Par6γ is at the mother centriole and controls centrosomal protein composition through a Par6α-dependent pathway

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Abbreviations: Par6: par-6 partitioning defective 6 homolog; PCM: pericentriolar material
Summary

The centrosome contains two centrioles that differ in age, protein composition and function. This non-membrane bound organelle is known to regulate microtubule organization in dividing cells and ciliogenesis in quiescent cells. These specific roles depend on protein appendages at the older, or mother, centriole. In this study, we present the identification of the polarity protein Par6γ as a novel component of the mother centriole. This specific localization required the Par6γ C-terminus, but was independent of intact microtubules, the dynein/dynactin complex and the components of the PAR polarity complex. Par6γ depletion resulted in altered centrosomal protein composition, with the loss of multiple proteins, including Par6α and p150Glued, from the centrosome. As a consequence, there were defects in ciliogenesis, microtubule organization and centrosome reorientation during migration. Par6γ interacted with Par3 or aPKC, but these proteins were not required for the regulation of centrosomal protein composition. Par6γ also associated with Par6α, which controls protein recruitment to the centrosome through p150Glued. Our study is the first to identify Par6γ as a component of the mother centriole and to report a role of a mother centriole protein in the regulation of centrosomal protein composition.
Introduction

As the major microtubule organizing center, the centrosome regulates a large number of critical cellular functions. During interphase, this non-membrane bound organelle arranges the radial array of microtubules that is important for cell shape, organelle positioning and protein transport. During mitosis, it contributes to the formation of spindle poles that control the accurate segregation of DNA into the two daughter cells. In cells that have exited the cell cycle, centrosomes form the base of primary and motile cilia, which are critical for signal transduction and the movement of cells, particles and cellular fluids (Cuschieri et al., 2007; Nigg and Stearns, 2011).

Centrosome organization and protein composition undergo dynamic change during the cell cycle. Soon after cell division, this complex organelle is composed of two barrel-shaped centrioles that are embedded in an electron-dense mass, the pericentriolar material (PCM). As cells progress through the cell cycle, the centrosome is actively remodelled with centrioles being duplicated and centriolar protein composition being modified through protein recruitment and removal (Khodjakov and Rieder, 1999; Casenghi et al., 2005). While a mass spectrometry analysis of purified interphase centrosomes has identified about 100 centrosomal proteins, database mining is suggesting the presence of as many as 383 proteins at the centrosome (Andersen et al., 2003; Nogales-Cadenas et al., 2009; http://centrosome.dacya.ucm.es). However, interactions between centrosomal proteins during specific stages of the cell cycle are only beginning to being understood (Fu and Glover, 2012).

Semi-conservative centrosome duplication in S-phase produces centrioles that differ in age, protein composition and function (Chretien et al., 1997; Bornens and Piel, 2002; Nigg and Stearns, 2011). During this process, a pre-existing centriole serves as a platform for the formation of a procentriole, which then elongates and matures (Strnad and Gonczy, 2008). As a consequence, one centriole of a pair is older by at least one cell cycle than its counterpart. This older centriole is decorated with functionally important protein appendages (Bornens, 2002; Bornens and Piel, 2002). Subdistal appendages recruit proteins, such as Cep170 and ninein, which control the anchoring of interphase microtubules (Guarguaglini et al., 2005; Lin et al., 2006). Distal appendages, in contrast, contain Cep164, which is proposed to play a role in the docking of the mother centriole with the plasma membrane and is required for ciliogenesis (Graser et al., 2007). Recently, additional mother centriole proteins have
been identified (Jakobsen et al., 2011), but the overall number of appendage-
associated proteins is not known.

The recruitment of proteins to the centrosome is poorly understood. Proteins, such as centrin and ninein, are delivered to the centrosome along microtubules by a dynein/dynactin-dependent process (Dammermann and Merdes, 2002). In contrast, AKAP450 and pericentrin contain localization domains that target these proteins to the centrosome in a microtubule-independent manner (Gillingham and Munro, 2000).

We have previously shown that the polarity protein Par6α controls centrosomal protein delivery (Kodani et al., 2010). Loss of Par6α affected microtubule-dependent and -independent centrosomal protein delivery alike, but the mechanism behind this process remains to be determined.

The PAR polarity complex is a known regulator of cell polarity. It is composed of three proteins, Par6, Par3 and aPkc and was first identified in a *C. elegans* screen for defects in cell polarity (Kemphues et al., 1988). This complex is assembled in response to activated Cdc42 and controls cell polarity, cytoskeletal rearrangement and tight junction assembly (Lin et al., 2000; Gao and Macara, 2004; Etienne-Manneville et al., 2005). Homologs of each PAR protein have been identified in other species, with a single version of each PAR complex component in *C. elegans* and *Drosophila*, but several isoforms in mammalian cells. For example, mammalian cells contain two Par3, two aPkc and three Par6 isoforms (Joberty et al., 2000; Noda et al., 2001).

