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Distinct cell cycle–dependent roles for dynactin and dynein at centrosomes

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Centrosomal dynactin is required for normal microtubule anchoring and/or focusing independently of dynein. Dynactin is present at centrosomes throughout interphase, but dynein accumulates only during S and G_2 phases. Blocking dynein-based motility prevents recruitment of dynactin and dynein to centrosomes and destabilizes both centrosomes and the microtubule array, interfering with cell cycle progression during mitosis. Destabilization of the centrosomal pool of dynactin does not inhibit dynein-based motility or dynein recruitment to centrosomes, but instead causes abnormal G_1 centriole separation and delayed entry into S phase. The correct balance of centrosome-associated dynactin subunits is apparently important for satisfaction of the cell cycle mechanism that monitors centrosome integrity before centrosome duplication and ultimately governs the G_1 to S transition. Our results suggest that, in addition to functioning as a microtubule anchor, dynactin contributes to the recruitment of important cell cycle regulators to centrosomes.

Introduction

The centrosome is one of the least well-understood organelles in the eukaryotic cell. Its protein composition and functions remain ill defined, but both show important variations among cell types and across the cell cycle. Centrosomes are required for cell cycle progression from G_1 into S phase and again as cells exit cytokinesis (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Piel et al., 2001). Throughout the cell cycle, centrosomes are the predominant site for microtubule nucleation, but only in certain cells, such as fibroblasts, do microtubules remain tightly focused in a radial array. In cell types such as neurons, muscle, and epithelia, most, if not all, microtubules are released to yield a noncentrosomal array whose final organization can take multiple forms.

The precise mechanisms by which microtubules remain focused and anchored at centrosomes in fibroblasts, and how this organization becomes altered in nonfibroblastic cells, are still being defined. In G_1 cells that contain only one centriole pair, microtubule-anchoring activity appears to be predominantly associated with the older of the two centrioles (designated the mother centriole; Piel et al., 2000). A number of proteins, including the proposed microtubule-anchoring protein, ninein, are selectively bound to the mother centriole (for review see Doxsey, 2001). We found previously that dynactin was necessary for maintenance of the normal radial microtubule array (Quintyne et al., 1999). Dynactin is concentrated at centrosomes (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993; Dictenberg et al., 1998), but it is not known with which centriole it associates.

Dynactin is best characterized as an "activator" of the minus end–directed microtubule motor, cytoplasmic dynein (Gill et al., 1991; Schroer and Sheetz, 1991). Dynactin facilitates dynein-based movement by acting as both a processivity factor (King and Schroer, 2000) and an adaptor that mediates dynein binding to subcellular cargoes and the cell cortex (for reviews see Karki and Holzbaur, 1999; Allan, 2000; Dujardin and Vallee, 2002). This dual function takes advantage of dynactin's bipartite structure. A projecting $p150^{Glued}$ sidearm binds both microtubules and dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; Waterman-Storer et al., 1995; Quintyne et al., 1999; Vaughan et al., 2001), whereas a backbone element comprised mostly of the actin-related protein Arp1 is thought to bind cargo (Schafer et al., 1994; for review see Allan, 2000; Muresan et al., 2001).

Cytoplasmic dynein and dynactin are found on endomembranes (Roghi and Allan, 1999; Habermann et al., 2001), the cell cortex (Dujardin and Vallee, 2002), and kinetochores and mitotic spindle poles (Pfarr et al., 1990; Steuer et al., 1990; Echeverri et al., 1996). The importance of the dynein–dynactin motor in microtubule minus end

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focusing at spindle poles (Compton, 2000; Heald, 2000) suggested that these proteins might provide a similar function at centrosomes during interphase. In keeping with this hypothesis, overexpression of a series of dominant negative inhibitors that interfered with dynein and dynactin function in distinct ways resulted in disorganization of fibroblastic microtubule arrays (Quintyne et al., 1999). All of the inhibitors prevented proper targeting of dynein to cargo, but none altered dynactin–cargo binding or, presumably, the ability of dynein itself to move on microtubules.

Dynamitin had the broadest effect on cellular architecture. Dynamitin disrupts the endogenous pool of cellular dynactin, yielding a "free" pool of $p150^{Glued}$ that can still bind dynein but not cargo. In addition to its expected effects on dynactin structure and the Golgi complex (Echeverri et al., 1996; Burkhardt et al., 1997), dynamitin overexpression causes defocusing of the radial microtubule array and a redistribution of the pericentriolar proteins γ-tubulin and dynactin. Full-length $p150^{Glued}$ or a dynein-binding fragment, $p150^{217–548}$, have no effect on endogenous dynactin structure, but act as competitive inhibitors of the dynein–dynactin interaction by binding dynein and preventing it from binding dynactin and cargo. Because these three inhibitors all interfere with dynein–cargo targeting, they have similar effects on endomembrane, microtubule, and centrosome organization (Quintyne et al., 1999).

Two other inhibitors, p24 and a second $p150^{Glued}$ fragment, $p150^{926–1049}$, are significantly more selective in their effects. Neither perturbs cytosolic or membrane-associated dynactin, dynein–dynactin binding, or dynein targeting, as endomembrane localization, motility, and dynactin structural integrity are unaffected. These inhibitors appear to disrupt only the centrosomal pool of dynactin, causing the loss of $p150^{Glued}$ from Arp1, which results in microtubule disorganization and compromised centrosome integrity (Quintyne et al., 1999). This suggested that centrosomal $p150^{Glued}$ was the dynactin subunit most important for microtubule anchoring and/or focusing during interphase. However, it was not clear whether $p150^{Glued}$ was acting directly by anchoring microtubules or indirectly by binding dynein, which could then focus microtubules.

Like the cell's genome, the centrosome must reproduce once per cell cycle. Centrosome doubling involves centriole pair splitting or disorientation during G_1, centriole duplication during S, and the complete separation of the two centriole pairs to yield spindle poles at the onset of mitosis (for reviews see Doxsey, 2001; Hinchcliffe and Sluder, 2001). In parallel with centriole duplication, the pericentriolar material (PCM)* becomes amplified. Some PCM components, such as pericentrin, γ-tubulin, and PCM-1, are recruited to the centrosome in a microtubule- and dynein–dynactin-dependent manner (for review see Zimmerman and Doxsey, 2000). A variety of other proteins, many of them regulatory kinases, are selectively recruited to the centrosome at particular stages of the cell cycle (for review see Lange, 2002), possibly via microtubule-based transport as well. The activities of such kinases and phosphatases are proposed to underlie the transition from G_1 to S and exit from cytokinesis, both of which require centrosomes (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Piel et al., 2001).

