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Plk4-Induced Centriole Biogenesis in Human Cells

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SUMMARY

We show that overexpression of Polo-like kinase 4 (Plk4) in human cells induces centrosome amplification through the simultaneous generation of multiple procentrioles adjoining each parental centriole. This provided an opportunity for dissecting centriole assembly and characterizing assembly intermediates. Critical components were identified and ordered into an assembly pathway through siRNA and localized through immunoelectron microscopy. Plk4, hSas-6, CPAP, Cep135, γ-tubulin, and CP110 were required at different stages of procentriole formation and in association with different centriolar structures. Remarkably, hSas-6 associated only transiently with nascent procentrioles, whereas Cep135 and CPAP formed a core structure within the proximal lumen of both parental and nascent centrioles. Finally, CP110 was recruited early and then associated with the growing distal tips, indicating that centrioles elongate through insertion of α-/β-tubulin underneath a CP110 cap. Collectively, these data afford a comprehensive view of the assembly pathway underlying centriole biogenesis in human cells.

INTRODUCTION

In animal cells, the centrosome orchestrates the formation of the cytoplasmic microtubule (MT) network during interphase and the mitotic spindle during M phase (Doxsey et al., 2005; Luders and Stearns, 2007; Nigg, 2004). The single centrosome present in a G1 phase cell comprises two centrioles embedded in a protein matrix, the socalled pericentriolar material (Bornens, 2002). Centrioles are cylindrical structures built of microtubules and closely related to basal bodies, which are essential for the formation of cilia and flagella (Dutcher, 2003; Azimzadeh and Bornens, 2004). The two centrosomal centrioles are structurally and functionally distinct in that only one is fully mature, as reflected by appendages at the distal end and a competence to initiate ciliogenesis (Ishikawa et al., 2005; Lange and Gull, 1996; Vorobjev and Chentsov Yu, 1982). How centrioles and basal bodies are assembled and how their numbers are controlled within cells constitute long-standing unresolved questions. In proliferating cells, centrosome duplication during S phase involves the formation of exactly one new centriole (procentriole) adjacent to each of the two pre-existing (parental) centrioles, so that a G2 cell then harbors two centrosomes, each containing two closely associated (engaged) centrioles. Upon entry into mitosis the two centrosomes separate from each other to form the spindle poles. Interestingly, the two centrioles present within each centrosome/spindle pole then disengage during exit from M phase, in response to the activation of the protease Separase (Tsou and Stearns, 2006b). This has led to an appealing model according to which centriole disengagement constitutes a licensing mechanism for ensuring that centrioles duplicate only once in every cell cycle (Tsou and Stearns, 2006a, Tsou and Stearns, 2006b; Wong and Stearns, 2003).

The formation of centrioles and basal bodies has been extensively studied by electron microscopy (Anderson and Brenner, 1971; Brinkley et al., 1967; Chretien et al., 1997; Dippell, 1968; Kuriyama and Borisy, 1981; Mizukami and Gall, 1966; Sorokin, 1968; Vorobjev and Chentsov Yu, 1982). These studies have suggested the existence of two fundamentally distinct assembly pathways. The simultaneous formation of multiple basal bodies was attributed to a "de novo" assembly mechanism, whereas the duplication of centrioles in proliferating cells was thought to require the pre-existing centrioles as "templates" for the formation of progeny (Beisson and Wright, 2003; Hagiwara et al., 2004). However, recent experiments have blurred these distinctions, and it now appears that pre-existing centrioles act primarily as solid-state platforms to accelerate the assembly process (Khodjakov et al., 2002; La Terra et al., 2005; Rodrigues-Martins et al., 2007; Uetake et al., 2007). Careful electron microscopic studies on centriole and basal body formation have revealed a filamentous corona forming around the proximal walls of parental centrioles and electron-dense material protruding into the proximal half of the elongating centriole (Anderson and

Brenner, 1971; Sorokin, 1968). Moreover, a characteristic fibrous structure displaying 9-fold symmetry (termed "cartwheel") has been proposed to serve as a scaffold for the assembly of centriolar MTs (Anderson and Brenner, 1971; Beisson and Wright, 2003; Cavalier-Smith, 1974).

Centriole biogenesis in mammalian cells remains poorly understood, but substantial progress has recently been made in invertebrate organisms. In *Caenorhabditis elegans*, a protein kinase, Zyg-1 (O'Connell et al., 2001), and four putative structural proteins, termed SPD-2, Sas-4, Sas-5, and Sas-6, are required for centriole duplication (Delattre et al., 2004; Kemp et al., 2004; Leidel et al., 2005; Leidel and Gonczy, 2003; Pelletier et al., 2004). Moreover, through elegant epistasis experiments and electron tomography the five proteins could be shown to assemble sequentially on nascent procentrioles (Delattre et al., 2006; Pelletier et al., 2006). Independently, the protein kinase Plk4 (also known as Sak [Fode et al., 1994; Swallow et al., 2005]) has been identified as a key regulator of centriole duplication in both *Drosophila* (Bettencourt-Dias et al., 2005) and human cells (Habedanck et al., 2005). Although the two kinases lack obvious sequence homology, it is plausible that Plk4 represents a functional homolog of *C. elegans* Zyg-1. When overexpressed in unfertilized eggs of *Drosophila,* Plk4 (Sak) induced the de novo formation of centrioles, demonstrating that this kinase is able to induce centriole biogenesis even in the absence of pre-existing centrioles (Peel et al., 2007; Rodrigues-Martins et al., 2007). Homologs of nematode Sas-4 and Sas-6 were also required for centriole biogenesis in *Drosophila* (Peel et al., 2007; Rodrigues-Martins et al., 2007) and a requirement for Sas-6 was demonstrated for human cells (Leidel et al., 2005), suggesting that fundamental aspects of centriole biogenesis have most likely been conserved during evolution.

We have previously shown that overexpression of Plk4 in human cells causes the recruitment of electron-dense material onto the proximal walls of parental centrioles (Habedanck et al., 2005), suggesting that Plk4 is able to trigger procentriole formation. Here, we have used a cell line allowing the temporally controlled expression of Plk4 to study the formation of centrioles in human cells. We show that Plk4 triggers the simultaneous formation of multiple procentrioles around each pre-existing centriole. These multiple centrioles form during S phase and persist as flower-like structures throughout G2 and M phase before they disperse in response to disengagement during mitotic exit, giving rise to a typical centriole amplification phenotype. Through siRNA-mediated depletion of individual centrosomal proteins, we have identified several gene products important for Plk4-controlled centriole biogenesis and assigned individual proteins to distinct steps in the assembly pathway. Finally, we have been able to correlate these functional data with morphological analyses using immunoelectron microscopy. Taken together, these results provide a first molecular analysis of centriole formation in human cells.

