Aurora A contributes to p150\textsuperscript{glued} phosphorylation and function during mitosis

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Introduction

The \textit{Drosophila melanogaster} aurora A gene was first identified through mutations resulting in either female sterility or maternal effect lethality. In both cases, mutations were associated with mitotic defects including failure for centrosomes to separate (Glover et al., 1995). Subsequently, it was shown to encode a protein kinase that is widely present in eukaryotic genomes (Giet and Prigent, 1999). \textit{Aurora A} is an essential gene in mammals and is expressed at elevated levels in a wide variety of tumor cells. Its overexpression is sufficient to trigger genetic instability and transformation in NIH3T3 mouse fibroblasts but not in normal cells, suggesting that this protein might behave as an oncogene under specific genetic backgrounds (Giet et al., 2005; Cowley et al., 2009). The multiple roles of aurora A protein kinase in centrosome function and mitotic spindle assembly in \textit{Drosophila}, \textit{Caenorhabditis elegans}, \textit{Xenopus laevis}, and human cells have been extensively studied (Rohgi et al., 1998; Hannak et al., 2001; Giet et al., 2002; Barr and Gergely, 2007). In many systems, the phenotypes caused by \textit{aurora A} loss of function suggest that \textit{aurora A} regulates the dynamics of astral microtubules. To do so, it has been shown that aurora A phosphorylates several microtubule-associated proteins, including the D-TACC–Msps microtubule-stabilizing complex. Indeed, after phosphorylation by aurora A, the D-TACC–Msps complex is targeted to the centrosome component, centrosomin. The Msps subunit of the complex (XMAP215 homologue) binds directly to microtubules to promote microtubule growth. It is thus proposed that phosphorylation of the D-TACC–Msps complex favors stabilization of newly nucleated microtubules at the centrosome (Giet et al., 2002; Terada et al., 2003; Barros et al., 2005; Zhang and Megraw, 2007). In mitotic \textit{X. laevis} egg extracts, aurora A phosphorylates the kinesin-related protein Eg5, hepatoma up-regulated protein, and its coactivators, TPX2. These proteins are required for bipolar mitotic spindle assembly and can be found in a complex with XMAP215 (Giet and Prigent, 1999; Wong et al., 2008). Furthermore, phosphorylation of the mitotic centromere-associated kinesin by aurora A induces its redistribution onto spindle microtubules, where it facilitates the establishment of spindle bipolarity (Zhang et al., 2008). Finally, the aster-associated protein, required...
for spindle assembly, is protected from degradation by the proteasome during mitosis after aurora A phosphorylation (Saffin et al., 2005; Venoux et al., 2008).

In this study, we show that aurora A can phosphorylate the p150
glued component of the dynein–dynactin complex (DDC) at the microtubule-binding domain (MBD) to prevent the accumulation of dynactin and its associated protein, dynein, on the spindle microtubules.

