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Role for NudC, a dynein-associated nuclear movement protein, in mitosis and cytokinesis

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Summary

***NudC*, a nuclear movement protein that associates with dynein, was originally cloned as a mitogen-inducible early growth response gene. NudC forms a biochemical complex with components of the dynein/dynactin complex and is suggested to play a role in translocation of nuclei in proliferating neuronal progenitors as well as in migrating neurons in culture. Here, we show that NudC plays multiple roles in mitosis and cytokinesis in cultured mammalian cells. Altering NudC levels by either small interfering RNA-mediated gene silencing or adenovirus-mediated overexpression resulted in multinucleated cells and cells with persistent intercellular connections and disorganized midzone and midbody matrix. These phenotypes suggest a failure in cytokinesis in NudC altered cells. Further, a key mitotic enzyme, polo-like kinase, is mislocalized from the centrosomes and the midbody in NudC altered cells. Gene silencing of *nud-1*, the *Caenorhabditis elegans* ortholog of NudC, led to a loss of midzone microtubules and the rapid regression of the cleavage furrow, which resulted in one-celled embryos containing two nuclei. The loss of midzone microtubule organization owing to silencing of the *NudC*/*nud-1* gene in two systems, coupled with the loss of Plk1 from mitotic structures in mammalian cells, provide clues to the cytokinesis defect and the multinucleation phenotype. Our findings suggest that NudC functions in mitosis and cytokinesis, in part by regulating microtubule organization at the midzone and midbody.**

Movies available online

Key words: NudC, Plk1, Cytokinesis, Midbody microtubule, Small interfering RNA, *Caenorhabditis elegans*

Introduction

NudC was first identified as a gene that regulates nuclear movement in the asexual reproductive cycle of the filamentous fungus *Aspergillus nidulans*. In *A. nidulans*, *nudC* is required for nuclear distribution, cell wall deposition, colony growth and viability (Osmani et al., 1990; Chiu et al., 1997). Other *nud* loci encode either components or proposed regulators of the minus-end-directed microtubule (MT)-dependent motor dynein and its activator dynactin (Morris, 2000). The mammalian homologues of NUD proteins include Lis1 (NUDF) (Reiner et al., 1995; Smith et al., 2000; Faulkner et al., 2000; Xiang et al., 1995), NudE/NudeL (NUDE) (Niethammer et al., 2000; Feng et al., 2000; Efimov and Morris, 2000), cytoplasmic dynein heavy (NUDA) (Xiang et al., 1994) and light (NUDG) chains (Beckwith et al., 1998), and the actin-related protein Arp1 (NUDK) (Xiang et al., 2000), which is a component of dynactin. In mammalian cells, we showed that NudC forms a biochemical complex with Lis1, associates with components of the dynein/dynactin complex and colocalizes with dynein/dynactin at the microtubule-organizing center (MTOC) in neurons and fibroblasts (Aumais et al., 2001). The colocalization of NudC with Lis1 in the proliferative zone of the developing neocortex (Morris et al., 1998) and in migrating neurons in culture (Aumais et al., 2001) suggest that the NudC/Lis1 complex plays a role in cell division and cell migration. The high degree of sequence conservation in NudC from fungus to human suggests an important function that has been conserved over evolution (Moreau et al., 2001). The functional interactions between NudC, Lis1, NudeL/NudE and dynein/dynactin are likely to contribute to motor functions and microtubule dynamics during such diverse cellular processes as nuclear movement (Xiang et al., 1994; Morris et al., 1998; Aumais et al., 2001), neuronal migration (Niethammer et al., 2000; Sasaki et al., 2000; Smith et al., 2000; Leventer et al., 2001; Aumais et al., 2001; Faulkner et al., 2000; Vallee et al., 2000), axonal transport (Liu et al., 2000), intracellular transport (Karki et al., 1998; Karki and Holzbaur, 1999; Swan et al., 1999) and cell division (Faulkner et al., 2000; Tai et al., 2002; Coquelle et al., 2002; Zhang et al., 2002; Liu et al., 1999; Liu et al., 2000).