RESULTS

Cell-Cycle Regulation of Plk4-Induced Centriole Biogenesis

To determine whether overexpression of Plk4 in human cells is capable of triggering the formation of multiple complete centrioles, we generated a cell line that allows the temporally controlled expression of this kinase and examined centriole formation during cell-cycle progression. As centrin constitutes an excellent marker for centriole formation in human cells (Bornens, 2002; Paoletti et al., 1996), anti-centrin antibodies (Baron et al., 1992) were used to monitor centriole assembly in these experiments (Figure 1). Already 16 hr after Plk4 induction, approximately 70% of asynchronously growing cells showed evidence of centriole amplification. They either displayed multiple scattered centrioles or multiple procentrioles arranged around each parental centriole, reminiscent of the petals of a flower (Figure 1A). Interestingly, flower-like structures could only be detected in cyclin A-positive S and G2 phase cells (upper row), but not in cyclin A-negative G1 cells, which instead contained multiple centrioles that appeared to be disengaged (lower row). Flower-like structures persisted during early stages of mitosis but then began to disassemble during late telophase (Figure 1B), consistent with the view that the disengagement of newly formed centrioles from parental centrioles occurs during exit from mitosis (Tsou and Stearns, 2006b). These data demonstrate that overexpression of Plk4 induces the assembly of multiple procentrioles during S phase. These then elongate during G2 and persist in an engaged state with their parental centrioles until disengagement at the end of mitosis causes centriole scattering.

We next asked whether the ability of Plk4 to induce the formation of multiple centrioles is regulated during the cell cycle and whether multiple procentrioles develop simultaneously or sequentially. After release from a nocodazole block in M phase, cells had to be incubated for 10–12 hr before flower-like structures could be seen (data not shown). In contrast, cells that were synchronized and held at the G1/S transition by aphidicolin responded to Plk4 induction by "flower" formation within 1–3 hr (Figure 2A). This indicates that cells need to reach a permissive cell cycle window (G1/S transition and early S phase) before they can respond to Plk4 activity. When using centrin staining as a marker for centriole assembly, the first visible evidence for Plk4-induced procentriole formation was the formation of a halo (or ring) around each parental centriole (see Figure 2B; 1 hr–Halo). Within these halos, a more intensely staining region could occasionally be discerned, suggesting that the one procentriole existing already at the onset of these experiments persisted on the parental centrioles. Halo formation could be seen in a significant fraction of cells already after 1 hr of Plk4 induction and was essentially complete after 3 hr (Figure 2A). At later times, each halo progressively resolved into a number of discrete nascent procentrioles. These new procentrioles appeared with very similar kinetics, indicating that they formed nearly simultaneously. A quantitative

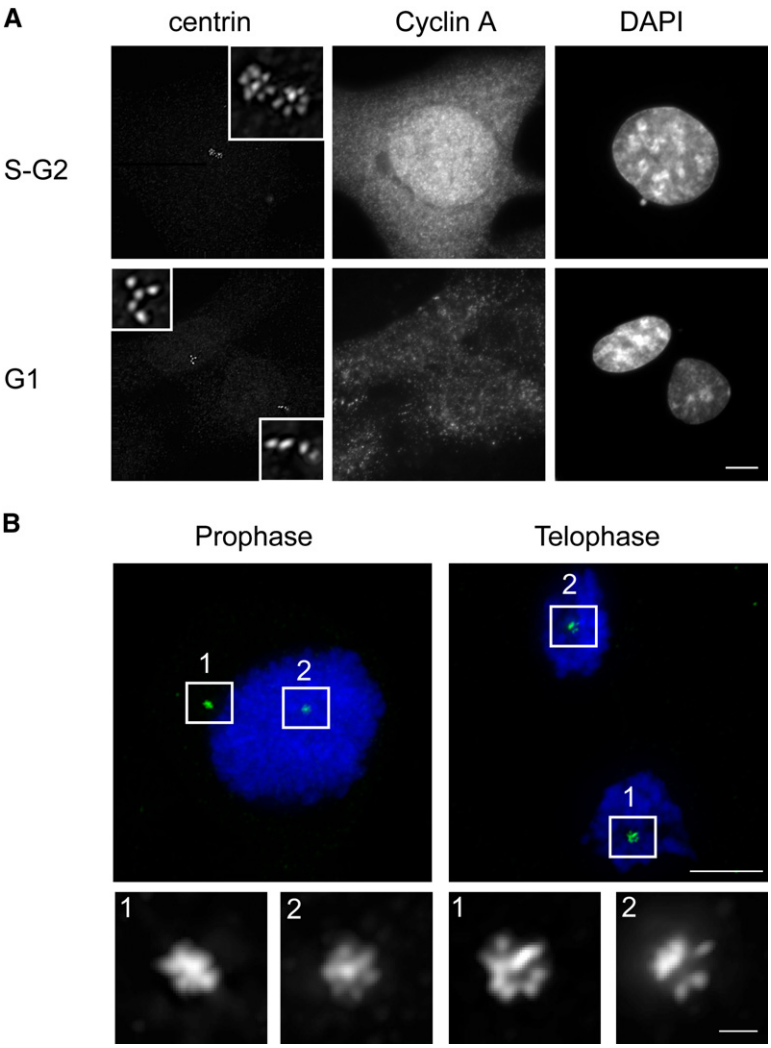


Figure 1. Plk4-Induced Centriole Biogenesis during Cell-Cycle Progression

Myc-Plk4 expression was induced for 16 hr in asynchronously growing U2OS cells before they were fixed and analyzed by immunofluorescence microscopy, using the antibodies indicated.

(A) Interphase cells costained for centrin (20H5) and Cyclin A, to indicate cell-cycle position, and DNA (DAPI). Note that Cyclin A-positive cells (in S or G2 phase; upper row) show multiple centrioles in a flower-like arrangement, whereas Cyclin A-negative cells (in G1; lower row) show scattered (disengaged) centrioles. Insets show enlarged views of centriole "flowers" and clusters, respectively.

(B) Mitotic cells costained for centrin (rabbit antibody) and DNA (DAPI). Upper panels show overviews of representative prophase and telophase cells; lower panels show higher magnifications of the two poles in each cell to visualize flower-like structures. Scale bars indicate 10 μM and 1 μM (higher magnifications).

analysis of flower-like structures 16 hr after Plk4 induction revealed that most of them contained 6 centrin-positive procentrioles, although some variation in number could be seen (Figure 2B). The limited spatial resolution of these experiments masks the exact events occurring during the conversion of a halo structure to individualized procentrioles, but analysis of the radial spacing of nascent procentrioles indicates that these structures formed randomly with regard to the circumference of the parental centriole (Figure 2B).

