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The PLK4–STIL–SAS-6 module at the core of centriole duplication

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Centrioles are microtubule-based core components of centrosomes and cilia. They are duplicated exactly once during S-phase progression. Central to formation of each new (daughter) centriole is the formation of a nine-fold symmetrical cartwheel structure onto which microtubule triplets are deposited. In recent years, a module comprising the protein kinase polo-like kinase 4 (PLK4) and the two proteins STIL and SAS-6 have been shown to stay at the core of centriole duplication. Depletion of any one of these three proteins blocks centriole duplication and, conversely, overexpression causes centriole amplification. In this short review article, we summarize recent insights into how PLK4, STIL and SAS-6 co-operate in space and time to form a new centriole. These advances begin to shed light on the very first steps of centriole biogenesis.

Introduction

As major microtubule-organizing centers of animal cells, centrosomes co-ordinate interphase microtubule networks and facilitate the formation of a bipolar spindle in mitosis. By influencing microtubule distribution as well as dynamics [1], centrosomes are important for a variety of cellular processes, including cell motility, shape, polarity and division, as well as intracellular signaling (reviewed in refs [2–4]). Each centrosome comprises a pair of centrioles, surrounded by pericentriolar material (PCM) [5]. The PCM contains a multitude of proteins organized in distinct layers [6–9] and γ-tubulin ring complexes important for nucleation of microtubules [1]. Centrioles are barrels composed of microtubule triplets arranged in nine-fold rotational symmetry [5,10,11]. They are important not only for the assembly of centrosomes, but indispensable also (as so-called basal bodies) for the formation of cilia and flagella [12,13].

Reflecting their diverse functions, centrioles, centrosomes and cilia are implicated in a variety of different diseases [14,15]. Defects in the centriole-ciliary apparatus result in a broad spectrum of diseases, collectively known as ciliopathies, that affect multiple tissues and organs [16,17]. Furthermore, mutations in several centrosomal proteins [including polo-like kinase (PLK4), STIL and SAS-6] result in primary microcephaly, a neurodevelopmental disorder characterized by drastically reduced brain size at birth, and/or dwarfism [18–21], reviewed in ref. [14]. Finally, centriole amplification as well as structural centrosome aberrations have long been associated with carcinogenesis [22–31].

To prevent chromosomal instability, centriole duplication must be strictly controlled during the cell cycle [32,33]. Conceptually, these controls must ensure, first, that centriole duplication occurs only once in every cell cycle and, second, that only one new procentriole is built per pre-existing centriole [34]. At the G1/S-phase transition, new procentrioles (daughter centrioles) form next to the proximal base of each of the two pre-existing (mother) centrioles. This key step in centriole biogenesis involves the formation of a cartwheel-like assembly platform that instructs the attachment of nine microtubule triplets to form the wall of the new procentriole [5]. How the physical attachment of a single new procentriole can repress the formation of additional procentrioles around the same mother centriole remains to be fully understood [35,36]. Likewise, it is not fully understood what mechanisms limit centriole duplication to once per cell cycle. However, suppression of duplication is apparently released as soon as procentrioles disengage from their mother centrioles at the end of mitosis, thus licensing

centrioles for a new round of duplication [35,37]. Importantly, suppressive mechanisms can be overridden by overexpression of any one of the core components of the PLK4–STIL–SAS-6 centriole duplication module (Figure 1A,B), indicating that tight control of the abundance of these proteins is crucial [38–43]. Here, we summarize recent progress in our understanding of how PLK4, STIL and SAS-6 are regulated, and how these three proteins co-operate to initiate centriole duplication.