We originally cloned mammalian *NudC* as an early mitogen-responsive gene in T cells and showed that, in synchronized cultures, *NudC* mRNA levels increase during mid-G1 and NudC protein levels double during G2/M (Axtell et al., 1995).

NudC expression is elevated in cells with a high growth rate (Gocke et al., 2000a) and in prostate tumors derived from transgenic mouse models (Aumais et al., 2000). Others have shown that NudC is highly expressed in clinical bone marrow isolates from patients with acute lymphoblastic or acute myelogenous leukemia (Miller et al., 1999; Gocke et al., 2000b). These studies show that NudC levels correlate with the proliferative status of various cell types, tissues and tumors. To address a role of NudC in mitosis, one study reduced NudC mRNA levels with an inducible-ribozyme strategy and showed an increase in the number of cells exhibiting multipolar spindles (Zhang et al., 2002). These results suggest that NudC plays a role in spindle formation during mitosis but the mechanism involved is unknown. Dawe et al. (Dawe et al., 2001) used *Caenorhabditis elegans* to silence *nud-1*, the nematode *NudC* ortholog, and showed that this led to embryonic lethality with defects in centrosome-pronuclear rotation at the time of the first embryonic cell division. In further support of a mitotic role for NudC, we recently demonstrated that NudC is phosphorylated at the onset of mitosis, and that NudC is both an in vitro and in vivo substrate for the mitotic polo-like kinase Plk1 (T. H. Zhou, J. P. Aumais, X. Liu, L.-y. Yu-Lee and R. L. Erikson, unpublished). Plk1 phosphorylates NudC in vitro on two serine residues, S274 and S326, in the highly conserved C terminus, and phosphorylation of NudC on these two sites in vivo appears to influence cytokinesis. How phosphorylated NudC mediates this effect is unclear.

To investigate further the role of NudC in mitosis and cytokinesis in mammalian cells, we altered NudC levels either by small interfering RNA (siRNA) silencing (Elbashir et al., 2001) to reduce endogenous levels of NudC, or adenovirus-mediated gene transfer to overexpress NudC ectopically. Here, we report that altering NudC levels inhibited cell proliferation and led to an increase in the proportion of multinucleated cells. Complementary studies using time-lapse video microscopy on *C. elegans* embryos extended our findings by demonstrating that cytokinetic furrows regressed when the *nud-1* gene was silenced by RNA-mediated interference (RNAi). In both NudC altered mammalian cells and *C. elegans* embryos, a remarkably similar and consistent phenotype was the loss of midzone microtubule organization, an event that probably contributes to the observed failure in cytokinesis. Further, altering NudC levels resulted in the mislocalization of Plk1 from various mitotic structures, including the midbody during cytokinesis. These results show that a proper level of NudC is crucial for mitotic progression and completion of cytokinesis, and further suggest that NudC might function by regulating the stability of the cytokinetic furrow and microtubule organization during cytokinesis.

Materials and Methods

Small interfering RNAs

Deprotected and double-stranded 21-nucleotide RNAs were synthesized by Dharmacon Research (Lafayette, CO) (Elbashir et al., 2001). The targeted sequence of rat *NudC* cDNA (accession no. X82445) was AACACCTTCTTCAGCTTCCTT, corresponding to the protein-coding region 88-108 bp relative to the first nucleotide of the start codon (Axtell et al., 1995). This sequence is conserved in rat, mouse and human *NudC* (Moreau et al., 2001). A firefly (*Photinus pyralis*) luciferase siRNA (GL2, accession no. X65324) served as a negative control. siRNAs were resuspended in RNase-free water at a final concentration of 20 pmol μl^{-1} and stored at –20°C until use.

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle Medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals), 100 U ml^{-1} of penicillin/streptomycin (Gibco), 50 $\mu g\ ml^{-1}$ Gentamycin (Sigma) and 1% (v/v) L-glutamine (Gibco). Cells were plated 24 hours prior to transfection in 12-well plates at a density of 25,000-30,000 cells per well. For immunocytochemical studies, cells were plated on poly-D-lysine-coated coverslips (Fisher). For each transfection, 200 pmol (10 μl) of the appropriate siRNA was diluted in 80 μl Opti-MEM I (Gibco). In a separate tube, 3 μl Oligofectamine (Invitrogen) was diluted in 7 μl Opti-MEM I and incubated at room temperature for 5 minutes. The diluted Oligofectamine was mixed gently with the diluted siRNA and incubated at room temperature for 20 minutes. RNA complexes were added to cells with 400 μl serum-free Opti-MEM I and incubated for 4 hours at 37°C, 5% CO_2, after which time FBS was added to the transfections to a final concentration of 4% (v/v) and a final volume of 0.75 ml.