Localization of Key Proteins in Centriole Assembly

Our ability to control centriole biogenesis by induction of Plk4 provided a unique opportunity for studying the assembly process in time and space. To identify centrosomal proteins required for Plk4-induced centriole biogenesis, we first depleted nearly 30 candidate proteins (Andersen et al., 2003) by siRNA before inducing Plk4 expression and examining (pro)centriole formation by immunofluorescence microscopy (Figure S1, see the Supplemental Data available with this article online). This siRNA screen identified hSas-6, CPAP (the putative homolog of *C. elegans* Sas-4), Cep135, and CP110 as being indispensable for centriole biogenesis. High-resolution immunofluorescence microscopy was then used to determine at what stages the above proteins contribute to centriole assembly. Following induction of Plk4, nascent flower-like structures as well as disengaged multiple centrioles were stained with antibodies against Plk4, Cep135, hSas-6, CPAP, and CP110 as well as α- and γ-tubulin. Simultaneously, centrin staining was used to visualize both procentrioles and mature centrioles (Paoletti et al., 1996). As summarized in Figure 3, different proteins displayed strikingly different localization patterns. Within the flower-like structures observed 16 hr after Plk4 induction (Figure 3A), Plk4 accumulated in a ring-like pattern around the parental centrioles (see also Figure 4, and Figures S3 and S4), and similar localizations were seen for Cep135, γ-tubulin, and hSas-6. However, compared to Plk4, Cep135 appeared to form a more compact structure, suggesting that it concentrates also within the lumen of the parental centriole (see below). In the case of hSas-6, the

ring structure was not as smooth as that seen with Plk4, suggesting that hSas-6 does not decorate the surface of the parental centriolar cylinder but rather associates with nascent procentrioles. Staining for CPAP, α-tubulin, and CP110 revealed star-like structures overlapping the nascent procentrioles, but, again, subtle differences were apparent. Compared to the localization of centrin, anti-CPAP antibodies clearly stained both the parental centriole and the proximal ends of nascent procentrioles, whereas α-tubulin was seen all along the length of the centrioles and CP110 could be detected primarily on the distal ends. Analysis of the multiple, Plk4-induced centrioles occurring in dispersed clusters (Figure 3B) revealed that Plk4, Cep135, CPAP, α-tubulin, γ-tubulin, and CP110 all colocalized with centrin-positive disengaged centrioles. In stark contrast, hSas-6 was undetectable on G1 phase centrioles (Figure 3B), indicating that this protein is transiently recruited to nascent procentrioles but subsequently displaced or degraded, possibly during centriole disengagement.

To examine the acquisition of centriole maturity markers during Plk4-induced centriole biogenesis, we also stained early flower-like structures and late disengaged centrioles for polyglutamylated tubulin, a marker for stabilized centriolar MTs (Bobinnec et al., 1998), and ODF-2, a marker for centriole maturation (Ishikawa et al., 2005). As demonstrated by staining with GT335 antibody (Wolff et al., 1992), only parental centrioles were polyglutamylated during early centriole biogenesis, whereas the newly assembled tubulin of nascent procentrioles lacked this modification (Figure 3C and Figure S2A). Likewise, ODF-2, a component of centriolar appendages (Ishikawa et al., 2005), could only be detected on one of the two parental centrioles, identifying it thereby as the fully mature parent (Figure 3C). At later stages, all centrioles in flower-like structures of mitotic cells (Figure S2B) and in centriole clusters of G1 phase cells (Figure 3D) stained positive for GT335, indicating that these centrioles were composed of polyglutamylated, stabilized MTs. In contrast, ODF-2 staining remained confined to only one centriole, the former parent, even in G1 cells with multiple disengaged centrioles (Figure 3D). This is consistent with expectation, as newly formed centrioles acquire appendages only during final maturation, which occurs in late G2 of next cell cycle (Bornens, 2002). Taken together, the above analysis demonstrates that overexpression of Plk4 induces the simultaneous formation of multiple complete centrioles.

Delineation of a Centriole Assembly Pathway

The above results suggested that Plk4 localizes early onto the wall of parental centrioles, where it may then trigger the recruitment of essential centriolar proteins and their incorporation into newly assembling procentrioles. To corroborate this conclusion, we used siRNA to deplete individual proteins implicated in centriole biogenesis and then monitored procentriole formation in response to Plk4 induction. This approach made it possible to establish dependencies among individual proteins and visualize assembly intermediates (Figure 4 and Figures S3–S5). Following depletion of either hSas-6, CPAP, Cep135, γ-tubulin, or CP110, Plk4 still accumulated around the parental centrioles, exactly as it did in GL2-treated controls (Figure 4A and Figures S3–S5, top rows). This demonstrates that Plk4 localization does not depend on any of the above proteins and supports the view that this kinase acts high up in a regulatory hierarchy. In contrast, the depletion of hSas-6 completely suppressed the Plk4-induced assembly of procentrioles and, as a consequence, all other proteins remained restricted to parental centrioles (Figure 4B and data not shown). Likewise, centriole biogenesis was completely suppressed in response to depletion of CPAP, Cep135, or γ-tubulin (Figures S3–S5). Some hSas-6 staining of parental centrioles could be seen in such cells, but since hSas-6 is not present within the lumen of parental centrioles (see Figures 3A, 3B and 6B), we presume that this signal reflects residual hSas-6 associated with the centriolar surface. Most interestingly, procentriole formation also failed upon depletion of CP110, as visualized by centrin staining (Figure 4C, left panels), but in this case, ring-like structures clearly stained positive for hSas-6 (Figure 4C, right panels). This indicates that procentriole formation was blocked downstream of hSas-6 recruitment. Taken together, these data indicate that hSas-6, CPAP, Cep135, and γ-tubulin are recruited early after Plk4 induction to form nascent procentrioles. The four proteins were mutually dependent on each other and similarly required for further development of procentrioles, at least within the temporal and spatial resolution of these experiments. In contrast, CP110 clearly functions at a later stage.

We also asked whether centrin proteins are required for procentriole formation. Centrin depletion was achieved by 72 hr treatments of cells with siRNA duplexes targeting centrins 2 and 3. These two isoforms are expressed ubiquitously in somatic cells, whereas centrins 1 and 4 are restricted to multiciliated and flagellated cells (Gavet et al., 2003; Hart et al., 1999; Laoukili et al., 2000; Middendorp et al., 1997). Considering that centrin 2 was previously reported to be required for centriole duplication in human cells (Salisbury et al., 2002), we were surprised to find that induction of Plk4 in cells depleted of centrins 2 and 3 still induced both the formation of α-tubulin-positive procentrioles and, at later times, multiple disengaged centrioles, indistinguishable from controls (Figure 5A). Furthermore, centrin depletion produced no detectable adverse effects on the recruitment of CPAP, Cep135, or CP110 to nascent procentrioles (Figure 5B). Efficient depletion was confirmed using an antibody (20H5; [Baron et al., 1992]) known to detect both centrins 2 and 3 (Middendorp et al., 1997; Paoletti et al., 1996). With regard to a possible compensatory role of other centrin isoforms, we emphasize that no human centrin 4 gene has yet been identified and RT-PCR revealed no evidence for expression of centrin 1 in the cells studied here (Figure S6). Thus, although centrins normally associate with nascent procentrioles early during assembly (Figure 3), our data provide no