A core module for centriole duplication

Although the centrosome was discovered more than 100 years ago [44,45], we are only beginning to understand the processes that underlie its duplication. The application of powerful genome-wide RNAi screens in *Caenorhabditis elegans* embryos led to the identification of five critical genes required for procentriole formation, and subsequent molecular and biochemical studies revealed that the products of these genes are loaded sequentially onto the proximal part of the mother centriole (reviewed in refs [46,47]). Most upstream in the assembly cascade acts SPD-2, which recruits the ZYG-1 kinase. ZYG-1 then triggers the association of the SAS-5/SAS-6 complex to form a central tube onto which microtubules are deposited in a SAS-4-dependent manner [48]. Subsequent studies in other organisms revealed that these duplication factors comprise an evolutionarily conserved core module for centriole duplication. Additional proteins were shown to act as duplication factors in other species, notably *Drosophila* and human cells [43,49,50].

Collectively, the available evidence indicates that PLK4 (ZYG-1 in *C. elegans*), STIL (SAS-5 in *C. elegans* and Ana2 in *Drosophila*) and SAS-6 (DSas-6 in *Drosophila*) are particularly important for the initiation of centriole duplication (Figure 1C). In support of this notion, the assembly of these three components on the wall of the mother centriole appears to mark the first steps of procentriole formation. Thus, in the following we describe some of the key characteristics of these three proteins.

Polo-like kinase 4

Much of our current understanding of the molecular mechanisms underlying centriole biogenesis follows from the identification of PLK4 as a master regulator of centriole duplication [42,51]. PLK4, originally termed SAK [52], is a distant member of the PLK family of serine/threonine kinases. All PLKs share a common protein topology consisting of an N-terminal kinase domain followed by two or more polo-box (PB) motifs organized in different domains. PLK4 is distinct from other PLK family members in that it carries three, rather than two, PBs (reviewed in ref. [53]). PBs 1 and 2 of PLK4 (formerly named cryptic PBs) are important for dimerization and centriole recruitment of PLK4 [54–59]. Dimerization of PLK4 and trans-autophosphorylation is important for both activation of the kinase and regulation of PLK4 levels. Activation of PLK4 depends on phosphorylation of a critical residue within the kinase's activation loop (or T-loop) [60–62], whereas ubiquitin-dependent proteolytic degradation, at the hands of the SCF complex β-TrCP, is triggered by phosphorylation of a destruction motif (DSG motif) within the so-called linker 1 (L1) region of the kinase [63–66]. These two important autoregulatory mechanisms contribute to control the abundance and activity of PLK4 in space and time.

SAS-6

Elegant recent work has identified SAS-6 as a key structural component important for imposing nine-fold symmetry to cartwheel architecture [67–69] (reviewed in ref. [5]). The cartwheel consists of an inner hub from which nine spokes emanate, thereby determining the nine-fold symmetrical arrangement of microtubule triplets within a newly forming centriole [70,71]. *In vitro* studies have shown that SAS-6 is able to homodimerize, resulting in the formation of an N-terminal globular head domain, and that circular oligomerization of globular head domains can result in the formation of structures that resemble the inner cartwheel hub, with the C-terminal coiled coil (CC) domains projecting outwards to form the spokes [67,68]. These exciting results indicate that oligomerization of SAS-6 probably plays a major role in conferring nine-fold symmetry to the centriolar cartwheel. This being said, recent studies suggest that *de novo* formation of centrioles can occur in the absence of SAS-6 self-oligomerization [72] and that cartwheel architecture also depends critically on the assembly of the microtubule wall [73].