Immunoblotting

Cells were harvested by scraping in 200 μl TEN (20 mM Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA and 0.5% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease-inhibitor cocktail (both from Sigma), and subjected to two cycles of freeze-thaw. Cell lysates were clarified by centrifugation. Protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA), and 5-10 μg total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were immunoblotted with affinity-purified rabbit anti-rat NudC C-terminus peptide (C peptide) antibodies or rabbit anti-rat MBP-NudC fusion protein antibodies (Morris and Yu-Lee, 1998) as indicated, anti-Plk1 monoclonal antibodies (Zymed), or mouse anti-β-actin monoclonal antibodies (AC-40, Sigma). Immunoblots were developed by enhanced chemiluminescence.

Immunocytochemistry

After 24-96 hours, cells were fixed for 20 minutes at room temperature in PEM (80 mM K-PIPES, 5 mM EGTA, 2 mM $MgCl_2$) with 4% (w/v) polyethylene glycol-6000 (PEG), 4% (v/v) ultrapure electron-microscopy-grade formaldehyde (Electron Microscopy Sciences, Ft Washington, PA) and 0.5% Triton X-100. To localize NudC to microtubule structures, cells were briefly treated with 0.5% Trion-X-100 in PEM/PEG for 1 minute at room temperature to remove soluble NudC and then fixed as described above. Cells were then blocked in PBS with 2% (w/v) bovine serum albumin (BSA) and 2% (v/v) normal goat serum (Sigma) and incubated with 0.5 $\mu g\ ml^{-1}$ anti-NudC C peptide, anti-β-tubulin (tub2.1, Sigma) or anti-Plk1 antibodies followed by incubation with Texas-red goat-anti-rabbit IgG (Molecular Probes) or FITC-conjugated goat-anti-mouse IgG (Southern Biotechnologies, Birmingham, AL). Slides were mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA) containing 0.2 mg ml^{-1} 4′, 6-diamidino-2-phenylindole (DAPI). Images were acquired using a RT Color Spot digital camera (Diagnostic Instruments) mounted on a Leica DME microscope with red, green or blue emission filters. Figures were compiled using Spot Software (version 3.0) and Photoshop (version 5.5; Adobe, Mountainview, CA).

Growth curves

Untransfected HeLa cells or HeLa cells transfected with 200 pmol

NudC or luciferase (*Luc*) siRNA were harvested at 24 hour intervals in 100 μl Trypsin/EDTA (Gibco) and resuspended in 400 μl growth medium. Cells were pipetted repeatedly to dislodge cell clumps and were counted in quadruplicates with a HY Lite Neubauer hemacytometer (Hausser Scientific) with Trypan blue exclusion. Values are presented as (cell number $\times 10^4$) ml^{-1}. HeLa cells transduced with recombinant adenovirus containing either the *NudC* or the *Luc* gene were also counted in a similar manner.

Recombinant adenovirus constructions and transduction

Full-length *NudC* was inserted into the adenovirus (Ad) shuttle vector pXCMV at the *Hin*dIII/*Not*I sites to generate pXCMV-NudC. The recombinant adenovirus Ad-NudC was generated by co-transfecting pXCMV-NudC and pJM17, which contains the adenoviral genome with the *E1* gene deleted, into 293 cells as previously described (Estrera et al., 2001). Recombinant virus (Ad-NudC or Ad-Luc) was transduced into HeLa cells at an M.O.I. of 1. After 24 hours, the growth medium was changed and the cells were cultured for 96 hours.

