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Cell Cycle-Dependent Localization of Dynactin Subunit p150^{glued} at Centrosome

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ABSTRACT

p150^{glued} is the largest subunit of dynactin protein complex, through which cargo vesicles link to the microtubule minus-end directed motor protein dynein. In addition, p150^{glued} also locates in the mother centriole where it organizes the subdistal appendage. The components of appendage are dynamically regulated throughout the cell cycle stages, but it is still unclear whether the centrosomal residency of p150^{glued} correlated with cell cycle progression. Here we found that p150^{glued} was located in the mother centriole during G1/S stage and its centrosomal residency was independent of microtubule transportation. However, the centrosomal p150^{glued} became blurred at G2/M phase and this event was not regulated by its phosphorylation. Entering into mitosis, p150^{glued} was robustly enriched in the mitotic spindle nearby the spindle poles but not in the centrosome. During serum starvation (G0 stage), p150^{glued} appeared at the base of primary cilium and its depletion attenuated starvation-induced primary cilium formation. We also checked its role in the maintenance of centrosome homeostasis and configuration, and found depletion of p150^{glued} did not induce centrosome amplification or splitting but inhibited U2OS cell growth. G1 arrest and reduced EdU incorporation were observed in p150^{glued} deficient U2OS cells. In addition, cyclin E was downregulated following p150^{glued} depletion. The p53/p21 signaling was activated indicating that CDKs were inactivated. The reduced cell growth was ameliorated in the p150^{glued} depleted cells when treated with p53 inhibitor. Thus, we have identified the centrosomal targeting of p150^{glued} in distinct cell cycle stage and uncovered its role in controlling G1/S transition. J. Cell. Biochem. 116: 2049–2060, 2015.

KEY WORDS: p150^{glued}; MOTHER CENTRIOLE; CENTROSOME; CELL CYCLE; MITOSIS

The centrosome is a non-membrane bound organelle juxtaposed to the nucleus. It is a tiny organelle composed of a pair of centrioles, mother and daughter centrioles, as well as the surrounding pericentriolar material (PCM), serving as the microtubule nucleation site that establishes microtubule networks during interphase [Doxsey, 2001]. At mitosis, the duplicated centrosomes form mitotic spindle poles that orchestrate the mitotic spindle for the proper separation of chromosomes into two daughter cells. Thus centrosome plays an important role in orchestrating microtubule networks and mitotic spindle.

Centriole is a microtubule-based cylinder structure in nine triplet array. Mother centriole, which is decorated with appendages, serves as microtubule anchoring and primary cilium nucleating site [Lange et al., 2000]. Based on the structure of the mother centriole, the appendages can be subdivided into distal and subdistal appendages depending on their decoration sites. Distal appendage is critical for the initiation of ciliogenesis [Schmidt et al., 2012], while the subdistal appendage functions as microtubule anchoring site to maintain microtubule array [Mogensen et al., 2000].

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Centriole duplicates coordinately with DNA replication [Hinchcliffe and Sluder, 2001]. Like DNA replication, the duplication of centriole is tightly restricted to once per cell cycle. When cells enter S phase, CDK2 triggers the replication processes of DNA and centrosome [Meraldi et al., 1999]. PLK4 is recruited to the basal–lateral side of the pre-existing centriole. By using the old centriole as a platform, the new pro-centriole grows in the orthogonal orientation when centriole starts to duplicate [Habedanck et al., 2005]. The components of centrioles, such as SAS6 and CPAP, are added into the new pro-centriole in a sequential order throughout the G2 phase to form a complete centriole [Kleylein-Sohn et al., 2007]. Entering into mitosis, centrosomes recruit more γ-tubulin-ring complexes to the PCM for facilitating microtubule nucleation and move to the opposite site of the nucleus where they orchestrate mitotic apparatus. At the end of mitosis, the orthogonal orientation of the centrioles is disengaged, permitting centrosome duplication in the next cell cycle [Tsou and Stearns, 2006].

Once centrioles duplicate, the old daughter centriole will be decorated with appendages and becomes the new mother before the end of mitosis. This process ensures each divided daughter cell contains one mother centriole. Entering into quiescent stage (G0 phase), the mother centriole functions as a nucleation site for the growth of primary cilium, which is a immotile cilium functions as an antenna to receive signals from the environment [Michaud and Yoder, 2006]. When cells are stimulated by growth factors, cells will re-enter cell cycle meanwhile the primary cilium will be dis-assembled. Defects in cilium disassembly lead to delay mitosis entry. Thus, the mother centriole plays many roles in microtubule nucleation, ciliogenesis, and cell cycle progression.

$p150^{glued}$, the largest subunit of dynactin, stably associates with microtubule-activated dynein ATPase and functions as a motor protein to transport intracelluar cargos [Gill et al., 1991; Paschal et al., 1993; Waterman-Storer et al., 1995]. The $p150^{glued}$-mediated perinuclear transportation is required for lysosome maturation and activation [Li et al., 2014]. In the neuron, the retrograde transportation of axonal organelles is driven by $p150^{glued}$, this event is critical for the prevention of neurodegenerative diseases [Perlson et al., 2010]. For example, p150 mutants found in patients impact the subcellular localization of p150 in motoneurons [Stockmann et al., 2013]. However, mutations within the microtubule-binding domain of $p150^{Glued}$ might be the contributory risk factors rather than a causative for Amyotrophic Lateral Sclerosis [Dixit et al., 2008]. In addition, $p150^{glued}$ also resides and maintains the structure of subdistal appendage of the mother centriole [Askham et al., 2002; Kodani et al., 2013]. Its depletion leads to disorganization of the subdistal appendages, microtubule disorganization, chromosome misalignment, and defect in prometa–metaphase transition [Askham et al., 2002; Ozaki et al., 2011; Lazarus et al., 2013].Thus $p150^{glued}$ regulates microtubule-based cellular transport and maintains the structure of subdistal appendage.

In this study, we found $p150^{glued}$ located to the centrosome in a cell cycle-dependent manner. At G1/S phase, $p150^{glued}$ was abundant in the centrosome. During G2/M phase, centrosomal $p150^{glued}$ became blurred and mainly located in the minus-end of microtubules; this process was independent of the phosphorylation status of $p150^{glued}$. When entering into mitosis, $p150^{glued}$ was highly enriched at the mitotic spindle, but absence from the spindle poles. In addition, depletion of $p150^{glued}$ attenuated ciliogenesis but did not affect centrosome homeostasis. We also found depletion of $p150^{glued}$ downregulated the expression of cyclin E and activated p53/p21 signaling followed by G1 arrest and reducing cell growth.

MATERIALS AND METHODS

CELL CULTURE

Human hTERT-immortalized retinal pigment epithelial RPE1 cells were grown in Dulbecco's modified Eagle medium (DMEM)-F12 medium and human osteosacroma U2OS cells were grown in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere at 5% CO_2. These cells were examined for the free of mycoplasma contamination by immunofluorescence regularly, and DAPI staining according to the guidelines. For cell cycle synchronization, U2OS cells were synchronized at G0/G1 phase by culturing in serum-free medium for 48 h and at G2/M phase by treating with nocodazole at the concentration of 1 μM for 18 h according to the published method [Cotto-Rios et al., 2011]. Before analysis, nocodazole were washed out and completed medium were added for an additional 1 h for clear microtubule staining.