The roles of the three mammalian Par6 proteins, which in humans are called Par6α, Par6β and Par6γ, have not been fully investigated. Several studies using dominant negative approaches support a general role for Par6 proteins in cell polarity, but have failed to distinguish between individual isoforms (Etienne-Manneville and Hall, 2003; Georgiou et al., 2008). However, Gao and Macara have examined the function of each individual Par6 isoform by expressing HA-tagged forms of mouse Par6A (the likely homolog of human Par6γ) and Par6B (the likely homolog of human Par6β) and human Par6C (which is Par6α) in polarized MDCK cells (Gao and Macara, 2004). While mouse Par6A associated with tight junctions, mouse Par6B was in the cytosol, and human Par6C at sites of cell-cell contact. In addition, there were differences in the requirement for these individual proteins in tight junction formation and in interactions with Pals1. We and others have used isoform-specific antibodies
and have detected Par6α at the centrosome of human epithelial cells and mouse neurons (Solecki et al., 2004; Kodani et al., 2010). In both systems, Par6α was essential for the regulation of centrosome organization and function.

The Par6 binding partners Par3 and aPkc have been linked to the centrosome through effects on ciliogenesis. Both Par3 and aPkc were found to stain the axoneme of primary cilia in kidney epithelia, colocalizing with acetylated tubulin (Fan et al., 2004). These proteins were also required for ciliogenesis in polarized MDCK cells and sea urchin, but their specific contributions to this process are not yet known (Sfakianos et al., 2007; Pruliere et al., 2011). Similar to Par6 proteins, functional differences between the isoforms of mammalian Par3 and aPkc in ciliogenesis have not been addressed.

In this study, we demonstrate that the Par6 protein Par6γ is an important regulator of centrosome organization and function. We establish the mechanism by which Par6γ associates with the mother centriole and describe a molecular pathway by which this polarity protein may control the centrosome.
Results

Par6γ is a novel component of the mother centriole centrosome

Our finding that Par6α is a component and regulator of the centrosome (Kodani et al., 2010) prompted us to examine the localization and function of the other human Par6 proteins, Par6β and Par6γ (Fig. 1A). Sequence alignment of the three proteins revealed a high degree of conservation between their N-termini, with 65% identity between Par6α and either Par6β or Par6γ and 71% between Par6β and Par6γ (Fig. 1B, Supplementary material Table S1). Their C-termini were less conserved, with only 25% identity between Par6β and either Par6α or Par6γ and 30% between Par6α and Par6γ (Fig. 1B, Supplementary material Table S1).

To understand the functional differences between these three Par6 family members, we took advantage of isoform-specific antibodies raised against their divergent C-termini. We detected Par6α at the centrosome and centrosomal satellites, which is consistent with our previous results (Kodani et al., 2010). Par6β localized to the nucleus, and this staining was specific as it was lost upon siRNA-mediated depletion of the protein (Supplementary material Fig. S1A,B). Intriguingly, Par6γ associated with the centrosome, where it colocalized with γ-tubulin at one of the two centrosomal foci (Fig. 2A). This staining for Par6γ was no longer present when we depleted Par6γ with either of two independent siRNAs in HeLa cells, indicating that this signal was specific (Fig. 2A,B). A similar staining was detected in the two human cell lines hTERT-RPE-1 and U2-OS, further confirming the centrosomal localization of Par6γ and the specificity of our antibody (Figs. 2C,S1C). Our results establish Par6γ as a component of the centrosome and suggest that a second member of the Par6 protein family may have a role at this important organelle. In addition, they indicate that there may be prominent functional differences between the three Par6 proteins in mammalian cells.

We next determined the precise localization of Par6γ within the centrosome. Co-staining with antibodies to centrin2, which is present at both centrioles (Salisbury et al., 2002), confirmed our earlier observation that Par6γ only stains one centriole of an interphase centrosome (Fig. 2A,D). The Par6γ-positive centriole corresponded to the mother centriole because it stained for Cep164 and ninein, which are known components of the distal and subdistal mother centriole appendages, respectively. There was extensive colocalization of Par6γ with ninein and another subdistal protein Cep170 (Figs. 2D,4A), but only partial overlap with Cep164 and HsSAS-6, which...
Par6γ associates with the proximal centriole end (Fig. 2D). While immunogold electron microscopy would be the best method to determine the exact localization of Par6γ at the mother centriole, our results indicate that Par6γ may associate with subdistal appendages. In summary, our results demonstrate that Par6γ is a novel component of the mother centriole, which is consistent with its localization to the basal body during ciliogenesis in hTERT-RPE-1 cells (Fig. 2E).

Par6γ associates with the mother centriole via its C-terminus

The sequence differences between the C-termini of the three Par6 proteins led us to hypothesize that the centrosomal localization signal for Par6γ is contained within its C-terminus. To test this hypothesis, we generated full length and deletion constructs of Par6γ with an N-terminal GFP tag and expressed them in HeLa cells (Fig. 3A). GFP was detected at one centriole of the interphase centrosome when we expressed full length Par6γ, demonstrating that exogenously-expressed full length Par6γ reproduces the mother centriole-specific localization of the endogenous protein (Fig. 3B,C). While a GFP-tagged form of the Par6γ C-terminus (amino acids 259 to 376) localized to the centrosome, GFP-tagged N-terminus (amino acids 1 to 258) or smaller C-terminal fragments (Fig. 3A-C) did not and were instead found in the cytosol. These results indicate that the entire Par6γ C-terminus is necessary and sufficient to target this protein to the mother centriole.