In our original analysis of centrosomal dynactin function we noted that centrosomes stained differentially for dynactin and dynein; most exhibited dynactin, but only some dynein. The present study is aimed at gaining a better understanding of the roles of centrosomal dynactin and dynein, specifically with respect to interphase microtubule organization and cell cycle progression. We find that dynactin is concentrated at centrosomes throughout interphase, but that dynein is detected only during S and G_2. Thus, maintenance of the G_1 microtubule array appears not to require centrosomally accumulated dynein. Dynactin is associated preferentially with the mother centriole in G_1 cells, providing further support for its proposed role as a microtubule anchor. The functions of centrosomal dynactin and dynein were probed further by dynactin subunit overexpression. As expected, based on their inhibitory effects on centrosomal dynactin, overexpression of certain inhibitors prevented dynein recruitment but did not affect cell cycle progression until mitosis. Inhibitors that cause just $p150^{Glued}$ to be lost from centrosomes did not block dynein accumulation, suggesting a novel mechanism for dynein recruitment. Surprisingly, these inhibitors caused abnormal centriole splitting in G_1 and delayed entry into S phase. Our findings suggest that the integrity of centrosomal dynactin contributes to proper centriole pairing and timely entry into S phase, and provide further evidence that S phase entry is regulated by centrosome-dependent events.

Results

Dynein binds centrosomes in a cell cycle–dependent manner

Because $p150^{Glued}$ can bind both microtubules and dynein, our previous study (Quintyne et al., 1999) did not clearly distinguish a novel microtubule-anchoring role for dynactin at centrosomes from its more common role as a dynein targeting factor. To resolve this ambiguity, we determined the prevalence of centrosomal dynactin and dynein in unsynchronized Cos-7 fibroblasts. The vast majority (80–95%) of cells exhibited centrosomal dynactin, whereas dynein was seen at centrosomes in only about two thirds of the cells in the population (Table I, top row). A possible explanation is that dynein binding to centrosomes is cell cycle dependent. To test this possibility, we synchronized cells at the G_1–S boundary using a double thymidine block, and then stained for dynein or dynactin at different times after thymidine washout (Fig. 1). Our criteria for cell cycle progression was centriole duplication, as indicated by the centriole-associated protein centrin. Centrosomal dynactin was observed at all cell cycle stages, but centrosomal dynein was detected only in cells that contained two centriole pairs, suggesting that dynein accumulates at centrosomes late in the cell cycle (starting in mid-S phase) and is lost immediately after mitosis. That high concentrations of centrosomal dynein do not appear to be required for microtubule organization during interphase further emphasizes the importance of centrosomal dynactin as a microtubule anchor.

*Abbreviations used in this paper: CNAP-1, centrosomal Nek2-associated protein-1; PAR, poly-ADP-ribose; PCM, pericentriolar material.

Table I. **Effects of dynactin subunit overexpression on centrosomal dynein and dynactin localization**

Overexpressed protein	Centrosomal p150	Centrosomal Arp1	Centrosomal dynein	Mitotic cells	Nuclear PAR
None (untransfected)	92 ± 4.3	83 ± 5.1	64 ± 3.1	5 ± 4.3	0.5 ± 0.3
Control (β-Gal)	93 ± 2.6	84 ± 1.4	66 ± 2.3	5 ± 3.2	ND
Dynamitin	19	40	7 ± 2.0	12 ± 5.1	ND
$p150^{217–548}$	22	30	7 ± 1.4	12 ± 1.4	1.0 ± 0.5
$p150^{926–1049}$	37	89	46 ± 3.6	5 ± 2.8	0.7 ± 0.3
p24	39	85	50 ± 4.2	0	8.8 ± 1.4

Unsynchronized cell populations were transfected with dynactin subunit expression constructs, processed for immunofluorescence, and scored for centrosomal $p150^{Glued}$, Arp1, dynein (intermediate chain), mitotic spindles (α-tubulin), or nuclear accumulation of the apoptotic cell marker PAR. Overexpressing cells were identified by GFP (dynamitin, $p150^{926–1049}$, and p24) or DsRed ($p150^{217–548}$) fluorescence. The percentage of cells scoring positive is provided for each condition. Values for $p150^{Glued}$ and Arp1 are from Quintyne et al. (1999). β-Gal, β-galactosidase.

Dynactin is localized to the mother centriole

Fibroblast microtubules appear to be anchored to the mother centriole exclusively (Piel et al., 2000). We therefore examined dynactin's distribution within centrosomes more closely (Fig. 2). Double staining of unsynchronized cells for dynactin and centrin revealed that dynactin localized to a single centriole. Cells were then stained for dynactin and ε-tubulin, a marker for the mother centriole in G_1 (Chang and Stearns, 2000). Both $p150^{Glued}$ and Arp1 staining showed close overlap with ε-tubulin. Deconvolution of these images (Fig. 2, bottom) suggested that dynactin enveloped the mother centriole in a horseshoe- or cup-shaped structure, similar to the localizations of the proposed microtubule anchors ninein and CEP100 (Mogensen et al., 2000; Ou et al., 2002).

Dynein and dynactin can bind centrosomes by different mechanisms

Overexpression of dynactin shoulder/sidearm subunits results in a loss of $p150^{Glued}$ from centrosomes (Quintyne et al., 1999). Because $p150^{Glued}$ is critical for dynein binding, it seemed likely that targeting of dynein to centrosomes would also be perturbed. To test this hypothesis, we analyzed dynein distribution in these cells (Table I; Quintyne et al., 1999). Overexpression of dynamitin or a dynein-binding fragment of $p150^{Glued}$ ($p150^{217–548}$; referred to as CC1 in Quintyne et al., 1999) was strongly inhibitory; only 7% of cells contained detectable centrosomal dynein in comparison with ~65% of controls. Surprisingly, overexpression of two other dynactin inhibitors, p24 or a second $p150^{Glued}$ fragment ($p150^{926–1049}$; referred to as CC2 in Quintyne et al., 1999), had only a minor effect on centrosomal dynein recruitment.

