Dynein drives nuclear rotation during forward progression of motile fibroblasts

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Summary

During directed cell migration, the movement of the nucleus is coupled to the forward progression of the cell. The microtubule motor cytoplasmic dynein is required for both cell polarization and cell motility. Here, we investigate the mechanism by which dynein contributes to directed migration. Knockdown of dynein slows protrusion of the leading edge and causes defects in nuclear movements. The velocity of nuclear migration was decreased in dynein knockdown cells, and nuclei were mislocalized to the rear of motile cells. In control cells, we observed that wounding the monolayer stimulated a dramatic induction of nuclear rotations at the wound edge, reaching velocities up to 8.5 degrees/minute. These nuclear rotations were significantly inhibited in dynein knockdown cells. Surprisingly, centrosomes do not rotate in concert with the nucleus; instead, the centrosome remains stably positioned between the nucleus and the leading edge. Together, these results suggest that dynein contributes to migration in two ways: (1) maintaining centrosome centrality by tethering microtubule plus ends at the cortex; and (2) maintaining nuclear centrality by asserting force directly on the nucleus.

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Introduction

Cell migration plays a central role in a variety of biological processes; tissue development, the immune response and wound healing all require directional migration. Microtubules (MTs) have been shown to contribute to several steps of cell migration, one of the most well characterized being polarity establishment. In migrating fibroblasts, the centrosome reorients between the nucleus and the leading edge, which in turn repositions the Golgi and is thought to establish and maintain cell polarity during migration (Mellor, 2004). Previous work has demonstrated a role for the microtuble motor dynein, its co-factor dynactin, and the dynein regulatory protein Lis1 in reorientation of the centrosome toward the leading edge (Etienne-Manneville and Hall, 2001; Gomes et al., 2005; Palazzo et al., 2001; Yvon et al., 2002). However, inhibition of dynein or dynactin after centrosome reorientation suppresses cell motility, indicating that there is an additional role for dynein in persistent fibroblast motility (Dujardin et al., 2003).

Several studies have suggested a role for dynein and Lis1 in both centrosome and nuclear positioning. During neuronal migration, nascent neurons extend a long leading process from the cell body. The centrosome then migrates into the leading process, and subsequently the nucleus moves forward in a saltatory manner (reviewed by Tsai and Gleeson, 2005). Inhibition of dynein or Lis1 disrupts nuclear migration and causes an increase in nucleus-centrosome distance in neurons (Shu et al., 2004; Tanaka et al., 2004; Tsai et al., 2005), suggesting that dynein and Lis1 couple centrosome and nuclear movements. Studies in lower organisms further support a role for dynein and Lis1 in nucleokinesis. In the filamentous fungus *Aspergillus nidulans*, nuclei migrate long distances toward the growing tip of the hyphae, a process that requires the fungal homologues of human dynein, dynactin and Lis1 (reviewed in Tsai and Gleeson, 2005). Studies of pronuclear migration in the one cell stage embryo of *Caenorhabditis elegans* have also indicated a role for orthologs of dynein heavy chain and Lis1 in nucleokinesis. In this system, the nuclear envelope protein Sun1 and dynein-interacting protein Zyg-12 are suggested to mediate attachment of dynein to the nucleus (Malone et al., 2003).

In contrast to migrating neurons, where there is a loose coupling between nuclei and centrosomes, nuclei and centrosomes in fibroblasts are closely associated. It has been hypothesized that the nucleus and the centrosome are statically tethered in fibroblasts, and that coupling of MTs to the advancing leading edge will pull the nucleus in the direction of migration (Neujahr et al., 1998).

In this study, we examine the role of dynein in both centrosome reorientation and nuclear dynamics in migrating fibroblasts. In addition to forward translocation of the nucleus during cell migration, we note a dramatic induction of nuclear rotations upon monolayer wounding. Both the forward translocation and the rotations are dynein dependent. Furthermore, the rotation of the nucleus is independent of centrosome positioning. These observations suggest a dynamic relationship between the nucleus and the centrosome, and reveal a role for dynein in coupling the nucleus to MTs, therefore maintaining its centrality in migrating cells.

Results

Dynein facilitates lamellar extension in migrating fibroblasts

In this study, we used an RNA interference (RNAi) approach to knockdown dynein heavy chain (DHC) and the p150^{Glued} subunit of dynactin, therefore allowing us to analyze the function of dynein and dynactin in migrating cells. NIH/3T3 fibroblasts were grown to confluence and then a wound-healing assay was used to initiate cytoskeletal redistribution and directional migration of cells towards the wound edge (Gundersen and Bulinski, 1988). Cells were mock transfected, transfected with small interfering RNA (siRNA) targeting

DHC or p150^{Glued}, or transfected with a scrambled oligonucleotide control; confluent cultures were scratch wounded. Six hours after wounding, the cells were fixed and protein lysates were collected. Western blot analysis revealed that target protein levels were consistently reduced 75-95% in knockdown cells when compared with cells that were mock treated (Fig. 1B) or transfected with a scrambled oligonucleotide (J.R.L. and E.L.F.H., unpublished). Observation of fixed cells via immunofluorescence demonstrates that ~90% of DHC knockdown cells and 88% of p150^{Glued} knockdown cells show decreased levels of protein targeted by siRNAs. Levels of dynein intermediate chain (DIC) decrease in DHC knockdown cells (Fig. 1B), suggesting that the dynein complex is being destabilized, as has been observed in other cell types (Caviston et al., 2007). However, dynactin is expressed at normal levels in DHC knockdown cells and dynein is expressed at normal levels in p150^{Glued} knockdown cells (Fig. 1B).

In mock-treated cells at the wound edge, the MT network is polarized in the direction of the wound (Gundersen and Bulinski, 1988), and dynein and dynactin accumulate in a patch-like pattern (Dujardin et al., 2003) at the tips of protrusions into the wound area (Fig. 1A). These cortical patches of dynein/dynactin may contribute to MT forces that maintain nuclear and centrosomal centrality. Dynactin demonstrates decreased accumulation in patches along the leading edges of dynein knockdown cells (supplementary material Fig. S1), indicating that dynein is required for dynactin accumulation at these patches.