Results and discussion

Most known aurora A substrates are associated with centrosomes and spindle microtubules (Barr and Gergely, 2007). Thus, to identify new aurora A substrates, we decided to ask whether they could be enriched in microtubule preparations. To this end, we prepared microtubule-associated proteins (MAPs) from Drosophila embryos (Fig. 1 A). We used these preparations as substrates for an aurora A in vitro kinase assay (see Materials and methods). We observed a prominent labeled band of 150 kD, which was analyzed by mass spectrometry as p150
glued. The white line indicates that intervening lanes have been spliced out. (C, left) Extracts from S2 cells stably expressing 3xFlag–aurora A were subjected to IP with preimmune (PI) or affinity-purified immune (I) anti-p150
glued antibodies. (right) Extracts from wild-type S2 cells (control) or S2 cells stably expressing 3xFlag–aurora A were subjected to anti-Flag IP. The precipitates were revealed with anti-p150
glued (top) or anti-Flag antibodies (bottom). Note the presence of p150
glued in 3xFlag–aurora A precipitates and, conversely, the presence of aurora A in p150
glued immunoprecipitates. (D) Scheme of the p150
glued fusion proteins used in the kinase assay. N- and C-terminal fragments of p150
glued are displayed in green and blue, respectively. (E) Recombinant MBP, MBP-Ct-Gl, and MBP-Nt-Gl were used for in vitro kinase assays using (+) or not using (∓) aurora A–(His)6 protein kinase in the presence of radio-labeled [γ-32P]ATP. The position of the aurora A–(His)6 band is indicated by arrows (+). MBP and MBP-Ct-Gl, indicated by open and closed arrowheads, respectively, are not phosphorylated, whereas MBP-Nt-Gl (asterisk) is strongly phosphorylated by aurora A. The Coomassie blue–stained gel (left) and the corresponding autoradiography (right) are shown. (F) Position of the eight phosphorylated Ser residues (yellow) in the p150
glued MBD (amino acids 0–200).
Phosphomapping by mass spectrometry revealed phosphorylation of eight Ser residues (Ser85, -109, -117, -135, -137, -138, -144, and -183) within a 200–amino acid N-terminal domain Fig. 1 D) in Escherichia coli to use in an in vitro kinase assay (Fig. 1 E). MBP alone and MBP-Ct-Gl were not phosphorylated by aurora A in vitro, whereas MBP-Nt-Gl was a good substrate. Phosphomapping by mass spectrometry revealed phosphorylation of eight Ser residues (Ser85, -109, -117, -135, -137, -138, -144, and -183) within a 200–amino acid N-terminal domain
Figure 3. A full-length p150\textsuperscript{glued} that can no longer be phosphorylated by aurora A binds more strongly to microtubules but cannot rescue \textit{glued} spindle assembly defect. (A) Scheme of the GFP fusion proteins expressed in S2 cell lines under the control of the metallothionein promoter (inducible by Cu\textsuperscript{++}). The constructs lack the \textit{glued} gene 3\textsuperscript{'} UTR (top) targeted by RNAi. (B) p150\textsuperscript{glued} (top) or actin (bottom) Western blot showing p150-GFP (left) and p150-SA8-GFP (right) protein levels after 0 and 18 h induction on the corresponding cell line. (C) p150-GFP (top), p150-GFP, p150-S\textit{A}8-GFP, and actin protein levels after \textit{glued} 3\textsuperscript{'} UTR RNAi (lanes 2 and 3). Note the knockdown of endogenous p150\textsuperscript{glued}, whereas p150-GFP protein level remains stable. (D) p150\textsuperscript{glued} Western blot on June 26, 2017.
corresponding to the MBD (Fig. 1 F). Most of these sites (except Ser137 and Ser138) matched the yeast and mammalian aurora phosphorylation known consensus site RXS/Tp (where Φ is a hydrophobic residue; Cheeseman et al., 2002; Ferrari et al., 2005). These phosphorylated sites were also detected with other sites after proteomic analyses of p150<sup>glued</sup>-isolated from Kc Drosophila cultured cells in vivo (http://www.sbeams.org/dev1/sbeams/cgi/Glycopeptide/peptideSearch.cgi). A purified recombinant MBD in which all phosphorylated Sers were mutated into Alas had lost its ability to be phosphorylated by aurora A in vitro (Fig. 2 A). Interestingly, these sites contribute to ~60% of total MBD phosphorylation by S2 cell extracts, confirming that other phosphorylation sites exist on p150<sup>glued</sup> MBD in vivo (Fig. S1 A). Furthermore, phosphorylation assays using an aurora A–depleted extract showed that aurora A by itself contributes to ~25% of total MBD phosphorylation observed in S2 cells (Fig. S1 B). p150<sup>glued</sup> MBD is responsible for the microtubule-binding properties of dynactin, and its deletion affects mitotic but not interphase functions of the protein (Kim et al., 2007). To analyze the relevance of those phosphorylation events in vivo, we examined the consequences of expressing either a GFP-tagged wild-type (MBD-GFP) or nonphosphorylatable variant of the p150<sup>glued</sup> MBD (MBD-SA8-GFP) in S2 cells (Fig. 2 B). We established stable cell lines in which such constructs were expressed at equivalent levels (Fig. 2 C). By immunostaining, both proteins were able to bind microtubules during interphase when aurora A was not active (Fig. 2 D). During early mitosis, MBD-GFP and MBD-SA8-GFP were strongly associated with centrosomal microtubules. However, the nonphosphorylatable MBD-SA8-GFP mutant protein was retained on the spindle microtubules during mitosis in all cells examined during metaphase but not MBD-GFP (n > 200; Fig. 2, compare E [middle] with F [middle]). We assessed the binding of recombinant MBD and MBD-SA8 to taxol-polymerized microtubules in vitro. The p150<sup>glued</sup> MBD affinity for microtubules was five times weaker after incubation with aurora A protein kinase and ATP. In these conditions, we found that the mutagenesis of the eight Sers into Alas restored the ability of p150<sup>glued</sup> MBD to bind to the microtubules (Fig. S1 C). Thus, the direct interaction of the p150<sup>glued</sup> MBD with the microtubules appears to be negatively regulated by aurora A phosphorylation in vitro and in vivo during mitosis.

