The Dynactin Complex Maintains the Integrity of Metaphasic Centrosomes to Ensure Transition to Anaphase*

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The dynactin complex is required for activation of the dynein motor complex, which plays a critical role in various cell functions including mitosis. During metaphase, the dynein-dynactin complex removes spindle checkpoint proteins from kinetochores to facilitate the transition to anaphase. Three components (p150Glued, dynamitin, and p24) compose a key portion of the dynactin complex, termed the projecting arm. To investigate the roles of the dynactin complex in mitosis, we used RNA interference to down-regulate p24 and p150Glued in human cells. In response to p24 down-regulation, we observed cells with delayed metaphase in which chromosomes frequently align abnormally to resemble a “figure eight,” resulting in cell death. We attribute the figure eight chromosome alignment to impaired metaphasic centrosomes that lack spindle tension. Like p24, RNA interference of p150Glued also induces prometaphase and metaphase delays; however, most of these cells eventually enter anaphase and complete mitosis.

Our findings suggest that although both p24 and p150Glued components of the dynactin complex contribute to mitotic progression, p24 also appears to play a role in metaphase centrosome integrity, helping to ensure the transition to anaphase.

Dynactin is composed of 10 subunit proteins that are required for dynein activation (2) and references therein. Three proteins among them, p150Glued (dynactin 1), dynamitin (p50 and dynactin 2), and p24 (dynactin 3) (3, 4), constitute a flexible and extendable structure (the projecting arm) that associates directly with microtubules and the dynein complex. Each dynactin molecule contains two copies of p150Glued and p24 and four copies of dynamitin. All three proteins are evolutionarily conserved from yeast to mammalian cells (5, 6), suggesting that these components are essential for the formation of a functional projecting arm. Within this substructure, p150Glued is sufficient for binding to dynein and for traversing the microtubule lattice, whereas dynamitin also plays a critical role in association with the dynein complex and in promotion of dynein-based movement. It is noteworthy that overexpression of dynamitin disrupts dynactin structure (7). Although the mechanism underlying this disruption is yet to be elucidated, dynamatin overexpression has been the major tool in molecular biology for down-regulation of dynactin function (2). Indeed, dynamatin overexpression was used to verify involvement of the dynactin complex in the spindle checkpoint silencing that induces metaphase arrest/delay (8).

In contrast to p150Glued or dynamatin, little is known about the role of the p24 subunit in mitosis. Although Ldb18 (a Saccharomyces cerevisiae homolog of p24) is essential for attachment of p150Glued to dynamatin and to the remainder of the dynactin complex (6), low amino acid identity between Ldb18 and human p24 (16.9%) does not favor speculation on the roles of mammalian p24.

RNAi is currently the most useful method for down-regulating the expression of a specific gene. Although several authors report successful suppression of p150Glued using siRNA or shRNA (8–10), their papers did not describe any mitotic abnormalities in cells expressing reduced levels of p150Glued. Moreover, there have been no reports of p24 down-regulation using the RNAi method. In this report, we use RNAi to down-regulate p24 and p150Glued proteins in human cells. Our results demonstrate that cells expressing reduced levels of either p24 or p150Glued both show severe metaphase delay but that other mitotic disturbances differ between the two suppressed genes.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection of siRNA—HeLa, U2OS, and HEK 293 cell lines and their derivative cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. siRNA oligonucleotides for p150Glued (siRNA-p150, 5'—
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GACTTCACCCTGTGATTA-3’; siRNA-p150b, 5’-CCACCCAGGUAAGAU-3’ (10) or p24 (siRNA-p24, 5’-CCGGTGGCATCCTGAT-3’; siRNA-p24b, 5’-GCACUUUGGCCACGUGAG-3’) were transfected at a concentration of 100 nM into HeLa(tc), a HeLa subline (11) or U2OS cells using Oligofectamine (Invitrogen), otherwise indicated in text and figure legends. Dead cells were identified using the trypan blue dye exclusion test. p24 and H2B-GFP were expressed using the pcDNA3 expression vector (Invitrogen).

