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Par6α Interacts with the Dynactin Subunit p150^{Glued} and Is a Critical Regulator of Centrosomal Protein Recruitment

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The centrosome contains proteins that control the organization of the microtubule cytoskeleton in interphase and mitosis. Its protein composition is tightly regulated through selective and cell cycle–dependent recruitment, retention, and removal of components. However, the mechanisms underlying protein delivery to the centrosome are not completely understood. We describe a novel function for the polarity protein Par6α in protein transport to the centrosome. We detected Par6α at the centrosome and centriolar satellites where it interacted with the centriolar satellite protein PCM-1 and the dynactin subunit p150^{Glued}. Depletion of Par6α caused the mislocalization of p150^{Glued} and centrosomal components that are critical for microtubule anchoring at the centrosome. As a consequence, there were severe alterations in the organization of the microtubule cytoskeleton in the absence of Par6α and cell division was blocked. We propose a model in which Par6α controls centrosome organization through its association with the dynactin subunit p150^{Glued}.

INTRODUCTION

The composition of the centrosome and its function in microtubule organization in interphase and mitosis are critical for cell homeostasis. A newly formed daughter cell contains two orthogonally arranged centrioles, which are characterized by a unique set of proteins at their proximal and distal ends (Strnad and Gonczy, 2008). The two centrioles are surrounded by electron-dense pericentriolar material (PCM), which contains proteins necessary for microtubule nucleation and anchoring (Bornens *et al.*, 1987; Bobinnec *et al.*, 1998). After centrosome duplication in S-phase, the two centrosomes move to opposite sides of the nucleus to form the poles of the mitotic spindle. Each daughter cell inherits one spindle pole that then becomes its centrosome. Abnormalities in centrosome number and organization promote chromosome segregation errors and aneuploidy and may contribute to the development of cancer (Nigg, 2006; Ganem *et al.*, 2009).

An interphase centrosome contains at least 100 proteins that are recruited by different mechanisms (Andersen *et al.*, 2003). AKAP450 and pericentrin contain specific localization motifs that are sufficient to target these large scaffold proteins to the centrosome without a requirement for intact microtubules (Gillingham and Munro, 2000). BBS6 and Poc5 also reach the centrosome in a dynein- and microtubule-independent manner, although specific localization domains within these centrosomal proteins have not yet been identified (Kim *et al.*, 2005; Azimzadeh *et al.*, 2009). In contrast, PCM-1, centrin, and ninein are transported along microtubules to the centrosome by the dynein–dynactin motor complex (Dammermann and Merdes, 2002). These centrosomal proteins are mislocalized if microtubules are depolymerized or if dynein function is disrupted (Balczon *et al.*, 1999; Kubo *et al.*, 1999; Dammermann and Merdes, 2002; Hames *et al.*, 2005). Studies with γ-tubulin have revealed a role for microtubules in the initial recruitment to the centrosome from the cytosol, but not in its retention at the centrosome (Khodjakov and Rieder, 1999; Young *et al.*, 2000), indicating that there are differential microtubule requirements during centrosomal protein localization.

The dynein–dynactin complex is involved in the regulation of centrosome organization and function. Dynein is a minus end–directed microtubule motor that depends on the multisubunit complex dynactin for the regulation of its processivity and cargo interaction (King and Schroer, 2000; Holleran *et al.*, 2001; Kardon and Vale, 2009). The dynein–dynactin complex is necessary for centrosomal delivery of proteins that maintain the radial organization of microtubules at the centrosome (Quintyne *et al.*, 1999; Dammermann and Merdes, 2002). Intriguingly, there are differences in the association of dynein and dynactin with the centrosome during the cell cycle (Quintyne and Schroer, 2002). Dynactin is detected at the centrosome throughout the cell cycle, whereas dynein localizes to the centrosome in S-phase and G2, but not during mitosis (Quintyne and Schroer, 2002). Both complexes control microtubule anchoring and retention via a mechanism that is distinct from the role of the dynein–dynactin complex in the delivery of microtubule-organizing proteins to the centrosome (Quintyne *et al.*, 1999; Quintyne and Schroer, 2002; Burakov *et al.*, 2008).

Centriolar satellites have been implicated in microtubule-dependent protein transport to the centrosome, but their exact role in this process remains unclear. These spherical cytosolic granules are often found in the vicinity of the centrosome and are characterized by the presence of PCM-1,

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BBS4, and Cep290 (Balczon *et al.*, 1994; Kubo *et al.*, 1999; Kim *et al.*, 2004; Kim *et al.*, 2008). PCM-1 is proposed to serve as a scaffold for centrosomal cargo proteins, including centrin, ninein, and Nek2 (Zimmerman and Doxsey, 2000; Dammermann and Merdes, 2002; Hames *et al.*, 2005). BBS4 and Cep290 may have roles in linking PCM-1 to the dynein motor complex through binding to the dynactin subunits p150^{Glued} and p50 dynamitin (Kim *et al.*, 2004; Kim *et al.*, 2008; Chang *et al.*, 2006). Thus, centriolar satellite components and the dynein–dynactin motor complex appear to form an interaction network that is important for protein delivery to the centrosome. In support of this model, inactivation of any one of these three centriolar satellite proteins causes defects in centrosomal protein trafficking and aberrant microtubule organization (Dammermann and Merdes, 2002; Kim *et al.*, 2004; Kim *et al.*, 2008). As centriolar satellites move toward the centrosome in a dynein- and microtubule-dependent mechanism (Kubo *et al.*, 1999; Zimmerman and Doxsey, 2000), it is possible that they promote the association of cargo with the dynein motor complex for efficient transport to the centrosome.

Par6α, a member of the Par6 family of polarity proteins, plays an important role in the control of centrosome organization and function (Solecki *et al.*, 2004). Par6 was identified in *Caenorhabditis elegans* zygotes as a critical regulator of asymmetric cell division (Watts *et al.*, 1996; Hung and Kemphues, 1999; Lin *et al.*, 2000), and homologues have been found in other species. There is a single Par6 gene in *C. elegans* and in *Drosophila melanogaster*, but mammalian cells contain four isoforms that are called Par6A–D (Joberty *et al.*, 2000; Gao and Macara, 2004). Only Par6B (Par6β) and Par6C (Par6α) are expressed in HeLa cells (Joberty *et al.*, 2000). It is well established that Par6 proteins regulate cell polarity, cytoskeletal rearrangement, and the assembly of tight junctions by forming a complex with the activated form of Cdc42, atypical protein kinase C (PKCζ) and the polarity protein Par3 (Lin *et al.*, 2000; Gao *et al.*, 2002; Etienne-Manneville *et al.*, 2005). Solecki and colleagues reported an additional role for Par6α in centrosome organization by showing that its depletion in neurons caused mislocalization of centrosomal proteins, alterations in the perinuclear tubulin cage and defects in centrosome movement during cell migration (Solecki *et al.*, 2004).

In this study, we present our results on the role of Par6α at the centrosome of epithelial cells. Par6α localized to the centrosome and centriolar satellites by a mechanism that involved microtubules and the dynein motor complex. RNAi-mediated depletion of this protein caused the mislocalization of specific centrosomal proteins, including regulators of microtubule anchoring. Thus, in the absence of Par6α, there were defects in microtubule organization in interphase, but also in mitosis, which affected cell cycle progression. Par6α associated with the dynactin subunit p150^{Glued} and was necessary for its localization to the centrosome. We propose that Par6α-mediated centrosome regulation involves the binding of Par6α to p150^{Glued}.

