Dynactin targets Pavarotti-KLP to the central spindle during anaphase and facilitates cytokinesis in Drosophila S2 cells

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Summary
The dynactin complex cooperates with the dynein complex in various systems for mitotic completion. Here we analysed the mitotic phenotype of Drosophila S2 cells following the knockdown of the dynactin subunit p150Glued. We found that p150Glued-depleted cells were delayed in metaphase and that the centrosomes were poorly connected to mitotic spindle poles. In addition, anaphase occurred with asynchronous chromosome segregation. Although cyclin B was degraded in these anaphase cells, Aurora B, MEI-S322 and BubR1 were not released from the non-segregating chromosomes. We also found that the density and organisation of the central spindle were compromised, with Aurora B and polo kinases absent from the diminished number of microtubules. Pavarotti-KLP, a component of the centralspindlin complex required for the formation of stable microtubule bundles, was not immediately targeted to the plus ends of the microtubules following anaphase onset as happened in controls. Instead, it accumulated transiently at the cell cortex during early anaphase and its targeting to the central spindle was delayed. These data suggest that the dynactin complex contributes to cytokinesis by promoting stable targeting of the centralspindlin complex to microtubule plus ends at anaphase onset. The contribution of the dynein-dynactin complex to synchronous chromosome segregation and cytokinesis is discussed.

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Key words: Dynactin, Mitosis, Cytokinesis

Introduction
Dynein is a microtubule motor that moves toward the minus end of microtubules as part of a large macromolecular complex (Collins and Vallee, 1989; Vallee et al., 1989). This complex has many functions. It is involved in the transport of molecules and organelles along microtubules during interphase and nuclear positioning (Karki and Holzbaur, 1999). Cytoplasmic dynein, which is present at the cell cortex, pulls on centrosome-connected microtubules and is responsible for the positioning of the centrosomes in the centre of the cell (Koonce et al., 1999; Vallee and Stehman, 2005). The same principle is used in yeast cells to pull newly duplicated nuclei into the bud and in mitotic vertebrate cultured cells, as well as in Drosophila and Caenorhabditis elegans somatic tissues, to pull the centrosomes apart during prophase (Busson et al., 1998; Carminati and Stearns, 1997; Koonce et al., 1999; Vallee and Stehman, 2005; Gonczy et al., 1999; Robinson et al., 1999; Schmidt et al., 2005).

The dynein complex can be isolated biochemically with dynactin, a 20S multi-protein complex containing at least ten proteins (Karki and Holzbaur, 1999). In vitro, the dyactin complex stimulates the dynein motor activity (Gill et al., 1991). It has been suggested that one major function of dynactin is to target dynein where it is required in the cell (Vallee et al., 1995).

The Glued gene encodes a 150 kDa protein that belongs to the dynactin complex. In fission yeasts and in humans, p150Glued regulates microtubule dynamics (Niccoli et al., 2004). In human cells, this regulation depends on association with EB1 and is essential to maintain the radial arrays of microtubules in interphase cells (Askham et al., 2002; Strickland et al., 2005). A recent study further proposed that the interaction between EB1 and p150Glued promotes the elongation of astral microtubules to facilitate furrow ingression during cytokinesis (Strickland et al., 2005).

When cells enter mitosis and prophase, dynein facilitates nuclear envelope breakdown for spindle formation: it pulls nuclear membranes and associated proteins poleward along astral microtubules leading to nuclear membrane detachment (Salina et al., 2002). During prometaphase, it has been clearly demonstrated in Drosophila and human cultured cells, that the dynein-dynactin complex (DDC) is continuously recruited to the kinetochores until they become properly attached to the mitotic spindle. Kinetochoore dynein then moves towards the spindle poles thereby removing checkpoint proteins and allowing the inactivation of the spindle checkpoint and the anaphase onset (Karess, 2005). In addition, mutations that affect dynein loading at the kinetochore, lead to asynchronous chromosome segregation and attenuated chromosome motion toward the poles (Savoian et al., 2000; Sharp et al., 2000). In
Drosophila, interfering with dynein-dynactin functions using antibodies or RNAi also induces the detachment of the centrosomes from the spindle poles (Goshima and Vale, 2003; Goshima and Vale, 2005; Morales-Mulia and Scholey, 2005).