As p150<sup>glued</sup> MBD is required for mitotic spindle assembly (Kim et al., 2007), it was tempting to speculate that phosphorylation at the sites we have mapped is required to control the overall dynactin accumulation on the mitotic spindle. To test this hypothesis, we checked whether a full-length p150<sup>glued</sup> GFP fusion protein in which the eight aurora A phosphorylation sites (Sers) had been replaced by Alas (p150-SA8-GFP) would (a) accumulate on spindle microtubules and (b) be able to complement p150<sup>glued</sup> depletion. For this purpose, we induced the expression of either the GFP-tagged p150<sup>glued</sup> wild-type protein (p150-GFP) or a mutant variant (p150-SA8-GFP; Fig. 3 A). After several hours of induction of their metallothionein promoter with copper, both GFP-fused proteins were detected by Western blot analysis (Fig. 3 B). Using a double-stranded RNA (dsRNA) specific to the glued 3′UTR gene (Fig. 3 A; Kim et al., 2007), we were able to deplete the endogenous p150<sup>glued</sup> but not the exogenous GFP-tagged protein (Fig. 3 C). In addition, sucrose gradient separation analyses revealed that both GFP fusion proteins were sedimenting together with endogenous p150<sup>glued</sup> in the 19S dynactin complex (Fig. 3 D). As reported previously in S2 cells, p150<sup>glued</sup>-depleted mitotic cells showed an obvious disconnection of centrosomes from the mitotic spindle in 67.3 ± 11.6% of the metaphase cells (Fig. 3 E, compare left with right), whereas only 22.4 ± 4% of the cells showed this phenotype in control cells (Goshima and Vale, 2003; Goshima et al., 2005b; Siller et al., 2005). Interestingly, we completely rescued this defect by expression of p150-GFP (26.7 ± 12%) but not p150-SA8-GFP (69.6 ± 6.9%; Fig. 3 E). In addition to that, the wild-type protein was weakly localized on spindle microtubules compared with the mutant, which showed strong association with these microtubules (Fig. 3 F). This localization of p150-GFP and p150-SA8-GFP was confirmed by high resolution time-lapse video microscopy. As seen in Fig. 4 (left; and Video 1), the p150-GFP protein weakly localized on the spindle region and microtubule plus ends (Fig. 4, triangles), whereas p150-SA8-GFP strongly decorated mitotic spindle fibers and microtubule plus ends (Fig. 4, right; and Video 2). The kinetochore localization (Fig. 4, arrows) remained identical for both constructs. All of these results led us to investigate whether aurora A was responsible for the dynamics of dynactin during mitosis. To do so, we generated a new p150<sup>glued</sup> antibody against the C-terminal fragment of p150<sup>glued</sup> (Fig. S2 A) to localize the p150<sup>glued</sup> protein during mitosis in Drosophila S2-cultured cells. Using this antibody, we observed a staining of the centrosomal microtubules during prophase and the early stages of spindle formation. The signal decreased during spindle maturation before becoming enriched once more at telophase, suggesting that spindle pole localization of p150<sup>glued</sup> was inhibited after nuclear envelope breakdown (Fig. 5 A). We also found a transient staining of the kinetochore regions of chromosomes during prometaphase that was replaced after chromosome congression by a weak staining of spindle fibers, which is similar to the findings of others (Siller et al., 2005).