MATERIALS AND METHODS

Antibodies

The following antibodies were used in this study: anti-Par6α (Dr. R. Lammers, University of Tübingen, Tübingen, Germany, and T-20 Lot L0406, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Par6α/β (Santa Cruz Biotechnology), anti-PCM-1 (Dr. A. Merdes CNRS Toulouse, Toulouse, France, and H-262, Santa Cruz Biotechnology), anti-centrin (Dr. J. Salisbury, Mayo Clinic, Rochester, MN), CPAP (Dr. P. Gönczy, EPFL, Lausanne, Switzerland), anti-Cep170 (Dr. G. Guarguaglini, University of Rome, Rome, Italy), anti-Cep192 (Dr. D. Sharp, Albert Einstein College of Medicine, New York, NY), anti-kendrin/pericentrin (Dr. M. Takahashi, University of Kobe, Kobe, Japan), anti-α-tubulin, anti-γ-tubulin and anti-FLAG (Sigma-Aldrich, St. Louis, MO), anti-γ-tubulin and anti-ninein (Abcam, Cambridge, MA), anti-hSAS6 and anti-β-PIX (Santa Cruz Biotechnology), anti-p150^{Glued} (BD Biosciences, San Jose, CA), anti-green fluorescent protein (GFP; Roche, Indianapolis, IN), anti-hemagglutinin (HA; Covance, Madison, WI), and anti-myc (Calbiochem, La Jolla, CA).

Cell Culture

HeLa, U2-OS, and hTERT-RPE-1 cells were cultured as previously described (Kodani and Sutterlin, 2008, 2009).

Immunofluorescence Microscopy

Immunofluorescence analysis was performed as described (Kodani and Sutterlin, 2008). To stain mitochondria, cells were cultured in complete media supplemented with 100 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) for 30 min followed by fixation and processing for antibody staining. Cells were imaged with a Zeiss Axiovert 200M microscope (Thornwood, NY) and analyzed with linear adjustments with the Zeiss Axiovision software. Photometric analysis was conducted using ImageJ (http://rsb.info.nih.gov/ij/) and Photoshop (Adobe, San Jose, CA). Centrosomal protein levels in control and Par6α-depleted cells were determined by quantifying the fluorescence intensity for each marker in 15 cells per experimental condition, normalizing it to γ-tubulin. These measurements were carried out in three independent experiments.

Molecular Biology

We depleted Par6α by targeting the sequence 5′-TGGACGTGCTACTTGGCTATA-3′ in the human Par6α cDNA (Frederick *et al.*, 2008). We depleted PCM-1 by using the targeting sequence 5′-GGCTTTAACTAATTATGGA-3′ in the human PCM-1 cDNA (Kim *et al.*, 2008). A scrambled sequence with similar nucleotide composition was used as a control (Sutterlin *et al.*, 2005). Small interfering RNA (siRNA) to Par6α or PCM-1 (Invitrogen, Carlsbad, CA) was transfected using Oligofectamine (Invitrogen) as described (Sutterlin *et al.*, 2005).

Human Par6α cDNA was obtained from Addgene (plasmid 15474; Cambridge, MA) and subcloned into the pEGFP-C2 vector (Clontech, Palo Alto, CA). For mitochondria targeting, the sequence encoding the N-terminal hydrophobic anchor of Tom20 (generously provided by Dr. C. Koehler, University of California, Los Angeles, Los Angeles, CA) was fused at the N-terminus of both GFP and GFP-Par6α. To induce large cytoplasmic aggregate formation a C-terminal truncation of PCM-1 (amino acids 1-1468) was expressed. Full-length GFP-tagged PCM-1 served as a control (generously provided by Dr. A. Merdes, CNRS, Toulouse, France). 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.

Immunoprecipitations

For coimmunoprecipitation reactions, HeLa cells were harvested with a cell scraper and lysed on ice for 10 min in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 1% NP-40 supplemented with protease inhibitors). For each reaction, 2 mg of total lysate was incubated with 5 μg of antibody at 4°C for 2 h and for 1 h 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.

Centrosome Isolation

Centrosomes were isolated from HeLa cells as previously described (Bobinnec *et al.*, 1998). In brief, 5×10^8 cells were treated with 2 μM nocodazole for 1 h at 37°C. Cells were then lysed in lysis buffer (1 mM HEPES, pH 7.2, 0.5% NP-40, 0.5 mM $MgCl_2$, and 0.1% β-mercaptoethanol, supplemented with protease inhibitors) and centrifuged at 2500 × *g* for 10 min. The resulting supernatant was filtered, incubated with 1 μg/ml DNase I, loaded onto a 60% sucrose cushion, and centrifuged at 10,000 × *g* for 30 min. Centrosome-enriched fractions were then centrifuged on a discontinuous sucrose gradient (70, 50, and 40% sucrose) at 40,000 ×*g* for 1 h. Gradient fractions were collected from the bottom and analyzed by SDS-PAGE and Western blotting.

RESULTS

Par6α Is a Component of the Interphase Centrosome

We examined the localization of Par6α, a 37-kDa isoform of the polarity protein Par6, in epithelial cells. Immunofluorescence studies with a specific antibody detected Par6α at the centrosome where it colocalized with the centriolar protein centrin (Figure 1A, left) and the centrosomal matrix protein γ-tubulin (Figure 1A, middle). Par6α also associated with centriolar satellites that are marked by PCM-1 (Figure 1A, right) and BBS4 (data not shown; Kubo *et al.*, 1999; Kim *et al.*,