STIL

The *STIL* gene originally attracted attention because of an implication of the *STIL* locus in a chromosomal aberration in T-cell acute lymphoblastic leukemia [74]. Furthermore, STIL was found to be essential for vertebrate development and overexpressed in several cancers, and the STIL protein was linked to mitosis and
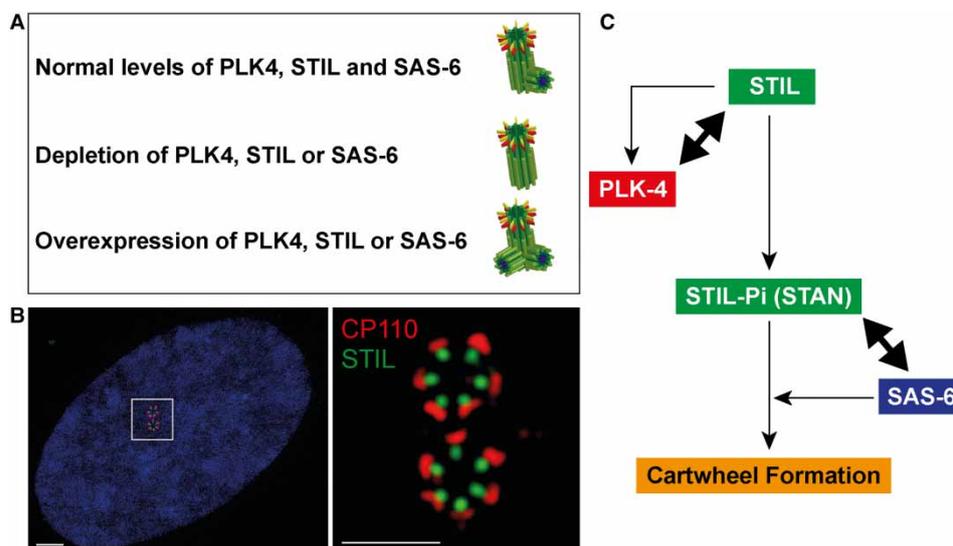
Figure 1. The PLK4-STIL-SAS-6 centriole duplication module.

(**A**) Abundance of PLK4, STIL or SAS-6 controls centriole numbers. Under physiological conditions, normal levels of PLK4, STIL and SAS-6 ensure the formation of only one procentriole per mother centriole (upper row). In contrast, depletion of either protein inhibits centriole formation (middle row) and, conversely, overexpression triggers the formation of several procentrioles per mother centriole (lower row). (**B**) Super-resolution immunofluorescence images illustrate the (near-) simultaneous formation of several procentrioles around each of the two mother centrioles of a U2OS cell conditionally overexpressing PLK4 [43]. Centrioles are stained for STIL (green) and CP110 (a marker for the distal ends; red). The right panel shows a ×10 magnification of the area marked in the left panel (DNA is shown in blue). Error bars denote 2 μM (left image) and 1 μM (right image). (**C**) Highly simplified scheme summarizing key interactions between components of the PLK4–STIL–SAS-6 module during cartwheel formation (for more detailed information see text and Figures 2 and 3).

cell proliferation [75–84]. Following the insight that human STIL likely represents the homolog of *C. elegans* SAS-5 and *Drosophila* Ana-2 [85], a direct role in centriole duplication was rapidly confirmed [39–41,86]. Although STIL is much larger than SAS-5 and Ana2, all three proteins contain a central CC domain and, additionally, share sequence similarity in a domain termed STAN (for STIL/Ana2) [85]. As described in more detail below, STIL co-operates with PLK4 and SAS-6 in cartwheel formation and also interacts with CPAP, the human homolog of *C. elegans* SAS-4, which is important for later steps of centriole formation [40,41,87,88].

STIL is a key substrate and downstream effector of PLK4

PLK4 has been reported to phosphorylate several proteins implicated in centrosome biology, including its own centriolar recruitment factors Cep152 and Cep192 [56,58], PCM1 (a constituent of centriolar satellites) [89], GCP-6 (a component of the γ-tubulin ring complex) [90] and the FBXW5 F-Box protein implicated in SCF-mediated protein degradation of SAS-6 [91]. However, several of these studies are based exclusively on *in vitro* kinase reactions, and the significance and functional consequences of these phosphorylations *in vivo* remain to be fully understood. In contrast, there is now strong evidence to indicate that STIL is one of the most important binding partners and physiological substrates of PLK4 [92–96].