RNAI AND PLASMID OVEREXPRESSION

$p150^{glued}$ of U2OS and RPE1 cells were depleted using annealed siRNA with the target sequence:

sip150: 5′-gccuugaacaguuccauca [dt] [dt]-3′ [Delgehyr et al., 2005]

Scrambled siRNA with the target sequence: 5′-gaucauacgugcg-aucaga [dt] [dt]-3′ was purchased from Sigma (Sigma, St. Louis, MO).

For siRNA transfection, 10 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were mixed first with 500 μl Opti-MEM medium (Life Technologies, Grand Island, NY) for 5 min, then with 2 μl siRNA (100 μM) in 500 μl Opti-MEM medium, incubated at room temperature for 20 min before the mixture was layered onto cells in 1 ml DMEM or DMEM/F12 medium (100–nM working concentration). Cells were harvested for immunofluroescence and immunoblotting 48 h after transfection.

For $p150^{glued}$ overexpression, Wild type $p150^{glued}$ fused with EGFP (GFP-WT-p150) was kindly provided by Dr. David Stephens (University of Bristol, [Watson and Stephens, 2006]). Mutant form of $p150^{glued}$ (T145-147E, GFP-Glu-p150) [Zhapparova et al., 2013] was amplified by site directed mutagenesis primers which substitute three threonines for glutamates (underlined):

(Forward) 5′- AGGCACCGACAGCCCGAAAGGAAGAAGAACGGC-GACCCAAGCCCACGCG -3′

(Reverse) 5′- CGCGTGGGCTTGGGTCGCCGTTCTTCTTCCTTTCGG-GCTGTCGGTGCCT -3′ and cloning primers that contain restriction site (XhoI/EcoRI, underlined):

(Forward) 5′- GATCTCGAGTGGCACAGAGCAAGAGGCAC -3′

(Reverse) 5′- GCAGAATTCTTAGGAGATGAGGCGACTGT -3′.

The PCR were then performed with Expand High Fidelity PCR System (Roche, Mannheim, Germany) by using WT $p150^{glued}$ as the template. The PCR product was then cut with XhoI and EcoRI (Promega, WI) and then inserted into pEGFP-C1 (Clontech, Mountain View, CA).

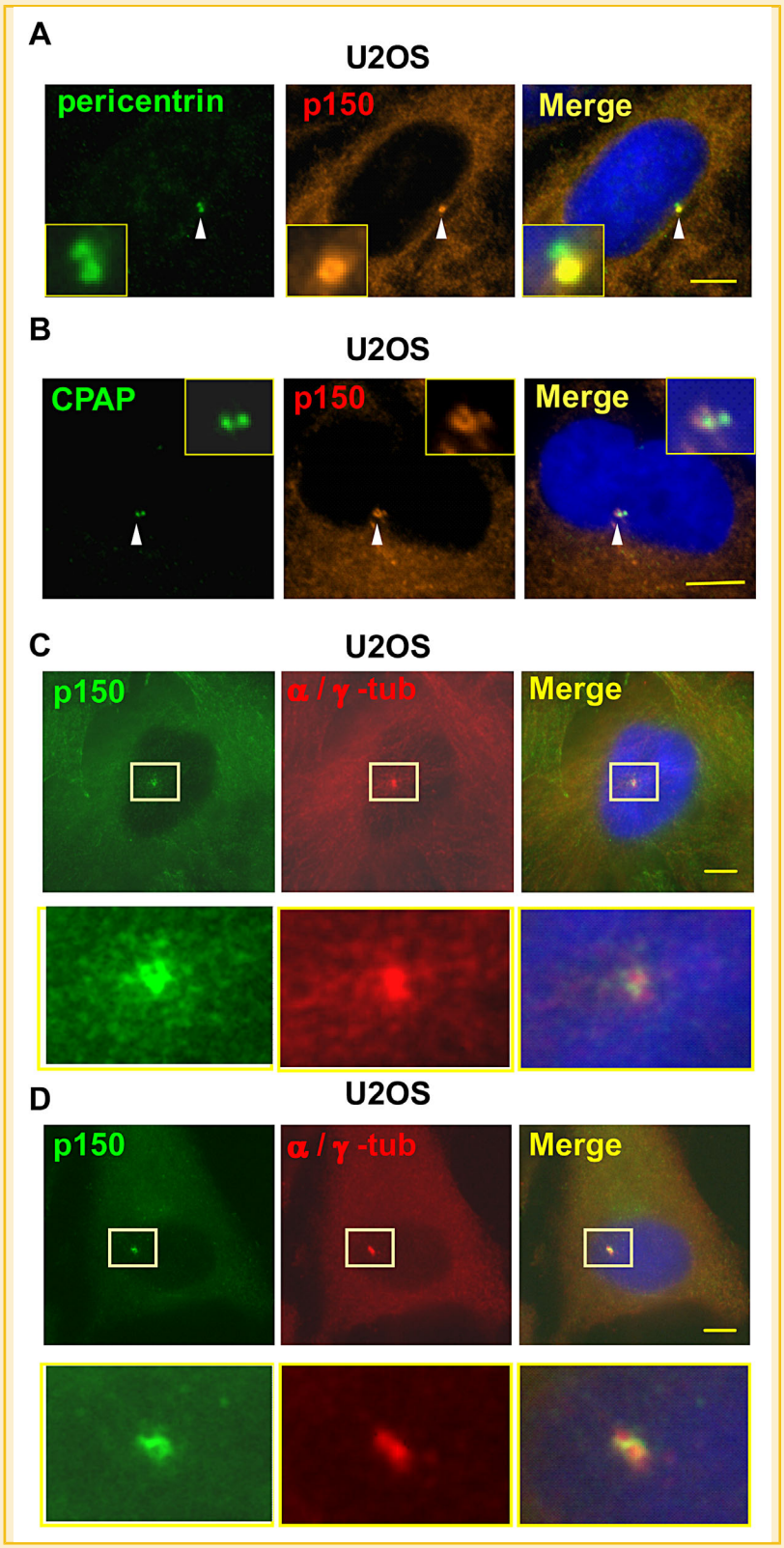

Fig. 1. Centrosomal targeting of $p150^{glued}$ does not depend on microtubule transport. (A–B) Asymmetric distribution of $p150^{glued}$ on the centrosomes (A) and centrioles (B). Immunostaining of U2OS cells with antibody against $p150^{glued}$ (p150) and pericentrin (A) or CPAP (B). Centrosomal targeting of $p150^{guled}$ was checked by double staining of normal (C) or microtubule-depolymerized U2OS cells (D) with antibodies against $p150^{guled}$ (p150, green) and α-tubulin and γ-tubulin (α/γ-tub, red). DNA was stained with DAPI. Scale bars are 5 μm. Lower panels are magnification of centrosome.