Here, using RNAi for the Glued gene in Drosophila S2 cells, we confirm that Glued is required for centrosome connection to spindle poles, the metaphase-to-anaphase transition, and synchronous chromosome-to-pole movement during anaphase. In addition, we report that the dynactin complex also facilitates cytokinesis by targeting Pavarotti-KLP, a component of the centralspindlin complex, to the plus ends of central spindle microtubules.

Results

p150Glued localises to centrosomes, mitotic spindle microtubules and kinetochores

Two antisera raised against the first 148 amino acids of the p150Glued polypeptide were produced in rabbits (Rb1477 and Rb1478). These two antisera recognised a common protein at 150 kDa, the expected size of the p150Glued protein in Drosophila S2 cell extracts as well as one or more other bands (Fig. 1A). After affinity purification, only a single 150 kDa protein could be detected (Fig. 1A, lane AP). The p150Glued protein signal disappeared following Glued RNAi of S2 cells whereas loading control proteins, cyclin B, aurora A, CP190 and dynein intermediate chain (DIC) remained stable (Fig. 1B, compare lanes – and lanes +), further demonstrating the specificity of our antibodies.

The anti-p150Glued affinity-purified antibody was used to stain cultured Drosophila S2 cells and embryos (Fig. 2). During prophase, the antibody decorated the two asters of microtubules (Fig. 2A, panel p). During metaphase, labelling was observed on centrosomes/asters as well as on spindle microtubules (panel m). From anaphase until cytokinesis, p150Glued was detected on the astral and central spindle microtubules (Fig. 2A, panels a,c). In interphase cells, we found a punctuated p150Glued labelling in the cytoplasm and along microtubule fibres (Fig. 2B) as observed previously in human cells (Vaughan et al., 2002). We further observed paired-dot-like staining on some metaphase chromosomes. This staining became more evident when microtubules were depolymerised with colchicine and furthermore, co-localised with polo kinase, a known kinetochore component (Fig. 2D). Thus, like the dynein motor protein, p150Glued associates with kinetochores. To further examine the distribution of p150Glued during mitosis, we stained syncitial embryos (Fig. 2C). In agreement with our findings in cultured cells, p150Glued first associated with prophase asters and the subsequent metaphase and anaphase spindles.

We next attempted to examine the dynamics of p150Glued in living cells using a GFP-tagged transgene. However, despite the use of the ubiquitin promoter to drive expression (see Materials and Methods) the fluorescence intensity of GFP-p150Glued was too weak to be detected in live material. Therefore a GFP antibody was used to stain fixed expressing cells. GFP labelling was detected on the centrosomal region and the spindle microtubules during mitosis, as well as at the midbody region during cytokinesis (supplementary material Fig. S1). Thus, a combination of GFP-tagging and immunofluorescence studies demonstrated that the p150Glued protein shares a common localisation in both S2 cells and syncitial embryos; it associates with centrosomes, microtubules and kinetochores. In addition, the p150Glued protein was also detected on the central spindle microtubules and the midbody during late telophase and cytokinesis.

Mitotic defects following Glued RNAi

To monitor the contribution of Glued during mitosis in Drosophila S2 cells, we performed RNAi against the Glued mRNA transcript, and mitotic cells were fixed and analysed by immunofluorescence microscopy (Table 1 and Fig. 3). Three to four days post-transfection with Glued dsRNA, p150Glued protein levels were greatly reduced as evidenced by western blot analyses (Fig. 1B). Mitotic indices were four times higher in p150Glued-depleted cells compared with control cells. Of these, the proportion of cells at metaphase (60.5%) was significantly greater than in controls (29.2%). In addition, the number of abnormal mitotic anaphase cells was five times greater in p150Glued knock down cells (see below).