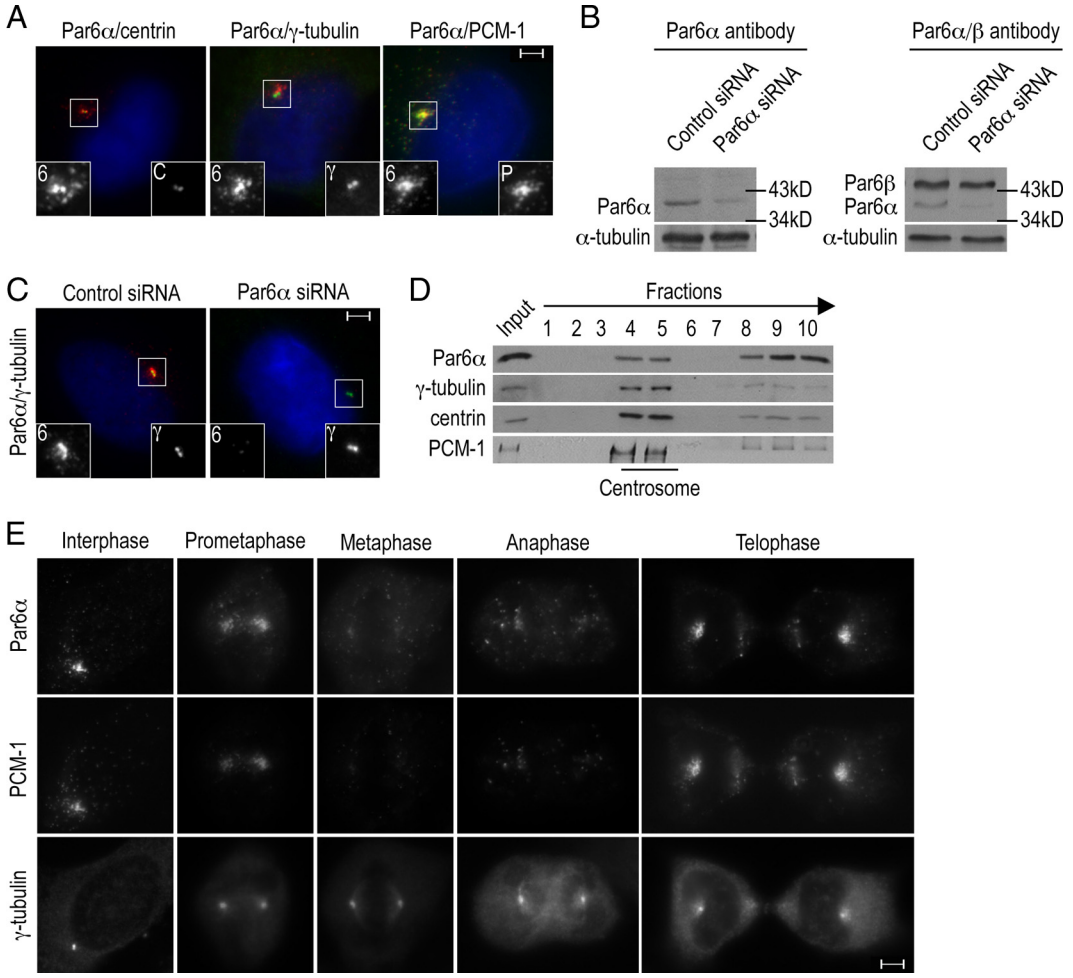

Figure 1. Par6α is a component of the centrosome and of centriolar satellites. (A) Immunofluorescence microscopy analysis of HeLa cells costained with antibodies to Par6α (red) and centrin, γ-tubulin or PCM-1 (all in green). The insets show magnified images of the boxed area. (B) Total lysates of control or Par6α-depleted HeLa cells were analyzed by Western blotting with antibodies that recognize only Par6α (left) or that detects both isoforms, Par6α and Par6β (right). α-tubulin served as a loading control. (C) Analysis of control and Par6α-depleted cells with antibodies to Par6α and γ-tubulin. (D) HeLa cell lysates were fractionated on sucrose gradients to enrich for centrosomes. Fractions were analyzed by Western blotting with antibodies to Par6α, centrin, γ-tubulin, and PCM-1. (E) Cell cycle–dependent localization of Par6α was determined by staining a nonsynchronous population of HeLa cells with antibodies to Par6α and PCM-1. The organization of γ-tubulin and DNA was used to mark the centrosome and to determine the specific cell cycle stage, respectively. Scale bars, (A, C, and E), 5 μm.

2004). Par6α staining in the centrosomal region was seen in HeLa cells and in other human cell lines, including U2-OS and hTERT-RPE-1 (data not shown). To verify the specificity of this staining pattern, we depleted Par6α by RNA interference (RNAi). This treatment led to the absence of a 37-kDa protein by Western blotting (Figure 1B) and the loss of centrosomal staining by immunofluorescence microscopy (Figure 1C). Because depletion of Par6α had no effect on the levels of the 45-kDa Par6β isoform (Figure 1B, right), we conclude that our antibody specifically recognizes Par6α.

To corroborate Par6α as a centrosomal component, we examined centrosome-enriched fractions for the presence of Par6α by Western blot analysis. These fractions were isolated by gradient centrifugation of total HeLa cell lysates and contained Par6α and the centrosomal marker proteins, centrin, γ-tubulin, and PCM-1 (Figure 1D). Par6α was also found in cytosolic fractions on top of the gradient, indicating that this protein also exists in the cytosol.

We next examined the association of Par6α with the centrosome and centriolar satellites during the cell cycle. We costained a nonsynchronous population of HeLa cells with antibodies to Par6α and PCM-1, marking the position of the centrosome by staining for γ-tubulin. Par6α colocalized with PCM-1 at the centrosome and at centriolar satellites during interphase and at spindle poles during prometaphase, followed by dissociation from spindle poles between metaphase and telophase (Figure 1E). These results suggest that the association of Par6α with the centrosome and centriolar satellites is regulated during the cell cycle in a manner similar to PCM-1 (Figure 1E, middle panel; Dammermann and Merdes, 2002).

Par6α Controls the Centrosome during the Cell Cycle

We analyzed the function of Par6α at the centrosome. As a measure of centrosome organization, we monitored the localization of known centrosomal proteins in cells depleted of Par6α by RNAi. We first looked at proteins that reach the centrosome by a microtubule-dependent mechanism (pericentrin, PCM-1, centrin, and ninein). The centrosomal levels of these proteins were substantially reduced in Par6α-depleted cells when compared with control cells transfected with scrambled RNAi (Figure 2, A and B). In contrast, the