Par6γ is essential for cell growth

We next determined the role of Par6γ in cell homeostasis. Par6γ depletion from HeLa cells for 24, 48 and 72 hours resulted in a time-dependent decrease in cell viability, as assessed by trypan blue staining (Supplementary material Fig. S2A). This effect was not seen in parallel untreated or control siRNA-treated cells. In addition, the frequency of cells with fragmented or deformed nuclei, as visualized by staining with the DNA dye Hoechst 33342, increased over time, with 82 ± 8% of cells exhibiting abnormal nuclei at 72 hours after knock-down (Supplementary material Fig. S2B,C). The same analysis was carried out with the second Par6γ-specific siRNA (siPar6γ#2) and we obtained the same results (unpublished data). These results show that Par6γ is critical for cell growth and normal morphology of nuclei. As Par6γ was efficiently depleted at 24 hours post silencing without affecting cell division and
nuclear morphology, we conducted all subsequent experiments at this early time point unless specified in the text.

**Par6γ controls the protein composition of the centrosome**

The prominent association of Par6γ with the mother centriole led us to investigate whether this protein has a role at the centrosome. We first examined centrosomal protein composition by comparing the levels of various centrosomal proteins in control and Par6γ-depleted cells. The levels of γ-tubulin, GCP2 or GCP-WD, which are all components of the γ-TuRC complex (Fant et al., 2009), were similar in control and Par6γ-depleted cells (Fig. 4A,B). In contrast, the levels of proteins of the centriole (HsSAS-6, STIL, Cep192, Cep152, CPAP and centrin2) and mother centriole appendages (Cep164, Cep170, ninein and Odf2) were dramatically decreased in the absence of Par6γ, when compared to control cells (Fig. 4 A,B). We also observed a significant reduction in the levels of centriolar satellite proteins, including PCM-1, Par6α, BBS4 and Cep290 (Fig. 4B). Thus, Par6γ plays an important role in the recruitment and maintenance of proteins at the centrosome.

**Par6γ controls the function of the centrosome**

We performed four assays to determine whether the altered centrosome in Par6γ-depleted cells is functional. First, we examined ciliogenesis, which depends on some of the centrosomal proteins that are mislocalized in the absence of Par6γ. While about 80% of control or control siRNA-transfected hTERT-RPE-1 cells contained primary cilia upon serum starvation, primary cilia were only detected in about 10% in Par6γ-depleted cells, indicating that Par6γ is required for ciliogenesis (Fig. 5A). Second, we monitored the organization of the microtubule cytoskeleton. Control cells displayed a radial array of microtubules that extended towards the cell periphery, but 77% of Par6γ-depleted cells contained randomly organized microtubules (Fig. 5B). Similar microtubule organization defects were observed when we overexpressed the C-terminal Par6γ localization domain (Fig. 5C). Under these conditions, ninein, a regulator of microtubule anchoring, was lost from the centrosome (unpublished data). These findings suggest that Par6γ is necessary for the normal organization of interphase microtubules. They also indicate that the C-terminal domain of Par6γ acts as a dominant negative, producing similar functional defects as Par6γ knockdown. As a third measure of centrosome function, we examined spindle organization and DNA
alignment during mitosis. 24 hours after the siRNA transfection, which is the time
point when we first detected the mislocalization of proteins from the interphase
centrosome, mitotic spindles of control and Par6γ-depleted cells were similar.
However, 24 hours later, we detected obvious defects in spindle formation, with 83%
of mitotic Par6γ-depleted cells displaying aberrant multipolar spindles. Consistent
with this observation, there was an increase in the percentage of mitotic cells from 3%
in control cells to 12% in Par6γ-depleted cells, suggesting that Par6γ-depleted cells
were arrested in mitosis (Fig. 5D). Finally, we examined cell motility, which requires
the reorientation of the centrosome towards the leading edge (Vaughan and Dawe,
2011). Cell motility was measured in a scratch wound, which we introduced into a
monolayer of control or Par6γ-depleted hTERT-RPE-1 cells. Monitoring the time-
dependent movement of cells into the wound area, we found that control cells covered
the wound area completely after 20 hours, whereas Par6γ-depleted cells were unable
to fill in the wound area (Fig. 5E). In addition, these cells were unable to reorient their
centrosome towards the wound edge as seen in the majority of control cells (Fig. 5F).
Altogether, these results indicate that the centrosome of Par6γ-depleted cells, which
lacks many important centrosomal proteins, is non-functional.