Cells overexpressing dynactin shoulder/sidearm subunits show relatively normal patterns of initial microtubule nucleation and PCM recruitment, but centrosomes and the microtubule array disintegrate over time (Quintyne et al., 1999). Given the unexpected behavior of dynein in cells overexpressing the latter two inhibitors, we thought it would be informative to examine centrosomal recruitment of dynactin and dynein under conditions of initial microtubule growth (Fig. 3). Cells whose microtubules have been depolymerized by nocodazole and cold no longer exhibit centrosomal dynactin (Paschal et al., 1993; Quintyne et al., 1999; Fig. 3, 0 min time points), but both dynactin and dynein reaccumulate after nocodazole washout (Fig. 3 A) with kinetics similar to microtubule regrowth (Quintyne et al., 1999).

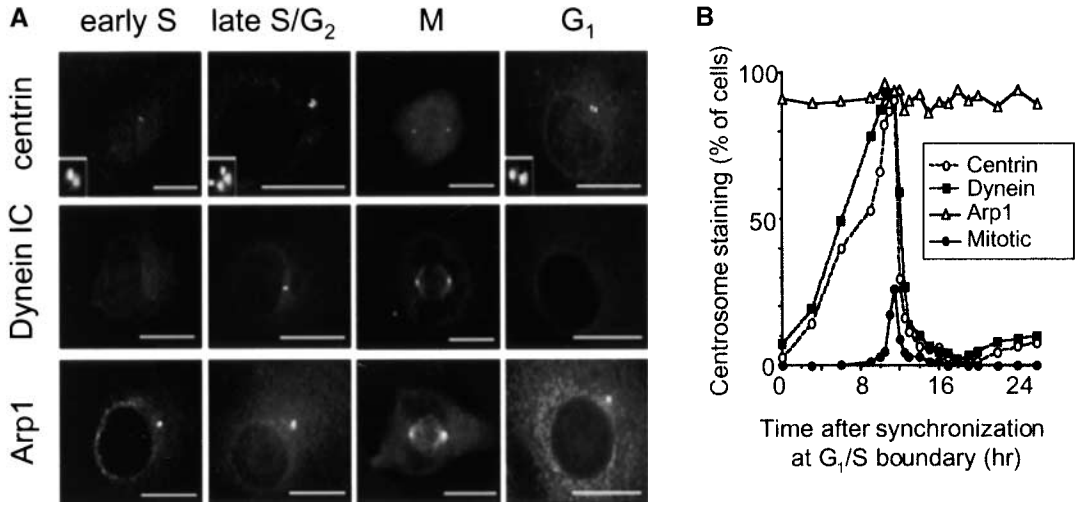

Figure 1. **Cell cycle localization of dynein and dynactin to the centrosome.** (A) Cells were synchronized using a double thymidine block and then released for increasing intervals before being fixed and labeled with Abs to the centriole marker, centrin, dynein (IC), or Arp1. Bars, 10 μm. (B) Cell populations were scored for either a centrosomal focus of dynein IC or dynactin subunit ($p150^{Glued}$ or Arp1) and for four centrin foci. At each time point, the number of mitotic cells was also determined on the basis of the characteristic mitotic patterns of centrin and dynein–dynactin staining (as in A). At least 400 cells were scored per time point in two independent experiments.

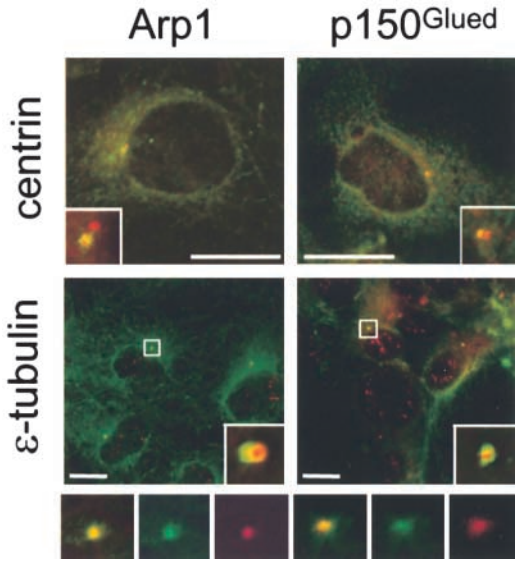

Figure 2. **Localization of dynactin at centrioles.** (Large panels) Cells were double labeled with Abs to $p150^{Glued}$ or Arp1 (green) and centrin or ε-tubulin (red); fiduciary markers were used to verify that the offset seen here is real. Insets show the boxed centrosome enlarged 5×. (Bottom) Deconvolution microscopy of the centrosome boxed in the panels above. Right panels, merge. Bars, 10 μm.

Overexpression of dynamitin or $p150^{217–548}$ completely prevented accumulation of dynactin and dynein at centrosomes (Fig. 3 B). This result was dramatic but expected. Because dynamitin and $p150^{217–548}$ are thought to block dynein–dynactin binding, they will prevent dynein-based transport of dynactin to the centrosome. Dynein is not expected to bind centrosomes that lack dynactin.

Overexpression of p24 or $p150^{946–1049}$ had no effect on dynactin accumulation at centrosomes initially, but over time $p150^{Glued}$ was lost whereas Arp1 remained behind (Fig. 3 C). After ~3 h, the steady-state condition was reached in which >85% of centrosomes labeled for Arp1 but only ~40% labeled for $p150^{Glued}$ (Table I). Dynein also accumulated at centrosomes, but much more slowly than in control cells (Fig. 3, compare A and C). This suggests that centrosomal dynein targeting to centrosomes in cells overexpressing p24 or $p150^{926–1049}$ occurs via a different mechanism from that operating under normal conditions. At even later time points (>210 min), we commonly observed centrosomes that stained for dynein but not $p150^{Glued}$ (unpublished data), suggesting that binding is $p150^{Glued}$ independent.