Table 1. Quantification of the mitotic defects in control or Glued RNAi S2 cells

|                  | Mitotic index | Prophase* | Metaphase* | Anaphase + telophase* | Cytokinesis* | Abnormal spindles* | Abnormal anaphases* |
|------------------|---------------|-----------|------------|-----------------------|-------------|---------------------|---------------------|
| Control RNAi     | 3.0±1.5       | 8.1±1.9   | 29.2±5.3   | 9.4±1.9               | 41.1±3.8   | 11.9±2.9            | 0.3±0.5             |
| Glued RNAi       | 12.0±3.9      | 4.5±1.3   | 60.5±4.8   | 2.0±1.7               | 23.7±1.2   | 4.5±1.9             | 4.9±1.2             |

*Values are the mean percentage of the total number of mitotic cells in each phase (± s.d.). †50% of these spindles show centrosome disconnection from spindle poles. ‡These cells include monopolar, monoaural bipolar and multipolar spindles.
Dynactin is a cytokinesis facilitator

In 50% of the cases, RNAi-treated metaphase cells had normal spindle morphology and could not be differentiated from control cells (Fig. 3B, panel 1). However, the other half of the metaphase cells displayed a weak connection or clear separation between the centrosomes and the spindle poles (Fig. 3B, panel 2), a phenotype also observed after dynein or dynactin interference (Goshima et al., 2005; Morales-Mulia and Scholey, 2005; Siller et al., 2005). The major defect observed following p150Glued RNAi occurred during anaphase, where 4.9% of cells were abnormal compared with 0.3% of control cells. These defects were manifest as gross morphological spindle defects and chromosome segregation errors. In most cases, the spindle appeared abnormally elongated with some chromosomes located at the spindle poles whereas others were still at the spindle equator, suggesting asynchronous chromosome separation. (Fig. 3B, lower panels 3 and 4). During early anaphase, as judged by the separation of the two chromatid masses, the central spindle microtubule density was less robust and lacked the symmetry observed in controls (Fig. 3B, middle panels 3 and 4). In telophase, a disorganised region of equatorial microtubules was able to form, but this region was lacking the well-organised stable anti-parallel microtubule bundles seen in control telophase cells (Fig. 3, compare B panel 5 with A panel 1). The FACS analyses showed the absence of polyploid cells in the Glued dsRNA-treated cells, but an increase of apoptotic and/or aneuploid cells was detected (Fig. 3C, arrow) suggesting that despite the formation of normal anaphase cells, cytokinesis occurred normally. In all depleted anaphase cells, the chromosomes showed a dot-like morphology suggesting an overcondensation of chromosomes and hence mitotic arrest, as expected from the elevated mitotic index (Table 1).

To confirm that these elongated spindles represented anaphase cells and not aberrant prometaphase cells with congression defects, we monitored the degradation of cyclin B in control and Glued dsRNA-treated cells at the metaphase-anaphase transition. Cyclin B levels were high in wild-type and Glued dsRNA-treated cells during metaphase and the protein accumulated strongly at centrosomes, spindles and, to a lesser extent, at some kinetochores (Fig. 4, left panels A and B).
During anaphase, cyclin B levels were strongly reduced in control cells but some signal was still detected at centrosomes as previously described (Mathe et al., 2004). We consistently found that cyclin B was degraded in Glued-depleted cells with elongated spindles and aberrantly positioned chromosomes (Fig. 4A, right panels), indicating that these cells had progressed into anaphase. In addition, we examined the distribution of the MEI-S322 antigen (the Drosophila homologue of mammalian shugosin) (Kerrebrock et al., 1995). This protein was released from the centromeres in control anaphase cells and was also lacking at polar-proximal chromosomes in Glued dsRNA-treated cells. However, the signal remained quite strong on those chromosomes lagging at the equator (Fig. 4C). Finally we investigated the status of the spindle assembly checkpoint in knockdown cells by staining for the BubR1 antigen. As expected in control cells, BubR1 protein levels were elevated at the kinetochores in early prometaphase cells and much lower in metaphase. The remaining BubR1 protein disappeared following anaphase entry (Karess, 2005). In those RNAi-treated cells exhibiting normal metaphase spindles, all kinetochores appeared to be labelled similarly to control cells. During anaphase, the kinetochores of polar-positioned chromosomes on elongated spindles showed no staining whereas those kinetochores at chromosomes at the centre of the spindle exhibited fluorescence (Fig. 4B). Taken together, these results reveal that the knockdown of p150Glued leads to elongated spindles upon which some chromosomes are able to segregate to the poles during anaphase while others lag at the spindle equator. These lagging chromosomes retain the MEI-S322 centromeric protein as well as the checkpoint component BubR1. Along with these chromosome segregation defects, the central spindle, which normally forms during late anaphase or telophase is greatly diminished, suggesting that p150Glued is needed for central spindle formation or stabilisation.