**Par6γ-dependent centrosome regulation does not involve the PAR polarity
complex**

As Par6 is a known component of the PAR polarity complex (Assemat et al.,
2008), we investigated the interaction pattern between the Par6, Par3 and aPkc
isoforms in our cells (Fig. 6A). Reciprocal co-immunoprecipitation experiments
revealed that Par6γ associated specifically with Par6α, but not with Par6β (Fig. 6B,C).
In addition, Par6α and Par6γ interacted with Par3α and with the two aPkc isoforms
Pkcι and Pkcζ (Fig. 6B,C). In order to determine the significance of these interactions
for centrosome regulation, we depleted Par3α and the two aPKCs from HeLa cells
and monitored effects on centrosomal protein composition. Interestingly, removal of
Par3α, Pkcι and Pkcζ by RNAi had no effect on the levels of any centrosomal
proteins, including centrosomal Par6α and Par6γ (Fig. 6D, Supplementary material
Fig. S3). This result, together with the finding that Par3 and aPKC isoforms are absent
from the interphase centrosome (unpublished data) suggests that Par6γ- and Par6α-
mediated centrosome regulation does not involve the PAR complex.
Par6γ controls the centrosomal localization of Par6α and p150<sup>Glued</sup>

The association between Par6γ and Par6α led us to examine whether Par6γ associates with the centrosome by a similar microtubule-dependent mechanism as Par6α (Kodani et al., 2010). Depolymerization of microtubules with nocodazole did not alter the centrosomal association of Par6γ (Fig. 7A). In contrast, Par6α, Cep164 and ninein, which reach the centrosome by dynein/dynactin-mediated transport along microtubules, were mislocalized from the centrosome under these conditions (unpublished data; Delgehyr et al., 2005; Graser et al., 2007; Kodani et al., 2010). We also disrupted the function of the dynactin complex by overexpressing the p150<sup>Glued</sup> and p50 subunits of dynactin (Quintyne et al., 1999), but did not detect any effects on Par6γ localization (Fig. 7B). We conclude that the centrosomal localization of Par6γ is independent of dynein/dynactin-dependent transport along microtubules.

We next tested whether Par6γ and Par6α depend on each other for their association with the centrosome. While loss of Par6α had no effect on Par6γ localization, the depletion of Par6γ or overexpression of the Par6γ C-terminus resulted in the mislocalization of Par6α from the centrosome (Figs. 6D,7C,D). Intriguingly, p150<sup>Glued</sup> was also lost from the centrosome in the absence of Par6γ (Fig. 7E), suggesting that Par6γ regulates the centrosomal localization of Par6α and p150<sup>Glued</sup>, which have both been implicated in the regulation of centrosomal protein delivery.

Altogether, our results support a model in which Par6γ controls the protein composition of the interphase centrosome by regulating the association of Par6α and p150<sup>Glued</sup> with the centrosome and centriolar satellites. Mislocalization of these important centrosome regulators results in similar alterations of centrosome organization and function as seen with the loss of Par6γ.
Discussion

In this study, we describe a novel function for the polarity protein Par6γ at the centrosome of epithelial cells. This poorly studied Par6 family member associated with the mother centriole, and was absent from the daughter centriole. Co-localization experiments with specific mother centriole markers suggested a possible association of Par6γ with subdistal appendages. The mother centriole localization of Par6γ required the C-terminus of this protein, but was independent of intact microtubules, the dynein/dynactin motor complex and Par6α. Depletion of Par6γ resulted in the mislocalization of a wide range of centrosomal proteins, including proteins of the centriole and of centriolar satellites. Centrosome-associated Par6α and p150Glued, which are known regulators of centrosomal protein composition, were also lost from the centrosome in the absence of Par6γ. As a consequence, there were defects in ciliogenesis, microtubule organization and cell migration, which are processes that require an intact centrosome. These results lead us to propose a model in which Par6γ controls centrosomal protein composition by regulating the localization of Par6α to the centrosome.

Four experimental findings support a role for Par6γ upstream of Par6α in the regulation of the centrosome. First, Par6γ dictates the centrosomal association of Par6α because Par6α was mislocalized in the absence of Par6γ or when we overexpressed the C-terminus of Par6γ. In contrast, Par6γ was unaffected by the loss of Par6α. Second, depletion of Par6γ produced a similar selective effect on centrosomal protein composition as the loss of Par6α (Kodani et al., 2010). For example, proteins such as centrin2, CPAP and PCM-1, were mislocalized from the centrosome, whereas the centrosomal association of γ-TuRC components was unaffected. Interestingly, Par6γ but not Par6α, was required for the centrosomal association of HsSAS-6 and Cep192, suggesting that Par6γ may control these specific factors by a Par6α-independent pathway. Third, there were similar effects on centrosome functions in the absence of these two polarity proteins, with defects in microtubule organization in interphase and mitosis, and in cell migration. We did not measure centriole duplication because markers commonly used to assess centriole number were mislocalized in the absence of Par6γ. Finally, Par6γ reached the centrosome in the absence of intact microtubules, which is in contrast to the microtubule-dependent delivery of Par6α.
The regulatory effect of Par6γ on Par6α localization may involve the interaction between these two proteins. We have observed a physical interaction between Par6γ and Par6α in our co-immunoprecipitation reactions, but it is not clear whether this association is direct or involves additional binding partners. Furthermore, it is unclear where inside a cell this interaction occurs as each of these proteins associates with physically distinct centrosomal structures: Par6γ is at the mother centriole and Par6α is predominantly found at centriolar satellites (Kodani et al., 2010). However, our immunofluorescence data do not exclude a mother centriole-associated pool of Par6α that can bind Par6γ. Alternatively, an association between these two Par6 proteins in the cytosol is conceivable.