Effects of the loss of centrosomal dynactin and dynein on progression through S, G_2, and M phases

Dynein accumulates at centrosomes during S and G_2 phases and is highly enriched at mitotic spindle poles, suggesting that it is recruited in preparation for mitosis. Inhibition of dynein–dynactin function profoundly affects spindle formation and pole maintenance in many systems (for reviews see Compton, 1998; Heald, 2000). Dynamitin overexpression causes cells to arrest in pseudoprometaphase with fragmented or monopolar spindles (Echeverri et al., 1996; Dujardin et al., 1998), consistent with our observation that such cells lack centrosomal dynein and the consequent loss

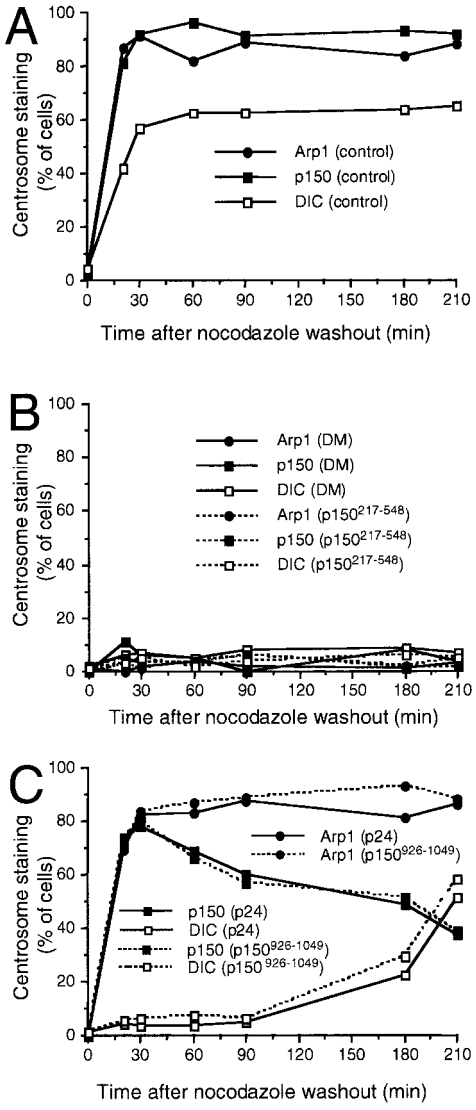

Figure 3. **Effects of dynactin subunit overexpression on accumulation of centrosomal dynein and dynactin during microtubule regrowth.** Microtubules in transiently transfected cells were depolymerized by nocodazole/cold treatment and then allowed to regrow (see Materials and methods for details). The appearance of a centrosomal focus of dynein intermediate chain (DIC; similar results were obtained using Abs to dynein light IC) or dynactin (Arp1 or $p150^{Glued}$) was analyzed by immunofluorescence. Overexpressing cells were identified by GFP (dynamitin, p24, and $p150^{926–1049}$) or DsRed ($p150^{217–548}$) fluorescence. (A) Nonexpressing cells in the transfected population. (B) Cells overexpressing dynamitin or $p150^{217–548}$. (C) Cells overexpressing p24 or $p150^{926–1049}$. DM, dynamitin.

of dynein focusing activity from spindle poles. $p150^{217–548}$ overexpression has very similar effects to dynamitin on microtubule, centrosome, and Golgi organization, so it seemed likely that it would also interfere with mitotic progression. When we examined mitotic index and spindle morphology in unsynchronized cells overexpressing $p150^{217–548}$, we noted an increased percentage of mitotic cells (Table I) with malformed spindles, as expected.

Dynein recruitment to centrosomes slightly precedes centriole duplication (Fig. 1), suggesting that dynein function

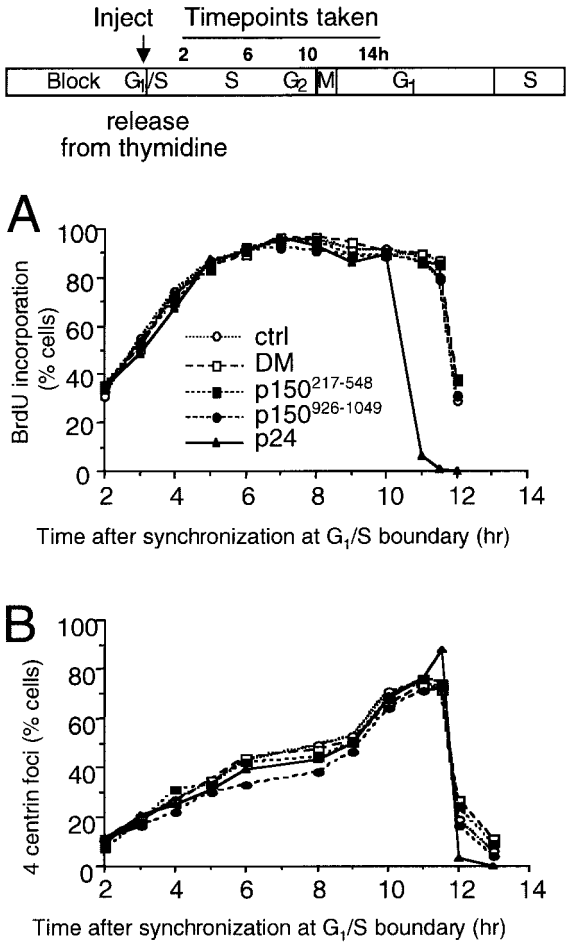

Figure 4. **Effects of dynactin subunit overexpression on S phase events.** Cells were synchronized using a double thymidine block, microinjected with cDNAs encoding dynactin subunits, and then released from the block and incubated at 37°C for increasing lengths of time (the experimental scheme is cartooned above the graphs). Some cells were fixed and labeled with Abs to centrin or treated with BrdU to label DNA before fixation and BrdU Ab labeling. The percent of cells in the population showing BrdU incorporation (A) or four centrioles (B) was determined. Overexpressing cells were identified by GFP or DsRed fluorescence. Ctrl, noninjected control cells on the coverslip; DM, dynamitin.

might also contribute to centrosome doubling or another late cell cycle event. To address this question we evaluated the effects of dynein–dynactin inhibitors on late cell cycle progression. Cells were synchronized at the G_1–S boundary by double thymidine block, microinjected with dynamitin or $p150^{217–548}$ cDNAs, and then released from the block (Fig. 4). Neither DNA synthesis, as assessed by BrdU incorporation, nor centriole duplication, as determined by centrin staining, was affected.