C. elegans culturing methods

Wild-type *C. elegans* strain N2 (Bristol variety) was cultured under standard conditions (Brenner, 1974). To perform RNAi studies, the full-length cDNA of *nud-1* (F53A2.4), the *C. elegans* ortholog of *nudC*, was cloned into the vector L4440 (Timmons et al., 2001) and transformed into HT115 (DE3). Transformed bacteria were grown for 14 hours at 37°C in 2 ml LB supplemented with 12.5 μg ml^{-1} tetracycline and 50 μg ml^{-1} ampicillin. These cultures were plated onto standard worm plates containing 0.8 mM IPTG and 50 μg ml^{-1} carbenicillin and allowed to grow overnight at room temperature. Dauer N2 larvae were then placed onto these plates and their offspring examined after 48 hours at 26°C (Kamath et al., 2000). Embryos analysed by time-lapse video microscopy were dissected in M9 medium from N2 worms treated with *nud-1* dsRNA and placed on a 2% agarose pad with a coverslip mounted on top. Early development was typically analyzed in wild-type and RNAi-treated embryos from shortly after fertilization through two rounds of cell division (~40 minutes).

Immunostaining of *C. elegans* embryos

Immunostaining of embryos was performed as previously described (Guo and Kemphues, 1995). Monoclonal anti-α-tubulin antibody (T9026, Sigma) was used at 1:400 and visualized using goat-anti-mouse AlexaFluor488 (Molecular Probes) at 1:800 dilution. DNA was visualized by staining with DAPI. Embryos were analysed using a Nikon Eclipse E800 epifluorescence microscope equipped with DIC optics and Endow GFP HYQ and UV-2E/C DAPI filter cubes (Chroma). Images were captured with a Spot RT CCD camera (Diagnostic Instruments). MetaMorph Software (Universal Imaging) was used for pseudocoloration of images and image overlays. Images were processed with Adobe Photoshop 7.0.

Results

NudC localization during M phase

To determine the subcellular localization of NudC in mitotic cells, randomly growing HeLa cells were stained with anti-NudC antibodies (red) (Morris and Yu-Lee, 1998), anti-β-tubulin antibodies (green) to visualize microtubule-containing subcellular structures and DAPI (blue) to visualize chromatin/DNA and to stage the cells in mitosis. NudC staining localized with β-tubulin staining on multiple mitotic structures that are important for M-phase progression (Fig. 1). NudC is localized at the microtubule organizing center (MTOC) during prophase, prometaphase and metaphase (Fig.