The STIL CC domain interacts with PLK4

The short central STIL CC domain is critical for self-oligomerization of STIL, which apparently results in tetramers in case of human STIL and *Drosophila* Ana2 or trimers in case of *C. elegans* SAS-5 [40,95,97–99]. Interestingly, the same CC domain is also required and sufficient to bind to PLK4 [92–95] (Figure 2A). Removal of the CC domain blocks the ability of STIL to localize to centrioles and to trigger centriole amplification, demonstrating that STIL binding to PLK4 and/or STIL self-oligomerization are
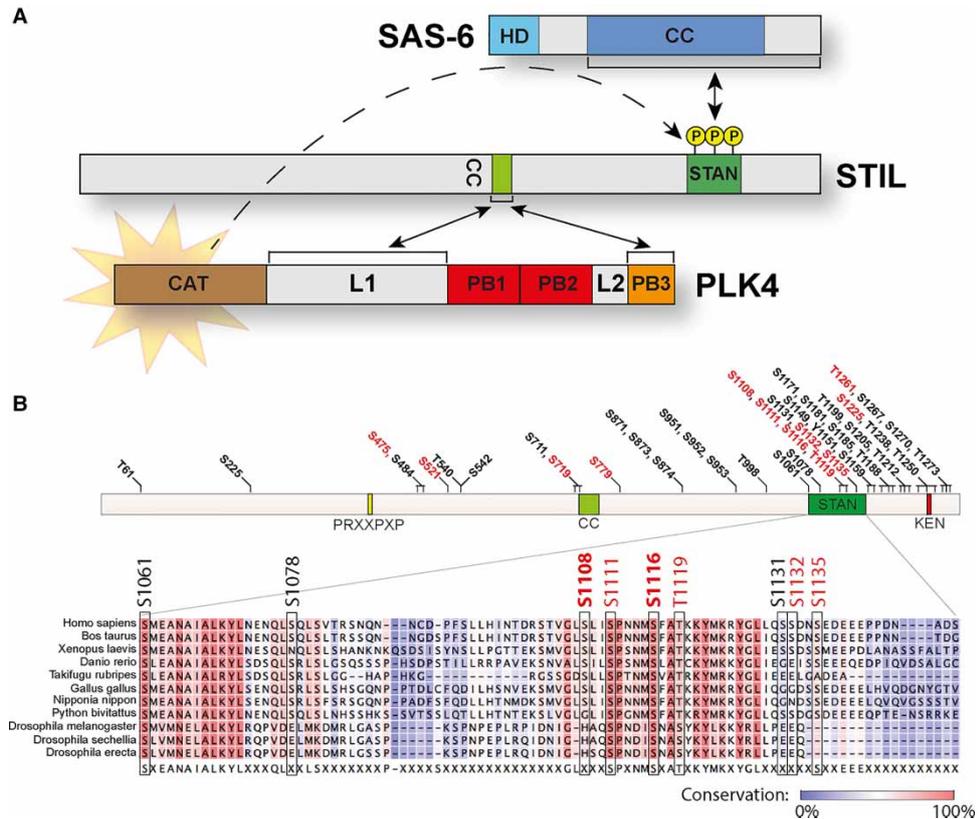
Figure 2. Precise interplay of PLK4, STIL and SAS-6 at the core of centriole duplication.

(**A**) Illustration of the co-operation between PLK4, STIL and SAS-6. The schematic emphasizes the interaction between the STIL CC domain and both the PLK4 PB3 and the L1 linker region. This interaction then triggers phosphorylation of key residues within the STIL STAN domain. Phosphorylation of the STIL STAN domain in turn enables recruitment of SAS-6. HD, head domain; CC, coiled coil; CAT, catalytic domain; L, linker; PB, polo-box; STAN, STIL/Ana2 domain. (**B**) Summary of PLK4 phosphorylation sites within STIL, as mapped by *in vitro* assays. Sites that have also been observed *in vivo* by mass spectrometry (www.phosphosite.org) [101] are marked in red. A sequence alignment of the STAN domain compiles several vertebrate STIL and *Drosophila* Ana2 sequences. Two PLK4 phosphorylation sites within this region (S1108 and S1116) are highlighted in bold, because they were shown to be phosphorylated by PLK4 *in vivo* by the use of site-specific phospho-antibodies [93]. PRXXPXP, CPAP-binding motif; CC, coiled coil; STAN, STIL/Ana2 domain; KEN, destruction motif (recognized by APC/C^{Cdh} [1]) important for STIL degradation.