Rescue Experiments—An siRNA-p24-resistant p24 cDNA was created by changing six nucleotides in the target sequence of siRNA-p24 that have no effect on amino acid sequence (CGGATAGCACATCCAGAC; underlined letters indicate replaced nucleotides). Because the target sequence for siRNA-p150 is in the 3’-UTR, we used a p150 cDNA 3’-UTR truncation (a gift of Dr. M. Katsuno and G. Sobue (12)) to generate a siRNA-p150-resistant p150 cDNA. To generate a pantropic retrovirus, HEK 293 cells were co-transfected with three plasmids: pHIT60 expressing murine leukemia virus gag pol (a gift of Dr. A. J. Kingsman (13)), pHCMV-G for vesicular stomatitis virus envelope pseudotypes (a gift of Dr. T. Friedmann (14)), and pMSCV (Clontech) driving expression of siRNA-resistant p24 or p150 cDNA and IRES-EGFP2 (15).

Analysis of mRNA and Protein Expression—Real-time quantitative RT-PCR was performed as described previously (16) using primer sets (p24 (forward, 5’-GAGTACATCGACCGATTGCCAC-3’ and reverse, 5’-TGTGAGTGAAC-3’), and p150Glued (forward, 5’-TGCAGGACCCGATCTACCCCTTG-3’ and reverse, 5’-GCATATTCTCTAGGACCAC-3’)). Immunostaining and image analyses were performed as described (11, 17). Signal specificity was tested by adding antigen for each antibody into the blocking solution. Relative fluorescence intensity was measured using ImageJ software. Immunoprecipitation and immunoblot analyses were performed according to standard procedures (18).

TUNEL—TUNEL assays were performed using the Fluorescence in Situ Hybridization (FISH) kit (Promega, Madison, WI). Briefly, cells fixed with paraformaldehyde and ethanol were incubated with fluorescein-dUTP and terminal deoxynucleotidyl transferase for one hour at 37 °C. Cells were propidium iodide-stained immediately prior to flow cytometry analysis (FACS-Calibur, BD Biosciences).

Fluorescence in Situ Hybridization (FISH)—HeLa cells cultured on coverglass slips were fixed with 3.7% formaldehyde, denatured on a heat block, and used directly for FISH analysis using a Myc probe complementary to chromosome band 8q24 (Dako, Glostrup, Denmark) according to the manufacturer’s protocol.

Reagents—Rabbit anti-p24 polyclonal antibodies were raised against a GST-p24(N) (amino acids 5–36) or a GST-p24(C) polypeptide (amino acids 145–186) and then affinity purified according to standard procedures (18). Commercial antibodies were purchased from the following suppliers:

p150Glued from BD Biosciences; actin (product no. 1378 996) from Roche Diagnostics; and α-tubulin (product no. T9026) and γ-tubulin (product no. T6557) from Sigma. Hoechst 33342 was purchased from Invitrogen.

RESULTS

Down-regulation of p24 and p150Glued Using siRNA—We initially used HeLa(tc) cells, a HeLa subline that allows high efficiency siRNA transfection (typically >90%) (11), for siRNA transfections. HeLa(tc) cells treated with siRNA (100 nM for 24 h) specific for p24 (siRNA-p24) or p150Glued (siRNA-p150) showed a 5-fold approximate decrease in mRNA expression levels relative to cells treated with scrambled control siRNA (control siRNA) (Fig. 1A).

We used rabbits to generate two polyclonal antibodies, p24(N) and p24(C), against different portions of p24 (see “Experimental Procedures”). Both antibodies recognized an endogenous p24 protein (Fig. 1B, lane 1) that migrated to the same position in SDS-polyacrylamide gels as exogenous p24 protein expressed from a eukaryote plasmid expression vector (lane 4). As previously reported, the apparent mass of p24 is ~21 kDa, slightly smaller than the mass predicted from the amino acid sequence (4). Cells treated with siRNA-p24 (100 nM) for 48 h expressed 10-fold lower levels of p24 protein relative to untreated cells or cells treated with control siRNA (lane 3). Immunoblot analysis using p150Glued antibody revealed a 20-fold reduction in p150Glued protein levels in cells treated with siRNA-p150 for 72 h (Fig. 1C).

Similar to previous reports (4), immunostaining of mitotic cells with p24(C) antibody showed p24 localized to kinetochores and centrosomes in prometaphase and metaphase cells with considerable signal remaining in early anaphase (Fig. 1D). Although the general localization of p150Glued (Fig. 1E) overlapped with p24, some specific differences were distinguished in cells doubly stained with p24 and p150Glued antibodies (Fig. 1F). First, staining of mitotic spindles with anti-p150 was strong, whereas only weak fluorescence was detected with anti-p24. Second, although both p24 and p150 signals were detected at prometaphase kinetochores, p150 signal intensity diminished rapidly in metaphase, whereas p24 signals were retained until anaphase. The contrast between the intense p24 immunofluorescence maintained in anaphase centrosomes with diminished p150Glued immunofluorescence that is barely detectable in early anaphase suggests a rapid efflux of p150Glued from centrosomes during metaphase.