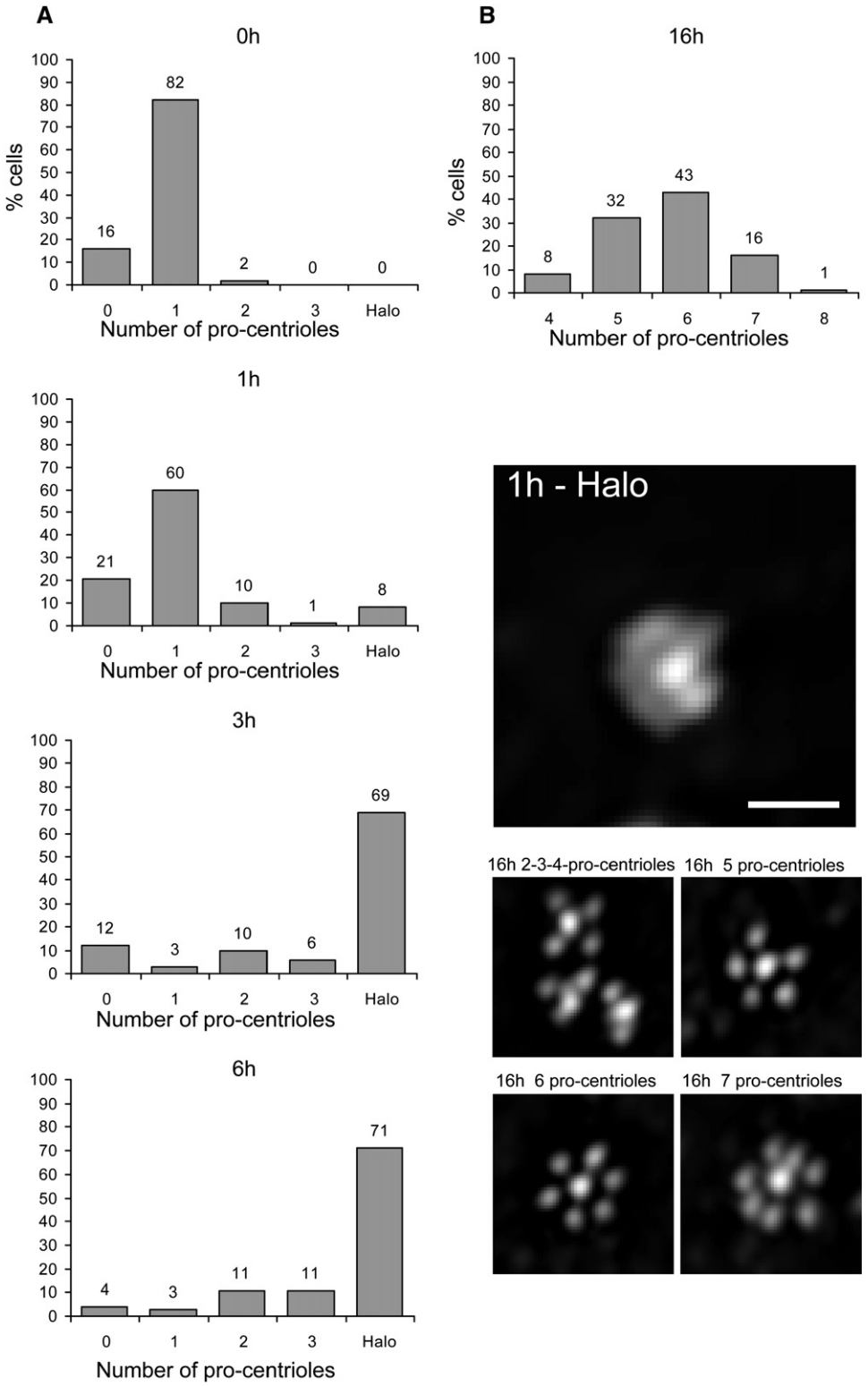


Figure 2. Multiple Procentrioles form Simultaneously in S Phase

(A and B) Cells were synchronized by aphidicolin treatment for 24 hr before Myc-Plk4 expression was induced for 1 hr, 3 hr, 6 hr (A), and 16 hr (B), and procentriole formation was visualized using anti-centrin 2 staining. Histograms in (A) and (B) summarize, for each indicated time point, the percentages of cells showing a halo surrounding each parental centriole or the indicated numbers of procentrioles, respectively. At each time point 100 cells were analyzed. Centrin stainings (B) are shown to illustrate the appearance of a typical halo (1 hr) as well as flower-like structures with two to seven

evidence to indicate that they are required for Plk4-induced centriole biogenesis.

Analysis of Centriole Biogenesis by Immunoelectron Microscopy

In a final series of experiments, immunoelectron microscopy was used to obtain more definitive insight into the localization of key proteins implicated in centriole biogenesis. As summarized in Figure 6, Plk4, hSas-6, CPAP, Cep135, CP110, and centrin 2 could be localized to distinct structures during early stages of procentriole assembly. Myc-tagged Plk4 could be seen on the outer wall of parental centrioles and at the interface between parental and nascent procentrioles (Figure 6A). A similar localization was also observed for endogenous hSas-6, although hSas-6 appeared to be associated more prominently with the nascent procentriole (Figure 6B and Figure S7). In contrast, CPAP and Cep135 were concentrated within the proximal lumen of both parental centrioles and procentrioles (Figures 6C and 6D). In particular, antibodies against Cep135 produced strong luminal staining within the proximal ends of centrioles as well as weaker staining along the centriolar surface (Figure 6D, right-hand panel). Such a staining pattern might be expected for a protein that forms part of a putative cartwheel structure (Anderson and Brenner, 1971; Cavalier-Smith, 1974). CP110 showed yet another, clearly distinct localization pattern. This protein was detected on the distal ends of both parental centrioles and nascent procentrioles (Figure 6E). Of particular interest, CP110 associated early with nascent procentrioles and then decorated the distal tips of all centrioles, regardless of their elongation state. This indicates that CP110 assembles into a cap-like structure early during procentriole formation and then remains on the distal end during centriole elongation, implying that α-/β-tubulin dimers are most likely inserted underneath a CP110 cap. Finally, centrin was seen within the lumen of both procentrioles and parental centrioles (Figure 6F), consistent with previous results (La Terra et al., 2005; Paoletti et al., 1996) and confirming that this protein constitutes a genuine marker for both nascent centrioles and mature centrioles.

DISCUSSION

Here we have shown that overexpression of Plk4 in human cells induces the near-simultaneous formation of multiple complete centrioles within a single S phase. Independently, *Drosophila* Plk4/Sak was reported to induce large numbers of centrioles even in a cell type (the unfertilized egg) that lacks a pre-existing centriole (Peel et al., 2007; Rodrigues-Martins et al., 2007). These studies thus identify Plk4 as a key regulator of centriole biogenesis and strengthen the notion that pre-existing centrioles represent "solid-state platforms" to facilitate centriole formation rather than genuine "templates" (Nigg, 2007).

Cell-Cycle Control of Plk4-Induced Flower-like Centriole Structures

Our analyses of synchronized cells revealed that Plk4 induced procentriole formation rapidly, provided that cells had reached a cell-cycle stage permissive for centriole formation. This requirement falls in line with previous studies indicating that centrosome duplication depends on traverse of G1/S, as reflected by phosphorylation of the retinoblastoma protein, activation of the E2F transcription factor, and activation of Cdk2 in a complex with cyclin E and/or A (Cowan and Hyman, 2006; Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). In response to Plk4 activation, nascent procentrioles initially grew off each parental centriole in an arrangement reminiscent of petals on a flower. Interestingly, these flower-like structures remained intact throughout S and G2 phase as well as most of M phase before they began to disassemble in late telophase, consistent with the proposal that centriole disengagement is triggered by Separase activity (Tsou and Stearns, 2006b). At present, there is no information on the dimensions of the first "seed" structures that form on the surface of parental centrioles. Thus, it is interesting that the cylinders of parental centrioles most frequently supported the formation of six procentrioles, most likely reflecting steric constraints imposed by the dimensions of nascent precursor structures and their vicinity to the "solid-state assembly platform.