After aurora A RNAi, 60% of the metaphase cells (n = 3; 200 cells) showed stronger staining of the spindle pole regions (Fig. 5 A, arrows; and Fig. S3). Interestingly, identical results...
were obtained after aurora A knockdown in human HeLa cells (unpublished data), suggesting that DDC regulation is conserved from Drosophila to humans. We further showed the same behavior for the dynactin-interacting protein dynein, which accumulated at the spindle poles after aurora A RNAi in S2-cultured cells (Fig. S2 B). We also asked whether dynein could become mislocalized in larval neuroblasts from S2-cultured cells (Fig. S2 B). We also asked whether dynactin could become mislocalized in larval neuroblasts from S2-cultured cells (Fig. S2 B). We also asked whether dynactin could become mislocalized in larval neuroblasts from S2-cultured cells (Fig. S2 B). We also asked whether dynactin could become mislocalized in larval neuroblasts from S2-cultured cells (Fig. S2 B).

The dynactin complex, composed of p150glued and at least 10 other polypeptides, cooperates with dynein to fulfill multiple roles during mitotic progression (Karki and Holzbaur, 1999; Goshima and Vale, 2003; Morales-Mulia and Scholey, 2005; Delcros et al., 2006). In Drosophila S2 cells, DDC is required for the centrosome connection to the mitotic spindle and for the metaphase/anaphase transition (Goshima and Vale, 2003; Morales-Mulia and Scholey, 2005). Our RNAi experiments in S2 cells confirm these previous studies, as centrosomes were disconnected from the poles in ~70% of the p150glued-depleted mitotic cells. Both RNAi experiments and mathematical models concur in the notion that kinetochore fibers are captured and focused at the poles by the DDC, which connects these fibers to the centrosome-nucleated microtubules. Thus, a lack of DDC can explain the fact that centrosomes are disconnected from the spindle (Maiato et al., 2004; Goshima et al., 2005a). Our study revealed that aurora A participates in the phosphoregulation of p150glued, but other kinases can contribute to this process on p150glued MBD itself (Fig. S1) or other DDC subunits (Huang et al., 1999; Vaughan et al., 2001, 2002). Interestingly, the variant that can no longer be phosphorylated by aurora A remains associated with spindle microtubules and cannot complement the glued loss of function. This suggests that DDC levels on spindle microtubules and at the poles need to be tightly controlled in Drosophila cells to avoid centrosome disconnection, a phenotype sometimes also observed in aurora A mutant embryos (unpublished data), but not in other cell types in which other kinases possibly participate in dyakinin limitation. The reason why DDC localization at spindle poles needs to be modulated remains unclear. It is possible that DDC function requires a moderate association of dynactin with spindle microtubules. It is also possible that overaccumulation of DDC (and cargoes) could create a traffic obstruction at spindle poles to compete with other MAPs. Consequently, this would prevent the connection between kinetochore fibers and astral microtubules. Further studies will be required to elucidate the complete DDC regulation during mitosis.

Figure 4. p150-SA8-GFP displays higher affinity for microtubules than wild-type p150-GFP protein during mitosis. S2 cells expressing equivalent levels of p150-GFP (left) or p150-SA8-GFP (right) were imaged by time lapse video microscopy (Videos 1 and 2). The p150-SA8-GFP protein is strongly associated with spindle fibers and microtubule plus ends, whereas p150-GFP shows moderate association with these structures (arrowheads). The kinetochore localization (arrows) is not affected. Bar, 10 µM.