Par6γ is a novel component of the mother centriole. This localization is specific because we no longer detected Par6γ at the mother centriole when we depleted the protein with two independent siRNAs. In addition, a GFP-tagged form of this protein labelled the centrosome. The Par6γ C-terminus, which is necessary and sufficient for targeting Par6γ to the mother centriole, shares little homology with the C-termini of other Par6 isoforms and is devoid of conserved sequence features (Fig. 1A; Joberty et al., 2000). It also does not contain any known centrosomal targeting motifs, such as the PACT domain, which is present in AKAP450 and pericentrin (Gillingham and Munro, 2000), or sequence homology with other mother centriole-specific protein, such as ninein or Cep170 (Guarguaglini et al., 2005; Lin et al., 2006; Huber et al., 2008). Thus, the Par6γ C-terminus appears to contain a novel mother centriole-specific sorting signal. Overexpression of this domain of 117 amino acids produced dominant negative effects, suggesting that Par6γ may bind to a receptor on the mother centriole. Future studies will focus on the identification of such a receptor and on determining the protein interaction network at the centriole.

Our data on Par6γ localization differs from a published study. Gao and Macara took the approach of overexpressing a tagged form of mouse Par6A (the likely homolog of human Par6γ) or a tagged human form of Par6C (Par6α) in MDCK cells and detected these proteins at sites of cell-cell contact (Joberty et al., 2000; Gao and Macara, 2004). Discrepancies with our data are likely due to the overexpression of tagged proteins, the use of different cell lines and the difficulty of simultaneously visualizing tight junctions and the centrosome. While we have observed the specific localization of Par6γ at the mother centriole, we cannot exclude the existence of a cytosolic or membrane-bound pool of this protein.
Protein recruitment to the centrosome remains poorly understood. It is well accepted that the centrosome contains at least 100 proteins (Andersen et al., 2003), but a global mechanism for the assembly of this complex organelle is unknown. There is microtubule-dependent and independent transport to the centrosome, but it is not known how individual cargo is selected (Dammermann and Merdes, 2002; Delgehyr et al., 2005; Graser et al., 2007). Interestingly, even proteins of the same centrosomal substructure are delivered to the centrosome by different mechanisms. For instance, ninein, Cep170, Odf2 and Par6γ are all components of the mother centriole, but ninein requires microtubules to reach the mother centriole (Dammermann and Merdes, 2002; Delgehyr et al., 2005; Graser et al., 2007), while the other factors do not (Guarguaglini et al., 2005; Huber et al., 2008). Similarly, there is no common delivery mechanism for related proteins because Par6α, but not Par6γ, is transported to the centrosome along microtubules. Several centrosomal localization domains have been reported, but a receptor for these signals has not been identified (Momotani et al., 2008; Ferguson et al., 2010; Wang et al., 2010). As loss of Par6γ or Par6α affects the centrosomal recruitment of a large number of proteins, our findings support an important role for these polarity proteins as general regulators of centrosomal protein composition.

It is not known how Par6γ controls centrosomal protein recruitment from its mother centriole position. Other appendage proteins have been identified (Ishikawa et al., 2005; Guarguaglini et al., 2005; Guo et al., 2006; Graser et al., 2007; Jakobsen et al., 2011), but only a few have been linked to specific cellular functions. For example, Cep164 at the distal appendages is proposed to mediate the docking of the mother centriole with the plasma membrane during ciliogenesis, and ninein at subdistal appendages has been implicated in microtubule anchoring (Graser et al., 2007; Singla et al., 2010). We provide support for a role of a mother centriole protein in the control of centrosomal protein recruitment, but the mechanism behind this process is not clear. It is conceivable that this regulatory activity involves effects on the formation or stability of mother centriole appendages. However, this possibility is rather unlikely because loss of appendages, as observed in Odf2-depleted cells, does not affect microtubule organization and cell cycle progression (Ishikawa et al., 2005). Alternatively, Par6γ may contribute to the maintenance of centriolar satellites, which have been implicated in centrosomal protein delivery (Dammermann and Merdes,
2002; Lopes et al., 2011). Future studies will investigate the mechanism by which a mother centriole protein controls the assembly of the entire centrosome.

Our studies reveal a novel mechanism for the function of Par6 proteins. Par6 proteins were generally found to function in concert with other components of the PAR polarity complex, but our data support a PAR complex-independent role for Par6 proteins in centrosome regulation. Par3α or either of the two aPkc was not required for the control of the centrosome because protein composition and function of this organelle were normal in their absence. In addition, these PAR complex components were not detected at the centrosome, suggesting that Par6 proteins do not depend on their normal binding partners to carry out this specific role at the centrosome. It is likely that Par6γ associates with Par3 and aPkc into the PAR complex at other cellular locations and to control other cellular functions. Interestingly, Par3 and aPkc have been detected at primary cilia, but their specific roles are not known (Sfakianos et al., 2007; Pruliere et al., 2011). Further studies are also required to examine whether the binding of Cdc42 to Par6α or Par6γ is important for Par6-mediated regulation of the centrosome.