Identification of Proteins Required for Centriole Biogenesis

To identify human centrosomal proteins required for centriole biogenesis, we carried out a siRNA-based phenotypic screen. This approach positively identified hSas-6, CPAP, CP110, Cep135, and γ-tubulin as indispensable for centriole formation. A requirement for hSas-6 and CPAP in centriole formation was expected in view of previous studies in invertebrates (Basto et al., 2006; Leidel et al., 2005; Leidel and Gonczy, 2003; Peel et al., 2007; Rodrigues-Martins et al., 2007). Likewise, γ-tubulin had previously been shown to be required for basal body formation in the ciliate *Paramecium* (Ruiz et al., 1999) and structural similarity has been noted between Cep135 and Bld10, a component of a putative cartwheel structure implicated in basal body formation in *Chlamydomonas* (Matsuura et al., 2004). In the case of CP110, no invertebrate or protozoan homolog has previously been described. However, human CP110 was originally identified as a Cdk2 substrate required for centrosome overduplication in S phase-arrested cells (Chen et al., 2002). So, to the extent that homologs of the various proteins studied here exist in invertebrates or protozoans, these are likely to play functionally analogous roles.

At first glance, it may appear surprising that depletion of both centrins known to be expressed in U2OS cells (centrins 2 and 3) did not detectably interfere with Plk4-induced

procentrioles (16 hr). Note that the flower-like structures harbouring two, three, and four centrioles were taken from a cell that fortuitously contained three parental centrioles. Scale bars denote 1 μM.

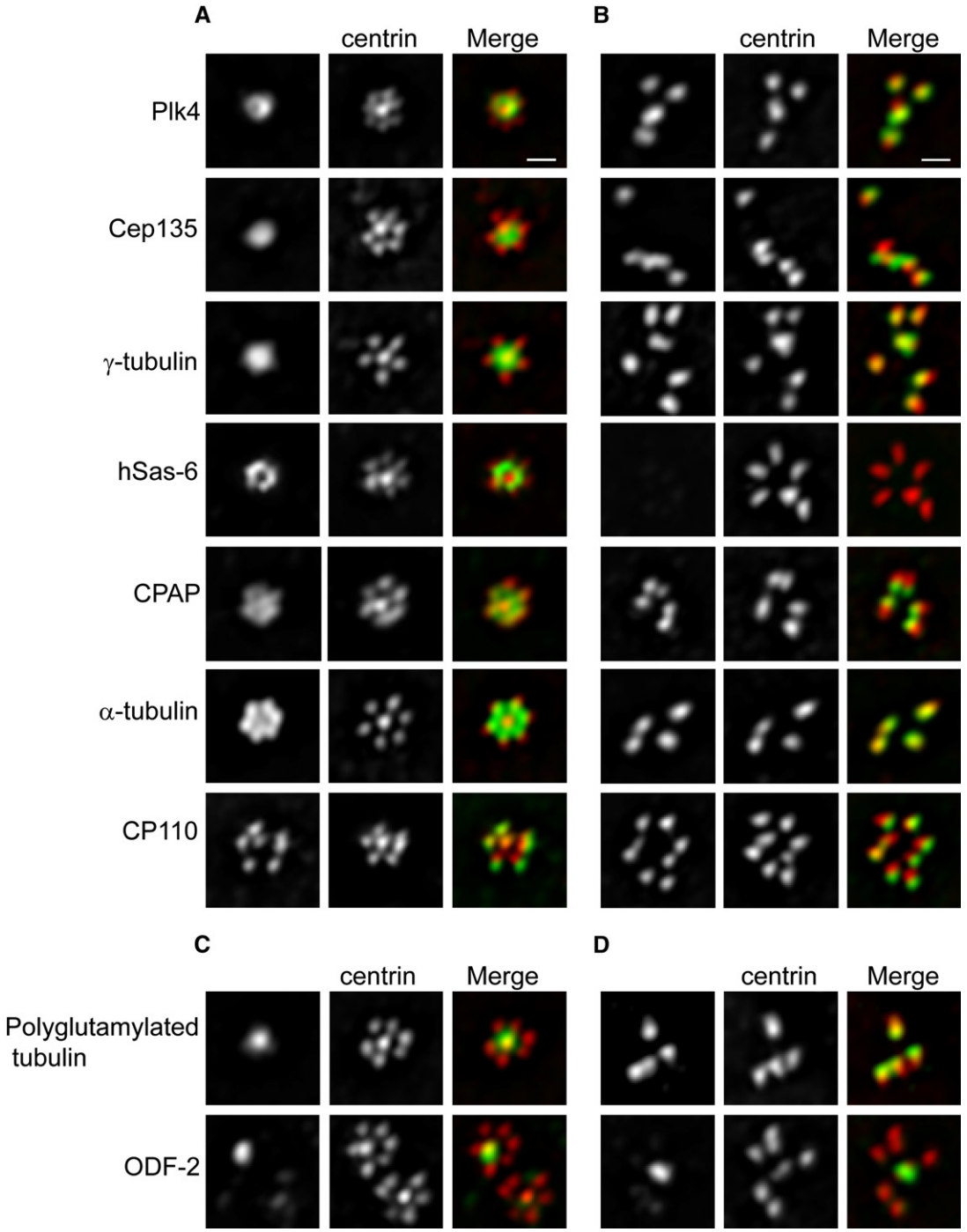


Figure 3. Localization of Proteins Identified as Essential for Centriole Biogenesis

(A–D) Assembly of multiple procentrioles was triggered by 16 hr Myc-Plk4-induction in U2OS cells. All cells were stained for centrin (red) to identify both parental centrioles and procentrioles and costained for the indicated proteins (green). Panels (A) and (C) show multiple procentrioles arranged in typical flower-like structures around parental centrioles (centers), whereas panels (B) and (D) show centriole clusters after disengagement. Scale bar denotes 1 μM.

centriole biogenesis. The yeast centrin homolog Cdc31p is clearly required for spindle pole body duplication in *Saccharomyces cerevisiae* (Paoletti et al., 2003; Spang et al., 1995), and a previous siRNA study had proposed an essential role for mammalian centrin 2 in centrosome duplication (Salisbury et al., 2002). As with all siRNA experiments, we cannot rigorously exclude that residual, albeit undetectable, centrin protein may have conferred some