**Aurora B and polo kinases are not recruited to the microtubules in Glued-depleted anaphase cells**

The failure to form or maintain the central spindle in p150Glued-depleted cells prompted us to investigate the distribution of two...
Dynactin is a cytokinesis facilitator central-spindle-dependent protein kinases. The first of these, Aurora B, is a chromosome passenger protein that localises to the centromeric region of the chromosomes during prophase until metaphase (Fig. 5A, panel 1). This protein then redistributes to the central spindle midzone during anaphase A (Fig. 5A, panel 2), where it remains during anaphase B (panel 3) and telophase (panel 4) and is needed to stabilise central spindle microtubules for successful cytokinesis (Giet and Glover, 2001).

In Glued RNAi-treated cells, Aurora B still localised to the centromeric region of mitotic chromosomes during metaphase (Fig. 5B, panel 1). In post-anaphase cells Aurora B was not detected on the centromeres of those chromosomes that had successfully migrated to the poles. However, it was retained at the centromeres of those chromosomes that failed to segregate (Fig. 5B, panels 2 and 3) and telophase (panel 4) and is needed to stabilise central spindle microtubules for successful cytokinesis (Giet and Glover, 2001).

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Although the failure of Aurora B to concentrate at the equator in a timely fashion was sufficient to explain the diminished central spindles observed following Glued RNAi, we checked the localisation of the polo kinase, another kinase that is crucial for central spindle formation and successful cytokinesis. In anaphase cells, polo localised to the kinetochores of the segregated chromatids (Fig. 6A,B) and at the overlapping plus ends of central spindle microtubules at the midzone. In Glued dsRNA-treated cells, polo was also found on segregated kinetochores, but was not detected at the plus ends of predicted central spindle microtubules (Fig. 6C,D). We
confirmed that the knockdown cells had progressed beyond metaphase by staining for Cyclin B (green), which further allowed us to discriminate the boundaries of the cell (shown in white). As can be seen in the figure, along with the spindle defects described above, knockdown cells have abnormal cortices that are distorted and buckled. This may reflect unregulated cortical contractions during this late mitotic stage.

**Glued RNAi cells lack stable microtubule bundles and Pavarotti-KLP**

To further define the central spindle defects associated with p150Glued depletion, we examined the distribution of Pavarotti-KLP. This motor protein is part of the evolutionarily conserved centralspindlin complex that has been implicated as a major player in both central spindle formation and cleavage furrow.

**Fig. 5.** Aurora B behaviour in p150Glued-depleted anaphases. Control (A) or Glued dsRNA-treated (B) S2 cells (B) were fixed and stained for aurora B (green and lower panels in monochrome), tubulin (red and monochrome in top right panel 4) and DNA (blue). In control or p150Glued-depleted metaphase cells (panel 1), aurora B is recruited to the centromeres. During anaphase A (A, panel 2), note the disappearance of aurora B from the centromeres and its progressive relocalisation to the central spindle during anaphase B (panel 3) and telophase (panel 4). In p150Glued-depleted anaphase cells (panels 2 and 3), aurora B is detected at the centromeres (white arrowhead) that have not segregated (see also supplementary material Fig. S2) and there is no recruitment to the microtubules. Panel 4 shows a p150Glued-depleted anaphase cell in which aurora B signal was overexposed (lower left panel) to show that aurora B signal is not detected on the few microtubules seen between the two chromatin masses. Bar, 10 μm.