When cells were transfected with Cy3-labeled siRNA-p24, the intensity of p24 immunofluorescence in Cy3 staining-positive cells was reduced (Fig. 1G, left panels). p150Glued signals were also reduced in response to treatment with siRNA-p150 (100 nM) for 72 h (right panels).

siRNA-p24 or -p150 Induces Mitotic Disturbances—Although we observed no obvious morphological differences in phase-contrast microscopy images of interphase HeLa(tc) cells treated for up to 72 h with control siRNA, siRNA-p24, or siRNA-p150 (100 nM) (data not shown), we did observe a significant increase in the mitotic index of cells 48 h after transfection with siRNA-p24 relative to control siRNA (11.1% relative to 4%) (p < 0.01, Chi-square test, Fig. 2A). In addition,
there was also a significant increase \( (p < 0.01) \) in the number of dead cells (determined by trypan blue dye exclusion) among the cells treated with siRNA-p24 for 48 and 72 h (7.2%, 15/207 and 17.1% 36/211, respectively) and siRNA-p150 for 72 h (8.0%, 16/202) relative to untreated cells (2.0%, 8/401).

Flow cytometric analyses of HeLa cells treated with siRNA-p24 for 48 h and stained with propidium iodide dem-
onstrate that siRNA-p24 induces mitotic delay/arrest, in that the G2/M-phase ratio (Fig. 2B, vertical axis) was significantly higher in treated cells (32.2%) than in cells without treatment (19.2%) However, the cell death induced by siRNA-p24 does not appear apoptotic because TUNEL assays showed only a small increase in DNA free 3’ ends (Fig. 2B, horizontal axis; see DNase-treated HeLa cell panel as a positive control) in siRNA-p24-treated cells.

To determine the effects of down-regulation of p24 and p150Glued on mitosis, we analyzed chromosome alignment in Hoechst 33342-stained mitotic cells. Although treatment of cells with control siRNA (100 nM) for up to 72 h did not affect their distribution (Fig. 2C), we observed that treatment with siRNA-p24 for 48 h specifically induced one of two distinct patterns of abnormal chromosome alignment in 39.8% (133/334) of mitotic cells (Fig. 2C). First, chromosomes in 120 mitotic cells treated with siRNA-p24 for 48 h aligned abnormally to resemble a figure eight pattern (Fig. 2D), whereas the same pattern almost never appeared (<1/200) in mitotic cells without siRNA treatment or those treated with control siRNA. Second, 3.9% (13/334) of mitotic cells treated with siRNA-p24 for 48 h demonstrated multipolar mitoses. Among the remaining 201 mitotic cells with normal-looking chromosome alignment (100% total) or abnormal mitosis were determined from observations of Hoechst 33342-stained nuclei. Asterisks indicate statistically significant changes in a particular mitotic phase (p < 0.01) relative to the no treatment control by Chi-square test. D, representative images of Hoescht 33342-stained chromosomes in a figure eight alignment. Bar, 5 μm. Pro, prophase; Prometa, prometaphase; Meta, metaphase; Ana, anaphase; Telo, telophase.

FIGURE 2. Down-regulation of p24 and p150 induces mitotic abnormalities in HeLa(tc) cells. A, cells were treated with each siRNA for periods indicated on the left. From a total of 300 cells counted/treatment, the relative percentages of mitotic, interphase, and dead cells determined by separation of floating cells (mitotic and dead versus interphase) and by trypan blue dye exclusion (live versus dead) are indicated by shading. Asterisks indicate a statistically significant (p < 0.01) increase relative to the no treatment control using Chi-square test. B, cells treated with siRNA-p24 for indicated periods were stained with propidium iodide (PI) just prior to flow cytometry and TUNEL analysis. FL1 values on the horizontal axis indicate the amount of dUTP polymerized by DNA free 3’ ends, whereas FL2 values on the vertical axis show the DNA content. C, from a total of 300 cells counted/treatment, the percentages of mitotic cells with normal-looking chromosome alignment (100% total) or abnormal mitosis were determined from observations of Hoechst 33342-stained nuclei. Asterisks indicate statistically significant changes in a particular mitotic phase (p < 0.01) relative to the no treatment control by Chi-square test. D, representative images of Hoechst 33342-stained chromosomes in a figure eight alignment. Bar, 5 μm. Pro, prophase; Prometa, prometaphase; Meta, metaphase; Ana, anaphase; Telo, telophase.
strated the figure eight chromosome alignment seen with siRNA-p24 (Fig. 2C). After an additional 24 h of treatment with siRNA-p150, however, we observed a significant increase in the ratio of prometaphase cells and a corresponding decrease in anaphase and telophase cells. In addition, small percentages of mitotic cells showed the figure eight chromosomal alignment (11.9%, 28/236) or multipolar mitoses (3.0%, 7/236).