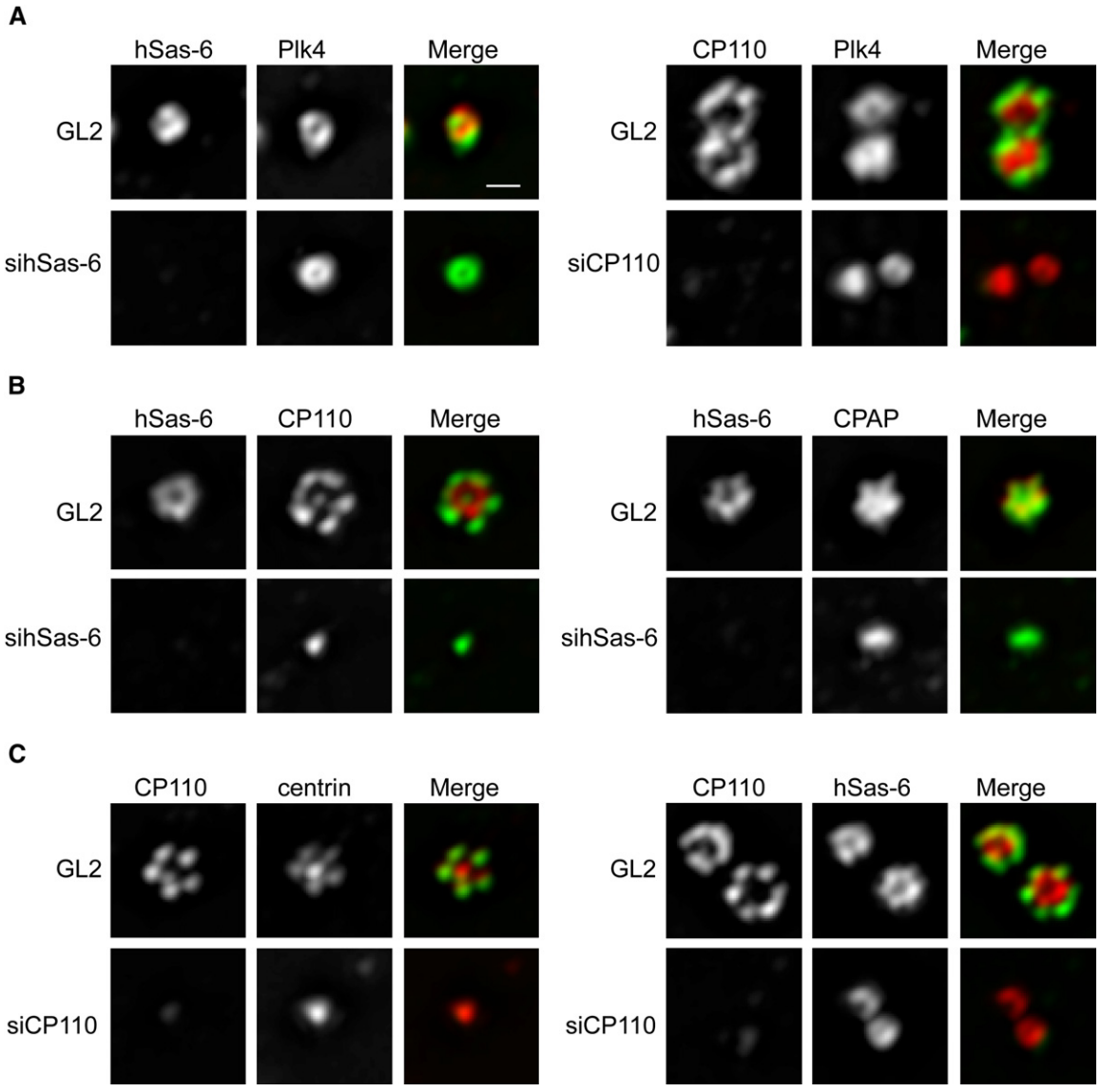


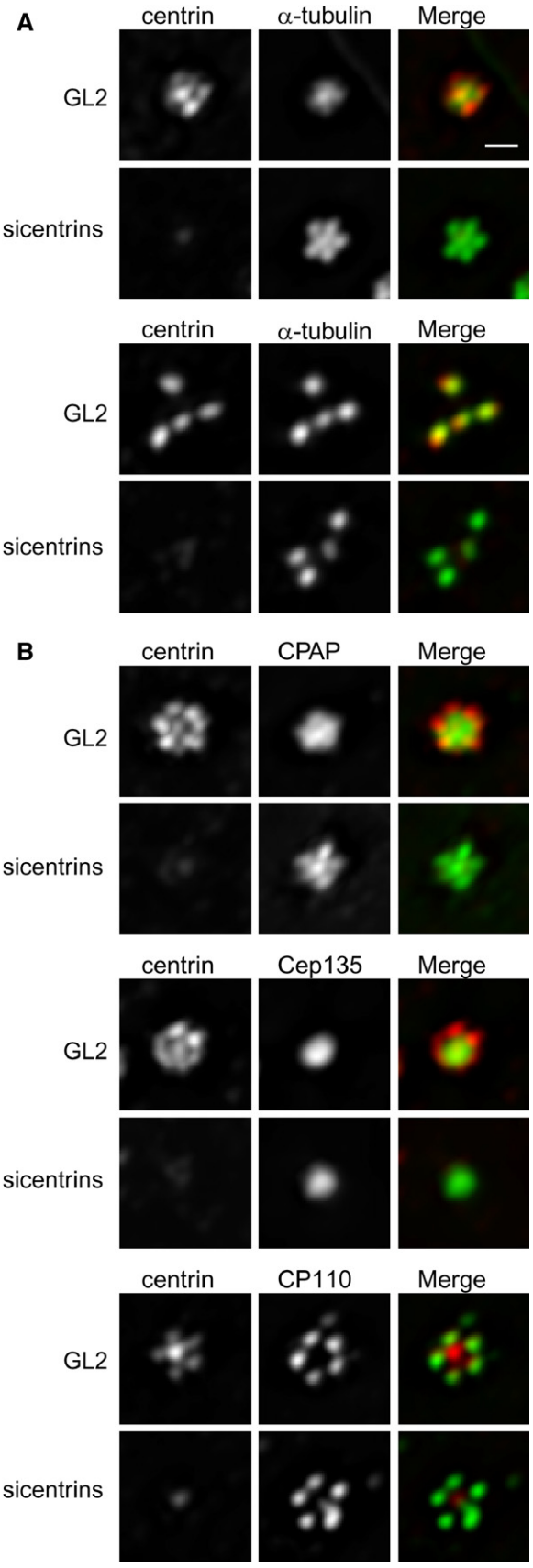
Figure 4. Delineation of a Centriole Assembly Pathway

(A–C) U2OS cells were transfected for 72 hr with siRNA duplexes targeting hSas-6 or CP110 or GL2 for control. Then, Myc-Plk4 was induced for 16 hr in the continued presence of siRNA duplexes, and cells were processed for immunofluorescence microscopy, using the antibodies indicated. (A), sihSas-6 or siCP110; (B), sihSas-6; (C), siCP110. Scale bar denotes 1 μM.

functionality in our experiments. However, we emphasize that there is presently no genetic evidence to support a role for centrin-related proteins in centriole duplication in *Drosophila* or *C. elegans* (Azimzadeh and Bornens, 2004), and studies in *Paramecium* indicate that centrins are required for basal body positioning rather than biogenesis (Ruiz et al., 2005).