Because dynein recruitment to centrosomes is altered in cells overexpressing $p150^{926–1049}$ or p24 (Fig. 3), we evaluated late cell cycle progression here as well. Unsynchronized populations of cells overexpressing $p150^{926–1049}$ progressed into and through mitosis just like controls (Fig. 4), exhibiting well-formed spindles in ~5% of the total population (Table I). Overexpression of p24 affected cells differently, as mitotic cells were never observed in unsynchronized populations (Table I; Karki et al., 1998). p24 overexpression appeared to drive cells into apoptosis, as judged by staining with the apoptosis marker poly-ADP-ribose (PAR; Table I), with cell death occurring just before mitosis. In synchronized cells, p24 overexpression did not inhibit DNA synthesis or centriole duplication, but significantly fewer p24-overexpressing cells remained as mitosis approached (Fig. 4) and those that remained stained positive for nuclear PAR (unpublished data). The timing of cell death did not seem simply to be the consequence of accumulation of toxic amounts of p24, as cells died just before mitosis regardless of the time after synchronization at which they were microinjected with p24 cDNA (unpublished data).

Loss of centrosomal $p150^{Glued}$ inhibits S phase entry and induces G_1 centriole splitting

Overexpression of dynactin shoulder/sidearm subunits profoundly destabilizes centrosomes (Quintyne et al., 1999), but progression through S and G_2 appears unaffected (Fig. 4). This is not necessarily surprising, because the centrosome-associated surveillance mechanism that governs S phase entry may already be satisfied in cells synchronized at the G_1–S boundary. To determine how the loss of centrosomal dynactin might impact this mecha-

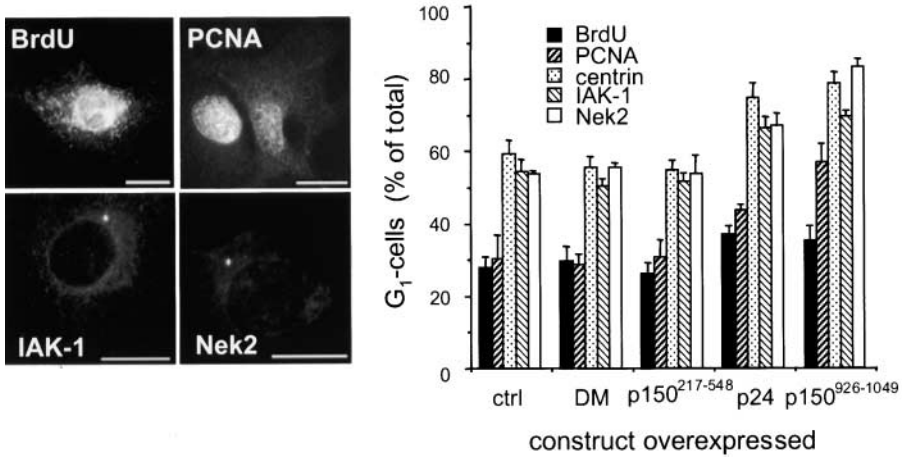

Figure 5. **Localization and prevalence of S phase markers in cells overexpressing dynactin subunits.** (Left) Typical S phase distribution of BrdU (nuclear staining), PCNA (nuclear accumulation), or the kinases IAK-1 and Nek2 (centrosomal accumulation). Bars, 10 μm. (Right) Unsynchronized cells were transfected with dynactin shoulder/sidearm expression vectors and scored for expression of S phase markers. Overexpressing cells were identified by GFP or DsRed fluorescence. Ctrl, nonexpressing control cells in the transfected population; DM, dynamitin.

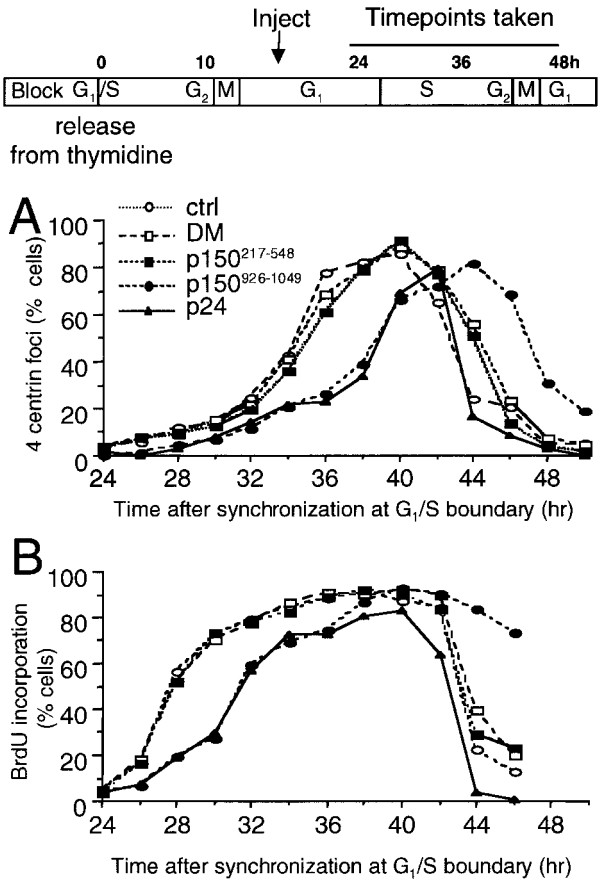

Figure 6. **Effects of dynactin subunit overexpression on cell cycle progression.** Cells were synchronized using a double thymidine block, released, and allowed to proceed through mitosis, and then microinjected with cDNAs encoding dynactin subunits. At different times after microinjection, cells were fixed and stained for centrin (A) or labeled with BrdU and fixed and stained with BrdU Abs (B). Ctrl, noninjected control cells on the coverslip; DM, dynamitin.

nism, we evaluated cell cycle progression from G_1 into S (Fig. 5). The proportion of unsynchronized cells in G_1 versus later in the cell cycle was determined by examining BrdU incorporation, centrin staining, PCNA (a protein that accumulates in S phase nuclei; Bravo and Celis, 1985), and the protein kinases Nek2 and IAK-1, two potential regulators of cell cycle progression that accumulate at centrosomes in S phase (Schultz et al., 1994; Gopalan et al., 1997). Approximately 30% of unsynchronized control cells revealed no BrdU incorporation or nuclear PCNA, indicating that they were still in G_1. 50–60% of cells showed no evidence of centriole duplication (two centrin foci or no centrosomal Nek2 or IAK-1). When we repeated this analysis in cells overexpressing dynactin subunits, dynamitin and $p150^{217–548}$ were seen to have no effect. However, significantly more cells overexpressing $p150^{926–1049}$ or p24 appeared to be G_1, as judged by the behavior of PCNA and the three centriole markers. $p150^{926–1049}$ had a particularly potent effect on centrosomal Nek2 recruitment; <20% of overexpressing cells stained for this marker compared with ~50% of controls. These results were strongly suggestive of a G_1–S delay in these cells.