We also treated cells with secondary siRNAs that target alternative sequences in the p24 or p150Glued genes. When cells were treated with siRNA-p24b, which only reduced p24 levels ~3-fold (Fig. 3A), few mitotic cells showed the figure eight chromosome alignment (Fig. 3B), suggesting that greater reductions in p24 protein levels are required to induce the figure eight alignment. In contrast, cells treated with siRNA-p150b reduced p150 to levels similar to siRNA-p150 (Fig. 3A) and showed similar mitotic disturbances, including an increased ratio of prometaphase cells, a decreased ratio of anaphase/telophase cells, and a few cells with figure eight chromosome alignment.

To exclude the possibility that the figure eight chromosome alignment is an off-target effect of siRNA-p24, we performed
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FIGURE 4. Time-lapse observations of mitotic cells. A, immunoblot analyses of lysates from U2OS cells using p24(C), p150Glued, or β-actin antibody. Cells were treated with siRNA indicated for 72 h. Ratios of relative intensity (p24 or p150/actin) measured by densitometry are indicated below each lane. An arrow marks the position of p24. B, U2OS cells expressing an H2B-GFP fusion protein were treated with control siRNA or siRNA-p150 for >48 h or with siRNA-p24 for >24 h. Percentages of cells demonstrating termination of mitosis in one of the manners listed on the right are indicated with shading. Asterisks indicate a statistically significant increase in duration (p < 0.05) relative to the no treatment control.

rescue experiments by expressing a siRNA-p24-resistant p24 mRNA from a pantropic retrovirus containing EGFP as a selection marker (see “Experimental Procedures”). EGFP-positive cells were sorted using flow cytometry and then treated with siRNA-p24.

Immunoblot analyses revealed similar levels of p24 in cells infected with retrovirus containing p24 cDNA or siRNA-resistant p24 cDNA (Fig. 3C, lanes 5 and 7). Following treatment with siRNA-p24 (100 nM for 48 h), uninfected cells, as well as those infected with empty virus or virus containing wild-type cDNA (Fig. 3C, lanes 2, 4, and 6) showed down-regulation of p24 protein. In contrast, there was no significant reduction in p24 levels in cells expressing the siRNA-resistant p24 cDNA (Fig. 3C, lane 8). Mitotic cells with the figure eight chromosome alignment were observed in cells infected with empty virus (73%) or virus containing wild type cDNA (68.5%) at virtually the same frequency as uninfected cells (77.3%) (Fig. 3D). Only cells infected with virus containing the siRNA-resistant cDNA showed a significant reduction in the frequency figure eight mitoses (34.5%, p < 0.01), suggesting that p24 is indeed effective in preventing chromosomes from the figure eight alignment.

We also expressed a siRNA-resistant p150 cDNA in HeLa cells using the same pantropic retrovirus system (Fig. 3E). Unlike uninfected cells or those infected with the empty virus, cells expressing siRNA-resistant cDNA showed little decrease in p150 levels following siRNA treatment. Similarly, there was no significant increase in prometaphase or decrease in ana/telophase ratios in cells treated with siRNA-p150 (Fig. 3F), and the ratio of cells with the figure eight chromosome alignment decreased.

Chromosomes Align into a Figure Eight after Metaphase Arrest—To further analyze the mitotic disturbances induced by p24 or p150Glued down-regulation, we established a U2OS cell line expressing a histone H2B-GFP fusion protein constitutively (19), transfected these cells with control, p24, or p150Glued siRNAs (efficiency typically 70%) and then collected time-lapse images of the transfected cells. In these cells, the magnitude in reduction of p24 or p150Glued protein expression levels by siRNA-p24 or siRNA-p150, respectively, were similar to those achieved in HeLa cells (Fig. 4A).