Materials and methods

Preparation of microtubules from Drosophila embryos for aurora A phosphorylation assays

500 µl Drosophila early embryos (0–4 h) were collected and lysed in 500 µl BRB80 buffer (80 mM Pipes, pH 6.8, 1 mM MgCl2, and 1 mM EGTA) supplemented with protease inhibitors (Roche) and 0.5% NP-40. The crude extract was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was centrifuged a second time at 100,000 g for 20 min at 4°C. 0.4 ml supernatant containing the soluble proteins was supplemented with 20 µM taxol and 1 mM GTP. Microtubule polymerization was induced for 20 min at 25°C. The microtubules were sedimented on a 0.4 ml BRB80 cushion supplemented with 20 µM taxol, 1 mM GTP, and 40% glycerol. The microtubule pellet containing the MAPs was washed twice in BRB80 and resuspended in BRB80. The MAP fractions were stored at −80°C.

Phosphorylation assay

20 µg MAPs fraction or 3 µg recombinant substrate proteins was incubated with 200 ng recombinant aurora A–(His)6 kinase, 1 µCi radio-labeled γ[32P]ATP (GE Healthcare), and 100 µM ATP in kinase buffer (Giet et al., 2002). The samples were separated by SDS-PAGE and stained by Coomassie blue. p150glued was identified by mass spectrometry on the excised band from the gel. To map the phosphorylation sites on the MBP-Nt-Gl protein, the kinase assay was performed without radio-labeled ATP, and the phosphorylated band was excised and sent for phosphopeptide identification to the Proteomic Platform of the Quebec Genomic Center (http://proteomique.crchul.ulaval.ca/en/equipment.html).
dsRNA production and constructs

The aurora A dsRNA production was described previously (Giet and Glover, 2001; Giet et al., 2002). In brief, an ~1,000-bp PCR product containing a T7 sequence at each end was used as a template to generate RNA using the Megascript kit (Promega). To obtain the glued template (for the 3' UTR region), an ~450-bp PCR product using the primers 5'-AATAATACGACTCACTATAGGGAAAAGGATCDTGTATCGTGGCA-3' and 5'-AATAATACGACTCACTATAGGGGAGTTATACAACATCAGCAAA-3' was produced. After isolation, the RNAs were boiled for 20 min and annealed by slow cooling overnight at room temperature. dsRNAs were analyzed by agarose gel electrophoresis and aliquoted at -80°C before use in RNAi experiments. To generate the expression construct encoding the full-length aurora A in fusion with 3xFlag at its N-terminal end, aurora A ORF was first cloned into the pENTR vector (to create an entry clone) using a cloning kit (TOPO; Invitrogen) and recombined using the gateway system (Invitrogen) into the pHFW containing the 3xFlag tag and a heat shock promoter. An aurora A kinase-dead mutant (K193/R) was generated by mutation of the conserved lysine of the kinase subdomain II into an arginine.

For the MBD expression constructs, each Ser to Ala mutation in the p150\text{glued} MBD was obtained by sequential PCRs. The wild-type (MBD) or nonphosphorylatable (MBD-SA8) domains were cloned directly into the pENTR vector (to create an entry clone) using a cloning kit (TOPO; Invitrogen) and recombined using the gateway system (Invitrogen) into the pHFW containing the 3xFlag tag and a heat shock promoter. An aurora A kinase-dead mutant (K193/R) was generated by mutation of the conserved lysine of the kinase subdomain II into an arginine.

For in vivo phosphorylation assay, 3 × 10⁶ control or aurora A–depleted cells were resuspended in 500 µl of lysis buffer containing protease inhibitors (10 mM Na₂VO₄ and 50 mM β-glycerophosphate; Giet et al., 2002). 5 µl of the extract was used to phosphorylate 5 µg MBD or MBD-SA8 in the presence of 2.5 µCi radio-labeled γ[³²P]ATP (GE Healthcare) and 100 µM cold ATP in kinase buffer. MBD and MBD-SA8 phosphorylation signals were quantified using a phosphoimager (Molecular Dynamics).