essential for centriole duplication. Mapping of the STIL interaction domain on PLK4 yielded somewhat conflicting results, but the bulk of the evidence indicates that STIL interacts with more than one domain on the kinase [92,94,95]. In particular, structural data demonstrate that the PB3 of PLK4 and the L1 linker region (connecting the kinase domain with the C-terminal PB region) are sufficient to bind to STIL (Figure 2A) [95]. Moreover, mutational analyses indicate that STIL binds to the PB3 and the L1 linker region through different types of interaction, suggesting that STIL can bind concomitantly to both domains [95]. Considering that the L1 linker region has been implicated in autoinhibition of PLK4 and PB3 in release of inhibition [100], these structural data raise the exciting prospect that STIL binding to PLK4 results in kinase activation (see below).

PLK4 phosphorylates STIL in the STAN domain to facilitate SAS-6 binding

Importantly, PLK4 not only binds to STIL, but also phosphorylates STIL on several residues within the so-called STAN domain [92–94] (Figure 2A), a region conserved within the C-terminus of human STIL, *Drosophila* Ana2 and *C. elegans* SAS-5. In particular, residues S1108 and S1116 within the STAN domain were
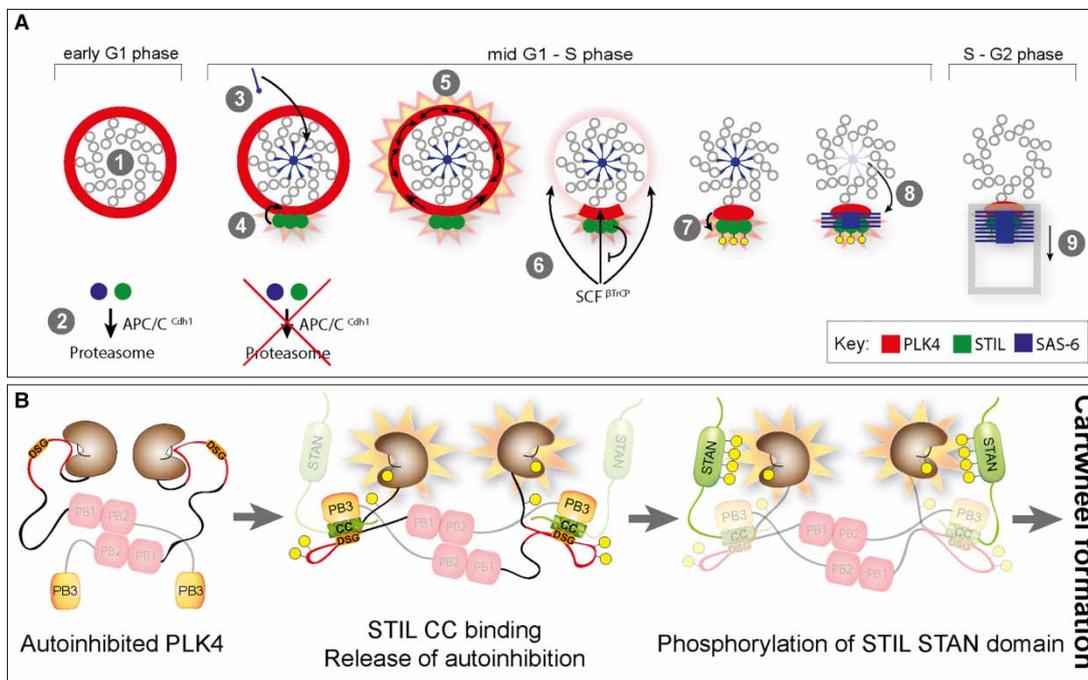
Figure 3. How PLK4, STIL and SAS-6 initiate centriole duplication.