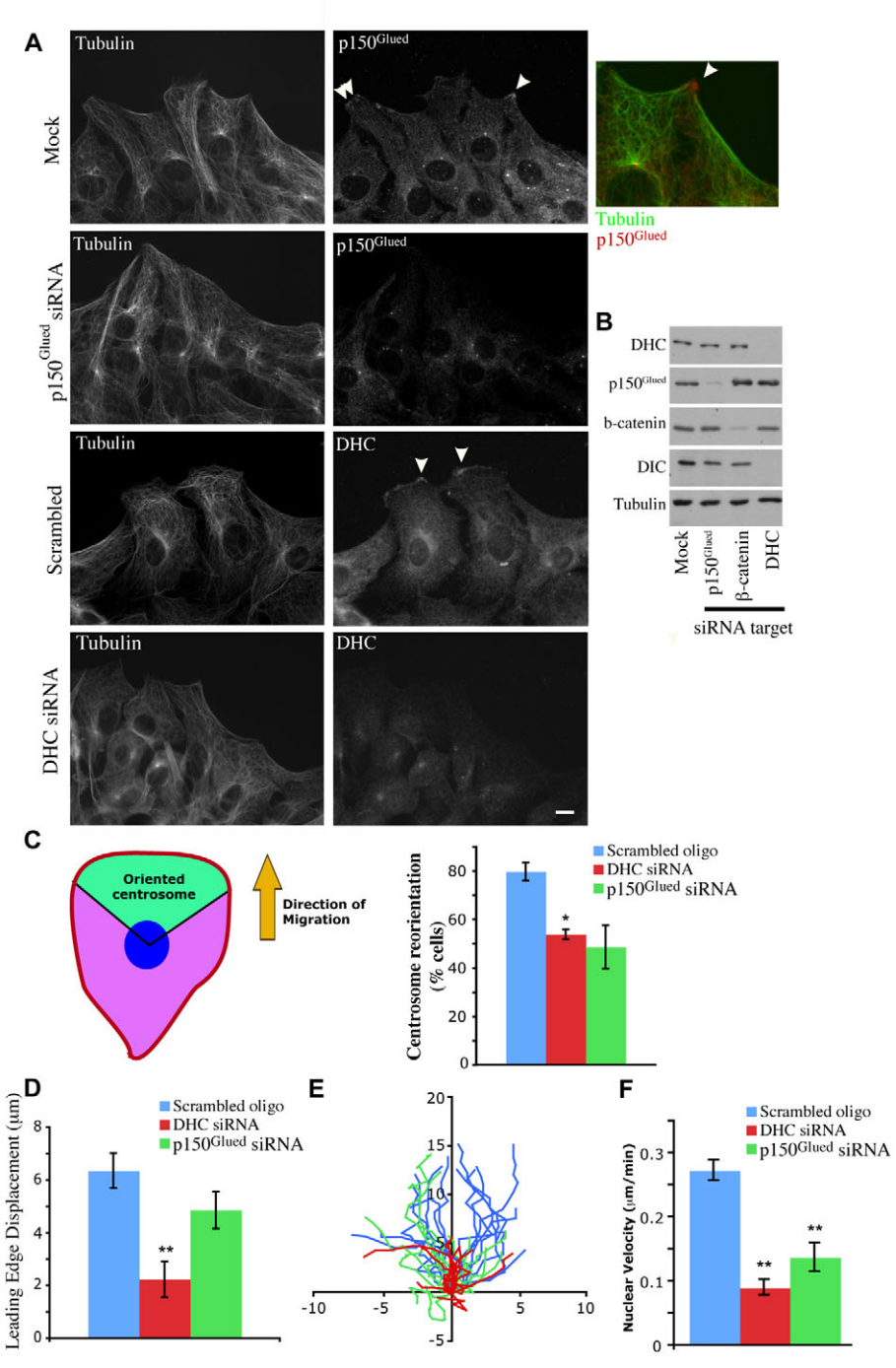

Fig. 1. Dynein and dynactin localize to the leading edge of migrating cells and promote centrosome reorientation, leading-edge protrusion and nuclear migration. (A) Dynein and dynactin concentrate in cortical patches at the leading edge of migrating cells. NIH/3T3 cells were mock transfected, transfected with siRNAs targeting p150^{Glued} or DHC, or transfected with a scrambled oligonucleotide control, then grown to confluency and the monolayer wounded. Six hours after wounding, cells were fixed and stained for α-tubulin and either p150^{Glued} or DIC. Mock-transfected cells show patches of dynein and dynactin at the leading edge of migrating cells (arrowheads). Inset, overlay of cortical patch of p150^{Glued} (red) and projecting MTs (green). Scale bar, 10 μm. (B) Western blot of protein lysates from cells that were mock transfected or transfected with siRNAs targeting p150^{Glued}, DHC or β-catenin consistently show knockdown of target protein levels by 75-95%. Levels of DIC decrease in DHC knockdown cells, suggesting the dynein complex is being destabilized. By contrast, knockdown of β-catenin, which binds to DIC but is not part of the dynein motor complex (Ligon et al., 2001), does not destabilize DIC. (C) Centrosome reorientation is inhibited in dynein and dynactin knockdown cells. Orientation of centrosomes 6 hours after wounding. Centrosome orientation was determined by measuring the position of centrosomes, as determined by γ-tubulin staining, in relation to Hoechst-stained nuclei. Centrosomes in the forward-facing 120 degree sector (green zone) were scored as reoriented. (D) Leading-edge extension is inhibited in dynein knockdown cells. Displacement of the leading edge over 45 minutes of wound healing was measured (n=15). (E) Tracks of nuclei centroids during migration of fibroblasts over 45 minutes. Paths are oriented so cells are migrating towards the top of the graph, each path starts at (0,0). Axis labels are in μm. (F) Rates of nuclear movement are decreased in dynein and dynactin knockdown cells. Nuclear velocity during migration was decreased in DHC and p150^{Glued} siRNA cells, compared with mock-transfected cells (n=15). Error bars indicate s.e.m.; *P<0.05; **P<0.005.

Mock-treated and siRNA-treated cells were fixed 6 hours after wounding, a timepoint several hours after centrosome reorientation occurs in these cells (Gundersen and Bulinski, 1988), and stained with an antibody to the centrosomal marker γ-tubulin. In mock-treated cells, the majority of centrosomes are perinuclear and positioned in the direction of the wound, whereas cells treated with siRNA against dynein or dynactin have centrosomes that are more evenly distributed around the nucleus (Fig. 1C), indicating they are defective in centrosome reorientation. This defect is consistent with previous observations of reduced centrosome reorientation after dynein or dynactin inhibition (Etienne-Manneville and Hall, 2001; Gomes et al., 2005; Palazzo et al., 2001; Yvon et al., 2002). Although inhibition of dynactin function by overexpression of individual dynactin subunits has previously been shown to interfere with MT anchoring at the centrosome (Quintyne et al., 1999), we observed no overt defects in microtubule organization in dynein or dynactin knockdown cells. This may be due to some residual dynactin remaining at the centrosome (Fig. 1A), as RNAi does not cause complete silencing of the target molecule.