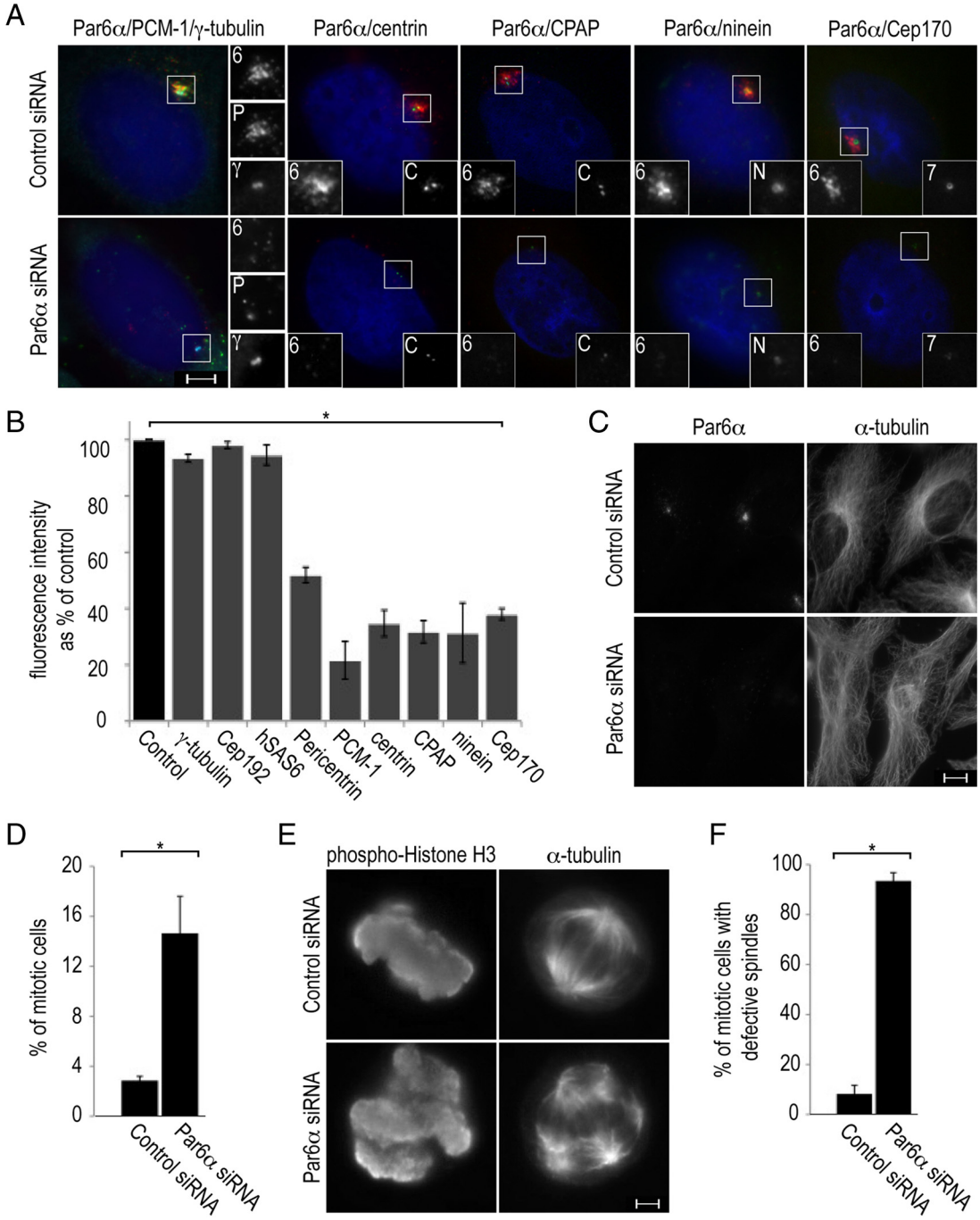

Figure 2. Par6α-depleted cells contain disorganized, nonfunctional centrosomes. (A) Control and Par6α-depleted cells were stained with antibodies to Par6α (red, 6) to confirm protein depletion, to γ-tubulin (γ, cyan) and to PCM-1 (P), centrin (C), CPAP (C), ninein (N), and Cep170 (7; all in green) to monitor centrosomal protein organization. Scale bar, 5 μm. (B) Quantifications of the fluorescence intensity at the centrosome are shown. The control was defined as 100% and represents the fluorescent signal detected for each marker protein at the centrosome of control siRNA-transfected cells. Fifteen cells were analyzed per experiment, and three independent experiments were performed. The p values for each experiment were <0.005 (paired *t* test). (C) Microtubule organization was examined by staining control and Par6α-depleted cells with antibodies to Par6α and α-tubulin. Scale bar, 10 μm. (D) Staining with a specific antibody to phospho-Histone H3 was used to determine the percentage of cells in mitosis (n = 3), *p < 0.001, paired *t* test. (E) Mitotic control and Par6α-depleted cells were stained with antibodies to phospho-Histone H3, and to α-tubulin and the DNA dye, Hoechst, to visualize spindle organization or DNA alignment, respectively. The percentage of mitotic cells with aberrant spindles is shown (n = 3), *p < 0.001, paired *t* test. Scale bar, 5 μm.

localization of γ-tubulin, whose retention at the centrosome is reported to be independent of microtubules (Khodjakov and Rieder, 1999), was not affected by the absence of Par6α (Figure 2, A and B). We also examined the effects of Par6α depletion on proteins of proximal (Cep192, hSAS6) and subdistal (Cep170) regions within centrioles. In addition, we analyzed the distribution of CPAP, which is likely to localize to the proximal and subdistal region of the centriole through

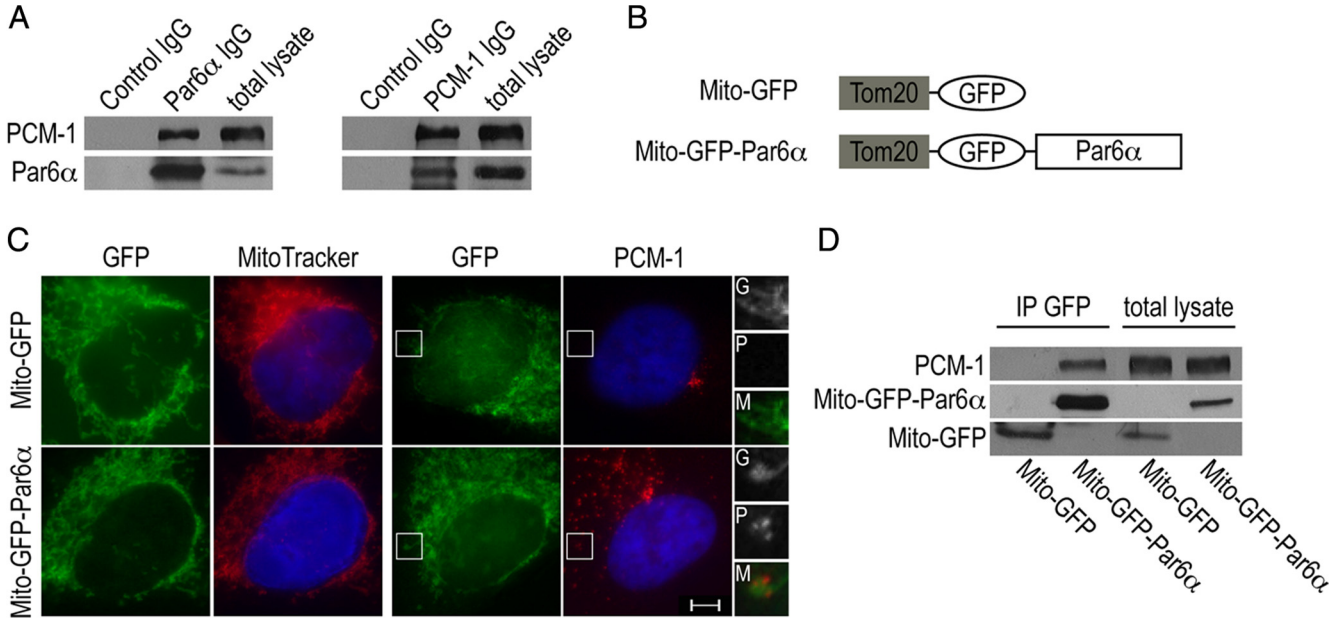

Figure 3. Par6α interacts with PCM-1 and directs its localization. (A) Total HeLa cell lysates were subjected to immunoprecipitations with polyclonal antibodies to Par6α (Par6α IgG), PCM-1 (PCM-1 IgG), or to β-PIX or the FLAG-epitope (control IgG) as negative controls. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with antibodies to Par6α and PCM-1. The input corresponds to 50 μg of total lysates, whereas each immunoprecipitation was performed from 2 mg of total lysate. (B) Schematic representation of the mitochondria-targeted constructs. (C) HeLa cells expressing mitochondrially-targeted GFP (Mito-GFP) or mitochondrially-targeted GFP-tagged full-length Par6α (Mito-GFP-Par6α) were incubated with MitoTracker or fixed and stained with antibodies to PCM-1. The localization of GFP (G), Par6α (P) and mitochondria (M) are shown at larger magnifications. Scale bar, 5 μm. (D) HeLa cells expressing Mito-GFP or Mito-GFP-Par6α were subjected to immunoprecipitations with a GFP-specific antibody, followed by Western blot analysis with antibodies to GFP and PCM-1.

an association with the subdistal centriole protein Protein 4.1R (Hung *et al.*, 2000; Krauss *et al.*, 2008; Kohlmaier *et al.*, 2009). Cep192 and hSAS6 levels were unaffected by Par6α depletion (data not shown), but the centrosomal levels of Cep170 and CPAP were significantly reduced in the absence of Par6α (Figure 2, A and B). These results indicate that Par6α controls protein recruitment to the centrosome, although its regulatory effects appear limited to specific subsets of proteins.