In summary, our study has identified a novel role for the polarity protein Par6γ in the assembly of the centrosome. This function is likely to involve the interaction of Par6γ with Par6α, but not with Par3α or aPkcα. Our study describes three important and novel observations. First, it identifies Par6γ as a novel component of the mother centriole. Second, it demonstrates that Par6 proteins can function independently of the PAR polarity complex, controlling processes that are not directly related to cell polarity. Finally, it provides evidence for a role of a mother centriole protein in the recruitment of centrosomal proteins, thereby defining a novel function for the mother centriole.
Materials and methods

Antibodies

Antibodies used in this study and their sources: BBS4 (immunofluorescence [IF]: 1/250), gift from Dr. M. Nachury, Stanford University, CA, USA; centrin2 (IF: 1/8000), a gift from Dr. J. Salisbury, Mayo Clinic, MN, USA; Cep152 (IF: 1/1000), Bethyl Laboratories, Inc.; Cep164 (IF: 1/500), Santa Cruz Biotechnology, Inc.; Cep170 (IF: 1/250), a gift from Dr. G. Guarguaglini, University of Rome, Italy; Cep192 (IF: 1/2000), a gift from Dr. L. Pelletier, University of Toronto, Canada; Cep290 (IF: 1/500), gift from Dr. H. Khanna, University of Massachusetts, MA, USA; HsSAS-6 (IF: 1/250) and CPAP (IF: 1/500), both gifts from Dr. P. Gönczy, EPFL Lausanne, Switzerland; GCP2 (IF: 1/500) and GCP-WD (IF: 1/500), both gifts from Dr. T. Stearns, Stanford University, CA, USA; HA (IF: 1/500), Covance; myc (IF: 1/1000), Calbiochem; ninein (IF: 1/2000) and STIL (IF: 1/1000), both from AbCam; Odf2 (IF: 1/500), Sigma-Aldrich; p150Glued (IF: 1/250), BD Biosciences; Par3α (IF: 1/250; western blot [WB]: 1/250) and Par3β (IF:1/100; WB: 1/250), Sigma-Aldrich; Par6α (IF: 1/100, WB: 1/250), a gift from Dr. R. Lämmers, Medical Clinic IV, Tübingen, Germany; Par6β (IF: 1/500; WB: 1/250), Santa Cruz Biotechnology, Inc.; Par6γ (IF: 1/1000), Sigma-Aldrich; α-tubulin (IF: 1/8000; WB, 1/50000) and γ-tubulin (IF, 1/1000), all from Sigma-Aldrich; PCM-1 (IF: 1/1000), Santa Cruz Biotechnology, Inc.; phospho Histone H3 (IF: 1/1000), Cell Signaling Technology; acetylated α-tubulin (IF: 1/4000), Sigma-Aldrich.

Cell culture, transfection, RNA and DNA constructs

HeLa and U2-OS (ATCC, Manassas, VA) cells were cultured in Advanced DMEM (Invitrogen), supplemented with 4% FBS, and 2 mM GlutaMAX-I (GIBCO, Rockville, MD). hTERT-RPE-1 (ATCC) cells were cultured in DMEM supplemented with 8% FBS and 2 mM GlutaMAX-I (GIBCO, Rockville, MD). For primary cilia induction, hTERT-RPE-1 cells were serum-starved for 48 hours. All of these cells were transiently transfected using oligofectamine (Invitrogen) for siRNA transfections, or Fugene HD (Roche) for plasmid transfections according to the manufacturer’s instructions.
Stealth RNAi™ siRNA duplex oligoribonucleotides (Invitrogen) are depicted in Supplementary material Table S2.

A GFP-tagged form of the human Par6γ C-terminus (pEGFP-C3-Par6γ-Cter) was generated by PCR and cloned via HindIII and BamHI into pEGFP-C3 vector (Clontech, Palo Alto, CA). This construct was used to express GFP-Par6γ Nter, GFP-Par6γ Cter (259-376), GFP-Par6γ Cter A (259-317) and GFP-Par6γ Cter B (312-376). Par6γ in pCMV6-AC-GFP (OriGene Technologies, Rockville, MD) was used to express GFP-Par6γ WT and served as a template for PCR reactions. The primers used for cloning are depicted in Supplementary material Table S3. To disrupt dynein and dynactin function, human HA-p150 Glued and human p50-myc (generously provided by Dr. M. Takahashi, University of Kobe, Japan) were overexpressed in HeLa cells.

**Wound-healing assay**

hTERT-RPE-1 cells were grown to confluence on glass coverslips. 24 hours after control or knock-down transfections, a wound was introduced in the center of the cell monolayer by gently removing attached cells with a sterile plastic scratcher. Cell debris was removed by washing with PBS. The ability of the cells to migrate into the wound area at several marked points along the wounded area was assessed at 0, 10 and 20 hours after wounding. The percentage of recovered wound area was calculated by dividing the recovered area after 10 or 20 hours by the initial wound area at zero time.

**Immunofluorescence microscopy**

Cells grown on coverslips were fixed in ice-cold methanol at -20°C for 4 minutes, permeabilized and blocked in 2.5% blocking buffer (0.1% Triton X-100, 2.5% FBS in PBS) for 1 hour. Cells were incubated for 1 hour at room temperature with primary antibodies, washed with PBS, incubated for 30 minutes with secondary antibodies, washed with PBS and mounted on glass slides using gelvatol (Sigma-Aldrich). Primary and secondary antibodies were diluted in 2.5% staining buffer (0.1% Triton X-100, 2.5% FBS in PBS). DNA was stained with Hoechst 33342 (Molecular Probes/Invitrogen) during incubation with secondary antibodies. Cells were imaged on a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) and analyzed with linear adjustments with the Zeiss Axiovision software. Images were processed in Adobe Photoshop CS3.
Quantifications of protein levels at the centrosome: the fluorescence signal for each protein in cells transfected with control siRNA was defined as 100%. The signal intensity in areas devoid of cells, in the cytosol and the nucleus were used to normalize the measurements from different coverslips. Twenty cells were analyzed per experiment and three independent experiments were performed.