In order to investigate a role for dynein and dynactin in persistent cell motility, we observed mock-treated or siRNA-treated cells after monolayer wounding by timelapse microscopy (supplementary material Movie 1). In both control and knockdown cultures, cells at the wound edge show robust lamellar ruffling, consistent with a motile phenotype. However, dynein and dynactin knockdown cells appear to advance into the wound at a slower rate than mock-treated cells. Leading edge displacement in the direction of the wound was measured by marking the change in position of the cell edge over 45 minutes of wound closure. We observed a significant decrease in leading edge extension into the wound in dynein knockdown cells (Fig. 1D). Dynactin knockdown cells showed similar rates of leading edge protrusion compared with control cells, which may indicate some differential dependence for dynein versus dynactin in lamellipodial extension (Fig. 1D).

Dynein and dynactin contribute to nuclear movements in migrating fibroblasts

Cell migration involves both lamellar extension and forward progression of the cell body, including the nucleus. In order to examine the dynamics of nuclei in the knockdown cells, we measured the velocity of nuclear movement by tracking the paths of nuclear centroids by timelapse microscopy (Fig. 1E,F). Nuclei in dynein and dynactin knockdown cells moved at a slower velocity and with less directed movement than nuclei in mock-treated cells (supplementary material Movie 1). These slower velocities were correlated with mislocalization of nuclei in dynein and dynactin knockdown cells. Following either dynein or dynactin knockdown, nuclei were significantly closer to the rear of the cells than nuclei in mock-treated cells (0.0098±0.0006 μm^{-1} in control cells, compared with 0.0048±0.0003 μm^{-1} in $p150^{Glued}$ knockdown cells and 0.0055±0.0005 μm^{-1} in DHC knockdown cells when normalized for cell area; n=75; P<0.005 for each). This suggests that dynein and dynactin have a role in coupling nuclear position to cell migration.

Surprisingly, many control cells demonstrated sustained nuclear rotations during motility (Fig. 2A; supplementary material Fig. S2). These rotations can be monitored by tracking the paths of individual nucleoli, which stay in position relative to each other during interphase (Chubb et al., 2002) (Fig. 2B; supplementary material Fig. S3). We observed rotations in both clockwise and counterclockwise directions in cells at the wounded edge of the monolayer, which reached angular velocities up to 8.5 degrees per

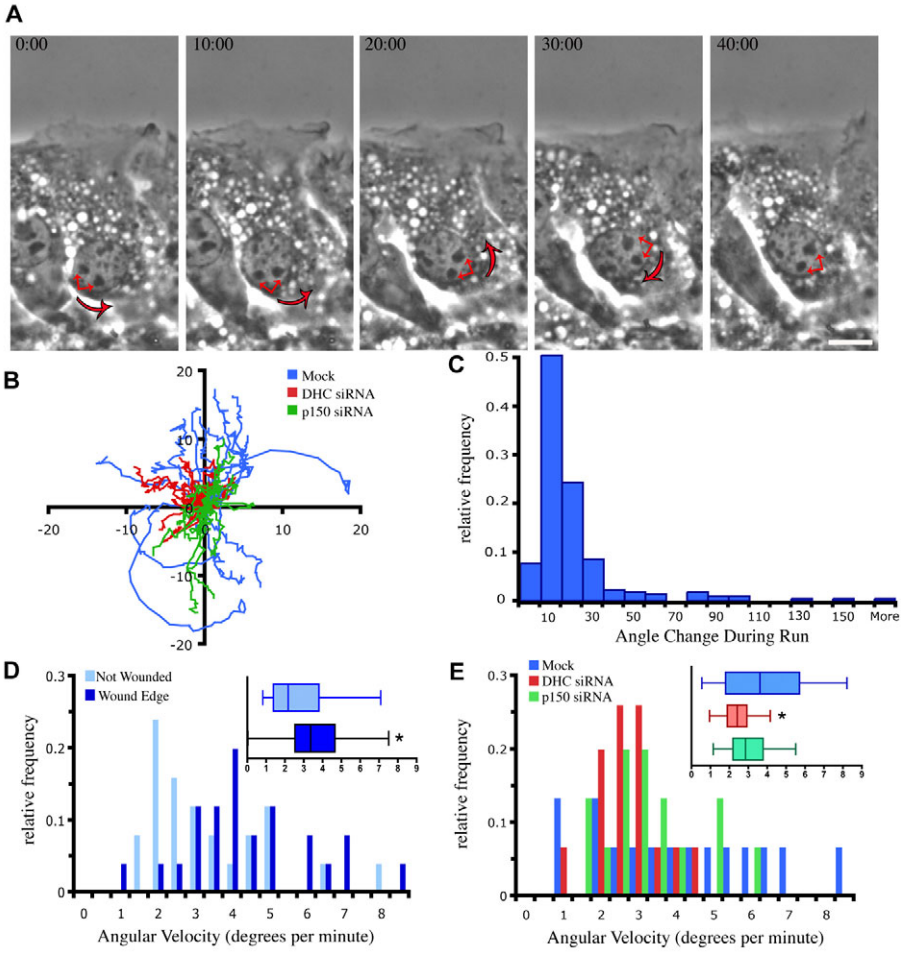

Fig. 2. Dynein-dependent nuclear rotations are enhanced after monolayer wounding. (A) A time series of a nucleus that rotates during cell migration. Red arrows indicate two nucleoli that stay in position relative to other nucleoli within the nucleus. This nucleus rotates counter-clockwise for the first 30 minutes, and then switches direction during the final 10 minutes of this timelapse. Scale bar, 10 μm. Time is minutes:seconds. (B) Tracks of nucleoli during migration of fibroblasts over 45 minutes. Large circular traces seen in mock-transfected cells demonstrate paths of nucleoli in nuclei that are rotating. These are rarely seen in DHC and $p150^{Glued}$ siRNA cells (n=15). Axis labels are in μm. (C) Particle tracking was used to track the duration of rotation of nuclei from cells at the wound edge during migration. A run was defined as rotation in a single direction without pausing (n=225 runs from 25 cells). (D) Angular velocity of nuclei in cells along the wound edge of wounded cultures is higher than that of nuclei in cells that have been grown to confluency, but not wounded (n=25). (E) Angular velocity of nuclei in mock-treated cells along the leading edge is greater than that of DHC and $p150^{Glued}$ siRNA cells, which rarely rotate and thus have a slower average angular velocity (n=15). Error bars indicate s.e.m.; *P<0.05.