ANTIBODIES

The following antibodies were obtained commercially: anti-γ-tubulin, anti-α-tubulin (mouse), anti-cyclin A2, anti cyclin D1, and anti-acetylated-tubulin (Sigma, St. Louis, MO), anti- $p150^{glued}$ (BD Biosciences, San Jose, CA), anti-Cep170 (Bethyl, Montgomery, TX), anti-Cep164 (Novus, Littleton, CO), anti-Arl13b (Proteintech, Chicago, IL), anti-cycin B1 (Cell Signaling, Beverly, MA), anti-centrin 20H5 (Millipore, Billerica, MA), anti-CP110 and anti-pericentrin (Abcam, Cambridge, UK), anti-Ku70 and anti-α-tubulin (rabbit) (Genetex, Trvine, CA), anti-actin (Santa Cruz Biotech, Santa Cruz, CA). The polyclonal anti-CPAP antibody has been described previously [Tang et al., 2009].

IMMUNOFLUORESCENCE MICROSCOPY

Cells were grown on glass coverslips at 37°C before fixation with ice-cold methanol at −20°C for 6 min. To depolymerize microtubules, cells were treated with 30 μM nocodazole on ice for 1 h, followed by fixation with ice-cold methanol for 5 min. After blocking with 5% BSA for 1 h, cells were incubated with antibodies for 24 h at 4°C. After extensive washing with PBS, cells were incubated with fluorescein isothiocyanate-conjugated and Cy3-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h in the dark. The nuclei were stained with 4′, 6-diamino-2-phenylindole (DAPI, 0.1 μg/ml) simultaneously. After extensive washing, the coverslips were mounted in 50% glycerol on glass slides. Fluorescent cells were examined with an AxioImager D2 fluorescence microscope (Zeiss, Switzerland). The number of centrosomes from more than 100 cells was counted under the microscope in three independent experiments and shown as mean ± standard deviation. Student's *t* test was performed to analyze the difference between different groups as indicated.

MTT ASSAY

Following siRNA transfection, cells were washed with PBS followed by adding 1 ml MTT solution (2 mg/ml in PBS) in each well. After incubation for 3 h at 37°C, 2 ml DMSO was added and incubation in the dark for additional 30 mins. Absorbance was measured at the wavelength of 570 nm.

CELL GROWTH ASSAY AND WESTERN BLOTTING ANALYSIS

U2OS cells (5×10^4) were grown on 3 cm dish followed by siRNA transfection. Then, 24 h after transfection, transfected medium were removed and new growth medium were added for an additional 24 h. Cells were trypsinzed and resuspended in PBS for cell number counting or centrifuged for further Western blotting analysis. Centrifuged cells were further lysed with lysis buffer containing 0.5% NP-40, 300 mM NaCl, 1mM EDTA, and the protease inhibitor cocktail (Roche, Mannhein, Germany) followed by centrifugation (15,000 rpm, 4°C). Supernatant was collected and further analyzed by Western blotting.

CELL CYCLE ANALYSIS

The cell cycle profile was analyzed by fluorescence-activated cell sorting (FACS) according to published method [Wang et al., 2015]. Briefly, cells were collected by trypsinization and re-suspended with PBS. Following centrifugation at 1,000 rpm for 5 min, cells were re-suspended with PBS-E (1 mM EDTA in PBS). After centrifugation, the pellet was re-suspended with 0.5 ml PBS-E and fixed with ice-cold 70% ethanol overnight at 4°C. Fixed cells were washed with PBS-E and stained with propidium iodide (PI, SouthernBiotech, Birmingham, AL) at room temperature for 1 h. DNA content of PI stained cells was measured by FACScan (Becton-Dickinson, San Diego, CA) and further analyzed by Kaluza software (Beckman Coulter, Brea, CA).

RESULTS

CENTROSOMAL TARGETING OF $P150^{GLUED}$ IS INDEPENDENT OF MICROTUBULE

It is known that $p150^{glued}$ is located in the microtubule and centrosome, especially on the subdistal appendage of the mother centriole. As microtubule arrays might affect targeting of centrosomal proteins, first we checked whether centrosomal localization of $p150^{glued}$ depended on the microtubule transport. During interphase, two pericentrin (marker of PCM) was observed but only one was decorated with $p150^{glued}$ (Fig. 1A). This asymmetric distribution of $p150^{glued}$ was further confirmed by CPAP (marker of centriole) staining (Fig. 1B). In addition, the targeting of $p150^{glued}$ to the mother centriole was confirmed by co-staining of p150glued with other mother centriolar markers Cep164 and Cep170 (Fig. S1A and B, G1/S phase). Thus $p150^{glued}$ was located in the mother centriole. To check whether centrosomal targeting of $p150^{glued}$ depended on microtubule, the microtubule networks was depolymerized by ice-cold treatment and the centrosomal targeting of $p150^{glued}$ was checked. Under normal condition, $p150^{glued}$ mainly located in the centrosome as shown by co-localized with γ-tubulin (Fig. 1C). When microtubule was depolymerized, however, $p150^{glued}$ was still localized in the centrosome indicating that centrosomal targeting of $p150^{glued}$ was independent of microtubule transport (Fig. 1D).

CENTROSOMAL $P150^{GLUED}$ IS REDUCED BEFORE CELLS ENTER MITOSIS

The centrosomal targeting of $p150^{glued}$ was further examined by different cell cycle stages. During G1/S phase (before centriole duplication), $p150^{glued}$ was co-localized with microtubule and enriched in the centrosome (Figs 2A and C). By G2/M phase, during which centrosomes were duplicated with the increased microtubule nucleation activity, the centrosomal $p150^{glued}$ became blurred and the signal of $p150^{glued}$ extended from the centrosome to the minus-end of microtubule in U2OS cells (Fig. 2B and C). This dynamic shift of $p150^{glued}$ was further confirmed by the double-staining of $p150^{glued}$ with Cep164 in RPE1 cells (Fig. S1A, G2/M phase). The centrosomal targeting of $p150^{glued}$ was also checked in synchronized cells. U2OS cells were arrested at G0/G1 stage by serum starvation for 48 h and found that $p150^{glued}$ localized to the centrosome (Fig. 3A). However, when cells were arrested in G2/M stage by nocodazole treatment for 18 h, centrosomal $p150^{glued}$ became blurred as compared with that of G0/G1 cells (Fig. 3B). These blurred $p150^{glued}$ signals were abolished when microtubule was depolymerized during G2/M transition in RPE1 cells (Fig. S1B–C). Thus, $p150^{glued}$ was concentrated in the centrosome during G1/S phase then shifted toward the minus-end of microtubule arrays by G2/M phase.