**Fig. 6.** Polo protein kinase behaviour in p150Glued-depleted anaphases. Wild-type (A,B) and Glued RNAi (C,D) cells during anaphase were fixed and stained for cyclin B (green), polo (red, in panels B and D, and lower panels in monochrome), and α-tubulin (red in panels A and C, and lower panels in monochrome. In control anaphase (B), polo localises to the kinetochores and on central spindle microtubules. In Glued dsRNA-treated cells (D), polo is detected on the kinetochores but not on the microtubules present between the two chromid masses. Note also the abnormal cortex contraction in this cell (white line). Bar, 10 μm.
Dynactin is a cytokinesis facilitator initiation in different organisms. This protein localises to microtubule plus ends after anaphase onset and is required for microtubule bundling and central spindle formation (Adams et al., 1998). In control cells, Pavarotti-KLP was detected on spindle microtubules during metaphase (Fig. 7A, panel 1). At anaphase onset, the protein concentrated at the plus ends of central spindle microtubules where it persisted until cytokinesis was completed (Fig. 7A, panels 2-4). Likewise, in Glued RNAi-treated cells Pavarotti-KLP was detected on the metaphase spindle (Fig. 7B, panel 1). Following the metaphase-to-anaphase transition, we found that Pavarotti-KLP did not concentrate into a tight aggregate at the plus ends of microtubules regardless of the presence or absence of lagging chromosomes. Interestingly, Pavarotti-KLP often appeared less intense at polar regions, although the significance of this remains unclear. We further found that in 50% of these anaphase cells, Pavarotti-KLP was detected at indentations of the cell cortex, suggesting that contraction had occurred (Fig. 7B, panel 2). In telophase-like cells, a diffuse staining was found at the defective central spindle region (Fig. 7B, panel 3). This Pavarotti-KLP signal, although weaker than that seen in control cells was present in some cells undergoing telophase (panel 4) and cytokinesis (panel 5). This signal was concomitant with microtubule bundling. This strongly suggests that in Glued RNAi cells, the recruitment of Pavarotti-KLP to the plus ends of microtubules and consequently the microtubule bundle formation were delayed rather than completely inhibited, leading to abnormal but successful cytokinesis.

Analysis of mitosis in live cells
Previous observations of fixed preparations suggested that the early recruitment of Pavarotti-KLP to the plus ends of central spindle microtubules, and the consequent microtubule bundling were delayed during early anaphase. However, it seemed that a minimal but sufficient amount of Pavarotti-KLP was able to promote central spindle formation during telophase, allowing cytokinesis to occur. Thus we decided to monitor mitosis in live cells expressing GFP-tagged α-tubulin following control or Glued dsRNA transfection.

In control cells, the time between nuclear envelope breakdown (NEBD) and anaphase onset was 31.1±7.1 minutes (n=16 cells, see also Fig. 8A and supplementary material Movie 1). Consistent with our fixed cell observations, we
frequently found (9 out of 15 cells filmed) centrosome disconnection from the spindle poles before anaphase (Fig. 8B and supplementary material Movie 2) following Glued dsRNA treatment. Filming further revealed a metaphase arrest and none of the 15 cells monitored entered anaphase within the 65-minute recording period. We therefore identified cells that were already in metaphase and began filming at that stage. Upon anaphase entry, spindles became distorted and buckled (Fig. 8C and supplementary material Movie 3). At no point did we observe the accumulation of large numbers of microtubules or microtubule bundles during anaphase (60:48). The cell is also subjected to strong cortex contraction but is able to organise a central spindle region and a midbody (115:4). The panels are maximum projections of Z series and note that at time 66:48 the two poles are not in the same focal plane. The time scale (minutes: seconds) is shown at the bottom of each panel. Bars, 10 μm.

Fig. 8. Following Glued RNAi, centrosomes are not properly attached to spindle poles during spindle formation and microtubule bundling is defective after anaphase. Selected frames from time-lapse series of a control cell (A, see also supplementary material Movie 1) or a Glued dsRNA treated cell (B,C and supplementary material Movies 2 and 3) expressing GFP-tagged α tubulin during mitosis. (A) Mitosis of a control cell. (B) Mitotic spindle formation following nuclear envelope breakdown until metaphase in a Glued dsRNA-treated cell. Note the poor centrosome connection to the spindle poles. (C) Metaphase-to-anaphase transition in a Glued dsRNA-treated cell. To avoid long light exposure and radiation damage, the acquisition started at metaphase. Note the lack of microtubule bundles during anaphase (60:48). The cell is also subjected to strong cortex contraction but is able to organise a central spindle region and a midbody (115:4). The panels are maximum projections of Z series and note that at time 66:48 the two poles are not in the same focal plane. The time scale (minutes: seconds) is shown at the bottom of each panel. Bars, 10 μm.