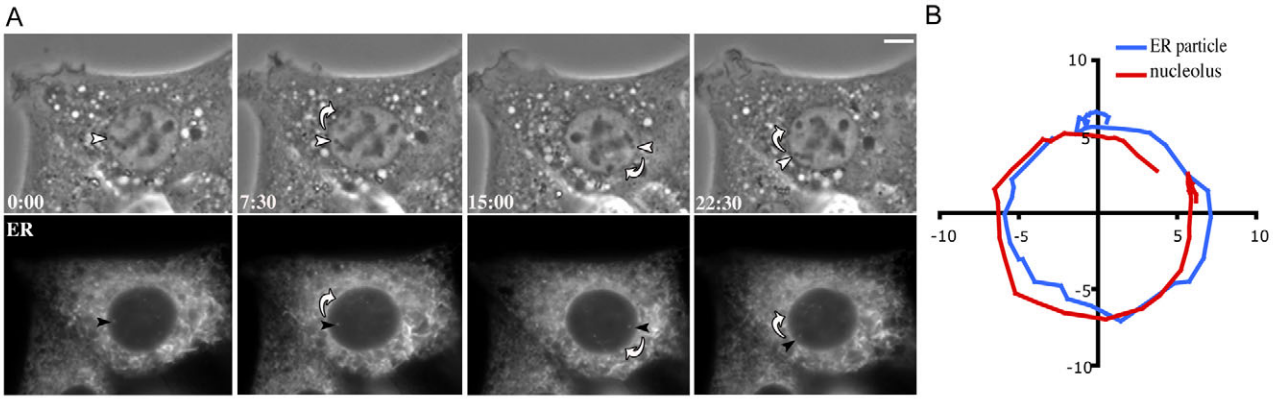

Fig. 3. Nuclear rotation includes ER closely associated with the nuclear membrane. (A) Cells transfected with dsRed2-ER were grown to confluency and wounded. The top panel shows a nucleus rotating clockwise during migration. White arrowhead indicates the position of a nucleolus during rotation. The bottom panel shows the morphology of the ER during the same timeframe. Some small fragments of the ER that are closely opposed to the nucleus rotate with it (black arrowhead, see also supplementary material Movie 3). Peripheral ER shows dynamic remodeling during the rotation. (B) Tracks of the paths of a single nucleolus and an ER particle within the cell shown in A during nuclear rotation over 45 minutes. Both follow the same circular path, indicating that they are both rotating in the same manner (see also supplementary material Movie 3). Time is minutes:seconds. Scale bar, 10 μm.

minute (Fig. 2B-E). Rotations were only observed in the plane of the substratum, as we would expect because of the flat ellipsoidal shape of fibroblast nuclei (Bolzer et al., 2005). This distinguishes the rotations we observe from rolling cell bodies that have been observed in motile fish keratocytes, an event that appears to be actin driven (Anderson et al., 1996). Although many rotations result in angle changes of hundreds of degrees, particle tracking at high temporal resolution reveals that most rotations are interrupted by short pauses or directional changes after runs of less than 20 degrees (Fig. 2C). Rotations were observed in cells at, and several rows back from, the wounded edge, but were less frequent in cells in monolayers that had not been wounded (Fig. 2D; supplementary material Movie 2). Nuclear rotations were observed in single migrating NIH/3T3 cells (J.R.L. and E.L.F.H., unpublished), suggesting that rotations are enhanced specifically in migrating cells, and may reflect increased dynamicity of the cytoskeleton during cell motility.

Depolymerization of MTs with high doses (16.6 μM) of nocodozole (NZ) also significantly decreased the velocity of nuclear rotations, demonstrating that nuclear rotations require an intact MT cytoskeleton (Fig. 6D,E; supplementary material Movie 6). Because dynein and dynactin are known to drive nuclear positioning, we examined the movements of nuclei in cells where DHC or $p150^{Glued}$ had been knocked down. Interestingly, cells in which dynein had been knocked down showed a significant reduction in the velocity of nuclear rotations (Fig. 2E; supplementary material Fig. S2, Movie 1). It is unlikely that this inhibition in nuclear rotation is secondary to a global disruption in the cellular cytoskeleton, as no overt changes were observed in the distribution of MTs (Fig. 1A), acetylated MTs (J.R.L. and E.L.F.H., unpublished), vimentin (J.R.L. and E.L.F.H., unpublished) or actin (supplementary material Fig. S4) in either dynein or dynactin knockdown cells.

It is possible that the rotations that we observe involve only components within the nucleus, but not the nuclear envelope. Because the outer nuclear membrane is continuous with the ER, we monitored ER dynamics using the fluorescent ER marker dsRed2-ER. We observed that segments of the ER that are most closely associated with the nucleus rotate in concert with the nucleus (Fig. 3; supplementary material Movie 3), suggesting that the entire nucleus, including the nuclear envelope, rotates in migrating cells. However, ER tubules extending farther from the nucleus, as well as most juxtanuclear ER, demonstrated dynamic remodeling over the time period of our observations (tens of minutes). In general the ER did not revolve in concert with the nucleus, nor did ER tubules appear to be trailing behind rotating nuclei, indicating that the majority of the ER network remodels as the nucleus rotates.

Nuclear rotations are not accompanied by centrosome movements

Previous studies have demonstrated that the centrosome precedes the nucleus in forward movement during cell migration, leading to the hypothesis that protein–protein interactions tether the centrosome to the nucleus, and that MT interactions with the cortex maintain centrosome and nuclear centrality (Gomes et al., 2005; Morris, 2003; Tanaka et al., 2004; Tsai and Gleeson, 2005). If this hypothesis were true, then we would expect the centrosome to move in concert with a rotating nucleus. Therefore, we transfected cells with 3×GFP-EMTB, a construct that brightly labels MTs without perturbing MT dynamics (Faire et al., 1999). Live cell imaging of both MTs and the nucleus, as visible by phase, demonstrates that the MT network does not follow the circular movements of rotating nuclei (supplementary material Movie 4). To look specifically at centrosome dynamics, we transfected cells with dsRed1-Centrin2 (Tanaka et al., 2004). The majority of transfected cells showed a single or two adjacent centrin-positive puncta close to the nucleus that are associated with the centrosome marker γ-tubulin (Fig. 4A). Visualization of MT regrowth after nocodazole washout shows that these puncta are capable of MT nucleation (Fig. 4A), corroborating evidence that Centrin2 is an accurate centrosome marker. Live cell imaging of dsRed1-Centrin2 and the nucleus, as visible by phase, demonstrates that the centrosome maintains its orientation between the nucleus and the leading edge, even in cells where the nucleus rotates (Fig. 4B-C; supplementary material Movie 5). Therefore, the centrosome is not in a static interaction with the nucleus as it rotates.

Previous work suggests that retrograde actin flow and myosin II contribute to nuclear positioning by pushing the nucleus rearward during centrosome reorientation in fibroblasts (Gomes et al., 2005).