We next investigated whether Par6α is necessary for centrosome function by visualizing microtubule organization in the absence of Par6α. Control cells in interphase contained a radial microtubule array that was nucleated at the centrosome (Figure 2C, top panel). In contrast, microtubules in Par6α-depleted cells were randomly arranged in the cytosol or on top of the nucleus (Figure 2C, bottom panel). We also assayed centrosome function in mitosis by examining the ability of Par6α-depleted cells to form bipolar spindles. Intriguingly, the percentage of mitotic cells, as detected by staining with an antibody to phospho-Histone H3, increased from 3% in control cells to 14% in Par6α-depleted cells, which is indicative of a cell cycle arrest in mitosis (Figure 2D). Of these mitotic Par6α-depleted cells, 95% contained multipolar spindles that were unable to align DNA in the metaphase plate (Figure 2, E and F). As a consequence of these spindle abnormalities and the mitotic arrest, Par6α-depleted cells eventually died (data not shown). We conclude that Par6α is critical for normal microtubule organization in interphase and mitosis, which are key functions of the centrosome.

Par6α and PCM-1 Associate with the Centrosome as a Complex

Because Par6α colocalized with PCM-1 throughout the cell cycle and controlled the localization of PCM-1 in the pericentriolar region, we tested if these proteins interact. We detected a specific association between Par6α and PCM-1 in reciprocal coimmunoprecipitation experiments (Figure 3A). To test the significance of this interaction for protein localization, we mislocalized Par6α to mitochondria and measured the effect on the intracellular distribution of PCM-1. We targeted Par6α to the mitochondria by fusing the transmembrane targeting motif of the mitochondrial protein, Tom20 (Kanaji *et al.*, 2000) to GFP-tagged Par6α (Mito-GFP-Par6α) or to GFP as a negative control (Mito-GFP; Figure 3B). We verified that each fusion protein associated with mitochondria by colocalization with the mitochondrial dye MitoTracker (Figure 3C, left panel). Intriguingly, PCM-1 was recruited to mitochondria of cells expressing Mito-GFP-Par6α, leading to the partial colocalization of these two proteins at mitochondria. Expression of Mito-GFP, in contrast, had no effect on PCM-1 localization (Figure 3C, right panel, top). Under these conditions, Mito-GFP-Par6α and PCM-1 interacted as shown in parallel coimmunoprecipitation experiments (Figure 3D). Our data indicate that the binding of PCM-1 to Mito-GPF-Par6α is sufficient to direct PCM-1 to mitochondria.

In a reciprocal experiment, we measured the effects of PCM-1 mislocalization on Par6α distribution. Instead of targeting PCM-1 to mitochondria, we expressed a truncated form of PCM-1, GFP-PCM-1ΔC (amino acids 1-1468), which is reported to induce the formation of large, PCM-1-containing aggregates in the cytosol (Dammermann and Merdes, 2002). Par6α colocalized with PCM-1 at these aggregates (Figure 4A), demonstrating that PCM-1 can also modify the intracellular localization of Par6α.

Because these results indicate that a complex of Par6α and PCM-1 is critical for the intracellular localization of these

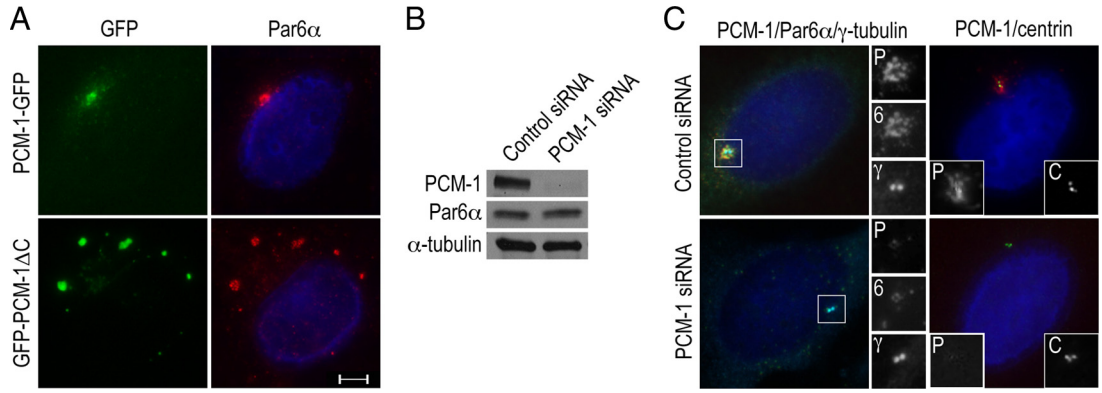

Figure 4. PCM-1 is required for the centrosomal localization of Par6α. (A) HeLa cells expressing GFP-tagged full-length PCM-1 (PCM-1-GFP) or an N-terminal portion of PCM-1 corresponding to amino acids 1-1468 (GFP-PCM-1ΔC) were fixed and stained with antibodies to Par6α. (B) Total lysates of control and PCM-1—depleted HeLa cells were analyzed by Western blotting with antibodies to PCM-1 and Par6α. α-Tubulin served as a loading control. (C) Staining of control and PCM-1—depleted HeLa cells with antibodies to PCM-1 (P, green) and Par6α (6, red). γ-tubulin (γ, cyan) served to visualize the centrosome in the same cell. Scale bar, 5 μm.

proteins, we tested the effect of PCM-1 depletion on the localization of Par6α to the pericentriolar region. In cells in which PCM-1 was depleted to 5% of control levels (Figure 4B), there was a significant reduction in the Par6α signal at the centrosome and centriolar satellites (Figure 4C). However, loss of PCM-1 had no obvious effects on centrosomal γ-tubulin or centrin levels (Figure 4C). In addition, microtubule organization and cell cycle progression were normal in the absence of PCM-1 (data not shown). Taken together, these results indicate that Par6α and PCM-1 associate with the centrosome as a complex, but that only Par6α is necessary for the regulation of centrosomal protein composition and function.

Localization of Par6α to the Pericentriolar Region Depends on Intact Microtubules and the Dynein–Dynactin Motor Complex

We examined the role of microtubules and the dynein–dynactin motor complex in the centrosomal localization of Par6α because these factors have been implicated in the delivery of centrosomal proteins via centriolar satellite (Dammermann and Merdes, 2002; Kim *et al.*, 2004; Kim *et al.*, 2008). We first depolymerized microtubules with nocodazole, which leads to the formation of cytoplasmic PCM-1-positive aggregates (Dammermann and Merdes, 2002). Under these conditions, Par6α was mislocalized from the pericentriolar region to these prominent cytosolic structures (Figure 5A). To test the involvement of the dynein–dynactin complex in Par6α localization, we blocked dynein function by overexpressing a tagged form of the dynactin subunit $p150^{Glued}$ (HA-$p150^{Glued}$). This treatment is reported to disrupt centrosome cohesion, producing two or more γ-tubulin–positive foci (Quintyne *et al.*, 1999). Interestingly, Par6α did not associate with any of these γ-tubulin foci (Figure 5B, left panel). We obtained similar results when we antagonized dynactin function by overexpressing myc-tagged p50-dynamitin (Figure 5B, right panel). In both experiments, cellular Par6α levels were unaffected (Figure 5C), which demonstrates that blocking dynein–dynactin motor function does not cause Par6α degradation but induces its redistribution into the cytosol. These results demonstrate that Par6α is recruited to the centrosome by a microtubule and dynein–dynactin-dependent mechanism.