Delineation of a Centriole Assembly Pathway in Human Cells

We have used siRNA approaches to establish mutual dependencies between individual proteins implicated in centriole biogenesis and, in parallel, studied their localization by both high resolution fluorescence and immunoelectron microscopy. The results of these studies afford a comprehensive view of the centriole assembly pathway, as summarized schematically in Figure 7. Following activation of Plk4 on the surface of the parental centriole cylinder, we observed the rapid recruitment of hSas-6, CPAP, Cep135, and γ-tubulin. Whether these proteins are recruited at exactly the same time could not be resolved. However, they are unlikely to form a single complex because hSas-6 was recruited exclusively to the nascent procentrioles, whereas CPAP and Cep135 could be seen within the proximal lumen of both parental and procentrioles (Figure 6) and similar intraluminal localization has also been described for γ-tubulin (Fuller et al., 1995). Whereas γ-tubulin is likely to nucleate centriolar



MTs, CPAP, and Cep135 probably play scaffolding roles in early centriole biogenesis. Once incorporated, these three proteins remained associated with centrioles. In contrast, hSas-6 was lost from centrioles, presumably in the course of centriole disengagement, either through displacement or degradation. Finally, the time of assembly and localization of CP110 indicates that centrioles do not grow by tubulin addition to distal tips, but rather by insertion of tubulin underneath a CP110-containing cap. Collectively, these findings strengthen the view that centriole and basal body formation are governed by an evolutionarily conserved mechanism (Delattre et al., 2006). However, some of the proteins described here do not have obvious homologs in invertebrates and, conversely, Sas-5 has so far been identified only in nematodes (Delattre et al., 2004). Thus, a better understanding of centriole biogenesis will undoubtedly benefit from the continued study of the underlying mechanism in multiple organisms.

Copy Number Control and Centriole Amplification in Tumor Cells

The Plk4-induced flower-structures described here emphasize that parental centrioles are competent to support the simultaneous formation of multiple centrioles, extending previous observations (Anderson and Brenner, 1971; Duensing et al., 2007; Vidwans et al., 2003). This clearly indicates that tight regulation of Plk4 activity is critical for controlling centriole numbers ("copy number control" [Nigg, 2007]) and raises the question of what mechanisms normally limit procentriole formation to one copy per preexisting centriole ? On the premise that parental centrioles constitute solid-state platforms to facilitate assembly rather than genuine "templates," one plausible scenario would be that Plk4 marks potential assembly sites on the parental centriole cylinder by phosphorylating yet-to-be-identified substrates. Plk4 activity is expected to be balanced by a counteracting phosphatase, and it will be crucial to determine whether mitogenic signaling and cell-cycle cues operate through activation of Plk4, inhibition of the antagonistic phosphatase, or both. Next, a protein (hSas-6 ?) or protein complex present in limiting amounts might be recruited to a site marked by Plk4, thereby forming a "seed" for a nascent procentriole. Under normal conditions, stabilization of a first seed (chosen at random) could constitute a rate-limiting step, whereas subsequent expansion of the nascent procentriolar structure would occur very rapidly, thereby consuming limiting material and preventing the utilization of secondary sites (akin to crystal growth). In response to excess Plk4 activity, however, multiple seeds can be stabilized simultaneously, leading to the concurrent formation of multiple

Figure 5. Formation of Multiple Centrioles in Centrin-Depleted Cells

(A and B) U2OS cells were treated as described in the legend to Figure 4, using siRNA duplexes targeting both centrin 2 and 3 or GL2 for control. Costainings were performed using the anti-centrin 2/3 reagent 20H5 and anti-α-tubulin (A) or the antibodies indicated (B). Scale bar denotes 1 μM.

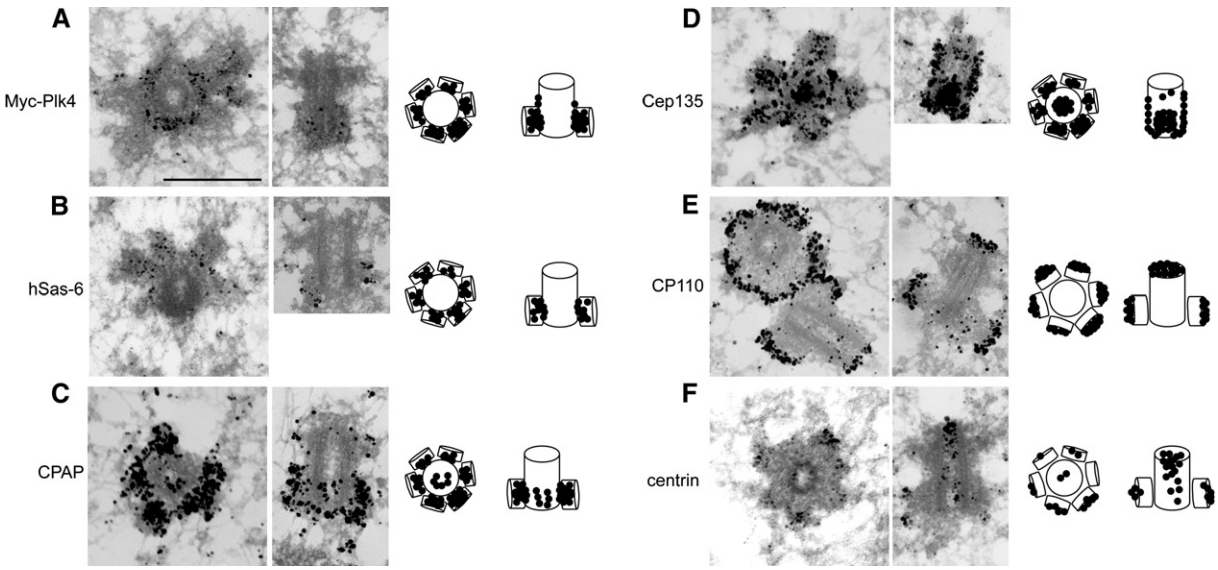


Figure 6. Analysis of Centriole Biogenesis by Immunoelectron Microscopy
(A–F) Myc-Plk4 was induced in U2OS cells for 16 hr before cells were processed for immunoelectron microscopy, using antibodies against the indicated proteins (B–F) and anti-Myc antibodies for visualization of Myc-Plk4 (A), followed by gold-labeled secondary α-rabbit or α-mouse antibodies. Schemes on the right indicate the localization of the individual proteins for clarity. Scale bar denotes 0.5 μm.

procentrioles, as described here. Interestingly, formation of multiple procentrioles can apparently occur also on parental centrioles that already harbor one procentriole (Figure 2). Live cell imaging will be required to determine whether the one pre-existing procentriole dissociates before the formation of multiple procentrioles or whether new procentrioles form next to the pre-existing one. In either case, the data show that excess Plk4 overrides an S phase control that normally limits procentriole formation to one per parental centriole (Nigg, 2007).