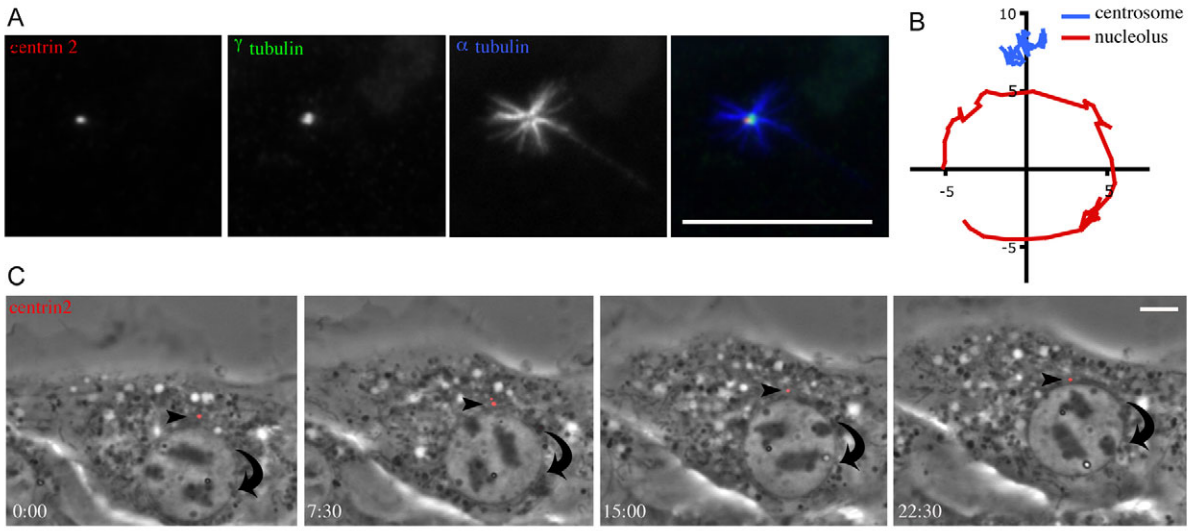

Fig. 4. Nuclear rotations are not coupled to centrosome rotation. (A) Exogenous Centrin2 closely associates with endogenous γ tubulin in puncta capable of MT nucleation, supporting its functional role as a centrosome marker. Cells transfected with dsRed1-Centrin2 (red) were subjected to nocodazole treatment. After a 5-minute wash, cells were fixed and stained for γ tubulin (green) and α tubulin (blue). (B) Tracks of a single nucleolus (red) and a centrosome (blue) within the cell shown in C during nuclear rotation over 45 minutes. The cell is migrating towards the top of the graph, and the nucleus rotates while the centrosome remains between the nucleus and the leading edge. (C) Cells transfected with dsRed1-Centrin2 (red), a centrosomal marker, were grown to confluency and wounded. The arrowhead indicates a centrosome that stays positioned between the nucleus and the leading edge while the nucleus rotates clockwise (black arrow, also see supplementary material Movie 5). Time is minutes:seconds. Scale bars, 10 μm.

In order to examine whether this force contributes to nuclear rotation during forward migration, we observed nuclear dynamics in cells treated with blebbistatin (BB), a myosin II inhibitor that has been shown to block actin retrograde flow (Ponti et al., 2004; Straight et al., 2003). As seen in fibroblasts with reduced levels of myosin II A, cells treated with BB demonstrate exaggerated membrane ruffling, enhanced migration, and reduced cell body retraction (Even-Ram et al., 2007; Vicente-Manzanares et al., 2007). Cell treated with BB showed no inhibition of nuclear rotations (Fig. 5; supplementary material Movie 6), indicating that myosin II and retrograde actin flow do not drive these movements. In fact, some cells treated with BB reached angular velocities above those observed in DMSO-treated cells, up to 9.8 μm/minute (Fig. 5B). This suggests that actomyosin may be dampening nuclear rotations in control cells, perhaps because actin/myosin II stress fibers act as a physical hindrance to dynamic nuclear movements.

Nuclear rotations do not depend on dynactin localized to either the leading edge or the Golgi complex of migrating cells

There are two locations in the cell that dynein may be actively contributing to migration: at the cell cortex or at the nucleus. Dynein and dynactin accumulate in patches (Dujardin et al., 2003) at the leading edges of migrating fibroblasts (Fig. 1A), where they may act to influence MT dynamics or exert force on MTs that project towards the cortex in order to polarize the centrosome during migration. Alternatively, dynein/dynactin may localize near the nucleus to contribute to migration: either interacting directly with components of the nuclear envelope (Malone et al., 2003; Salina et al., 2002) or accumulating at the perinuclear Golgi (Fath et al., 1994).

In order to specifically inhibit dynein/dynactin accumulation at the leading edge, we treated NIH/3T3 cells with a low dose (100 nM) of NZ. Although high doses of NZ are known to depolymerize MTs, concentrations in the nanomolar range have been shown to inhibit MT dynamics without causing disassembly (Vasquez et al., 1997). After treatment with low-dose NZ for 30 minutes, cells have normal MT distribution but accumulation of dynactin patches at the leading edge is inhibited (Fig. 6A,B), suggesting that the formation and/or maintenance of these patches are dependent on dynamic MTs. Centrosome reorientation is also inhibited in the presence of low-dose NZ (J.R.L. and E.L.F.H., unpublished) (see also Gomes et al., 2005; Magdalena et al., 2003; Yvon et al., 2002), which may indicate a role for dynactin patches at the leading edge in coupling MT plus-ends to the cortex. Dynamic MT plus-ends may act to deliver molecules to the cortex that act to maintain dynactin patches. One possible candidate is dynein, which is necessary for the formation of dynactin patches at the leading edge (supplementary material Fig. S1). This system would parallel observations in both budding yeast and *Aspergillus nidulans*: dynein is off-loaded from MT plus ends to the cortex, where it exerts pulling forces on MTs (Lee et al., 2003; Sheeman et al., 2003; Xiang and Fischer, 2004).

We used low doses of NZ to assess nuclear rotations in the absence of dynein/dynactin accumulation at the leading edge. Cell monolayers were wounded and allowed to recover for a minimum of 4 hours to allow for centrosome reorientation towards the wound edge prior to addition of 100 nM NZ. Timelapse microscopy demonstrates that nuclear rotations occurred even in absence of dynactin patches at the leading edge at angular velocities similar to those observed in control, DMSO-treated cells (Fig. 6D,E; supplementary material Movie 6). It should be noted that although nuclear rotations were not disrupted, the cells did not progress forward after low doses of NZ were applied, as has been seen previously (Liao et al., 1995). This may be caused directly by the reduction in MT dynamics or indirectly by a change in MT dynamics because fewer dynamic MTs reach cortical patches of dynein/dynactin.