We then evaluated progression through G_1 and S in more detail. To do this, we synchronized cells by double thymidine block, allowed them to complete mitosis and enter G_1, and then microinjected them with cDNAs (Fig. 6). Dynamitin or $p150^{217–548}$ overexpression had no effect on S phase entry, as judged by the onset of centriole duplication and DNA synthesis. $p150^{926–1049}$ or p24 overexpression, however, caused a noticeable delay in S phase entry.

In this experiment, and in our analysis of unsynchronized cells (above), we observed that centrin foci in G_1 cells expressing $p150^{926–1049}$ or p24 were no longer tightly coupled (Table II), in contrast to centrioles in the same cells in S phase. This behavior differed from that of controls or cells overexpressing other dynactin inhibitors that showed tightly paired or clustered centrin foci throughout the cell cycle. We extended these findings by evaluating the behavior of centrosomal Nek2-associated protein-1 (CNAP-1), a Nek2 substrate that is proposed to be a component of the centrosome "bridge" that underlies centrosome cohesion G_2 (Fry et al., 1998; Mayor et al., 2000; Uto and Sagata, 2000). The pattern of CNAP-1 staining across the cell cycle was identical to centrin. Apparently, the loss of centriole cohesion we see is not due to the absence of CNAP-1, as centriole pairs and clusters were stained in all cells. Taken together, our results suggest that centriole coupling must be achieved before cells are permitted to pass from G_1 into S phase. An imbalance of centrosome-associated dynactin subunits interferes with coupling and results in delayed S phase entry.

Table II. **Effects of dynactin subunit overexpression on centrosome separation**

Overexpressed protein	Distance between centrioles (μm)				
	centrin (2 foci)	centrin (4 foci)	CNAP-1	CNAP-1 ($BrdU^-$)	CNAP-1 ($BrdU^+$)
None (untransfected)	0.7 ± 0.3	0.6 ± 0.5	0.6 ± 0.4	0.6 ± 0.2	0.4 ± 0.2
Control (β-gal)	0.6 ± 0.3	0.6 ± 0.4	0.6 ± 0.4	ND	ND
Dynamitin	0.8 ± 0.5	0.6 ± 0.3	0.7 ± 0.42	0.6 ± 0.3	0.5 ± 0.2
$p150^{217–548}$	0.7 ± 0.5	0.6 ± 0.3	0.5 ± 0.4	0.6 ± 0.2	0.4 ± 0.3
$p150^{926–1049}$	1.5 ± 0.6	0.7 ± 0.6	1.6 ± 0.7	1.8 ± 1.6	0.6 ± 0.3
p24	1.7 ± 0.6	0.8 ± 0.6	1.7 ± 0.7	1.4 ± 0.7	0.5 ± 0.2

Centriole spacing was measured between the centers of centrin or CNAP-1 foci (on TIFFs). For cells with four centrin foci, distance was measured between the larger of each pair. Cell cycle phase was determined by BrdU incorporation. Overexpressing cells were identified by GFP or DsRed fluorescence. At least 70 cells were scored for each condition. Averages and standard deviations were calculated from centrioles with spacing of 4 μm or less, but spacings of up to 20 μm were observed in $p150^{926–1049}$ and p24 overexpressers in G_1.

Discussion

We showed previously (Quintyne et al., 1999) that centrosomal dynactin is required for maintenance of the radial microtubule arrays in fibroblasts. However, it has been assumed by many that centrosomal dynactin functions solely to bind dynein, which then maintains microtubule organization by providing focusing activity. In the present study, we show that dynein and dynactin bind centrosomes differently across the cell cycle; dynactin is present at centrosomes at all times, whereas centrosomal dynein is only detected during S and G_2 phases and at mitotic spindle poles. This finding suggests that dynein and dynactin provide distinct functions at centrosomes and that dynactin may serve multiple roles during the cell cycle. Dynein is most likely recruited to provide microtubule focusing activity at spindle poles. Importantly and unexpectedly, we found that perturbation of dynactin specifically at centrosomes results in abnormal centriole splitting and a delay in S phase entry, suggesting that dynactin contributes in some way to the surveillance mechanism that governs centrosome duplication and the G_1 to S transition (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001).

Targeting of dynein to centrosomes

The dynein–dynactin motor is of critical importance for mitotic spindle pole stability (for review see Compton, 2000), but the lack of dynein accumulation at centrosomes during G_1 and early S suggests that its microtubule focusing activity is not required across the cell cycle. A number of structural and regulatory proteins, some of which are required for the G_2 to M transition or early mitotic events, are recruited to centrosomes during S and G_2 phases. Given its importance in mitosis, it comes as no surprise that dynein also binds centrosomes in a cell cycle–dependent manner. This may involve modification of dynein, dynactin, or some other component of the PCM.

When analyzed at steady-state, cells overexpressing some dynactin inhibitors can target dynein to centrosomes despite the absence of centrosomal $p150^{Glued}$ (Table I), but do so more slowly (Fig. 3), suggesting a different mechanism. The "slow" mode of binding may involve pericentrin, a centrosomal protein that can bind dynein directly (Purohit et al., 1999; Tynan et al., 2000). Any pericentrin-dependent binding mechanism must be complex because centrosomal dynein is not observed in cells overexpressing other dynactin inhibitors whose centrosomes contain pericentrin (Quintyne et al., 1999). For example, dynein binding might utilize pericentrin that is recently trafficked to centrosomes via the dynein–dynactin motor itself (Young et al., 2000). In any case, our data indicate that dynactin provides the primary mechanism by which dynein associates with centrosomes under normal circumstances.