(**A**) Hypothetical model summarizing the first steps in centriole duplication. For further explanation, see text. (**B**) Detail of the above model, with focus on the release of PLK4 autoinhibition upon binding of STIL CC to both PB3 and L1 linker domains of PLK4, and the phosphorylation of the STIL STAN domain. Note that binding of STIL CC to L1 may mask the DSG motif, thereby protecting PLK4 from degradation.

shown to be directly phosphorylated by PLK4 *in vivo* using phospho-specific antibodies [93]. Mutation of PLK4 phosphorylation sites within the STAN domain of STIL abolishes both STIL/SAS-6 interaction and centriole (over-)duplication. This demonstrates that phosphorylation of the STAN domain promotes the recruitment of SAS-6 to STIL (Figure 2A), and thus probably constitutes a crucial step in the formation of the cartwheel [92–94]. In addition, no fewer than 43 sites within STIL have been described to be phosphorylated by PLK4 *in vitro* [92–94], and 12 of these were shown to be phosphorylated *in vivo* by mass spectrometry approaches [101] (Figure 2B). As many of these phosphorylation sites are localized outside the STAN domain (Figure 2B), it may be rewarding to definitively identify the kinase(s) that act(s) on these sites *in vivo* and explore their functional relevance.

Depletion of PLK4 or chemical inhibition of the kinase results in rapid loss of STIL from centrioles, suggesting that PLK4 activity is required to maintain STIL at the site of centriole formation [93,102]. In this conclusion, mutation of five PLK4 phosphorylation sites within STIL's STAN domain to non-phosphorylatable alanine residues significantly interferes with STIL's ability to localize to centrioles [93]. In striking contrast, removal of the entire STAN domain does not interfere with STIL accumulation [93–95]. Taken at face value, this suggests that the STAN domain is not strictly required for centriolar recruitment of STIL, but rather that the STAN domain exerts a negative effect on localization, and that this effect is overcome by phosphorylation at the site of centriole duplication [93]. One way to test this hypothesis would be to combine PLK4 inhibition with expression of the STIL ΔSTAN mutant: according to the above model, inhibition of PLK4 should interfere with localization of STIL wild type, as has been observed [93,102], but STIL ΔSTAN should remain stably associated with centrioles.

In contrast with human cells, where PLK4 phosphorylation of the STAN domain seems to constitute a prerequisite for the formation of a robust STIL–SAS-6 complex, SAS-5 can interact with SAS-6 in the absence of ZYG-1 in *C. elegans* [103]. In this context, we note that phosphorylation of the STAN domain by ZYG-1 has not been reported. Although an early study had attributed a key role to SAS-6 phosphorylation (by ZYG-1) for cartwheel formation [104], subsequent work showed that SAS-6 is recruited to the site of centriole

formation through a direct, phosphorylation-independent interaction with ZYG-1 [105]. In *Drosophila*, Ana2 (STIL) and DSas-6 may also be able to interact in the absence of PLK4, at least as suggested by yeast two hybrid studies [85]. However, the addition of PLK4 to *in vitro* binding assays strongly enhances the STIL–SAS-6 interaction [96]. Furthermore, simultaneous overexpression of PLK4, Ana2 and DSas-6 in *Drosophila* spermatocytes results in assembly of highly ordered structures that bear resemblance to cartwheels, confirming that these three components co-operate in centriole duplication [106]. As observed for human STIL, PLK4 phosphorylates Ana2 in the STAN domain and mutation of major phosphorylation sites results in complete loss of SAS-6 binding and centriole duplication [96]. However, in contrast with human cells, abrogation of PLK4 phosphoacceptor sites within the STAN domain does not interfere with centriole localization of Ana2. In summary, complex formation between STIL/Ana2/SAS-5 and SAS-6/DSas-6/SAS-6 constitutes an evolutionarily conserved key step in the assembly of a cartwheel structure, although, not unexpectedly, subtle aspects of regulation differ between organisms.