Simultaneous formation of multiple centrioles could represent one important mechanism for rapid centrosome

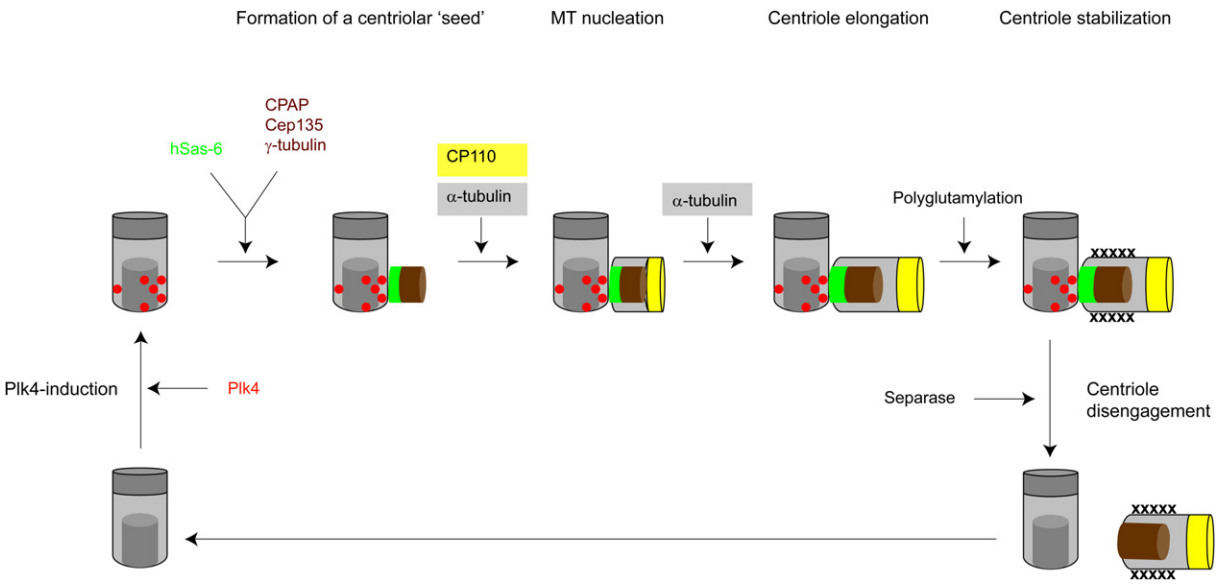


Figure 7. Model of Centriole Assembly in Human Cells
This scheme summarizes the salient features of the centriole assembly pathway that emerge from our siRNA and immunoelectron microscopy studies. Nascent procentriolar structures are depicted coding Plk4 in red; hSas-6 in green; CPAP, Cep135, and γ-tubulin in brown; α-tubulin in gray; and CP110 in yellow. Polyglutamylation is indicated by x. For simplicity the parental centriole is depicted in gray, polyglutamylation on the parental centriole is omitted, and only one nascent procentriole is shown. For detailed explanation see main text.

amplification in tumor cells (Duensing et al., 2007). Thus, it will be interesting to examine how frequently Plk4 and/or other positive regulators of centriole biogenesis are upregulated in tumors. At first glance it may seem paradoxical that Plk4$^{+/-}$ mice are prone to form tumors comprising supernumerary centrosomes (Ko et al., 2005), since reduced levels of Plk4 are known to impair rather than enhance centrosome formation (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). However, the centrosome amplification seen in Plk4$^{+/-}$ cells may be explained by the cell division failures that occur upon depletion of Plk4, possibly as a consequence of abnormal spindle formation (Habedanck et al., 2005). With the identification of Plk4 as a key regulator of centriole biogenesis, the stage is now set for studying its cell-cycle regulation in both normal cells and tumor cells. Moreover, a key challenge for the future will be to identify the physiological substrates of this kinase.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

Plasmids encoding CPAP (Hung et al., 2000) and hSas-6 (Leidel et al., 2005) were kindly provided by Dr. T. Tang (Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan) and P. Gönczy (Swiss Institute for Experimental Cancer Research, ISREC, CH-1066 Epalinges/Lausanne, Switzerland), respectively. Rabbit polyclonal antibodies were raised at Charles River laboratories (Elevages Scientifique des Dombes, Charles River Laboratories, Romans, France) against GST-Plk4 (aa 888-970), His-centrin 2, His-CPAP (aa 1071-1338), and His-Cep135 (aa 649-1140) and then purified according to standard protocols. A monoclonal mouse antibody of the IgG2a subtype was raised against hSas-6 (aa 404-657) and used as hybridoma supernatant. Rabbit anti-Cyclin A antibodies were described previously (Maridor et al., 1993), and antibodies against α-tubulin-FITC and γ-tubulin were purchased from Sigma (Taufkirchen, Germany). AlexaRed-555 and AlexaGreen-488 labeled secondary anti-mouse and anti-rabbit antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-centrin 20H5 and anti-CP110 antibodies were generously provided by Drs. J. Salisbury (Mayo Clinic Foundation, Rochester) and B. Dynlacht (New York University School of Medicine, New York), respectively. To simultaneously visualize Cep135 and CPAP or CP110, α-Cep135 antibodies were covalently coupled to the fluorophore AlexaRed-555, using the Alexa Fluor 555 Antibody Labeling Kit (Invitrogen).

Cell Culture and Transfections

The tetracyclin-inducible cell-line expressing Myc-tagged Plk4 was generated by transfection of U2OS-Trex cells (Invitrogen). Stable transformants were established by selection for 2 weeks with 1 mg ml^{-1} G418 (Invitrogen) and 50 μg ml^{-1} hygromycin (Merck, Darmstadt, Germany). U2OS cells were cultured as described previously (Habedanck et al., 2005), and Myc-Plk4 expression was induced by the addition of 1 μg ml^{-1} of tetracyclin.

siRNA-Mediated Protein Depletion

Centrosomal proteins were depleted using siRNA duplex oligonucleotides (see Table S1), using the luciferase duplex GL2 for control (Elbashir et al., 2001). Transfections were performed using Oligofectamin (Life Technologies, Karlsruhe, Germany). Because hSas-6 is absent in G1 cells, all cells were synchronized by the addition of aphidicolin (1.6 μg ml^{-1}) during the last 24 hr of siRNA treatment before Myc-Plk4 was induced for 16 hr in the continued presence of aphidicolin. We emphasize that rigorous interpretation of hSas-6 results required aphidicolin addition, but in all other instances results were indistinguishable when aphidicolin was omitted.

Microscopic Techniques

To maximize visualization of centrioles, cytoplasmic MTs were depolymerised by a 1 hr cold treatment (4°C) before cells were permeabilized and fixed by incubation for 30 s in PBS, 0.5% Triton X-100, followed by 10 min methanol (−20°C). Antibody incubations and washings were performed as described previously (Meraldi et al., 1999). Slides were analyzed using a Deltavision microscope on a Nikon TE200 base (Applied Precision, Issaquah, WA) equipped with an APOPLAN x100/1.4 n.a. oil-immersion objective. Serial optical sections obtained 0.2 μm apart along the Z axis were processed using a deconvolution algorithm and projected into one picture using Softworx (Applied Precision). For electron microscopy, cells were grown on cover slips, fixed with 4% Paraformaldehyd for 10 min, and permeabilized with PBS+0.5% Triton X-100 for 2 min. Blocking in PBS+2% BSA was performed for 30 min, primary antibody incubations were performed for 60 min and followed by incubation with goat-anti-mouse/rabbit IgG-Nanogold (1:50 Nanoprobes) for 50 min. Nanogold was silver enhanced with HQ Silver (Nanoprobes). Cells were further processed as described previously (Fry et al., 1998).

Supplemental Data

Supplemental Data include seven figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/13/2/190/DC1/.

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