Dynein and dynactin are known to localize to the Golgi membrane (Fath et al., 1994) and to maintain the perinuclear

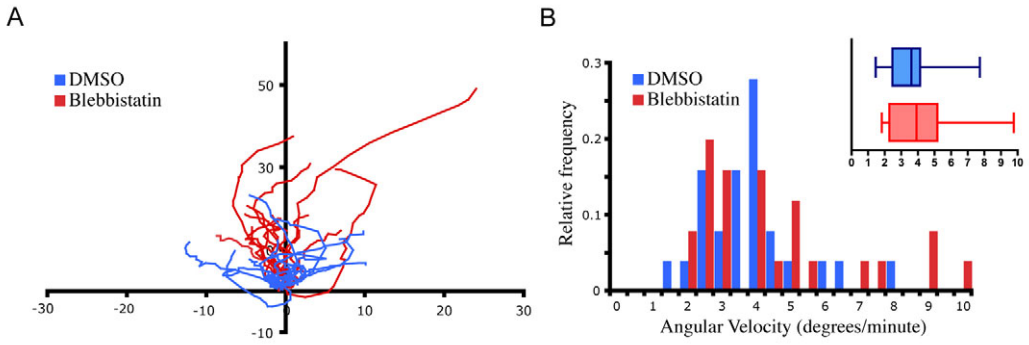

Fig. 5. Nuclear rotations are independent of myosin II activity. (A) Tracks of nucleoli during migration of DMSO or blebbistatin-treated fibroblasts over 45 minutes (n=15). Axis labels are in μm. (B) Angular velocity of nuclei in the presence of myosin II inhibitor blebbistatin (n=25).

localization of the Golgi complex (Corthesy-Theulaz et al., 1992). It is possible that the enrichment of dynein/dynactin at the Golgi could drive nuclear rotations. To investigate this, we treated cells with Brefeldin A (BFA) to disrupt the Golgi complex. In cells that recovered from wounding in the presence of BFA, fragments of Golgi were dispersed throughout the cytoplasm with no perinuclear stacks remaining (Fig. 6C). Centrosomes were polarized normally, and dynactin patches accumulated at the leading edge (J.R.L. and E.L.F.H., unpublished). After monolayer wounding, cells demonstrated nuclear rotations that were robust, even in the presence of BFA (Fig. 6D; supplementary material Movie 6). The angular velocities of these rotations were comparable with rotations observed in control, DMSO-treated cells (Fig. 6E).

DISCUSSION

Dynein and dynactin have been shown to contribute to centrosome reorientation and polarity establishment during both directed cell migration and immunological synapse formation (Combs et al., 2006; Etienne-Manneville and Hall, 2001; Palazzo et al., 2001; Yvon et al., 2002). However, microinjection of wound edge cells with inhibitory antibodies to dynein after centrosome reorientation will cause the injected cell to fall behind in the field of migrating cells (Dujardin et al., 2003), suggesting that dynein has an additional role in directed cell motility. Here, we identify a role for dynein in driving nuclear movement during fibroblast migration.

Wounded cell monolayer cultures provide a system in which the kinetics and direction of cell movement can be synchronized throughout a population of cells. An early step in wound healing involves reorientation of the centrosome to a position between the nucleus and the leading edge (Gundersen and Bulinski, 1988), a process that is dynein dependent (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001; Yvon et al., 2002) and cell-type specific, as epithelial cells do not demonstrate centrosome reorientation (Danowski et al., 2001; Yvon et al., 2002). The predominant model for centrosome reorientation involves patches of cortical dynein tethering MT plus ends to the cortex, which maintains the centrosome in the center of the cell. Concurrently, actin retrograde flow moves the nucleus towards the rear, so that it is between the centrosome and the trailing edge of the cell (Gomes et al., 2005). The involvement of cortical dynein in this process is supported by experiments that show that low doses of nocodazole, which block accumulation of cortical dynein, inhibit centrosome reorientation (Fig. 6A,B) (Gomes et al., 2005; Magdalena et al., 2003; Yvon et al., 2002).

Studies of neuronal motility suggest that dynein has roles in both centrosome and nuclear positioning. Inhibition of dynein and Lis1 increases the nuclear–centrosomal distance, suggesting impaired nucleokinesis during neuronal migration (Tanaka et al., 2004). Unlike the saltatory movement of nuclei in neurons, migration of fibroblast nuclei are temporally coupled to both leading edge extension and centrosome movement. Additionally, imaging of GFP-tagged tubulin in migrating NIH/3T3 cells demonstrates that the centrosome and the nucleus are physically close to one another (Gomes and Gundersen, 2006). Such observations have led to the hypothesis that, in fibroblasts, the interaction of MTs with the leading edge couples centrosome movement to leading edge protrusion, and that a physical interaction of the centrosome with the nucleus passively pulls the nucleus forward.

We now propose a role for dynein in driving nuclear movement along perinuclear MTs in migrating fibroblasts, similar to the role for dynein and Lis1 in neuronal nucleokinesis. Knockdown of dynein in fibroblasts inhibits centrosome reorientation at the initiation of fibroblast motility, but defects in nuclear migration during later phases of migration were also observed: nuclear displacement in the direction of migration (Fig. 1F), as well as the velocity of nuclear rotations that are induced upon wounding, were reduced in dynein knockdown cells (Fig. 2E). Imaging of the centrosome during migration revealed that although the nucleus and centrosome are physically close to one another, they are not statically associated, as nuclear rotations are not accompanied by centrosome revolutions (Fig. 4). These data support previous observations of GFP-γ-tubulin in wound edge cells, which demonstrated that motion of the nucleus and the centrosome were uncoordinated (Yvon et al., 2002). Although centrosome positioning appears to be mediated by cortical dynein (Morris, 2003), selective disruption of leading edge dynein patches did not inhibit nuclear rotations (Fig. 6D,E), suggesting that nuclear rotations are driven by dynein at the nuclear membrane. Such a mechanism could dynamically couple the nucleus to perinuclear MTs, therefore maintaining its centrality during cell migration.