**Lysate preparation and Western blot analysis**

Cells were harvested by direct lysis in lysis buffer (50mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitors (aprotinin, leupeptin and pepstatin) for 10 min on ice. Lysates were cleared and protein concentration was determined by Bradford protein assay (Biorad). 25 µg of total cell lysate/lane were resolved on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Pall Corporation). Membranes were blocked for 1 hour at room temperature in 5% non-fat milk in TBS and 0.05% Tween 20. Blots were incubated overnight with primary antibodies at 4°C, followed by incubation with the appropriate HRP-conjugated secondary antibodies (Jackson Immuno Research Labs, Inc) for 1 hour at room temperature. Proteins were detected using ECL reagents (100mM Tris-HCl, pH 8.5, coumaric acid, luminal, 0.3% peroxide) and visualized by exposure to X-ray film.

**Co-immunoprecipitations**

HeLa cells were harvested by direct lysis on ice for 30 min in lysis buffer (20 mM Tris, pH 7.4, 50 mM NaCl, and 0.5% NP-40 supplemented with protease inhibitors). For each reaction, 1 mg of total lysate was incubated with 5 µg of antibody at 4°C for 3h and for 1h with protein G Sepharose (GE Healthcare, Uppsala, Sweden). Immunocomplexes were washed with lysis buffer, separated by SDS-PAGE, transferred to nitrocellulose or PVDF membranes (Whatman, Clifton, NJ), and analyzed by Western blotting.

**Protein sequence analysis**

The multiple sequence alignment of Par6α (accession number Q9NPB6.1), Par6β (accession number Q9BYG5.1) and Par6γ (accession number Q9BYG4.1) from the National Center for Biotechnology Information (NCBI) was performed using DNAman software (Lynnon Biosoft Copyright©). The sequence file was in Clustal format and was analyzed for the percentage of identity and similarity.
Statistical analysis

All values are expressed as mean±s.e.m. Values were compared using multifactorial analysis of variance followed by the Student-Newman-Keul’s test for multiple comparisons. A P < 0.05 was considered to be significant.
Acknowledgements

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References


Figure Legends

Fig. 1. The Par6 family proteins. (A) Domain map of Par6α, Par6β and Par6γ; the numbers refer to positions of amino acid residues. (B) Sequence alignment of the three human Par6 proteins. Protein-protein interaction domains PB1 type II and PDZ are highlighted. Identical or closely related residues are shown in black or grey, respectively.

Fig. 2. Par6γ localizes to the mother centriole. (A) HeLa cells were stained with antibodies to γ-tubulin (green) and Par6γ (red). (B) Western blot analysis of total protein lysates from HeLa cells that were untreated (Control), transfected with control siRNA (siControl) or specific siRNA to Par6γ (siPar6γ and siPar6γ#2) for 24 hours. α-tubulin served as the loading control. (C) hTERT-RPE-1 and U2-OS cells were stained with antibodies to Par6γ (red) and γ-tubulin (green). (D) HeLa cells were stained with antibodies to Par6γ (red) and one of centrin2, Cep164, ninein or HsSAS-6 (green). The cartoon indicates the specific localization of these centrosomal proteins; M refers to mother centriole, D to the daughter centriole, PCM, pericentriolar material. The histograms present the percentage of colocalization of Par6γ with the indicated proteins. The percentage of colocalization was determined by comparing the pixel density at the centrosome in the merged channel to the sum of pixel densities in the same area in each individual channel. Ten cells were analyzed/experiment; n=3. (E) Serum-starved hTERT-RPE-1 cells were stained with antibodies to Par6γ (red) and acetylated-α-tubulin (green). Scale bars: A-E, 10 µm.

Fig. 3. The Par6γ C-terminus is necessary and sufficient for the centrosomal localization. (A) Schematic representations of Par6γ (Par6γ WT), GFP-Par6γ WT, GFP-Par6γ Nter, GFP-Par6γ Cter (259-376), GFP-Par6γ Cter A (259-317) and GFP-Par6γ Cter B (312-376). (B) HeLa cells expressing GFP-tagged Par6γ constructs described in A (red) were stained with antibodies to centrin2 (green). Merged images are also shown. Scale bar, 10 µm. (C) Table summarizing the localization of these Par6γ constructs in HeLa cells.

Fig. 4. Par6γ regulates the protein composition of the centrosome. (A) Control and Par6γ-depleted HeLa cells were stained with antibodies to Par6γ (red) and one of γ-
tubulin, Cep152 or Cep170 (green). Merged images with DNA stained by Hoechst 33342 dye are shown. Insets show the enlarged area of the centrosome. Scale bar, 10 µm. (B) Quantification of the fluorescence intensity of centrosomal proteins in Par6γ-depleted HeLa cells, such as those of A, in comparison to control cells. *, P < 0.01.