Discussion

Contribution of DDC to synchronous chromosome segregation

Here we show that in Drosophila cultured cells, knockdown of p150Glued leads to a metaphase arrest and after the eventual transition into anaphase, disrupts chromosome segregation and central spindle formation. Previous studies have shown that perturbation of other DDC subunits can disengage the centrosomes from metaphase spindles in both tissue culture cells and mutant neuroblasts (Goshima et al., 2005; Morales-Mulia and Scholey, 2005; Siller et al., 2005). Likewise our study confirms a role for the DDC in timely anaphase entry. We found that half of the p150 knock down cells shared a common phenotype during karyokinesis. In addition, spindles were long and some chromosomes were clustered at the poles while others remained at the spindle equator. While this phenotype is suggestive of cells with chromosome congression defects, several lines of evidence suggest that these are aberrant anaphases: (1) we consistently found that such abnormal cells had cyclin B levels that were indistinguishable from anaphase controls; (2) the centromeric protein MEI-S322, which is lost from anaphase centromeres, was not detected on those chromosomes which were adjacent to the spindle poles, but was detectable on chromosomes lagging at the equator; (3) the chromosome passenger protein Aurora B, which redistributes to the spindle equator following anaphase onset for successful cytokinesis (see below), was lacking on polar proximal chromosomes but could still be detected on the centromeres of those chromosomes which were adjacent to the spindle poles, but was detectable on chromosomes lagging at the equator; (3) the chromosome passenger protein Aurora B, which redistributes to the spindle equator following anaphase onset for successful cytokinesis (see below), was lacking on polar proximal chromosomes but could still be detected on the centromeres of those chromosomes which were adjacent to the spindle poles, but was detectable on chromosomes lagging at the equator; (3) the chromosome passenger protein Aurora B, which redistributes to the spindle equator following anaphase onset for successful cytokinesis (see below), was lacking on polar proximal chromosomes but could still be detected on the centromeres of those chromosomes which were adjacent to the spindle poles, but was detectable on chromosomes lagging at the equator; and (4) when cells were stained for the spindle checkpoint protein BubR1, the signal intensity at kinetochores found near the spindle poles was identical to those observed in control anaphase cells, whereas the fluorescent signal on the kinetochores seen at the spindle equator was similar to that observed during metaphase. Taken
together, these data strongly suggest that the depletion of p150Glued by RNAi distorts anaphase, allowing some chromosomes to undergo their normal maturation (i.e. loss of checkpoint and centromeric proteins followed by disjunction and segregation to the poles), while others are inhibited from initiating this series of events.

Our observations indicate that the disappearance of Aurora B, MEI-S322 and BubR1 from the centromeres/inner kinetochores may be not be fully dependent on the global loss of MPF activity (note that most cyclin B is degraded in Glued anaphase cells). This suggests that DCC does not influence the initial accumulation of cyclin B on the spindle or kinetochore regions, and is not involved in the degradation of the spindle-associated pool. However, the retention of Aurora B, MEI-S332 and BubR1 at kinetochores in Glued-depleted cells suggests that the DCC is needed for removal of these proteins and for anaphase onset/chromatid disjunction. How the DCC regulates this is unclear. A pool of BubR1 localises to the inner region of the kinetochore (Buffin et al., 2005; Jablonski et al., 1998) where it interacts with Aurora B (Lampson and Kapoor, 2005). In addition, MEI-S322-dependant phosphorylation by aurora B is required for chromatid cohesion (Resnick et al., 2006). Thus, the loss of p150Glued and DCC function may prevent these interactions and the loss or redistribution of key proteins for chromatid cohesion and subsequent chromosome segregation. In addition, previous studies have revealed that a primary function of the DCC, loaded to the kinetochore via the ZW10/Rod complex, is to generate at least in part, poleward chromosome movement during anaphase in many organisms (Savoian et al., 2000; Schmidt et al., 2005; Sharp et al., 2000). Thus, depletion of p150Glued may lead to lagging chromosomes by two methods, in the first, the molecules needed for chromatid disjunction fail to be redistributed and second, there is a failure to secure polewards force generating dynein motor molecules.