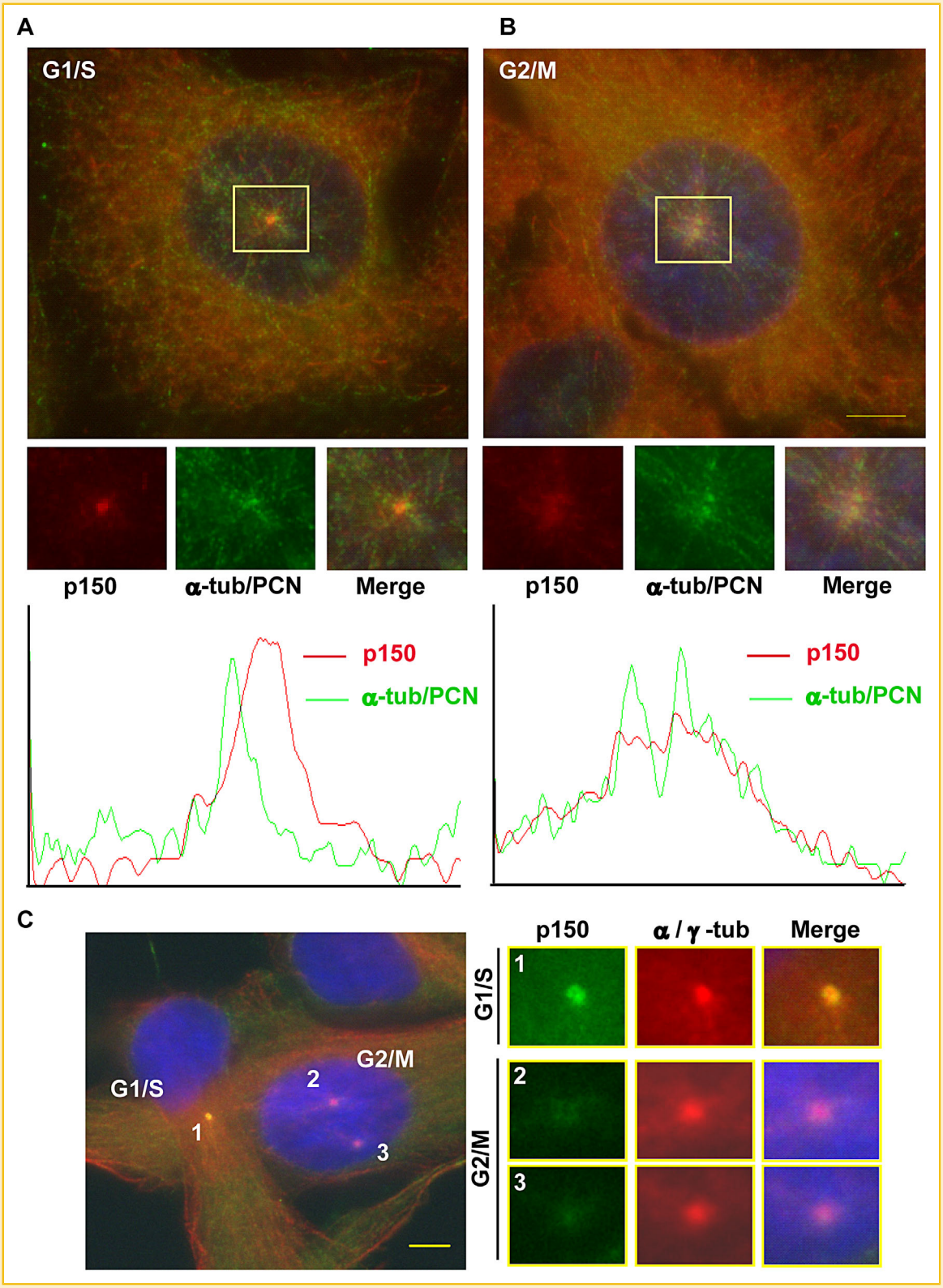

Fig. 2. p150^{glued} disappear from the centrosome during G2/M phase. The expression pattern of p150^{glued} was examined by immunostaining with antibodies against p150^{glued} (p150), α-tubulin and γ-tubulin (α/γ-tub, red) in G1/S (A, C) or G2/M (B, C) stages. The signals of p150^{glued}, α-tubulin, and γ-tubulin in (A) and (B) were quantified by Image J (lower panel). DNA was stained with DAPI. Scale bars are 5 μm. Lower panels are magnification of centrosome region.

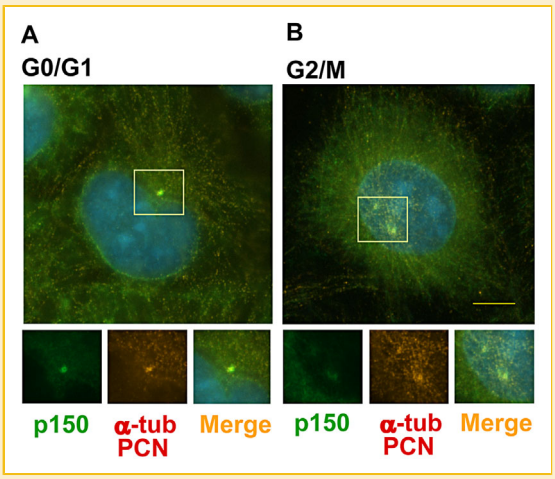

Fig. 3. The signal of p150^{glued} was checked in synchronized cells. U2OS cells were synchronized in G0/G1 (A) or G2/M (B) stages by serum starvation and nocodazole treatment, respectively. Centrosomal targeting of p150^{glued} was checked by immunostaining with antibodies against p150^{glued} (p150), α-tubulin and γ-tubulin (α/γ-tub, red). DNA was stained with DAPI. Scale bar is 5 μm. Lower panels are magnification of centrosome region.

It has been shown that p150^{glued} binds to the mitotic spindle [Rome et al., 2010], we further checked its localization in more detail. Entering into mitosis, p150^{glued} was colocalized with the mitotic spindle but was not associated with the centrosome (mitotic spindle poles, Fig. 4A–B). To further confirm this, the microtubule was depolymerized by ice-cold and found p150^{glued} was present throughout the cytoplasm (Fig. 4C). Thus p150^{glued} was located in the centrosome from G1 to G2 phase and the majority of centrosomal p150^{glued} released from the centrosome to the microtubule networks before G2/M transition. During mitosis, the microtubule-associated p150^{glued} was robustly recruited to the microtubule-based mitotic spindle and absent from the centrosome.

REDUCED CENTROSOMAL P150^{GLUED} DURING G2/M PHASE IS INDEPENDENT OF ITS PHOSPHORYLATION STATUS.

It is shown that the phosphorylation status of p150^{glued} affects its centrosomal targeting [Zhapparova et al., 2013]. The Ste20-like kinase phosphorylates p150^{glued} on several residues including Thr 145–147, phosphorylations on these residues prompt p150^{glued} to associate with centrosome. Thus we tested whether phosphorylation status of p150^{glued} affects its centrosomal targeting during G2/M phase. Phospho-mimetic p150^{glued} (GFP-Glu-p150) was generated by mutating Thr 145–147 into Glu followed by checking its centrosomal targeting in different cell cycle stages. Either wild-type (GFP-WT-p150) or GFP-Glu-p150 located in the centrosome during G1/S phase (Fig. 5A–B, upper panel). By G2/M phase, GFP-WT-p150 was reduced and became blurred in the centrosome, this phenomenon was also observed in GFP-Glu-p150 expressing cells (Fig. 5A–B, lower panel). Thus, even when p150^{glued} was forcibly phosphorylated, it was still released from the centrosome indicating that the dynamic of p150^{glued} during G2/M phase was independent of its phosphorylation status.