Fig. 5. Par6γ controls the function of the centrosome. (A) Serum starved control and Par6γ-depleted hTERT-RPE-1 cells were stained with antibodies to Par6γ and acetylated α-tubulin. The histogram shows the percentage of cells with primary cilia. Arrows indicate the localization of Par6γ (middle panel) and the primary cilia (right panel). (B) Control and Par6γ-depleted HeLa cells were stained with antibodies to Par6γ (red) and α-tubulin (green). The histogram shows the percentage of cells with abnormal microtubule organization. (C) HeLa cells overexpressing GFP-Par6γ WT (red) or GFP-Par6γ Cter (red) were stained with antibodies to α-tubulin (green). The histogram shows the percentage of cells with abnormal microtubule organization. (D) Control and Par6γ-depleted HeLa cells were stained with antibodies to phospho-histone H3 (P-H3, red) and α-tubulin (green). The histograms show the percentage of mitotic cells and the percentage of mitotic cells with defective spindles at 24 and 48 hours post transfection. (E) Wound area in control and Par6γ-depleted hTERT-RPE-1 cells at 0, 10 and 20 hours after wounding. The percentage of cell surface recovery was quantified for each time point. (F) Control and Par6γ-depleted hTERT-RPE-1 cells were stained with antibodies to γ-tubulin (red) and α-tubulin (green) 10 hours after wounding. Arrows show the localization of the centrosome, the dotted lines show the edge of the wound. The histogram shows the percentage of cells in which the centrosome is not reoriented. (A-F) Merged image with DNA stained by Hoechst 33342 dye are shown. 100 cells were analyzed/experiment; n=4; *, P < 0.05, **, P < 0.01. Scale bars: A, F, 20 µm; B-D, 5 µm; E, 100 µm.

Fig. 6. Centrosomal Par6γ localization and function does not involve PAR polarity complex proteins. (A) Domain map of components of the PAR polarity complex; the numbers refer to positions of amino acid residues. (B) Total HeLa cell lysates were used for co-immunoprecipitation experiments with antibodies to Par6α or Par6γ. Flag antibody was used as negative control (Control). Par6α, Par6β, Par6γ, Par3α, Par3β, Pkci and Pkcz were detected with specific antibodies. (C) Schematic representation of the potential interaction network of Par6α and Par6β with members
of the PAR polarity complex. (D) Table summarizing the changes in fluorescence intensity at the centrosome of various centrosomal proteins in HeLa cells, in the Par6α or Par6γ interactors identified in B depleted by siRNA.

Fig. 7. Par6γ localizes to the centrosome by a microtubule and dynein/dynactin-independent mechanism and regulates the centrosomal recruitment of Par6α and p150Glued. (A) HeLa cells treated with DMSO or nocodazole were stained with antibodies to Par6γ (red) and α-tubulin (green). The histogram shows the fluorescence intensity of centrosomal Par6γ in comparison to control cells. 25 cells were analyzed/experiment. n = 3; *, P < 0.01. (B) HeLa cells were transfected with HA-tagged p150Glued (left) or with myc-tagged p50-dynamitin (right) to inhibit dynein or dynactin function, respectively. Empty vectors served as negative controls. Cells were fixed and stained with antibodies to HA or myc (green), Par6γ (red) and γ-tubulin (cyan). (C) Par6α- and Par6γ-depleted HeLa cells were stained with antibodies to Par6γ (red), γ-tubulin (green) and Par6α (cyan). (D) HeLa cells expressing GFP-tagged Par6γ constructs (red) were stained with antibodies to Par6α (green) and γ-tubulin (cyan) (E) Control and Par6γ-depleted HeLa cells were stained with antibodies to Par6γ (red), γ-tubulin (cyan) and p150Glued (green). The histogram shows the quantification of the fluorescence intensity of p150Glued at the centrosome in control and Par6γ-depleted cells. n = 3; *, P < 0.01. Scale bars: A-E, 5 µm.
**A**

![Diagram showing protein expression and localization](image)

**B**

![Images of GFP and centrin2 localization](image)

**C**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Centrosome</th>
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<tbody>
<tr>
<td>GFP-Par6 γ WT</td>
<td>yes</td>
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<tr>
<td>GFP-Par6 γ Nter</td>
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<tr>
<td>GFP-Par6 γ Cter (259-376)</td>
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</tr>
<tr>
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</tr>
<tr>
<td>GFP-Par6 γ Cter B (312-376)</td>
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</tr>
</tbody>
</table>
A

Control

Par6γ  γ-tubulin  Par6γ  Cep152  Par6γ  Cep170

siPar6γ

Par6γ  γ-tubulin  Par6γ  Cep152  Par6γ  Cep170

B

γ-TuRC complex

Fluorescence intensity (% of Control)

Control  γ-tubulin  GCP2  GCP-WD

Centriolar proteins

Fluorescence intensity (% of Control)

Control  CPAP  STIL  centrin2  hSAS-6  Cep192  Cep152

Appendage proteins

Fluorescence intensity (% of Control)

Control  Cep164  Cep170  ninein  Orf2

Centriolar satellite proteins

Fluorescence intensity (% of Control)

Control  PCM-1  Par6q  BBS4  Cep290