STIL is an upstream regulator of PLK4
STIL promotes PLK4 kinase activity
In the absence of STIL, PLK4 protein levels increase significantly, both in the cytoplasm and at the centrosome [93,95]. Considering that PLK4 levels are controlled by PLK4 activity, as described above [63–66], this prompted the hypothesis that STIL might be an activator of PLK4 [93,95]. In support of this view, PLK4 abundance is similarly increased upon mutational ablation of the degradation motif or inhibition of PLK4 kinase activity by a small molecule inhibitor [93,107,108]. Furthermore, overexpression of STIL triggers autophosphorylation in a non-degradable version of PLK4, resulting in retarded electrophoretic mobility and enhanced phosphorylation of T170 within the activation loop [93], indicative of enhanced kinase activity [60–62].

These observations indicate that the interaction of STIL and PLK4 not only triggers downstream events relevant for cartwheel formation, but also generates a positive feedback loop to enhance PLK4 kinase activity. This feedback mechanism likely contributes to ensure that PLK4 only reaches full activity once it has met its substrate STIL. How exactly STIL binding activates PLK4 kinase remains to be elucidated. Elegant work by Rogers and co-workers provides an attractive working model [100]. By studying the regulation of PLK4 in *Drosophila*, these authors found that PLK4 exists in an autoinhibited state, reminiscent of PLK1 [109–111], and that autoinhibition likely depends on an interaction between the L1 linker and the adjacent T-loop activation domain [100]. They also showed that the C-terminal PB3 domain of PLK4 is required for relieve of autoinhibition, and suggested that an additional binding partner might be required for triggering a conformational change in the kinase. In light of recent structural data demonstrating that STIL binds to both the PB3 domain and the L1 linker region, STIL appears to be ideally suited for triggering the relieve of PLK4 autoinhibition [95]. Thus, STIL likely plays a key role in the spatial and temporal control of PLK4 activation during initiation of centriole duplication.

STIL protects PLK4 from degradation
Prior to the onset of centriole duplication, PLK4 seems to form a ring around parental centrioles [57,94], but as soon as procentriole formation is initiated, this pattern resolves into a dot-like structure that coincides with the localization of STIL and SAS-6, presumably reflecting cartwheel formation [8]. Subsequently, and for the entire duration of interphase, PLK4, STIL and SAS-6 then co-localize exclusively at the newly formed procentriole. Holland and co-workers reported that STIL activates PLK4 and that this activation then results in destabilization of both centrosomal and cytoplasmic pools of PLK4 [93]. Activation-induced degradation is entirely plausible [63–66], but the question then arises of how PLK4 can coexist with STIL at the site of procentriole formation without being destroyed. One plausible answer is that STIL not only activates PLK4, but also locally protects it from degradation. This hypothesis is supported by observations made in cells overexpressing STIL, where multiple procentrioles form near-simultaneously around each mother centriole. Under such circumstances, PLK4 forms a ring around mother centrioles, and, importantly, a ring is observed also in response to overexpression of an STIL mutant that lacks the STAN domain and is unable, therefore, to trigger centriole amplification. This suggests that the presence of STIL is sufficient to stabilize PLK4, independently of downstream events such as SAS-6 recruitment and daughter centriole formation [94,95]. What mechanisms locally tip the balance between STIL-mediated activation and consequent degradation of Plk4 versus STIL-mediated protection against degradation remains to be elucidated. Given that STIL binds to the L1 linker region of PLK4 that

also contains the DSG motif, one possibility is that STIL interferes with β-TrCP recognition and/or phosphorylation of this degradation motif.

Conclusions and prospects

Recent insights into how PLK4, STIL and SAS-6 co-operate to initiate the formation of a new procentriole enables us to depict a tentative step-by-step model that holds promise to explain the initial events of centriole duplication at a molecular level (shown in Figures 1C and 3). Several key features of this model are well established, but other aspects remain hypothetical and will require further experimental scrutiny. Also, it should be noted that Figure 3 ignores regulatory steps occurring at the level of transcription [112–114].