DEPLETION OF P150^{GLUED} INHIBITS PRIMARY CILIA FORMATION

Since mother centriole is the nucleation site of primary cilia [Kobayashi and Dynlacht, 2011], we further test whether p150^{glued} was still localized to mother centriole when primary cilia is formed. Primary cilia was induced by serum starvation of RPE1 cells for 24 h and the primary cilia was grown as shown by the staining of Arl13b (Fig. 6A). During ciliogenesis (G0 stage, under serum starvation), p150^{glued} was located in the base of primary cilia even when microtubules were depolymerized indicating that p150^{glued} was targeted to the mother centriole during G0 stage and this process was in a microtubule-independent manner. The effect of p150^{glued} on primary cilia formation was also checked by depletion of p150^{glued}. The abundance of p150^{glued} was reduced dramatically in siRNA-transfected RPE1 cells (Fig. 6B). 48 h after p150^{glued} depletion followed by serum starvation for an additional 24 h (Fig. 6C), the ciliogenesis was reduced in p150^{glued} deficient RPE1 cells (Fig. 6D). Thus p150^{glued} was recruited to the mother centriole during G0 phase and its depletion inhibited the formation of primary cilia.

We further checked the centrosome homeostasis as deficient of centrosomal proteins might induces centrosome amplification and centriole splitting [Lai et al., 2011; Wang et al., 2013, 2014]. The p150^{glued} was depleted for 72 h and the population of cells with multiple centrosomes (more than 2 γ-tubulin spots) was counted (Fig. 6E). Depletion of p150^{glued} by siRNA transfection in U2OS cells did not induce multiple centrosomes (Fig. 6F) or centriole splitting (data not shown). Thus, depletion of p150^{glued} did not affect centrosome copy numbers and configuration.

DEPLETION OF P150^{GLUED} LEADS TO G1 PHASE ARREST

Since centrosome is also important for the proper progression of cell division cycle, we further examined the growth of U2OS cells when p150^{glued} was depleted. Following p150^{glued} depletion for 72 h, the cell numbers were reduced dramatically; and this was further confirmed by MTT assay (Fig. 7A–B). Since p150^{glued} depletion affected cell growth, the cell-cycle profiles was further examined by flow cytometry after p150^{glued} depletion for three days. Three days after p150^{glued} depletion, the proportions of subG1 (apoptotic cells) and polyploid cells (>4N) were not changed indicating that depletion of p150^{glued} did not induce cell death or polyploidy (Fig. 7C). However, the proportion of G1 cells were increased, whereas cells in S and G2/M phase were reduced. These data indicated that 3-days depletion of p150^{glued} led to G1 arrest but did not induce apoptosis or genomic instability.

To further confirm the effect of p150^{glued} depletion on cell cycle progression, EdU cell proliferation assay was performed. The population of EdU positive cells was reduced in p150^{glued} deficient cells (Fig. 7D). In addition, G1 arrest often associate with decrease in percentage of mitotic positive cells. As expected, depletion of p150^{glued} blocked mitosis entry as shown by reduced mitotic index (Fig. 7E). Thus, p150^{glued} depletion led to reduced cell growth by increasing G1 arrest.

Cell cycle progression is regulated by the activity of CDKs, which are activated or inactivated by its binding partners or the inhibitors, respectively [Morgan, 1995]. The cell cycle-specific cyclins and inhibitors were checked following p150^{glued} depletion in U2OS cells. The levels of cyclin A and D were not changed (Fig. 8A). However,

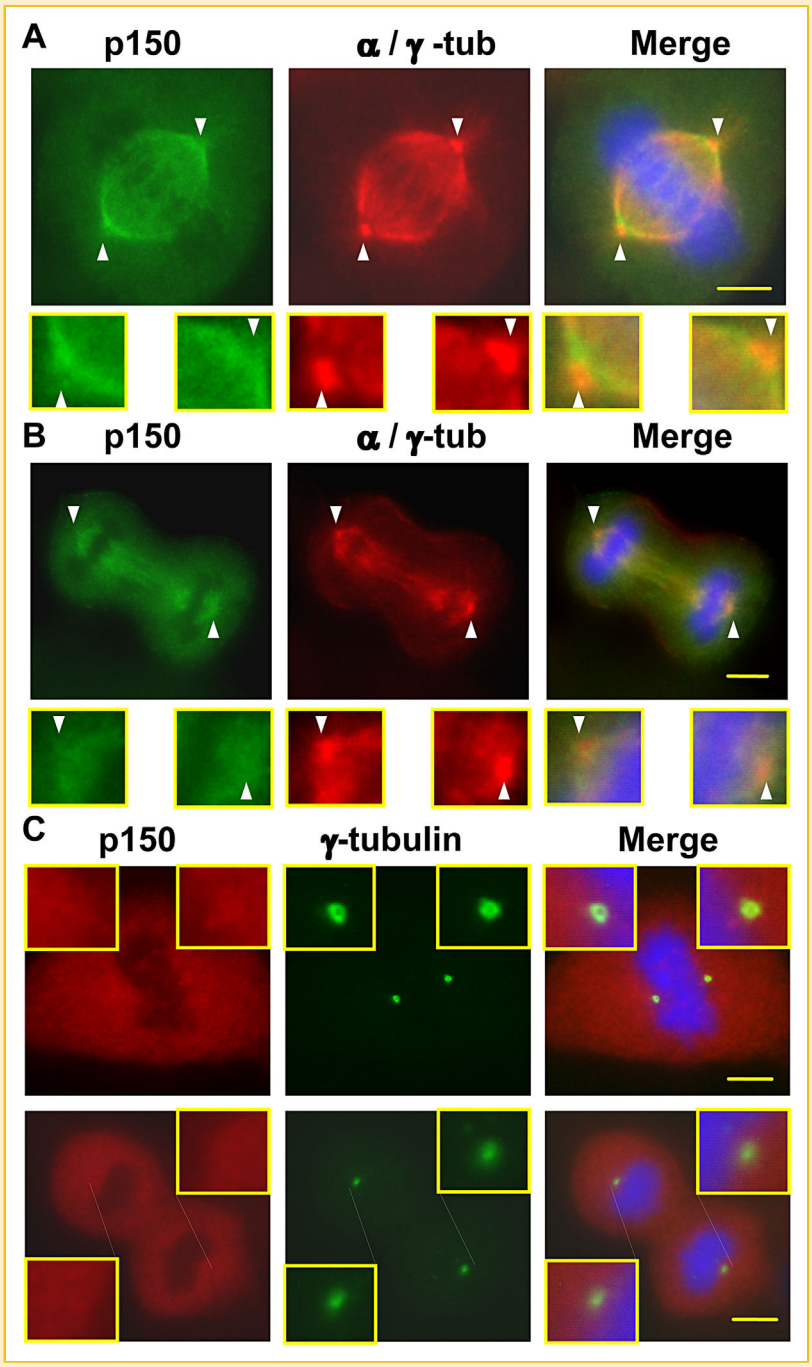

Fig. 4. $p150^{glued}$ does not locate to the centrosome during mitosis. (A–B) The expression pattern of $p150^{glued}$ was detected by double staining of U2OS cells with antibodies against $p150^{glued}$ (p150) and γ-tubulin during metaphase (A) or late mitosis (B). Lower panels are magnification of arrow head (mitotic spindle poles). (C) The expression pattern of $p150^{glued}$ was detected as that of in (A) and (B) when microtubule was depolyemerized by ice-cold treatment. Inserts are magnification of centrosome region. DNA was stained with DAPI. Scale bars are 5 μm.