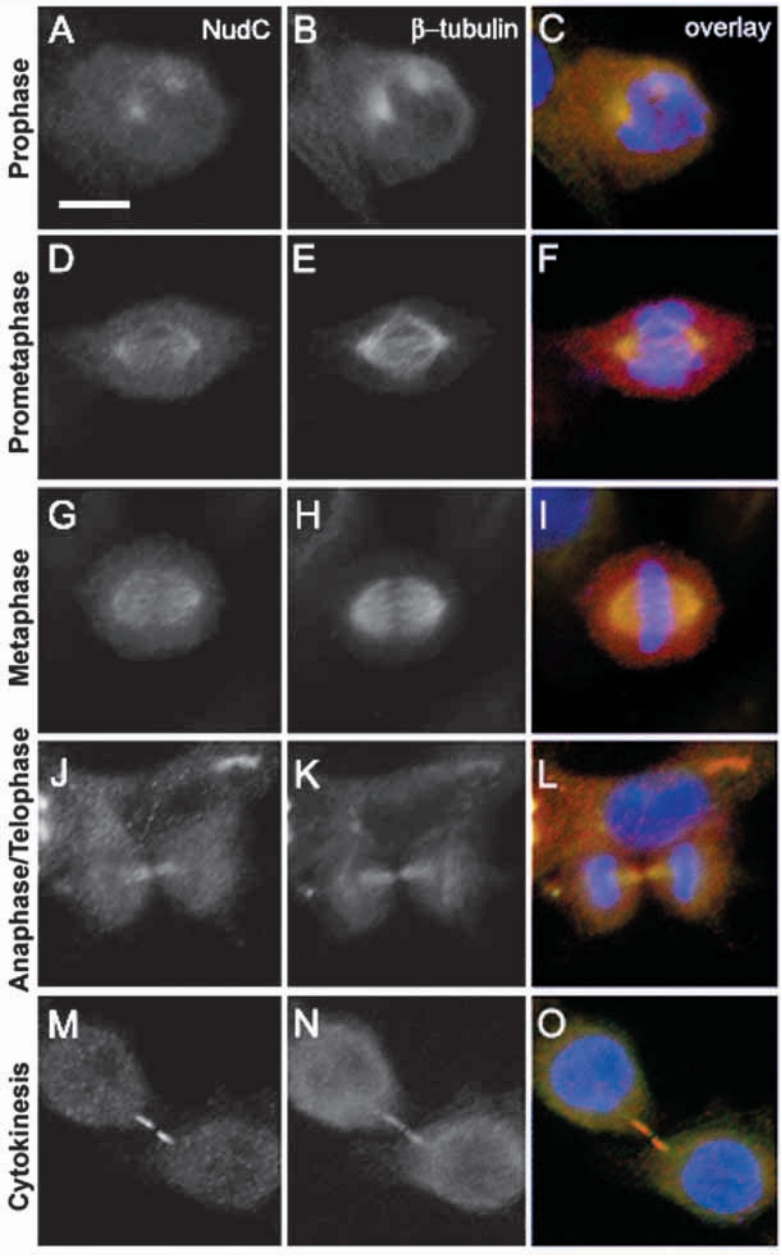

Fig. 1. NudC localization during M phase. Randomly growing HeLa cells were treated briefly with detergent, as described in Materials and Methods, to remove the soluble portion of NudC, and stained with anti-NudC C peptide antibodies (red) and anti-β-tubulin antibodies (green). The DAPI staining pattern of DNA is used to stage the cells during mitosis (M phase). The first two columns are shown in black and white for contrast. The last column is shown in color to indicate NudC localization to microtubule-containing mitotic structures (overlay, yellow). (A-C) Prophase. (D-F) Prometaphase. (G-I) Metaphase. (J-L) Anaphase/Telophase. (M-O) Cytokinesis. Bar, 10 μm for all panels.

1A-I) and on the mitotic spindles during prometaphase and metaphase (Fig. 1D-I). NudC is also found on the midzone microtubules at anaphase/telophase (Fig. 1J-L) and at the midbody during cytokinesis (Fig. 1M-O). These results suggest that NudC is associated with microtubule-based structures important for mitosis progression and cytokinesis.

NudC downregulation by siRNA inhibits cell proliferation

To determine the role of NudC during cellular replication in mammalian cells, we downregulated endogenous *NudC* by RNAi (Elbashir et al., 2001) and examined the effects of *NudC* gene silencing on NudC protein levels and cell growth. HeLa cells were transfected with either *NudC* siRNA or a firefly luciferase *Luc* siRNA, which served as a control against nonspecific gene silencing. Total cell lysates were prepared 24 hours, 48 hours, 72 hours and 96 hours post transfection and immunoblotted with anti-NudC antibodies. Cells transfected with *NudC* siRNA showed a reproducible and significant (80-90%) reduction in the amount of steady-state 42 kDa NudC protein 48 hours after transfection (Fig. 2A, lane 3). The reduction in NudC levels was sustained for up to 96 hours after transfection (Fig. 2A, lanes 4-5). By contrast, NudC levels in cells transfected with *Luc* siRNA were unaffected over 96 hours of incubation (Fig. 2A, lanes 6-9) and remained comparable to those found in the untransfected control cells (Fig. 2A, lane 1). Total lysates were also blotted with an anti-β-actin antibody to show equal loading of proteins (Fig. 2A, lower panel). Several doses of *NudC* siRNA were applied over a 3-day period to determine whether repeated applications of the siRNA duplex could enhance *NudC* gene silencing. Repeated dosing with *NudC* siRNA was no more efficient in depleting NudC levels than a single initial application (data not shown). Therefore, the regimen for subsequent experiments was a single dose of *NudC* siRNA transfection followed by a 72-hour incubation. *NudC* siRNA transfected cells showed reduced proliferation after 72-96 hours post transfection when compared with *Luc*-siRNA-treated or untransfected control cells (Fig. 2B). These results support the idea that NudC plays a role in mitosis and cell proliferation.