Contribution of DDC to central spindle formation
Along with the karyokinetic defects described above, we found that the depletion of Glued also disrupted central spindle formation. This microtubule-based structure consists of overlapping anti-parallel arrays of bundled microtubules and a variety of associated proteins and is crucial for successful cytokinesis (D’Avino et al., 2005). Indeed in many instances, only weak indications of a central spindle could be found. In contrast to control cells which always contained robust central spindles and stained positive for Aurora B and polo, the early forming central spindle structures in Glued RNAi cells lacked detectable levels of these kinases. These molecules are essential for successful cytokinesis (Adams et al., 1998; D’Avino et al., 2005; Echard et al., 2004; Gatti et al., 2000; Giet and Glover, 2001; Somma et al., 2002), leading to the expectation that cleavage should fail in Glued RNAi-treated cells. We failed to detect any octapod cells or elevated numbers of binucleated cells following Glued RNAi in our study, even after a double Glued RNAi treatment. In addition, several RNAi screens recently performed in Drosophila S2 cells, did not identify any DDC encoding genes essential for cytokinesis (Echard et al., 2004; Eggert et al., 2004) although DDC proteins are found in midbody preparations (Skop et al., 2004). It is thus possible that DDC facilitates central spindle stabilisation and contributes to cytokinesis without being absolutely necessary for this process in flies. Indeed, we found that later-staged cells did localise Aurora B and polo to their central spindle structures, indicating that the DDC facilitates the recruitment of these kinases to the central spindle. In agreement with our findings, a recent study revealed that cytokinesis was not abolished but likewise delayed in sea urchin embryos injected with anti-p150Glued antibodies (Strickland et al., 2005).

Our study further revealed that the DDC is needed for the recruitment of Pavarotti-KLP to the spindle midzone. This kinase is involved in central spindle formation, furrow formation and ingression by forming a complex with RacGAP50c to form the centralspindlin complex, a master regulator of cytokinesis (D’Avino et al., 2005). Pavarotti-KLP, which concentrates at the overlapping plus ends of microtubules at anaphase onset in control cells, failed to concentrate at the midzone in p150Glued knockdown cells. However, like Aurora B and polo kinases, Pavarotti-KLP did ultimately concentrate on the central spindles and midbodies of later-staged cells. We further observed that Pavarotti-KLP concentrated at the cell cortex in RNAi-treated cells. This may explain the abnormal contractions seen in both fixed and living preparations because it has previously been demonstrated that ectopic expression of this motor protein can promote the recruitment of other furrow components for furrow ingression (Minestrini et al., 2003). Thus, the DDC appears to be responsible for an efficient localisation of Aurora B and polo kinases as well as of Pavarotti-KLP to the central spindle for a timely onset of furrowing.

In summary, we report here for the first time that p150Glued part of the evolutionarily conserved DDC, is needed for the localised redistribution of the centromeric and/or kinetochore proteins MEI-S332, BubR1 and Aurora B for chromatid disjunction and subsequent segregation. We further show for the first time that p150Glued is involved in cytokinesis. Although not required for successful cell cleavage, our data reveal that Glued increases the efficiency of Aurora B and polo kinase recruitment to the midzone for central spindle formation and stabilisation. We further demonstrate that the DDC is needed for the efficient recruitment of Pavarotti-KLP, a member of the centralspindlin complex, which is essential for cell cleavage.