cyclin E, which is required for G1/S transition, was reduced dramatically (Fig. 8B). In addition, p53 was also phosphorylated in $p150^{glued}$ deficient cells. This activation of p53 was important since the downstream transcript of p53, p21, the CDK inhibiotr, was also upregulated when $p150^{glued}$ was depleted (Fig. 8B). Thus, reduction of cycin E and upregulation of p53 induced G1 arrest in $p150^{glued}$ deficient U2OS cells. To further confirm the role of p53 in controlling cell growth upon $p150^{glued}$ depletion, pifithrin-α, the p53 specific inhibitor (p53i), was treated when $p150^{glued}$ was knocked down. Treatment of p53i itself did not affect cell growth; however, the reduction of cell growth was ameliorated in the $p150^{glued}$ depleted cells when treated with p53i (Fig. 8C). Thus, $p150^{glued}$ depletion induced cell cycle arrest was contributed by activation of p53 signaling.

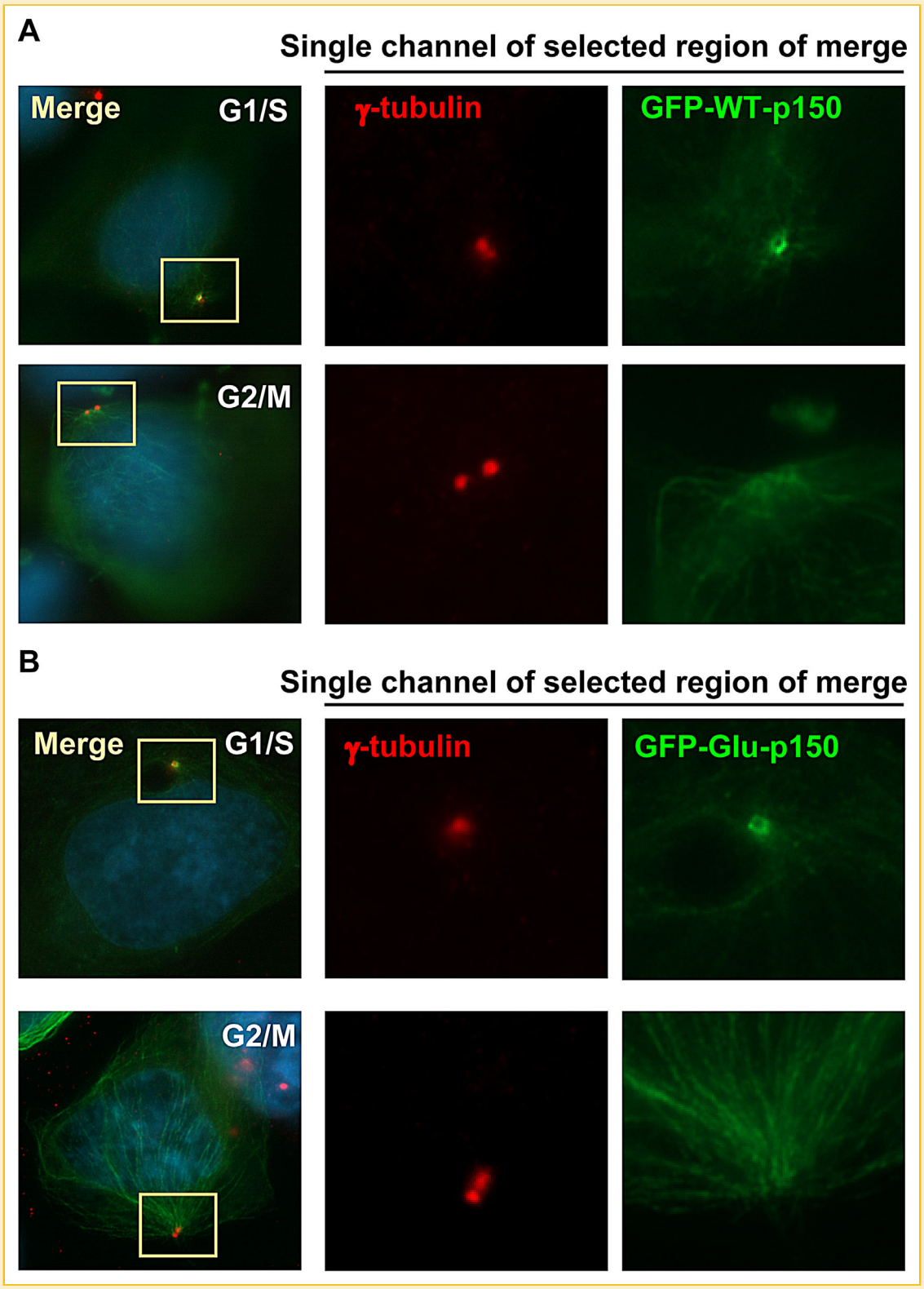

Fig. 5. Phosphorylation status of $p150^{glued}$ does not affect its release from centrosome during G2/M stage. U2OS cells were transfected with (A) wild-type (GFP-WT-p150) or (B) constitutive phosphorylated (GFP-Glu-p150) $p150^{glued}$ followed by double staining with antibodies against GFP and γ-tubulin at G1/S (upper panel) or G2/M (lower panel) stage. DNA was stained with DAPI.