In mitosis, Cdk1/cyclin B was recently shown to bind to the CC region of STIL, thereby preventing premature interaction with PLK4 [115]. Then, in early G1 phase, when PLK4 forms a ring around each parental centriole [94], it likely exists in an autoinhibited form, because kinase activity is inhibited by binding of the linker L1 region to the catalytic domain [100] (Figure 3A — step 1 — and Figure 3B). At this stage, STIL and SAS-6 proteins are not yet present, due to their degradation by APC/C^{Cdh} [1] (Figure 3A, step 2) [38–40]. With the silencing of the APC/C at the G1/S transition, however, the stage is set for the initiation of procentriole formation. According to one provocative proposal, SAS-6 is first recruited to the proximal lumen of the mother centriole, where it assembles into cartwheel-like structures through interactions with the luminal wall (Figure 3A, step 3) [116] (see also ref. [117]). Meanwhile, STIL binds PLK4 on the circumference of the mother centriole, resulting in the formation of an STIL–PLK4 complex marking the site where the new centriole is going to be built (Figure 3A, step 4) [8,92–96]. This concentration of an STIL–PLK4 complex to one particular site is triggered when binding of the STIL CC domain to both the PB3 and the L1 linker of PLK4 releases PLK4 autoinhibition (Figure 3B). This then stimulates autophosphorylation of the PLK4 T-loop activation site (Thr170), resulting in a further increase of kinase activity [60,61,93]. We postulate that close proximity then enables activated PLK4 to transactivate neighboring non-STIL-bound PLK4 molecules, resulting in activation of PLK4 over the entire ring (Figure 3A, step 5). As a result of activation, PLK4 dimers will trans-autophosphorylate their DSG motifs, triggering binding of SCF β-TrCP and proteasome-mediated degradation (Figure 3A, step 6). A central tenet of the proposed model is that those PLK4 molecules that interact with STIL are protected from degradation and thus define the site of centriole duplication. This notion falls in line with super-resolution microscopy data, demonstrating the early formation of a PLK4 dot on the circumference of the mother centriole [8]. Importantly, the proposed mechanism suggests ways for preventing the formation of more than one procentriole per parental centriole: as long as one procentriole containing active PLK4 persists close to the wall of the parental centriole, one would in fact predict that newly recruited additional PLK4 will be activated and hence degraded. The next crucial step in centriole biogenesis is the phosphorylation of STIL, by PLK4, within the STAN domain (Figure 3A — step 7 and Figure 3B), which then enables STIL–SAS-6 interaction (Figure 3A, step 8) [92–94,96]. Assuming that SAS-6 oligomers are first assembled within the lumen of the mother centriole [116], some mechanism must exist to allow their transfer to the procentriole construction site, marked by PLK4–STIL, on the side of the mother centriole cylinder (Figure 3A, step 8). Following cartwheel formation, presumably under participation of Cep135, CPAP and other proteins, microtubules then attach to the ends of the cartwheel spokes to start the formation of the new centriolar wall (Figure 3A, step 9).

In future, it will be important to further study the regulation of the PLK4–STIL–SAS-6 module and the role of additional proteins in centriole duplication. Moreover, continued structural work will be required to elucidate the precise interaction of key components in centriole biogenesis. It seems safe to predict that additional substrates of PLK4 await identification, and it will also be interesting to explore the role of further posttranslational modifications in centriole biogenesis. These points are exemplified by a proposal implicating the F-box protein (and putative PLK4 substrate) FBXW5 in the control of SAS-6 levels [91] and a report implicating a prolyl-hydroxylase, acting on Cep192, in centriole duplication [118]. Finally, much remains to be learned about the coordination of centriole biogenesis with cell cycle progression [112,119–121]. These questions will undoubtedly keep attention focused on centriole duplication for many years to come.

Abbreviations

CC, coiled coil; CPAP, centrosomal P4.1-associated protein; GCP-6, gamma-tubulin complex component 6; L, linker; PB, polo-box; PCM, pericentriolar material; PLK, polo-like kinase; SAS-5, spindle assembly abnormal protein 5; SAS-6, spindle assembly abnormal protein 6; SCF, Skp-Cullin-F-box; SPD-2, spindle-defective protein 2; STAN, STIL/Ana2; ZYG-1, zygote defective protein 1.

Competing Interests
The Authors declare that there are no competing interests associated with the manuscript.

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