Previous studies have demonstrated an interaction of dynein with isolated nuclei from cerebellar granule neurons (Tanaka et al., 2004) and with the nuclear envelope of NRK cells (Salina et al., 2002) and *C. elegans* embryonic and gonadal cells (Malone et al., 2003). The p62 subunit of dynactin facilitates the association of dynein with the nuclear envelope (Salina et al., 2002); the nuclear envelope component that facilitates this interaction in vertebrate cells is

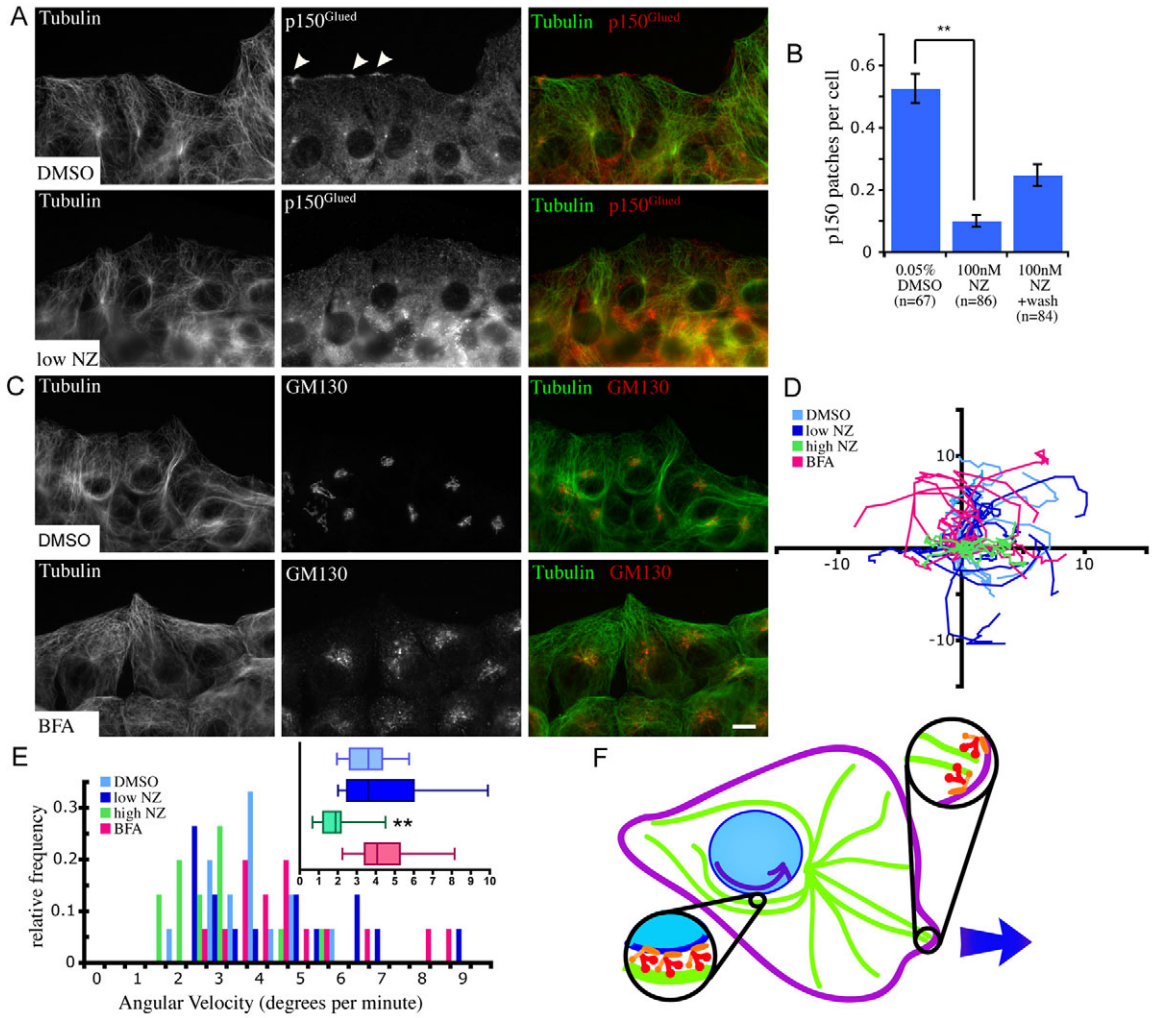

Fig. 6. Nuclear rotations are independent of leading-edge and Golgi-localized dynein. (A) Cells treated with low doses of nocodazole (NZ) 5.5 hours after wounding maintain the MT network morphology (green), but lose patches of p150^{Glued} (red) at the leading edge. (B) Dynactin patch accumulation is inhibited in cells treated with low doses of NZ. Patches are partially rescued 30 minutes after NZ washout. (C) Cells treated with BFA 5.5 hours after wounding maintain MT network morphology (green) but have fragmented Golgi (red, Golgi marker GM130). Scale bar, 10 µm. (D) Tracks of nucleoli treated with DMSO, BFA or NZ during migration of fibroblasts over 45 minutes. Axis labels are in µm. (E) Angular velocity nuclei of cells treated with DMSO (control), NZ or BFA (n=15). (F) Scheme of the two roles of dynein and dynactin in cell motility: (1) dynein and dynactin accumulate in cortical patches at the leading edge, where they interact with MTs and mediate centrosome and MT orientation during motility; and (2) dynein and dynactin interact with the nuclear envelope and transport the nucleus along MTs on the sides of the nucleus. Error bars indicate s.e.m.; **P<0.005.

unknown, but may include SUN-domain proteins (Worman and Gundersen, 2006).

Do nuclear rotations promote migration? The spatial positioning of chromatin is proposed to play an important role in gene regulation, but it is generally believed to be the position of the gene with respect to the nucleus, rather than the entire cell, which influences transcription (Meaburn and Misteli, 2007). Additionally, nuclear rotations are often sustained for more than 360 degrees, making it unlikely that they act to redirect a specific domain of the nucleus to a specific position within the cell. Only a subset of cells at the wound edge show sustained rotations in a single direction while migrating, but these cells do not migrate faster or with more directional persistence than cells without rotating nuclei (J.R.L. and E.L.F.H., unpublished data). It is therefore likely that the rotations themselves do not contribute to migration, but that the rotations reveal the mechanism by which dynein maintains nuclear centrality in migrating cells. Observation of wound-edge cells with nuclei that are not rotating in a sustained manner reveals many stochastic nuclear movements that appear to be very short rotations that frequently switch direction. This ‘jostling’ seems to center nuclei in cells as they move forward. In support of this idea, knockdown of dynein is accompanied by a rearward shift in nuclei position, so that they become situated in the back of migrating cells. This may be a passive shift in position, owing to the forward movement of the cell body when uncoupled to the nucleus. Alternatively, retrograde actin flow may be actively pushing the nucleus rearward, as has been shown to occur during centrosome reorientation (Gomes et al., 2005). In support of this idea, rotations were robust in cells treated with BB (Fig. 5).