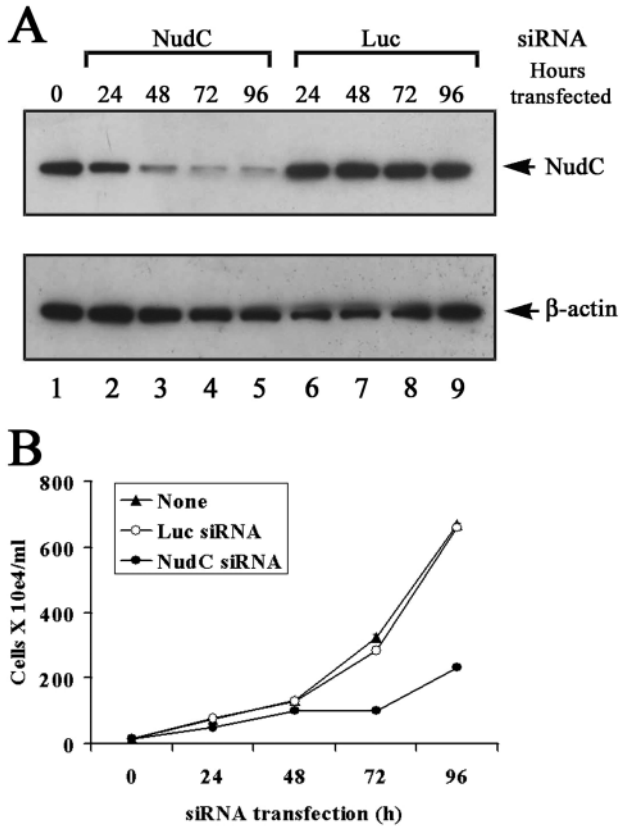

Fig. 2. NudC downregulation by siRNA-mediated gene silencing. HeLa cells were transfected with *Luc* siRNA or *NudC* siRNA for 24-96 hours. (A) NudC protein levels were determined by immunoblotting with anti-NudC C peptide antibodies (top). Equal protein loading was determined by immunoblotting with anti-β-actin antibodies (bottom). A representative of four independent experiments is shown. (B) The proliferation of HeLa cells transfected with either *Luc* siRNA or *NudC* siRNA, or left untransfected was determined over 96 hours. The total cell number $\times 10^4$ in 1 ml of growth medium was determined. Identical results were obtained counting triplicate samples in two independent experiments.

NudC depletion induces multinucleation and results in a failure in cytokinesis

To investigate the basis of the growth inhibition of NudC-depleted cells, HeLa cells were transfected with either *Luc* siRNA or *NudC* siRNA for 72 hours and examined by immunofluorescence microscopy. siRNA-mediated *NudC* gene silencing was observed in over 90% of cells as determined by a lack of NudC staining (red) (Fig. 3B), which agrees with reduced NudC protein expression by western-blot analysis (Fig. 2A). In control cells, NudC staining was generally observed in all the cells, with higher levels in mitotic cells (Fig. 3A, arrow), in agreement with previous studies showing that NudC protein levels double during mitosis (Morris and Yu-Lee, 1998; Aumais et al., 2000). Downregulation of *NudC* by siRNA led to several morphological phenotypes. MTOC structures were not distinct in *NudC*-siRNA-treated cells, in contrast to control cells, which showed MTOCs located characteristically on one side of the nucleus of interphase cells (Fig. 3A, asterisk). A significant (sixfold) increase in the number of large, flat cells containing multiple nuclei (Figs 3B, arrows) was observed in *NudC*-siRNA-treated cells (12%) compared with *Luc*-siRNA-treated controls (2%) (Fig. 3C). A 60% increase in the number of cells connected to each other by cytoplasmic bridges was found in *NudC*-siRNA- compared with *Luc*-siRNA-treated cells (Fig. 3B, arrowheads), suggesting a delay or inhibition in cleavage, cell separation or cytokinesis.

