarity. Recent work has shown that cells can easily develop resistance to a drug that affects KIF11 by increasing the expression levels of KIF15 (Tanenbaum et al., 2009). Taken together, these results confirm once more the view that bipolarity is not so easy to eliminate.

Conclusions and future directions

For more than a hundred years, the centrosome has been exclusively seen as a cell division organelle with essential functions in the assembly of a bipolar spindle during mitosis. However, several recent studies have revisited the exact function of the centrosome during cell division and suggested that under some circumstances, the centrosome is actually dispensable for bipolar spindle assembly (Basto et al., 2006; Heald et al., 1996; Khodjakov et al., 2000; Khodjakov and Rieder, 2001). Obviously, it is better for a cell to have centrosomes than not (Gonzalez, 2008) especially, if the mitotic spindle needs to be correctly positioned or a cilium generated in the following cell cycle. However, from an optimistic point of view, this also means that we have now the possibility to focus on other (perhaps even more) complex, interesting and unexpected functions of the centrosome.

At the moment, it appears to be essential to understand why and how centrosome amplification can be tolerated in some cells, while having a negative impact in other cell types. Could such differences depend on the cell or tissue function, or alternatively, on the architecture of the cell? Or could it be possible that the developmental stage or adult age influence the capacity of a cell to deal with extra centrosomes? Importantly, even if mitosis is the obvious phase to investigate the effects of centrosome amplification, it is also crucial to consider the possible impact of centrosome amplification in cilliogenesis and, thus, in the signalling processes that depend on primary cilia, either during development or during processes that are required to maintain tissue homeostasis at adult stages.

The observation that centrosome amplification can lead to CIN in vertebrate cells, together with the fact that centrosome amplification is at the origin of tumour formation in flies, argue in favour of a detailed analysis of the consequences of centrosome amplification in a mammalian model organism. It will be important to clarify whether centrosome amplification in mammals leads to CIN and/or spindle positioning defects, as is the case in *Drosophila*. Mutations in centrosome components are known to cause primary autosomal recessive microcephaly [(MCPH), see Table 1], a neuro-developmental disorder that is characterised by decreased brain size at birth. Importantly, defects in spindle positioning of progenitor neural stem cells have been considered to be at the origin of brain-size reduction (Thornton and Woods, 2009). Therefore, it will be important to determine the consequences of centrosome amplification during neuro-development.

A global understanding of the consequences of centrosome amplification is only starting to emerge. Future work, developed both at the cell and organism level, will be essential to ascertain the impact of centrosome amplification during development and how it can contribute to disease.

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