Centrosomal dynactin function

Our findings suggest that centrosomal dynactin plays important roles in microtubule anchoring, dynein binding, and recruitment and maintenance of cell cycle regulators. That dynein cannot be detected at centrosomes during G_1 strongly suggests that $p150^{Glued}$ anchors microtubules directly. Aside from dynactin, few candidate microtubule anchors exist (for review see Bornens, 2002). The γ-TuRC can nucleate and cap microtubule minus ends but is not thought to serve as an anchor (Doxsey, 2001). Other proteins that are selectively associated with the mother centriole include ninein (Mogensen et al., 2000), ODF2/cenexin (Nakagawa et al., 2001), and ε-tubulin (Chang and Stearns, 2000). Ninein is a large coiled-coil protein that lacks defined microtubule binding motifs (Bouckson-Castaing et al., 1996). Although the existing data support our hypothesis that $p150^{Glued}$ provides a key microtubule-anchoring activity at centrosomes, it is possible that dynactin is just one component of a microtubule-anchoring complex or matrix that contains other structural and/or regulatory components. Overexpression of the dynactin inhibitors used here would interfere with the recruitment of any protein that is targeted to centrosomes via $p150^{Glued}$, so the exact nature of the anchoring mechanism remains an open question.

Centrosome duplication involves amplification of the PCM, a process that depends on dynein–dynactin-dependent transport (for review see Zimmerman and Doxsey, 2000). Overexpression of inhibitors of the dynein–dynactin interaction would be predicted to interfere with the centrosome cycle but, remarkably, they have no effect until mitosis. Even more surprising is the fact that dynactin inhibitors that have no measurable effect on dynein-based motility (Quintyne et al., 1999) somehow delay S phase entry. Overexpression of p24 also drives cells into apoptosis just before mitosis. This may reflect a normal biological function of p24, but is more likely an artifact of overexpression.

Centrosomal dynactin, centriole duplication, and S phase entry

The daughter centriole in some cells moves independently of the mother in G_1 but the two become linked during S and G_2 (Piel et al., 2000), demonstrating that formation of a single, coherent centrosomal unit correlates with centriole duplication. Our observations suggest that centriole coupling is required for centriole duplication and S phase entry. That the centrosome must behave as a single copy organelle during duplication is an appealing notion, as this would allow concerted and efficient recruitment of PCM components and ensure that centrosome-associated signaling molecules (for review see Lange, 2002) become equally apportioned via the spindle poles into the two daughter cells.

Insight into how overexpression of different dynactin subunits might cause such distinct effects on the cell cycle can be gained by considering how each class of inhibitor affects centrosome structure and dynamics (Fig. 7; Quintyne et al., 1999). In control cells, microtubules are anchored by dynactin, centrosome components are transported to the centrosome via dynein as usual, and G_1–S progression occurs normally. When dynein–dynactin binding is blocked, microtubule nucleation persists but microtubules are no longer retained. Dynein-based trafficking of other centrosomal components is prevented, although some PCM proteins may reach their target in other ways. Despite this, the centriole coupling mechanism is maintained, centriole duplication proceeds, and cells enter S phase at the expected time. In

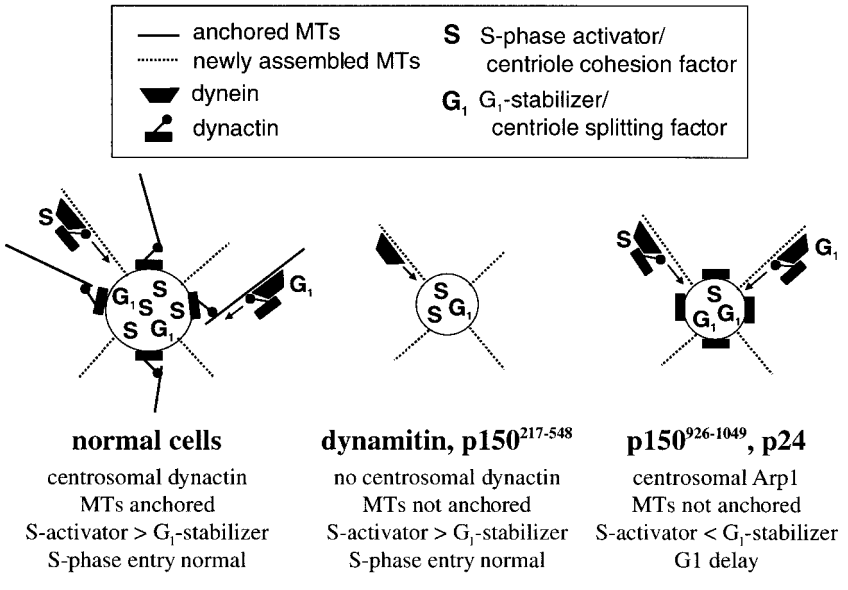

Figure 7. **Model figure summarizing the effects of dynactin subunit overexpression on microtubule anchoring and centrosomal protein targeting on G_1 centrosomes.** For simplicity, the centrosome is depicted as a single object and mother and daughter centrioles are not shown. In dynamitin and $p150^{217–548}$ overexpressers, dynein-based delivery of proteins to centrosomes is blocked, but an acceptable balance of S phase activators and G_1 stabilizers is maintained to allow cells to enter S phase at the normal time. In p24 and $p150^{926–1049}$ overexpressers, dynein-based transport brings the normal complement of cell cycle regulators to the centrosome, but G_1 stabilizing activities predominate due directly or indirectly to the localized perturbation of dynactin integrity. S phase entry is delayed.

cells overexpressing $p150^{926–1049}$ or p24, the story is more complex. Centrosomes nucleate microtubules and dynein-based movement of dynactin and other centrosome components continues on the newly assembled pool (Fig. 3; Quintyne et al., 1999). However, $p150^{Glued}$, microtubules, and any associated proteins are gradually released, leaving behind the naked Arp1 filament and residual PCM. This may result in an inappropriate balance of centriole cohesion factors, centriole splitting factors, G_1 stabilizers, and/or S phase activators.