To investigate further the basis of multinucleation, we examined more closely the phenotypes of *NudC*-downregulated cells during M-phase progression. During prometaphase, spindles appeared to be less focused in *NudC*-siRNA-treated cells (Fig. 4A,E). Misaligned chromosomes were seen during metaphase in *NudC*-siRNA-treated cells (Fig. 4F, arrow and inset). Furthermore, striking abnormalities in the midzone/midbody structure at anaphase/telophase and cytokinesis were observed. Midzone/midbody microtubules were either missing or poorly organized (Fig. 4G-I, arrowheads) compared with control cells (Fig. 4C,D, arrowheads) as revealed by β-tubulin staining (green). Many cells had divided and re-entered interphase, as judged by the

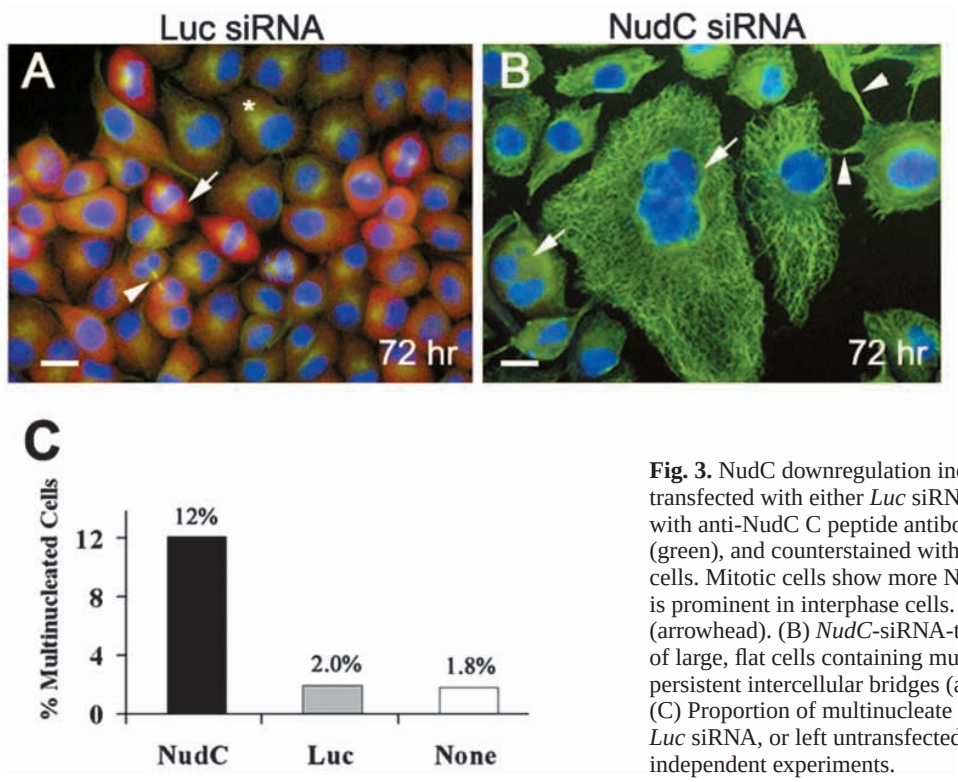

Fig. 3. NudC downregulation induces multinucleation. HeLa cells were transfected with either *Luc* siRNA or *NudC* siRNA for 72 hours and stained with anti-NudC C peptide antibodies (red) and anti-β-tubulin antibodies (green), and counterstained with DAPI (blue). (A) *Luc*-siRNA-transfected cells. Mitotic cells show more NudC staining (arrow). The MTOC (asterisk) is prominent in interphase cells. Cells undergoing cytokinesis are indicated (arrowhead). (B) *NudC*-siRNA-transfected cells. An increase in the number of large, flat cells containing multiple nuclei (arrows) and cells connected by persistent intercellular bridges (arrowheads) is seen. Bar, 10 μm. (C) Proportion of multinucleate cells after transfection with *NudC* siRNA or *Luc* siRNA, or left untransfected. Results are essentially identical in two independent experiments.