Par6α Regulates the Centrosomal Localization of $p150^{Glued}$

Because $p150^{Glued}$ was necessary for the localization of Par6α to the centrosome and centriolar satellites, we examined whether Par6α can bind to $p150^{Glued}$. In reciprocal coimmunoprecipitation experiments, we detected an interaction between Par6α, $p150^{Glued}$ and PCM-1 (Figure 6A), suggesting that these three proteins form a trimeric complex. As Par6α and $p150^{Glued}$ coimmunoprecipitated in the absence of PCM-1 (Figure 6B), we conclude that PCM-1 is not necessary for Par6α - $p150^{Glued}$ binding and that Par6α is more closely linked to $p150^{Glued}$ than PCM-1. Thus, Par6α and $p150^{Glued}$ appear to form the core of a protein complex that is important for centrosomal protein delivery.

To determine the functional significance of Par6α binding to $p150^{Glued}$, we tested the effects of Par6α depletion on $p150^{Glued}$ localization. Loss of Par6α caused a significant reduction in centrosomal $p150^{Glued}$ levels to 37.4 ± 1.2% of control levels (Figure 6, C, left panel, and D). In contrast, centrosomal $p150^{Glued}$ levels were unaffected in PCM-1—depleted cells and $p150^{Glued}$ levels remained at 94.8 ± 1.1% of control levels (Figure 6, C, right panel, and D). These experiments demonstrate that Par6α is critical for the centrosomal localization of $p150^{Glued}$, which itself is important for microtubule anchoring at the centrosome (Quintyne *et al.*, 1999).

DISCUSSION

In this study, we have identified a novel role for Par6α in the delivery of centrosomal proteins that function in microtubule organization during interphase and mitosis and ultimately control cell cycle progression. Our findings support a model in which Par6α binds to selected centrosomal cargo proteins, to the dynactin subunit $p150^{Glued}$, and to PCM-1 at centriolar satellites (Figure 7). This complex is then delivered to the centrosome via microtubule-dependent transport. On arrival, cargo proteins dissociate from the complex, integrate into centrioles and the pericentriolar matrix, and function in the control of microtubule organization (Figure 7A). In the absence of Par6α, PCM-1 and cargo proteins cannot associate with dynactin (Figure 7B). As a consequence, proteins with critical roles in centrosome function are not delivered to the centrosome, leading to abnormalities

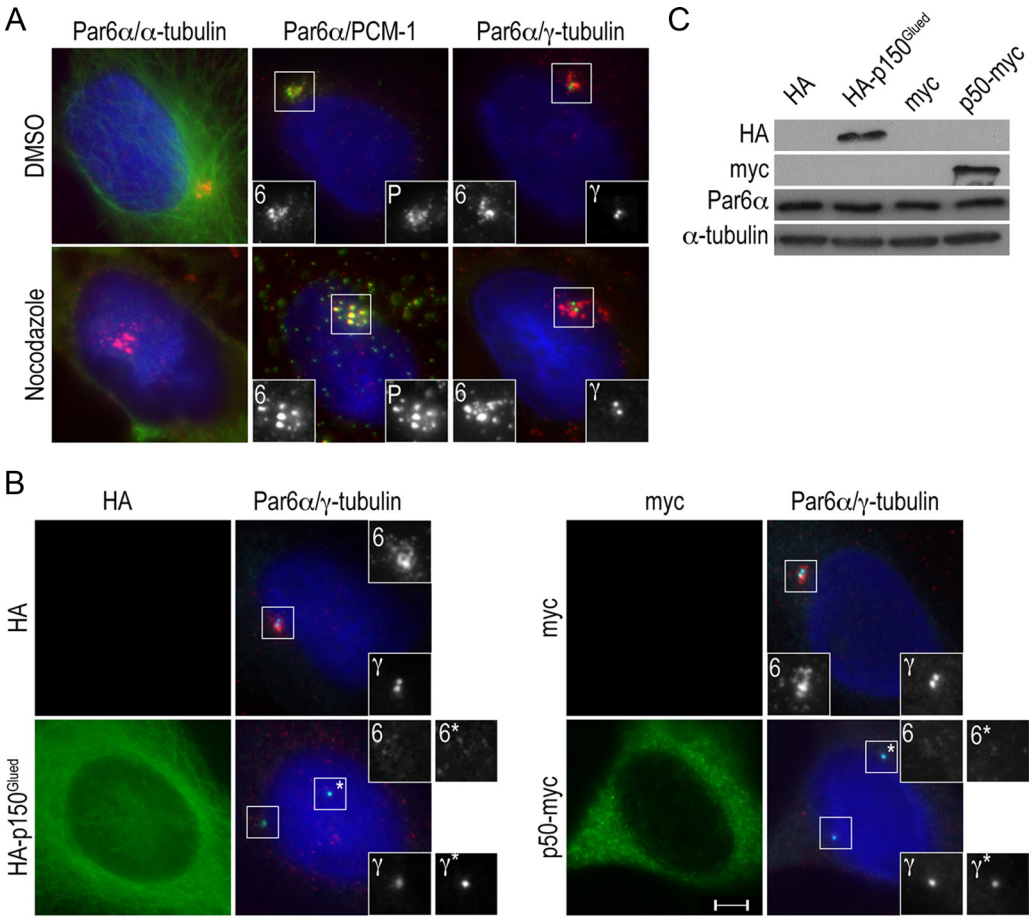

Figure 5. The centrosomal localization of Par6α depends on microtubules and the dynein–dynactin complex. (A) HeLa cells treated for 4 h with the microtubule-depolymerizing agent, nocodazole, or DMSO as a negative control were fixed and stained with antibodies to α-tubulin to verify microtubule depolymerization and to Par6α (6, red), PCM-1 (P, green) and γ-tubulin (γ, green) to monitor centrosomal protein organization. Insets show a magnification of the boxed area in the large image. (B) HeLa cells were transfected with HA-tagged p150^{Glued} (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) and Par6α (6, red). We also stained with antibodies to γ-tubulin (γ, cyan) to mark the position of the centrosome. The two highlighted areas in the main images show the two disconnected γ-tubulin foci which are magnified in the insets. Insets that are marked by an asterisk are magnified images of the boxed area that is labeled with the asterisk. Scale bar, 5 μm. (C) Transfected cell lysates were analyzed by Western blotting with antibodies to HA, myc, and Par6α. α-Tubulin served as a loading control.

in microtubule organization. In the absence of PCM-1, in contrast, Par6α, p150^{Glued}, and cargo molecules form a complex that is transported to the centrosome. However, because Par6α depends on PCM-1 for its retention at the centrosome, Par6α is mislocalized to the cytosol under these conditions (Figure 7C). Our model implicates Par6α with a role in centrosomal protein delivery in contrast to PCM-1, which may function in the retention of Par6α at the centrosome.

Our study provides strong support for Par6α as a centrosomal component in epithelial cells. We detected this specific Par6 isoform at the centrosome and centriolar satellites in three human cell lines using an antibody against an internal peptide that is unique to Par6α and not present in other Par6 isoforms (Gao and Macara, 2004). In addition, we verified the association of this protein with the centrosome by four different approaches. First, we probed HeLa cells with an antibody against the two Par6 isoforms, Par6α and Par6β, that revealed immunofluorescence staining at the centrosome (data not shown). Next, RNAi-mediated depletion of Par6α resulted in the specific loss of the 37-kDa Par6 isoform Par6α and the absence of Par6α at the centrosome. Third, Par6α colocalized with known centrosomal proteins when we fractionated whole HeLa cell lysates on a sucrose density gradient. Finally, Par6α interacted with centrosomal proteins, such as PCM-1, in coimmunoprecipitation experiments and in a mass spectrometry screen (Kodani, Huang, and Sütterlin, unpublished results). These findings are consistent with published reports on the role of this polarity protein in the regulation of centrosome function in neuronal and neutrophil-like cells (Solecki *et al.*, 2004; Xu *et al.*, 2007). However, our results differ from a study by Cline and Nelson (2007), who detected a Par6 isoform in the nucleus and cytosol of HeLa cells. Although Cline and Nelson named this Par6 isoform Par6α (or Par 6), it is likely that their analysis focused on the 45-kDa Par6 isoform Par6β, instead of the 37-kDa isoform Par6α.