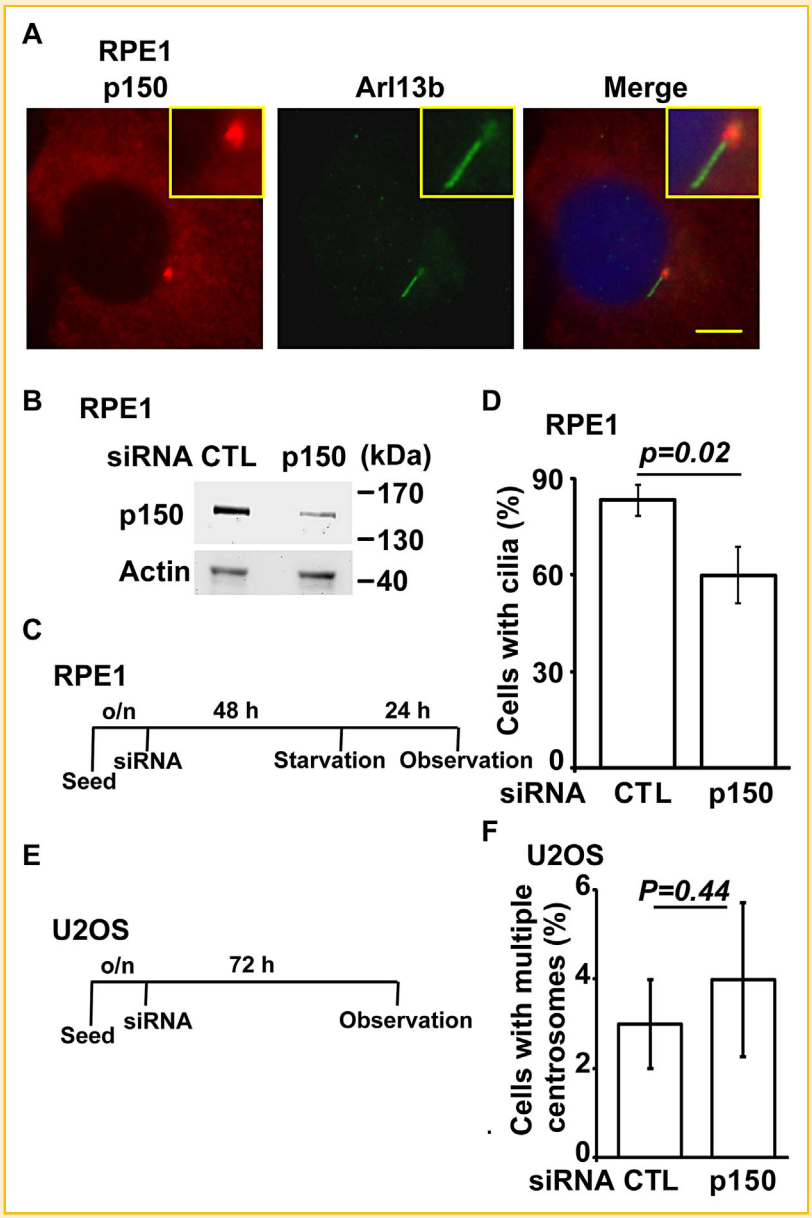

Fig. 6. Depletion of p150^{glued} does not affect primary cilium formation in RPE1 cells. (A) p150^{guled} located to the base of primary cilium. Double staining of U2OS cells with antibodies against p150^{glued} (p150) and Arl13b during serum starvation in RPE1 cells. DNA was stained with DAPI. Scale bars are 5 μm. Insects are magnification of primary cilium region. (B) p150^{guled} was depleted by siRNA efficiently. Extracts of RPE1 cells transfected with scrambled control (CTL) or p150^{guled} (p150) siRNA were analyzed by immunoblot with antibodies against p150^{guled} (p150) and actin. (C and E) Schematic representations of the experimental procedure used in D and F, respectively. Quantification of a population of siRNA-transfected RPE1 cells containing primary cilium (D) or U2OS cells with multiple centrosomes (F). These results are mean +/− SD from three independent experiments; more than 300 cells were counted in each individual group.

DISCUSSION

In this study, we found the dynamic association of p150^{glued} with centrosome in distinct cell cycle stages. In addition to microtubule, p150^{glued} was also recruited to the mother centriole at the G1/S phase and this centrosomal targeting of p150^{glued} was not abolished even when microtubule was depolymerized. However, the centrosomal p150^{glued} signal became blurred and released from the centrosome toward the minus-end of microtubule at G2/M phase. During mitosis, the signal of p150^{glued} was robustly emanated from, but not located in, the centrosome and extended along the spindle. In addition, we also found that reduced centrosomal p150^{glued} was independent of its phosphorylation status, since constitutive phosphorylated p150^{glued} (GFP-Glu-p150) still disappeared from the centrosome during G2/M phase. Furthermore, p150^{glued} was required for proper cell growth as depletion of p150^{glued} led to G1 arrest in a p53-dependent manner. Thus, p150^{glued} not only participated in the organization of radial microtubule, but affected proper cell cycle progression.

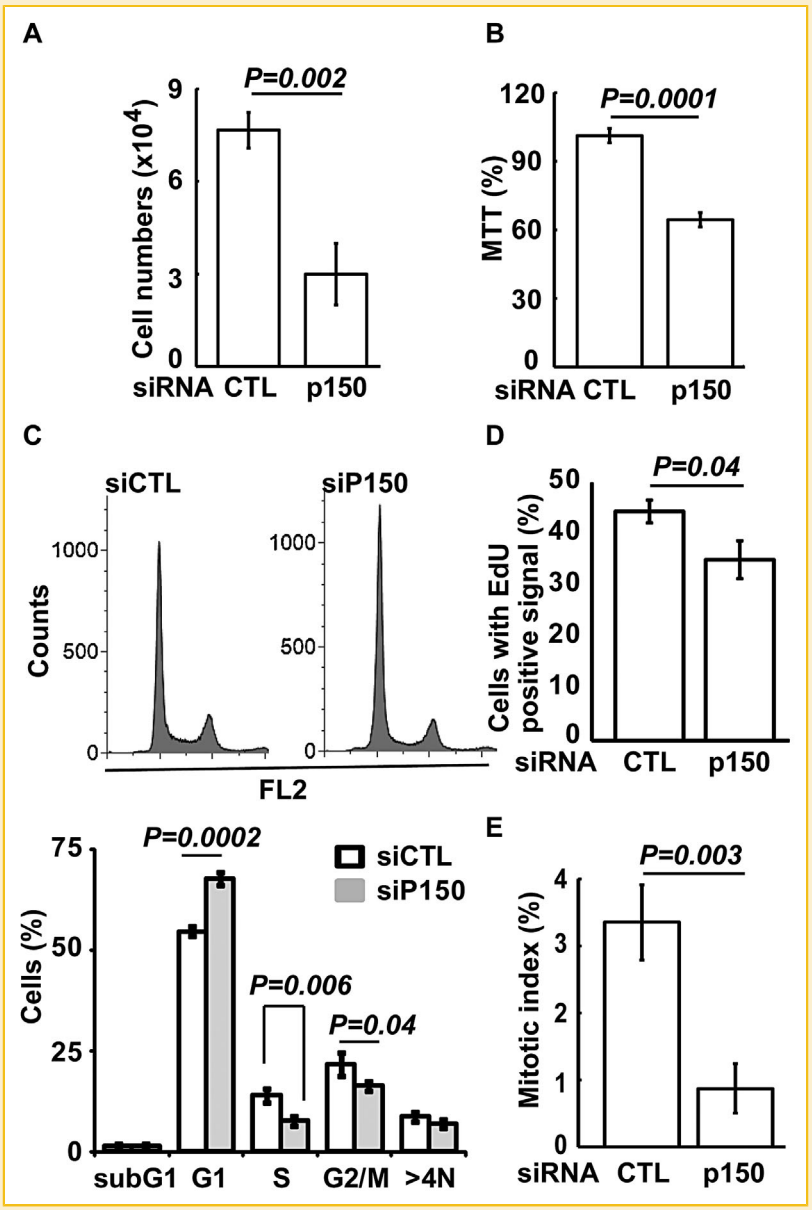

Fig. 7. Depletion of p150^{glued} reduces U2OS cells growth. (A–C) Depletion of p150^{guled} reduced U2OS cell growth. The percentage of cell numbers (A), cell viability (B), and cell cycle profiles (C) were quantified in scramble control (CTL) or p150^{glued} (p150) siRNA-transfected U2OS cells. (D) Depletion of p150^{glued} did not affect centrosome homeostasis. The population of cells with multiple centrosome (more than two centrosomes) was counted in scramble control (CTL) or p150^{glued} (p150) siRNA-transfected U2OS cells. These results are mean +/− SD from three independent experiments. (D and E) EdU incorporation (D) or mitotic index (E) were reduced in p150^{glued} depleted U2OS cells. Quantification of EdU incorporation (D) or mitotic index (E) in scramble control (CTL) or p150^{glued} (p150) siRNA-transfected U2OS cells. These results are mean +/− SD from three independent experiments; more than 1000 cells were counted in each individual group.