More than 95% of mitotic cells without siRNA treatment or treated with control siRNA completed mitosis (Fig. 4B, also see supplemental Movie 1). Cells treated with control siRNA demonstrated a significant elongation of metaphase (46 min in control siRNA-treated cells to compare with 29 min in untreated cells, p < 0.05), whereas the durations of prophase, prometaphase, and anaphase and telophase combined (ana/telophase) were not affected (Fig. 4C). These data suggest that U2OS cells treated with scrambled siRNA and/or cationic liposome experience a delayed progression through metaphase. Only one of 180 (0.55%) untreated cells and zero of 156 cells treated with control siRNA died while in mitosis.

When cells were treated with siRNA-p24 (100 nM) for >24 h, we observed a significant delay in metaphase (average of 66 min) relative to control siRNA-treated cells (average of 46 min, p < 0.05, Fig. 4C). There was no elongation in the duration of prometaphase or ana/telophase. We also observed cell death more frequently in siRNA-p24-treated cells: 3% (10/333) or 6.5% (22/333) of mitotic cells underwent cell death during prometaphase or metaphase, respectively (Fig. 4B). Intriguingly, in 21.5% (72/333) of siRNA-p24-treated mitotic cells, chromosomes aligned at the metaphase plate during prolonged metaphase broke up into a figure eight pattern (supplemental Movie 2). This similarity to the abnormal chromosome alignment observed in siRNA-p24-treated HeLa cells (Fig. 2D) indicates that these are not prometaphase cells but rather post-metaphasic cells that fail to enter anaphase. All 72 cells with figure eight chromosome alignment underwent cell death eventually (an average of 164 min after breakup of chromosome alignment at the metaphase plate) (supplemental Movie 2). Overall, 31% (104/333) of siRNA-p24-treated mitotic cells underwent cell death, whereas these cells in interphase rarely underwent apoptosis (≤ 0.1%).

In contrast to control siRNA- or siRNA-p24-treated cells, cells treated with siRNA-p150 for >48 h demonstrated delayed prometaphase (average of 23 min to compare with 12 min in control siRNA-treated cells, p < 0.05, Fig. 4C), during
which 2.4% (4/163) underwent cell death. Moreover, the metaphase delay induced by siRNA-p150 (average 85 min, Fig. 4C, see Video 3) was more severe than in cells treated with siRNA-p24 and 6.7% (11/163) of siRNA-p150-treated mitotic cells underwent cell death during metaphase. These findings are consistent with previous experiments in which HeLa cells treated with siRNA-p150 showed increased ratios of cells in prometaphase and metaphase relative to cells in anaphase and telophase (Fig. 2C). Unlike cells treated with siRNA-p24, none of these mitotic cells showed chromosomes aligned in a figure eight pattern, and all cells that survived through prometaphase and metaphase entered anaphase.

Cells with Figure Eight Chromosome Alignment Share Features with Prometaphase—Our findings demonstrate that down-regulation of either p24 or p150Glued induces severe metaphasic delays. This phenotype is analogous to a previous report in which HeLa cells treated with siRNA-p150 showed increased ratios of cells in prometaphase and metaphase relative to cells in anaphase and telophase (Fig. 2C). Unlike cells treated with siRNA-p24, none of these mitotic cells showed chromosomes aligned in a figure eight pattern, and all cells that survived through prometaphase and metaphase entered anaphase.

Despite their similarities in appearance to prometaphase (rather than anaphase) cells, the figure eight chromosome alignment is only seen in cells that have progressed through prometaphase and metaphase and entered anaphase.

**FIGURE 5. Mechanisms of figure eight chromosome alignment.** A, B, and D, HeLa cells were treated with the siRNA indicated and immunostained with the antibody indicated on the left. DNA was stained with Hoechst 33342, and the mitotic phase of cells are labeled above or within each image. Bars, 10 μm (A and B) and 5 μm (D). C, HeLa cells treated with siRNA-p24 were fixed with formaldehyde, denatured, and hybridized with a c-myc probe for FISH analysis. DNA was stained with DAPI. Arrows mark pairs of dots that indicate sister chromatids. Bar, 5 μm. E, HeLa cells were treated with siRNA-p24 and immunostained with the antibody indicated on the left. Relative fluorescence intensity of proteins in either metaphase (black bars) or figure eight (open bars) chromosomes (right panels). The mean (± S.D.) intensity of 50 centrosomal areas was measured, and background levels were subtracted. Bar, 5 μm. Prometa, prometaphase; Meta, metaphase; Ana, anaphase.