Our findings implicate Par6α with the regulation of centrosome organization and function. Specific centrosomal proteins, including PCM-1, pericentrin, ninein, and Cep170, which have known roles in the organization and dynamics of centrosomal microtubules (Li *et al.*, 2001; Dammermann and Merdes, 2002; Guarguaglini *et al.*, 2005), were unable to associate with the centrosome in the absence of Par6α. Consistent with this mislocalization phenotype, Par6α-depleted cells contained severely disorganized interphase microtu-

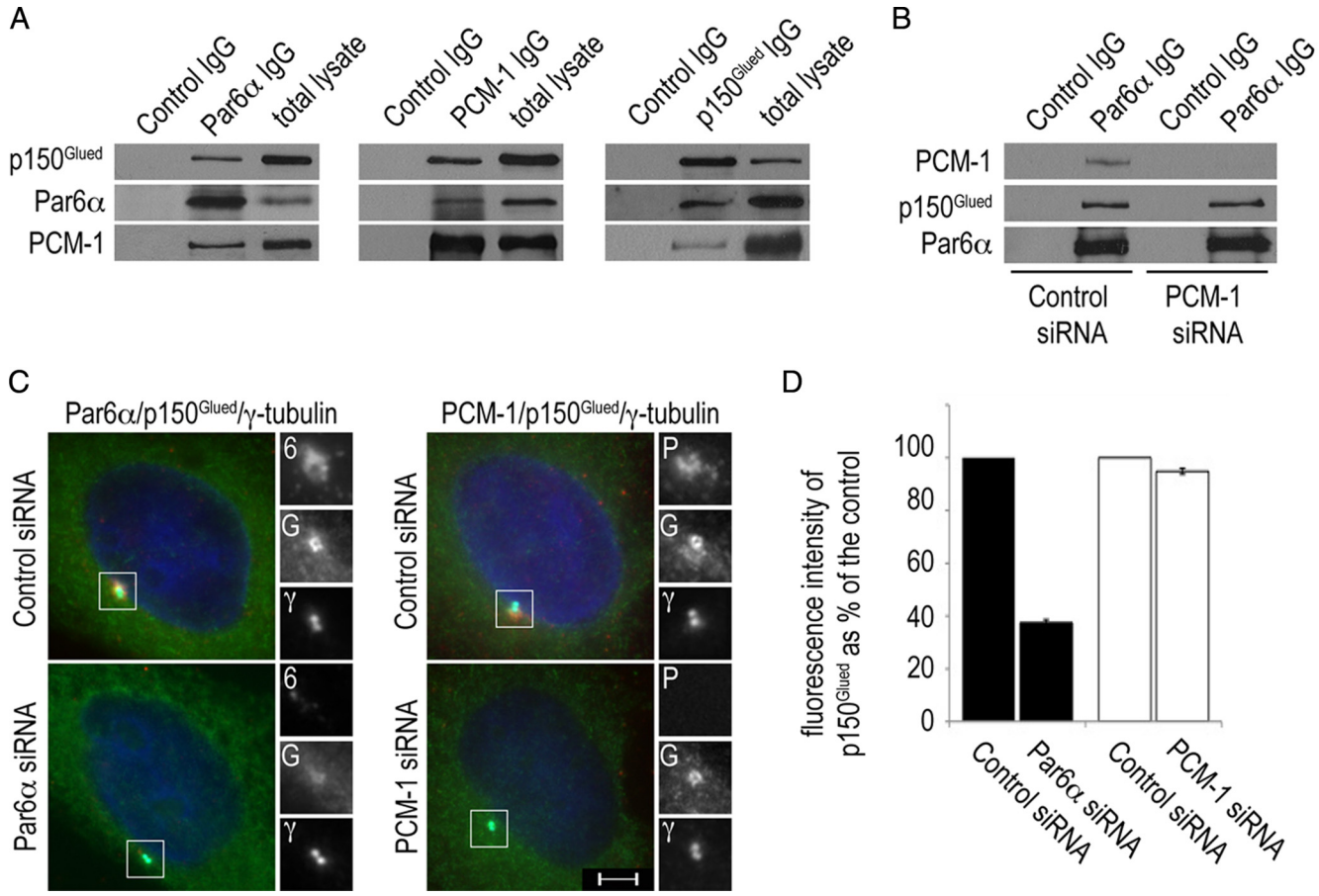

Figure 6. Par6α interacts with p150^{Glued} and is required for its centrosomal localization. (A) Total HeLa cell lysates were subjected to immunoprecipitations with antibodies to Par6α (Par6α IgG), PCM-1(PCM-1 IgG) p150^{Glued} (p150^{Glued} IgG). Antibodies to β-PIX or the FLAG-epitope (control IgG) were used as negative controls. Par6α, PCM-1, and p150^{Glued} in these samples were detected with specific antibodies. (B) Par6α, p150^{Glued}, or β-PIX as a negative control, were immunoprecipitated from control or PCM-1—depleted HeLa cells. Immunoprecipitates were analyzed by Western blotting with antibodies to Par6α, p150^{Glued}, and PCM-1. (C) Control or Par6α-depleted HeLa cells were stained with antibodies to Par6α (red) and to p150^{Glued} (green). γ-Tubulin staining (cyan) revealed the position of the centrosome in the same cell (left panel). In a parallel experiment, control or PCM-1—depleted cells were stained with antibodies to PCM-1 (red), p150^{Glued} (green), and γ-tubulin (cyan). Scale bar, 5 μm. (D) Quantifications of the fluorescent intensity are shown as percentage of the fluorescent signal at the centrosome of control siRNA-transfected cells. Fifteen cells were analyzed per experiment, and three independent experiments were performed (*$p < 0.005$).

bules. We also observed dramatic effects of Par6α depletion on mitotic microtubule organization with abnormal spindles in more than 90% of mitotic cells. Because Par6α does not associate with mitotic spindle poles, it is likely that these spindle defects are the consequence of centrosome defects in interphase. Mislocalized centrosomal proteins in the cytosol may be sufficient to induce the formation of acentrosomal asters that may be able to form extra spindle poles upon entry into mitosis. In support of this model, we previously found that dysregulation of centrosome organization can produce spindle abnormalities (Kodani and Sutterlin, 2008; Kodani *et al.*, 2009).