Factors that might impact centrosome cohesion, and thus duplication and S phase progression, include kinases and protein phosphatase 1α (PP1) that have been linked to centriole separation (for review see Hinchcliffe and Sluder, 2001; Meraldi and Nigg, 2001). Current models state that centriole separation is triggered by phosphorylation of the PCM component nucleophosmin, which targets it for degradation (for reviews see Doxsey, 2001; Hinchcliffe and Sluder, 2001). An imbalance in centrosome-associated kinase/phosphatase activities may allow premature nucleophosmin degradation and thus aberrant splitting. Alterations in centrosomal Cdk2/cyclin A/E (a putative nucleophosmin kinase), Nek2, and the Nek2 binding protein PP1 (protein phosphatase 1α) (Helps et al., 2000) all induce premature centriole separation (Meraldi and Nigg, 2001). Nek2 is proposed to trigger degradation of the centrosome "bridge" during G_2 by phosphorylating CNAP-1, so conditions that interfere with Nek2 accumulation would be predicted to delay centrosome separation. Our results contradict the above simple model for Nek2 function, but support the idea that the activity of this kinase favors assembly of the centrosome bridge. Centriole splitting is also observed in cells treated with nocodazole (Jean et al., 1999; Meraldi and Nigg, 2001), in strong support of the notion that centrosome cohesion depends on microtubule-dependent transport of structural components and regulators. Additional analysis of the effects of dynactin inhibitors on centrosome composition should facilitate identification of relevant molecules.

Materials and methods

Antibodies

The following antibodies were used in this study: ($p150^{Glued}$) mAb P41920 (BD Biosciences) and pAb UP502 (E. Holzbaur, University of Pennsylvania, Philadelphia, PA); (Arp1) mAb 45A (Schafer et al., 1994) and rabbit antibody to recombinant Arp1 (gift from J. Lees Miller, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); (dynein IC) mAb 74.1 (Chemicon); (dynein LIC) pAb JH92 made against recombinant dynein LIC-A (Gill et al., 1994); (tubulin) α-tubulin mAb DM1A (Sigma-Aldrich), affinity-purified rabbit antibody against peptide KVEGEGEEEGEEY (gift from E. Karsenti, EMBL), and 650952 rabbit anti-tubulin (ICN Biomedicals); (γ-tubulin) mAb GTU88 (Sigma-Aldrich) and rabbit antiserum pAb (Sigma-Aldrich) against peptide EEFATEGTDRKDVFFYK; (ε-tubulin) ε-1 polyclonal antibody (Chang and Stearns, 2000); (centrin) mAb 20H5 (Sanders and Salisbury, 1994); (BrdU) mAb 3D4 (BD Biosciences); (CNAP-1) anti–CNAP-1 pAb (Fry et al., 1998); (PCNA) mAb P56720 (BD Biosciences); (IAK-1) mAb I71320 (BD Biosciences); (Nek2) mAb N52120 (BD Biosciences); and (PAR) mAb 10H (BD Biosciences).

Expression constructs

To make DsRed-$p150^{217–548}$, a fragment containing $p150^{217–548}$ was amplified from CMV-p150 (Quintyne et al., 1999) by PCR, inserted directly into the pTA vector, and then subcloned into pDsRed-N1 (CLONTECH Laboratories, Inc.) using EcoRI. $p150^{926–1049}$–GFP was made by subcloning the region encoding $p150^{926–1049}$ from CMV-$p150^{926–1049}$ (Quintyne et al., 1999), using BglII and EcoRI, into pEGFP-C1 (CLONTECH Laboratories, Inc.). The clones were transiently transfected into Cos-7 cells to confirm that the effects of overexpression of these constructs were identical to those previously reported with untagged $p150^{217–548}$ and $p150^{926–1049}$ (Quintyne et al., 1999) by scoring for microtubule disruption, Golgi dispersal, γ-tubulin fragmentation, and centrosomal dynactin. cDNAs encoding p24–GFP and dynamitin–GFP were described previously (Quintyne et al., 1999).

Cell culture, transfection, and microinjection

COS-7 cells were grown in DME (GIBCO BRL; Life Technologies) supplemented with 10% FCS (Atlas). For transient transfections, cells were electroporated and seeded as previously described (Quintyne et al., 1999). For microinjection, cells were seeded onto gridded 18 × 18 mm^2 coverslips (Bellco) and either grown overnight or synchronized as described below. Dynactin subunit expression vector cDNAs (0.1 mg/ml in buffer containing 2 mM KH_2PO_4, 8 mM K_2PO_4, and 100 mM KCl) were injected into nuclei using an Eppendorf micromanipulator. Cells were incubated at 37°C for 4–24 h before being fixed and processed for immunofluorescence. Overexpression could be detected by GFP fluorescence after 2 h, and the characteristic effects of overexpression on Golgi complex morphology could be detected as early as 4.5 h after injection. For cell cycle experiments, cells were either injected 2–5 h before release from thymidine block (Fig. 4), or between 14 and 18 h after release (Fig. 6).

Immunofluorescence microscopy

Immunofluorescence was performed as previously described (Quintyne et al., 1999). In brief, cells were fixed for 5 min in −20°C methanol, treated with blocking solution, treated with primary antibodies, washed, and then treated with secondary antibodies and DAPI. Samples were scored using an Axiovert 35 microscope (ZEISS). For experiments involving electroporated or synchronized cells, at least 200 overexpressing (or control) cells were scored per construct per experiment or time point, and each experiment was repeated at least twice. For experiments involving microinjected cells, 50–70 cells were scored per construct per time point, and each experiment was repeated at least twice. Stacks for deconvolution were acquired and processed using a DeltaVision deconvolving microscope system (Applied Precision). All images were imported into Adobe Photoshop® (Adobe Systems) as TIFFs for contrast manipulation and figure assembly.

Microtubule regrowth assay

Microtubule regrowth assays were performed as previously described (Quintyne et al., 1999). In brief, transfected cells were seeded on coverslips, grown overnight, and treated with 33 μM nocodazole (Sigma-Aldrich) on ice for 25 min to depolymerize microtubules. Cells were washed with nocodazole-free medium, refed, and incubated at room temperature for varying times before being fixed and processed for immunofluorescence.

Cell synchronization and release

Cells were seeded onto coverslips at an initial density of 1.5×10^7 cells per 10-cm dish and grown overnight. A double thymidine block was performed by treating cells with fresh DME containing 2 mM thymidine (Sigma-Aldrich) for 12 h, releasing for 12 h in normal medium, and then incubating them again in 2 mM thymidine for 12–14 h. Essentially, all cells were synchronized at the G_1–S boundary, as determined by the presence of two centrin foci, before release from the block. For BrdU incorporation, cells were incubated in DME + 10 μM BrdU (BD Biosciences) at 37°C for 3 h before fixing and processing for immunofluorescence as described above.

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