Figure 5. \(p_{150}^{\text{glued}}\) accumulates at spindle poles of aurora A RNAi-deficient S2 cells and aurora A mutant neuroblasts. (A) Control S2 cells (top) were fixed and stained for tubulin (red), \(p_{150}^{\text{glued}}\) (green), and DNA (blue). The mitotic phases are displayed at the top. \(p_{150}^{\text{glued}}\) localization at spindle poles is clearly detected during prophase and cytokinesis, whereas the signal is very weak during prometaphase and metaphase. In parallel, ~60% of aurora A–depleted cells (bottom) exhibit \(p_{150}^{\text{glued}}\) accumulation at spindle poles (arrows). (B) Wild-type (left) or aurora A\(^{E209/D}[3R]T61\) (right) mutant neuroblasts were fixed and stained for tubulin (green; monochrome in middle), \(p_{150}^{\text{glued}}\) (red; monochrome in bottom), and DNA (blue). In control metaphase cells, \(p_{150}^{\text{glued}}\) is faintly detected along spindle fibers. In aurora A mutant neuroblasts, \(p_{150}^{\text{glued}}\) accumulates on spindle fibers and spindle poles (arrowheads). Bars, 10 µm.
pET112/TOPO bacterial expression vector or pEENTR to generate entry clones. The latter clones were recombined into pDEST17 to generate bacte-
rial expression constructs and in pAWG to allow S2 cell expression of either MBP-MBD or MBD-SAB-GFP under the control of the actin 5c pro-
totector. Both pAWG and pPHW were obtained from the Carnegie Institution of Washington.

T-N and C-terminus domains of p150glued were amplified by PCR and cloned into the pMal-C2E expression vector using KpnI and BamHI restriction sites. All constructs were verified by sequencing.

Full-length p150glued was cloned into pMTGFP-Ct (provided by M. Savoian, University of Cambridge, Cambridge, England, UK) using the gateway system (through an entry clone) to generate p150-GFP expression construct. p150-SAB-GFP was subsequently obtained with the QuickChange Mutagenesis kit (Agilent Technologies). The pAct5C-Cherry–tubulin construct was provided by G. Hickson (University of California, San Francisco, San Francisco, CA).

Production of the recombinant proteins and antibody purification

Aurora A–(His)6, aurora A–K/R-(His)6, MBD-(His)6, or MBD-SA8-(His)6 was expressed in E. coli BL21(DE3) pLysS (EMD) for 4 h at 25°C, purified on an amyllose column, and dialysed against PBS and stored at −80°C before use for aurora A kinase assay. The purified MBP-Ct-Gl protein was also used to immunize rabbits. Obtained anti-p150 antibody was affinity purified on a nitrocellulose membrane, and the purified antibodies were stored at −80°C. For use as described previously (Montembault et al., 2007).

In vitro microtubule-binding assay

1 µg purified recombinant p150glued MBD or MBD-SAB proteins in fusion with a hexahistidine tag was incubated with either 200 ng aurora A or aurora A–K/R proteins in kinase buffer containing 1 mM ATP in a total volume of 10 µl for 20 min. The kinase reaction was then incubated with 25 µl (100 µg) of taxol-stabilized microtubules for 10 min at 37°C (Roghi et al., 1998). The microtubule pellet and the supernatant containing microtubule-unbound proteins were separated by sedimentation at 100,000 g for 20 min at 37°C (TLA-100; Beckman Coulter). The proteins present in the pellets and supernatants were analyzed by Western blotting or Coomasie blue staining.