We propose that Par6α binding to p150^{Glued} is important for protein recruitment to the centrosome. There are at least three different mechanisms to explain how this interaction could promote centrosomal protein delivery. First, Par6α binding to p150^{Glued} may link centrosomal cargo proteins to the dynein–dynactin motor complex. Thus, Par6α would function as a cargo receptor, which is consistent with the observed mislocalization of specific centrosomal proteins in the absence of Par6α. However, it is not known whether Par6α has direct interactions with specific centrosomally targeted proteins. Alternatively, Par6α may bind p150^{Glued} to modify interactions with microtubules, dynein, or the centriolar satellite proteins BBS4 and Cep290. As each of these factors contributes to centrosomal protein delivery (Quintyne and Schroer, 2002; Kim *et al.*, 2004; Burakov *et al.*, 2008), it is possible that Par6α-dependent regulation of these interactions may promote the assembly of proteins at the centrosome. Finally, Par6α–p150^{Glued} interaction may control the localization and retention of p150^{Glued} and the entire dynactin complex at the centrosome, which is critical for centrosome organization and function (Quintyne *et al.*, 1999; Quintyne and Schroer, 2002). Additional studies on Par6α and its binding partners will be necessary to define the specific role of this protein in centrosomal protein delivery.

Although we have identified a complex of Par6α, PCM-1 and p150^{Glued}, many features of this complex remain to be determined. For example, it is not known if Par6α binds PCM-1 and p150^{Glued} directly or if this complex contains additional factors. Good candidates include the centriolar satellite proteins BBS4 and Cep290 (Kim *et al.*, 2004; Kim *et al.*, 2008). BBS4 binds to PCM-1 and p150^{Glued}, is required for centrosome organization and function (Kim *et al.*, 2004), and is mislocalized in Par6α-depleted cells (data not shown). Similarly, Cep290 is reported to bind to PCM-1 and the dynactin subunits, p150^{Glued} and p50 (Chang *et al.*, 2006;

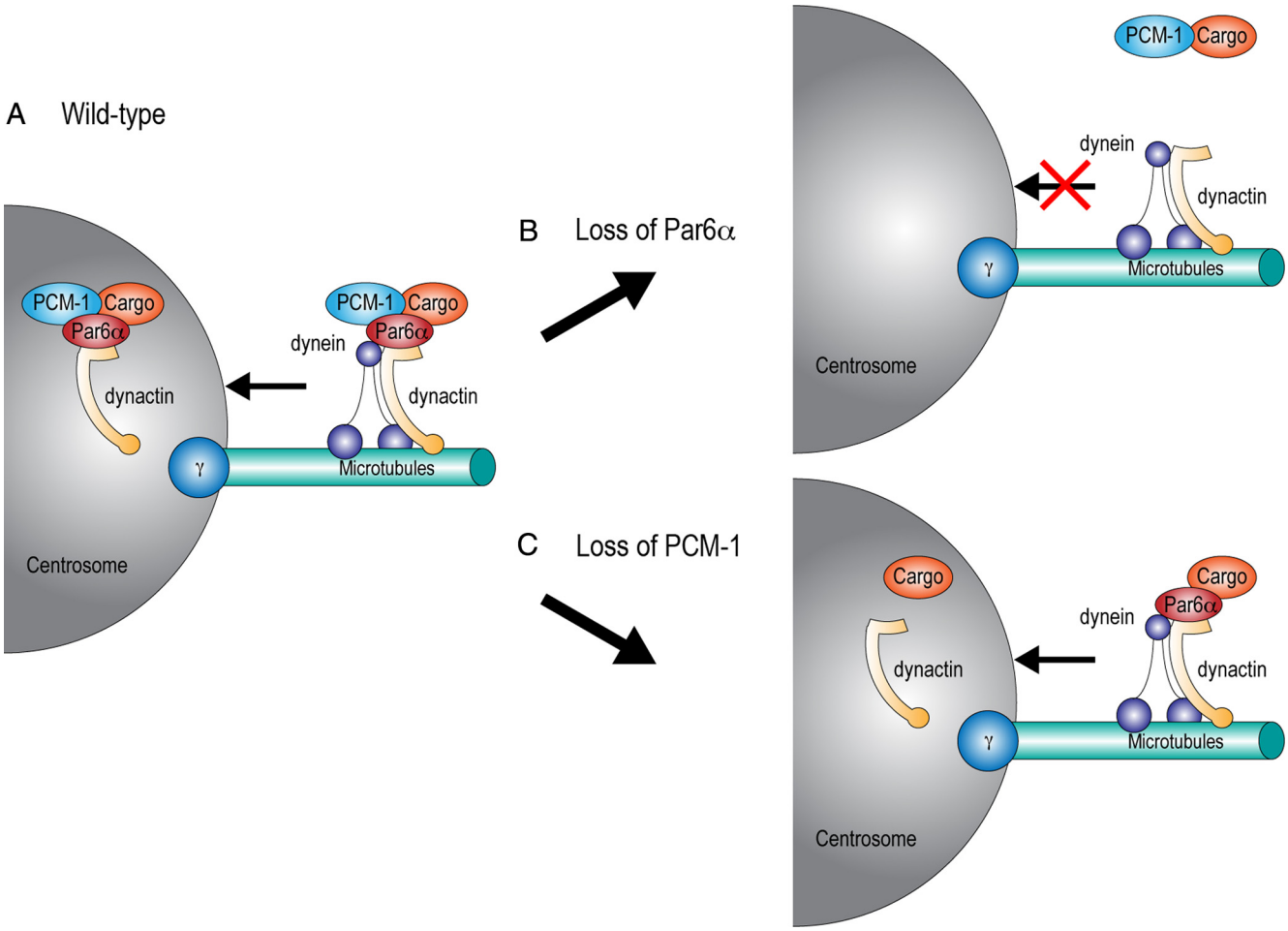

Figure 7. Model of Par6α function at the centrosome. (A) Par6α associates with PCM-1, the dynactin subunit, p150^{Glued}, and cargo proteins at centriolar satellites. This complex is loaded onto the dynein motor complex for transport to the centrosome. (B) In Par6α-depleted cells, PCM-1 and p150^{Glued} are unable to associate with the dynein motor complex resulting in a bloc in cargo delivery to the centrosome. (C) In PCM-1—depleted cells, Par6α associates with p150^{Glued} and cargo proteins, which can associate with dynein for transport to the centrosome. However, in the absence of PCM-1, Par6α is not retained at the centrosome.

Kim *et al.*, 2008). Other possible components include known Par6α-interacting proteins, such as Par3 and aPKC. Although a role for these proteins in centrosome assembly has not been tested, a recent study reported an association between Par3 and dynein, which is important for the regulation of microtubule dynamics and centrosome orientation during cell migration (Schmoranzer *et al.*, 2009).

Our data argue against a direct role of PCM-1 in Par6α-dependent centrosome regulation. We found that Par6α, but not PCM-1, binds to p150^{Glued}. In addition, we showed that efficient depletion of PCM-1, for 48 h with siRNA against a published sequence in the PCM-1 cDNA (Kim *et al.*, 2008), had no effect on centrosome organization and function and, thus, was not equivalent to Par6α depletion. Our findings, however, are in disagreement with reports by Dammermann and Merdes (2002), who detected defects in centrosome composition and microtubule organization when they used a different siRNA sequence to deplete PCM-1 for 96 h. A possible explanation for these discordant results is that prolonged knockdown of PCM-1 has an indirect effect on centrosome assembly by inducing the mislocalization of Par6α.

In summary, our results support a role for Par6α in the regulation of centrosome assembly, which is important for centrosomal microtubule organization during the cell cycle. We propose a model in which this novel function of Par6α depends on the interaction of Par6α with the dynactin subunit p150^{Glued}. However, further analysis of the Par6α interaction network will be necessary to understand the precise role of this polarity protein in centrosomal protein delivery.

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