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chromosome band 8q24 indicate that sister chromatids in cells with figure eight alignment have yet to segregate because four pairs of dots are visible on chromosomes in figure eight cells (Fig. 5C, arrows; FISH with chromosome 8-specific centromeric probes revealed that HeLa(tc) cells have four chromosome 8 (data not shown)). These data suggest that cells with figure eight chromosome alignment are most like prometaphase cells.

Because the alignment of chromosomes on the metaphase plate is mediated by tension between the spindle poles, reversal of the mitotic process by siRNA-p24 interference may be due to reductions in spindle tension. Using β-H9251-tubulin immunostaining, we compared the density and morphology of spindles in cells at different stages of mitosis. In cells displaying a figure eight configuration (Fig. 5D, panel 3), the robust fluorescence characteristic of metaphase mitotic spindles (panel 2) reverted to a shape and intensity more characteristic of mitotic spindles in prometaphase (panel 1). Moreover, the marked decrease in immunostaining signal intensity for β-H9251-tubulin and CG-NAP (pivotal components of the β-H9251-tubulin ring complex that provides microtubule nucleation sites) in figure eight-stage cells relative to metaphase cells (Fig. 5E) suggests that p24 is required to maintain the integrity of metaphase centrosomes.

**p24 Levels Relate to Figure Eight Configuration**—
In parallel experiments conducted in two different cell lines (HeLa or U2OS), metaphase arrest/delay was induced similarly following treatment with either siRNA-p24 or siRNA-p150; however, siRNA-p24 was significantly more effective than siRNA-p150 in inducing the figure eight chromosome alignment during metaphase (Figs. 2C and 4, B and C). We did not observe any change in expression of p150Glued and p24 mRNAs following treatment with siRNA-p24 or siRNA-p150, respectively (Fig. 1A). However, both of these siRNAs did reduce protein expression levels of p24, p150Glued, and dynamitin in HeLa(tc) cells (Fig. 6A). Similar results were obtained in U2OS cells (data not shown). Similar to previous reports demonstrating that overexpression of dynamitin disrupts dynactin structure (2), our results with p24 and p150Glued suggest that balanced availability of all
components is required to maintain the stability of the dynactin complex.

To test whether changes in p24 expression levels are sufficient to induce the figure eight chromosome alignment, we treated cells with siRNAs and then measured the expression levels of dynactin components and observed alterations in chromosome alignment. When cells were treated with siRNA-p150 for 24 or 48 h, expression levels of p24, dynamitin, and p150Glued relative to control cells were ~25% or higher (Fig. 6B), but no mitotic abnormalities were observed (Fig. 2, A and C). Treatment with siRNA-p150 for 72 h reduced the expression levels of dynamitin and p150Glued to <10% and p24 to ~20%, and this treatment induced metaphase arrest/delay and a few cells with the figure eight chromosome alignment (Fig. 2C). In contrast, treatment of cells with siRNA-p24 for 24 or 48 h reduced the expression levels of p24 to less than 15%, while maintaining relatively high (>25%) levels of dynamitin and p150Glued. In this case, more mitotic cells showed a figure eight chromosome alignment (Fig. 2C).

We also treated HeLa(tc) cells with a combination of both siRNA-p24 and siRNA-p150 (50 or 100 nM each) for 72 h. Although this procedure reduced both p24 and p150 expression levels to ~10% of those in control cells (Fig. 6C, lanes 2 and 3), the ratio of cells in the figure eight alignment (42–45%) was virtually the same as cells treated with siRNA-p24 alone (43%, Fig. 6D), in which p24 and p150 expression are reduced to ~10 and 25% of control levels, respectively (Fig. 6C, lane 4). Once again, cells treated with siRNA-p150 alone that expressed p24 at 25% and p150 at 10% of control cells (lane 5) showed only a small number (~10%) of mitotic cells with the figure eight configuration (Fig. 6D). These data demonstrate that among all components of the dynactin complex, manipulation of p24 expression levels is most likely to result in cells assuming the figure eight configuration.