RNAi, transfections, drug treatment, stable line generation, and rescue experiments

Drosophila S2 cells were grown and processed for RNAi as described previ-
ously (Clemens et al., 2000). In brief, 106 cells were incubated with 10 µg/ml dsRNA in media without sucrose. Alternatively, 10 µg transfection reagent (Transfast; Promega) was added together with 3 µg dsRNA following the manufacturer’s instructions. After 1 h, fresh media were added to the cells. At 48 h after transfection, the cells were fixed and analyzed for mitotic defects (Giet and Glover, 2001). 100–200 mitoses were scored and analyzed per experiment, and each experiment was repeated at least three times.

To make S2 stable lines expressing 3xFlag–aurora A, MBD-GFP, or MBD-SAB-GFP, p150-GFP, and p150-SAB-GFP, 5 µg of each expression construct was cotransfected in those cells. At 4 days after transfection, the cells were fixed and analyzed for mitotic defects (Giet and Glover, 2001). 100–200 mitoses were scored and analyzed per experiment, and each experiment was repeated at least three times.

Immunofluorescence analysis

S2 cells were fixed in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 4 mM MgCl2) containing 3.7% formaldehyde and 0.1% Triton X-100. In some cases, the cells were plated on concanavalin A–coated coverslips for 1 h and fixed in methanol at −20°C for 10 min. The fixed cells were briefly washed in PBS and blocked for 1 h in PBS containing 0.1% Triton X-100 and 1% BSA (PBSTBSA). Primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated for 1 h at room temperature in PBSTBSA. DNA was stained with Hoechst 33258. Slides were mounted in Prolong gold (Invitrogen) and observed with a microscope (DMRXA2; Leica) using a 63× 1.3 NA objective. Images were acquired with a camera (CoolSnap HQ; Photometrics) and processed with MetaMorph software (Universal Imaging). Alternatively, images were acquired with an inverted confocal microscope (SP2; Leica).

Time-lapse imaging

S2 cells expressing p150-GFP or p150-SAB-GFP were incubated with CuSO4 (300 µM final concentration) for 18 h and plated for 1 h on concanavalin A–coated incubation chambers (Labtek; Sigma) before imaging. Images were acquired using a spinning-disk system mounted on an inverted microscope (Eclipse Ti; Nikon) using a 100× 1.4 NA objective. Images were acquired every 5 or 10 s with a camera (CoolSnap HQ2; Photometrics) controlled by the MetaMorph acquisition software.

Antibodies and Western blotting

The YL1/2 rat anti-tyrosinated tubulin antibody (1:1,000) was obtained from Millipore, and the GTU-88 mouse anti-α–tubulin antibody (1:1,000) was obtained from Sigma-Aldrich. The mouse anti-dynein clone 1H4 antibody (1:100) was provided by T. Hays, and the anti-GFP monoclonal antibodies (1:1,000) were obtained from Roche or Invitrogen. Affinity-purified antibodies against aurora A were used at 0.5 µg/ml (Giet et al. 2002). The affinity-purified anti-p150glued antibody was purified as described previously (Montembault et al., 2007) and used at 2 µg/ml. Secondary peroxidase-conjugated antibodies were obtained from Jackson Immunoresearch Laboratories, Inc., and Alexa Fluor–conjugated secondary antibodies were obtained from Invitrogen. For Western blotting, ECL reagent was purchased from Thermo Fisher Scientific.

Online supplemental material

Fig. S1 shows the contribution of aurora A and other unknown kinases to p150glued MBD phosphorylation in vivo. It also shows that aurora A kinase inhibits MBD binding to microtubules in vitro. Fig. S2 shows p150glued anti-
body Western blotting in control or aurora A–depleted cells together with dynein localization in control or aurora A–depleted S2 cells. The colocal-
ization of dynein and dynactin is also displayed in wild-type or aurora A mutant neuroblasts. Fig. S3 shows the distribution (line scans) of p150glued and tubulin in control or aurora A–depleted cells. Videos 1 and 2 show the behavior of wild-type and nonphosphorylatable p150glued proteins fused to GFP during metaphase. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201001144/DC1.

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