Nuclear rotations have been observed previously in cell culture (Englander and Rubin, 1987; Paddock and Albrecht-Buehler, 1986; Paddock and Albrecht-Buehler, 1988; Pomerat, 1953) and in vivo during the early stages of brain development in zebrafish embryos (Herbomel, 1999). Rotations involve both the interior and exterior

of the nucleus, and require an intact MT cytoskeleton (Ji et al., 2007). Here, we show nuclear rotations are induced upon stimulation of cell migration. We also observe a large reduction in rotations in the presence of nocodazole (Fig. 6D,E). Furthermore, dynein was identified as the driving force for rotations in migrating NIH/3T3 fibroblasts. The repolarization that occurs with onset of migration may induce rotations by causing a redistribution of dynein associated with the nucleus. An asymmetric distribution will result in an imbalance of force leading to sustained rotation in a single direction. Stochastic redistribution of dynein motors during motility may lead to the pauses and directional reversals we also observed. The observation that some rotations persist beyond 360 degrees also suggest that multiple dynein molecules act to drive rotations, so that as one dynein may lose its attachment to an MT, other molecules may maintain nuclear–MT attachment and minus-end-directed movement.

Several studies have observed nuclear rotation in response to perturbation of intermediate filaments, including vimentin and lamin B1 (Hay and De Boni, 1991; Ji et al., 2007). The results presented here, in which induction of rotations was observed in response to stimulation of cell migration, might suggest that certain nuclear/cytoskeletal connections are relaxed when cells transition from a sedentary to migratory state. Intriguingly, disruption of actin stress fibers and myosin II activity with blebbistatin stimulated rotations that reached higher velocities than those observed in control cells (Fig. 5), although the differences were not statistically significant. Nonetheless, these data raise the possibility that actin/nuclear connections or actin stress fibers dampen dynein-driven rotations. Alternatively, rearward nuclear movement driven by actin retrograde flow (Gomes et al., 2005) may act as an opposing force to forward dynein-driven nuclear movement. The balance of these forces may act to maintain the nucleus in the center of migrating cells.

In conclusion, we propose a model wherein dynein has two distinct functions in migrating fibroblasts (Fig. 6F). First, cortical patches of dynein interact with MT plus-ends to facilitate centrosome reorientation. Second, nuclear envelope-associated dynein interacts with the nucleus and walks along perinuclear MTs to maintain nuclear centrality as the cell translocates. These two roles for dynein make it a central component in both polarity establishment and cell body translocation during fibroblast migration.

Materials and Methods

Cell culture, transfection and wounding

GFPTub3T3 cells (a kind gift from G. Gundersen) (Gomes et al., 2005), were maintained in DME containing 10% newborn calf serum (NCS). For siRNA transfections, 60 nM RNA oligonucleotides (Dharmacon) were transfected using Lipofectamine RNAiMAX (Invitrogen). The following target sequences were used: for DHC, DHC #1, GAAAUCAACUUGCCCGAUA and DHC #2, CCAAAUACCUACAUUACUU); for p150^{Glued}, p150 #1, CGAGCUCAC-CACAGACCUG and p150 #2, CCUACGCAAUCCGACCGAG; for β-catenin, CAGGGUGCUAUUCCACGACUA (alternate: CAGAUAGAAAUGGUCCGA); scrambled against p150 #1, CCUACGCAAUCCGACCGAG. For DNA transfections, Amaxa Nucleofector program U-30 was used to transfect 5 μg of DNA into ~1×10^6 cells using Nucleofector Kit R. pDsRed1-Cent2 was a kind gift from J. Gleeson (Tanaka et al., 2004); pDsRed2-ER (Clontech) was a kind gift from A. Akhmanova; 3×GFP-EMTB was a kind gift from J. C. Bulinski (Faire et al., 1999). For drug treatments, cells were incubated with 0.05% DMSO, 16.6 μM (high dose) or 100 nM (low dose) NZ (Sigma), 2.5 μg/ml BFA (Sigma) or 50 μM BB (Tocris).

Cells were grown to confluency and monolayers were wounded using a ~25 μm glass scribe or p200 micropipette tip. Cells were fixed in methanol with 1 mM EGTA for 10 minutes at –20°C, air-dried and blocked with PBS containing 1% BSA and 5% normal goat serum; coverslips were incubated with mouse primary antibodies to α-tubulin (clone DM1A, Sigma), p150^{Glued} (BD Biosciences), DIC (Chemicon), β-catenin (BD Biosciences), GM130 (BD Biosciences), acetylated tubulin (Sigma) and vimentin (Sigma) or rabbit primary antibodies to DHC (Santa Cruz), γ-tubulin (Covance) and p150^{Glued} (Tokito et al., 1996). Coverslips were incubated with Alexa-conjugated secondary antibodies from Molecular Probes and mounted with ProLong Gold (Molecular Probes). Alternatively, cells were fixed in 4% paraformadahyde, permeabilized in 0.25% Triton X-100, and stained with TRITC-conjugated phalloidin (Sigma) for visualization of actin.

Cells were lysed in 10 mM Tris with 25 mM NaCl, 0.25% IGEPAL and 5 mM EDTA, denatured in gel sample buffer, and protein levels were analyzed using SDS-PAGE and the above antibodies, followed by chemiluminescence detection (Western Lightening, Perkin Elmer).

Image acquisition

Images were acquired using a Leica DMIRBE microscope with 40×/0.70NA, 63×/1.32NA and 100×/1.4NA objectives and an Orca ER CCD camera (Hamamatsu) with OpenLab software (Improvision). For time-lapse imaging, cells were seeded on glass bottom dishes (World Precision Instruments), imaged in phenol red-free DMEM containing 10% NCS, 25 mM HEPES and 1% OxyFluor (Oxyrase), and sealed with mineral oil (Sigma). The microscope stage was maintained at 37°C. Time-lapse recordings were written with OpenLab software. Figures were prepared using Adobe Photoshop.

Data analysis

All data analysis was carried out with ImageJ software. p150^{Glued} leading edge patches were defined as areas of fluorescence intensity at the leading edge that were 1.5-fold greater than the fluorescence intensity of the area adjacent to the patch away from the wound edge after linearly adjusting the contrast of the image to 0.5% saturated pixels. Centrosome orientation in fixed cells was measured by calculating the angle of the centrosome, as determined by anti-γ-tubulin antibody staining, relative to a line bisecting the nucleus and parallel to the wound. Leading edge displacement was determined by measuring the distance the lamella moved perpendicular to the direction of the wound over the course of a 45-minute movie. Leading edge displacement was measured at five randomly selected points along the wound edge per time-lapse. The rates of forward movement of the entire nucleus were analyzed by tracking the movements of the nucleus centroid over time. Measurement of nuclear position within the cell was analyzed by drawing a line perpendicular to the direction of the wound, starting at the rearmost point of the nucleus and ending at the trailing edge of the cell. Nucleoli and ER particle tracking was performed with the 'Manual Tracking' plug-in for ImageJ submitted to http://rsb.info.nih.gov/ij/ by Fabrice P. Cordelieres. Angular velocity was calculated by measuring the distance traveled by a particle relative to the centroid of the nucleus over time.

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