Materials and Methods

dsRNA production and constructs
To generate Glued dsRNA, a cDNA fragment was amplified by PCR using the oligonucleotides 5'-ATGTAAGACACTCACATATAGGGCGAATGTCCGAGAA-AACCTGAAAAGT-3' and 5'-ATGGTATACGCACTCACTATAGGGCGAAGCGAAGCTGACCCCATC-3', containing a T7 promoter sequence at each end. The 1100 bp PCR product was used as a template to generate RNA using the Megascript kit (Promega). After isolation, the RNAs were boiled for 20 minutes and annealed by slow cooling overnight at room temperature. dsRNA was analysed by agarose gel electrophoresis and aliquoted at −80°C before use in RNAi experiments. To generate a construct for expressing GFP-tagged p150Glued in Drosophila cells, a PCR fragment was amplified using the oligonucleotides 5'-AGTGGATTAGCGACCA-AGCCTCAGTTATACGTGC-3' and 5'-TTGGTACCATTTACCTTTAATATA-3', containing a T7 promoter sequence at each end. The 1100 bp PCR product was used as a template to generate RNA using the Megascript kit (Promega). After isolation, the RNAs were boiled for 20 minutes and annealed by slow cooling overnight at room temperature. dsRNA was analysed by agarose gel electrophoresis and aliquoted at −80°C before use in RNAi experiments. To generate a construct for expressing GFP-tagged p150Glued in Drosophila cells, a PCR fragment was amplified using the oligonucleotides 5'-AGTGGATTAGCGACCA-AGCCTCAGTTATACGTGC-3' and 5'-TTGGTACCATTTACCTTTAATATA-3', containing a T7 promoter sequence at each end. The 1100 bp PCR product was used as a template to generate RNA using the Megascript kit (Promega). After isolation, the RNAs were boiled for 20 minutes and annealed by slow cooling overnight at room temperature. dsRNA was analysed by agarose gel electrophoresis and aliquoted at −80°C before use in RNAi experiments. To generate a construct for expressing GFP-tagged p150Glued in Drosophila cells, a PCR fragment was amplified using the oligonucleotides 5'-AGTGGATTAGCGACCA-AGCCTCAGTTATACGTGC-3' and 5'-TTGGTACCATTTACCTTTAATATA-3', containing a T7 promoter sequence at each end. The 1100 bp PCR product was used as a template to generate RNA using the Megascript kit (Promega). After isolation, the RNAs were boiled for 20 minutes and annealed by slow cooling overnight at room temperature. dsRNA was analysed by agarose gel electrophoresis and aliquoted at −80°C before use in RNAi experiments.

Production of recombinant proteins and antibody purification
p150Glued-Nter(His)6 protein was expressed in E.coli BL21(DE3)pLyS3 (Novagen) for...
4 hours at 25°C. The protein was purified on a Ni-NTA-agarose column (Qagen) following the manufacturer’s instructions. The purified proteinwas dialysed overnight against PBS (136 mM NaCl, 26 mM KCl, 2 mM Na2HPO4, 2 mM KH2PO4, pH 7.2) and used to immunise rabbits to generate Rab1477and Rab1478 antisera.

Rabbit anti-p150Glued antibodies were affinity purified on nitrocellulose membrane. 1 mg p150Glued recombinant protein was immobilised on a nitrocellulose membrane. The membrane was incubated for 10 minutes at room temperature in 100 mM glycine-HCl pH 3 to remove unbound protein, then 2 hours in PBS containing 5% BSA and 0.5% Tween 20 (PBST-BSA) for blocking. The membrane was incubated overnight in 20 ml PBST-BSA containing 10% antisera Rab1477 AND Rab1478. After extensive washings in PBST-BSA, specific anti-p150Glued IgG were eluted with 2 ml of 100 mM Glycine-HCl pH 3 for 10 minutes, neutralised with 200 μl of 1M Tris solution, concentrated using a centricron 30 (PALL) and stored at −80°C at 1 μg/ml.

RNAi, transformations, drug treatment and stable line generation

Drosophila S2 cells were grown and processed for RNAi as described previously (Clemens et al., 2000). Briefly, 106 cells were incubated with 10 μg/ml dsRNA in serum-free medium. Alternatively, 10 μg Transfast transfection reagent were added together with 3 μg dsRNA following the manufacturer’s instructions (Promega). After 1 hour, fresh medium was added to the cells. At 4 days post transfection, the cells were fixed and analysed for mitotic defects (Giet and Glover, 2001). 100-200 cells were analysed using a Coulter Epics Elite Flow Cytometer and stored at −80°C at 1 μg/ml.

DNA transfections, drug treatment and stable line generation

Drosophila S2 cells were grown and processed for RNAi as described previously (Clemens et al., 2000). Briefly, 106 cells were incubated with 10 μg/ml dsRNA in serum-free medium. Alternatively, 10 μg Transfast transfection reagent were added together with 3 μg dsRNA following the manufacturer’s instructions (Promega). After 1 hour, fresh medium was added to the cells. At 4 days post transfection, the cells were fixed and analysed for mitotic defects (Giet and Glover, 2001). 100-200 cells were analysed using a Coulter Epics Elite Flow Cytometer and stored at −80°C at 1 μg/ml.

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