Cell cycle progression is driven by the activity of CDK [Morgan, 1995]. The CDK is activated when cyclin associates with the kinase, while the activated CDK will be inhibited when the cyclin is degraded or binds to the inhibitors. Upon mitogen stimulation, cyclin E binds to and activates CDK2 that promotes G1/S transition. In p150^{glued} deficient cells, the level of cyclin E was decreased thus attenuated the activity of CDK2 led to G1 arrest. In addition, we also found p53/p21 signaling was activated when p150^{glued} was depleted. When unstress, p53 binds to MDM2 followed by ubiquitin proteasome degradation pathway [Pant and Lozano, 2014]. When centrosomal proteins are depleted, p53 is phosphorylated and accumulated in turn activating its dowstream target gene, p21, the inhibitor of CDK1 and 2 [Stewart et al., 1999; Satyanarayana et al., 2008]. Inactivation of CDK2 induced G1 arrest, while p21 also inhibited CDK1, a kinase critical for G2/M transition, thus preventing mitotic entry.

Here we show that depletion of p150^{glued} inhibits primary cilia formation during serum starvation. The Kif3a recruits several subdistal components such as ninein, Cep170, and p150^{glued} to organize the structure of subdistal appendage of mother centriole [Kodani et al., 2013]. Depletion of Kif3a leads to disruption of ciliary

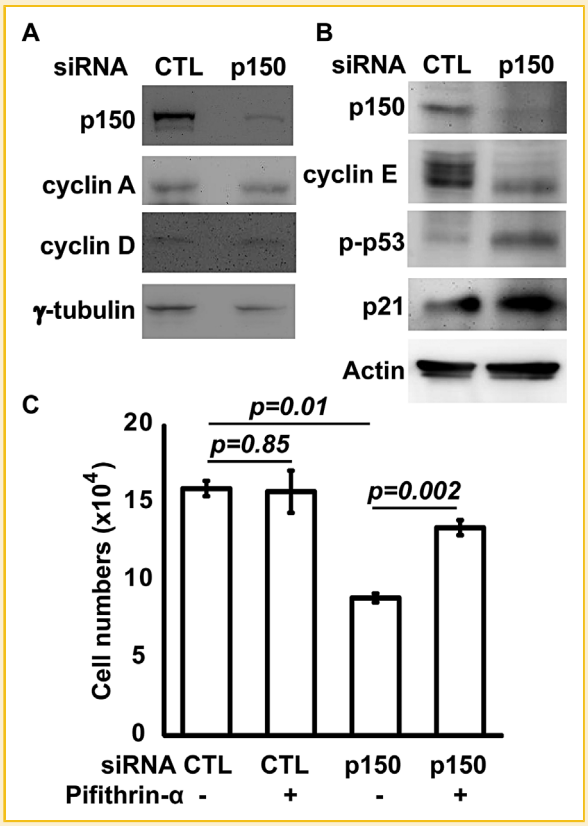

Fig. 8. Depletion of p150^{glued} activates p53/p21 signaling. (A–B) Depletion of p150^{glued} reduced the level of cyclin E but activated p53/p21 signaling. Extracts of scramble control (CTL) or p150^{glued} (p150) siRNA-transfected U2OS cells were analyzed by immunoblotting with antibodies against p150^{glued} (p150), cycin E, cyclin A, cyclin D, phosphorylated p53 on Ser15 (p-p53), p21, and γ-tubulin. (C) The percentage of cell numbers was quantified in scramble control (CTL) or p150^{glued} (p150) siRNA-transfected U2OS cells in the presence or absence of p53 inhibitor, Pifithrin-α. These results are mean +/− SD from three independent experiments.

basal foot (subdistal appendages) followed by defect in ciliogenesis indicating that the structure of subdistal appendage is important for primary cilia formation. Thus, it is reasonable that, in our study, depletion of p150^{glued}, the main component of subdistal appendage, inhibits primary cilia formation.

The recruitment or removal of centrosomal components is a tightly controlled event. Here we demonstrated that, like other centrosomal proteins, p150^{glued} showed dynamic centrosomal residency in different cell cycle stages. The centrosomal targeting of p150^{glued} is regulated by EB1, the microtubule tip-associated protein [Askham et al., 2002]. The C terminus of EB1 is required for its centrosomal targeting. Overexpression of C terminal domain of EB1 inhibits γ-tubulin and p150^{glued} locate to the centrosome indicating that these two protein may share the same EB1-regulated machinery for their recruitment to centrosome. In addition, p150^{glued} itself is also critical for the recruitment of Par6α, a member of polarity protein, to the centrosome [Kodani et al., 2010]. Overexpression of p150^{glued} disrupts the function of dynactin leading to Par6α fails to be recruited to the centrosome. Since p150^{glued} localizes to the centrosome in a cell-cycle depended manner, it will be interesting to examine whether EB1 and Par6α are also dynamically regulated in different cell cycle stages.

The centrosomal targeting of p150^{glued} is contributed by the phosphorylations of p150^{glued} at Thr 145-147 [Zhapparova et al., 2013]. We check whether constitutive phosphorylated p150^{glued} still locates to centrosome during G2/M phase and find, just like wild-type p150^{glued}, phospho-mimetic p150^{glued} still release from the centrosome indicating that phosphorylations of p150^{glued} at Thr 145-147 does not involved in its G2/M dynamics. It is known that the conserved microtubule-binding domain of p150^{glued} can be phosphorylated by Aurora A. When the function of Aurora A is compromised, p150^{glued} become enriched on mitotic spindle [Rome et al., 2010]. In addition, Plk1 interacts and phosphorylates p150^{glued} at Ser 179. The expression of phosphor-deficient p150^{glued} (S179A) shows G2 arrest and delays nuclear envelop breakdown [Zhapparova et al., 2013]. So far it is still unclear whether these phosphorylation sites affect the centrosomal targeting of p150^{glued}, it would be interesting to test the subcellular localization of these variants and check the role of Aurora A and PLK1 in controlling centrosomal residency of p150^{glued} during G2/M stage.

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