Further evidence in support of this correlation was observed when treated cells were immunostained simultaneously for p24 and p150Glued. p24 immunofluorescence at centrosomes of mitotic HeLa(tc) cells treated with siRNA-p24 was markedly less intense in cells with the figure eight chromosome alignment relative to metaphasic cells in the same microscopic field (Fig. 6E, panel 3). Intriguingly, p150 immunofluorescence at centrosomes in most (23/25) of these figure eight cells was as bright as the signal in metaphasic cells (Fig. 6E, panel 5). In contrast, in cells treated with siRNA-p150, both p24 and p150 immunofluorescence signals in figure eight cells were markedly reduced (Fig. 6E, panels 4 and 6) in all cells observed (25/25). These results suggest that failure of p24 to accumulate in mitotic centrosomes induces figure eight chromosome alignment.

**DISCUSSION**

In this report, we treated HeLa and U2OS cells with siRNAs specific for p24 or p150Glued, two components of the projecting arm of the dynactin complex. Time-lapse observations reveal that treatment of siRNA-p24 induces severe metaphase delay. In >40% of metaphase cells struggling to enter anaphase, chromosomes aligned on the metaphase plate break away and assume a configuration resembling a figure eight. In time, all cells with the figure eight alignment die without completing mitosis. In contrast with p24, cells treated with siRNA-p150 demonstrate severe delays in metaphase, but most cells do not assume the figure eight alignment and ultimately progress to anaphase. Neither p24 nor p150Glued appears to be involved in the progression of anaphase and telophase.

Reductions in p24 or p150Glued protein levels induce metaphase arrest/delay (Fig. 4C and supplemental Movies 1–3). This is consistent with a previous report in which cells overexpress dynamin, which down-regulates dynactin function, also undergo metaphase arrest/delay (8). Failure to remove spindle checkpoint proteins such as BubR1 from kinetochores (Fig. 5A) results in metaphase delay because checkpoint proteins inhibit cdc20, a specific activator of the anaphase-promoting complex/cyclosome (reviewed in Ref. 20). Because anaphase-promoting complex/cyclosome functions as an E3 ubiquitin ligase, mitotic checkpoint proteins that are remain on kinetochores delay ubiquitin-dependent degradation of cyclin B1 (Fig. 5B) or securin, which then inhibits cohesin hydrolysis and blocks chromosome segregation (Fig. 5C).

Nonetheless, the transition of chromosomes from a metaphase to a figure eight configuration does not appear to be a direct result of spindle checkpoint protein retention at kinetochores. Because there is a marked reduction of γ-tubulin and CG-NAP signals at mitotic centrosomes in cells with the figure eight chromosome alignment (Fig. 5E), insufficient amounts of γ-TuRC in metaphase centrosomes appear to be one of the major factors driving the phenotype. Thus, we hypothesize that loss of spindle microtubule tension (Fig. 5D) is the defect that induces chromosomes to break away from alignment at the metaphase plate.

The two functions of the dynactin complex are to remove spindle checkpoint proteins from kinetochores and to maintain centrosome integrity until entry into anaphase. Although we have observed that neither of these functions are disturbed when expression levels of p24, dynamitin, or p150Glued remain at least 25% of control levels (siRNA-p150 for 48 h, see Figs. 2C and 6A), we did note that a 10-fold reduction in the expression of either p24 or p150Glued impaired the removal of checkpoint proteins, resulting in metaphase delay. In contrast, chromosome integrity and the resulting formation of figure eight cells was only induced with a 10-fold reduction in p24 levels but not with a reduction in p150Glued. Immunofluorescence experiments also emphasized a correlation between accumulation of p24 (but not p150Glued) in centrosomes and figure eight chromosome alignment (Fig. 6E). These data suggest that p24 acts independently of p150Glued in metaphase centrosomes, where it helps maintain chromosome integrity and prevents breakaway until entry into anaphase.

Although it is generally accepted that p24, dynamitin, and p150Glued function together in the dynactin projecting arm (2), we and others (4, 21) have observed that the localization of different dynactin components during mitosis varies considerably. For example, p24 immunofluorescence is strong at centrosomes relative to mitotic spindles or kinetochores. This is particularly evident during early anaphase (Fig. 1, E and F)
when p150<sup>Glued</sup> immunofluorescence is barely detectable. In conclusion, our results suggest that p24 operates independently from the dynactin complex in binding to metaphase centrosomes and maintaining their integrity. To explore this hypothesis further, we are currently investigating the function of p24 in metaphase centrosomes in greater detail.

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