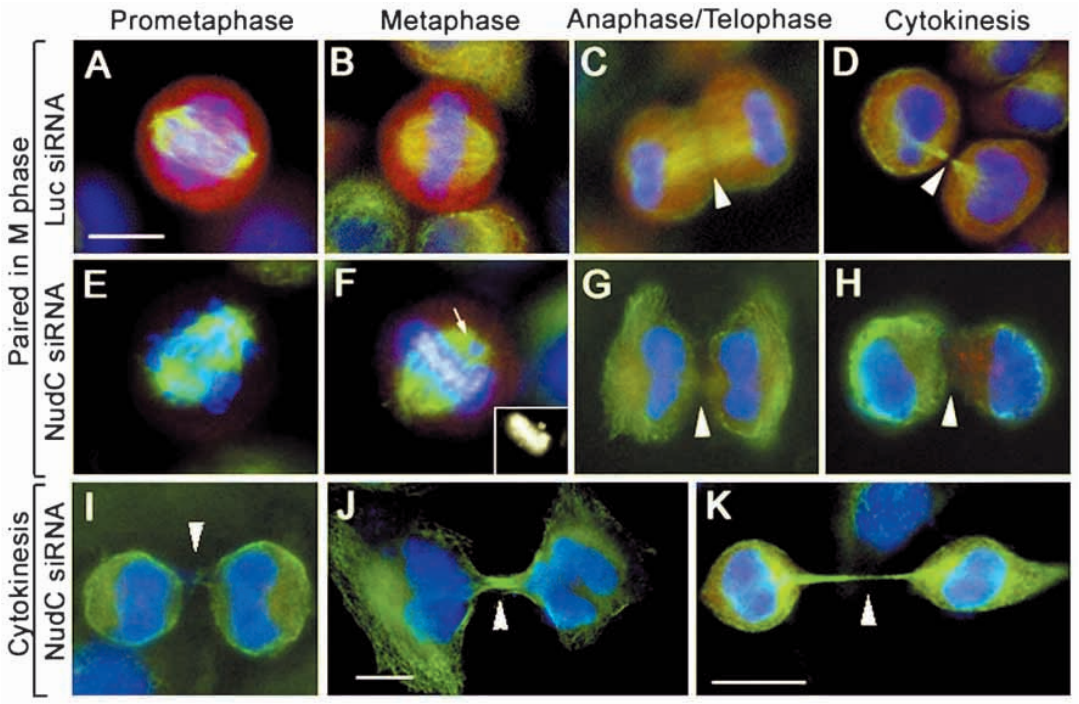

Fig. 4. NudC downregulation induces mitotic and cytokinetic phenotypes. HeLa cells prepared as in Fig. 3 were analysed for mitotic phenotypes. (A-D) Control siRNA-treated cells in different M phases. (A) Cell in prometaphase. (B) Cell in metaphase. (C) Cleavage-furrow (arrowhead) and midzone microtubules forming during anaphase/telophase. (D) Cell undergoing cytokinesis. (E-K) *NudC*-siRNA-treated cells. (E) Mitotic spindles appear to be less focused during prometaphase in *NudC*-siRNA-treated cells. (F) Cells exhibit misaligned chromosome (arrow; see inset) in metaphase. (G) Midzone microtubules are missing (arrowhead) in *NudC*-siRNA-treated cells in anaphase/telophase. (H,I) Midzone microtubules are missing or disorganized (arrowhead) in cytokinesis. (J,K) Midbody structure is unusually thick or very elongated (arrowhead) between divided cells, suggesting problems in cell separation in *NudC*-siRNA-treated cells. A much reduced though still detectable level of NudC was observed in *NudC*-siRNA-treated cells undergoing mitosis. NudC, red; β-tubulin, green; DNA, blue. Bar, 10 μm in all panels. Scale is the same in A-I.

decondensed DNA, but they remained connected to each other by either thick (Fig. 4J) or very long and thin (Fig. 4K) intercellular cytoplasmic bridges. These cells failed to separate, resulting in a cell structure containing two or more nuclei of abnormal size and shape (Fig. 4J). These results suggest that multinucleation in *NudC*-downregulated cells might be due to incomplete cytokinesis.

NudC downregulation results in mislocalization of the mitotic kinase Plk1

To determine the consequence of a lack of microtubule organization at the midzone/midbody in NudC-depleted cells, we examined the presence of proteins that are known to localize in the midzone/midbody matrix and to play a role in cytokinesis (Field et al., 1999). One such factor is the mitotic kinase polo-like kinase (Plk1) (Glover et al., 1998), which we have recently shown to interact with and phosphorylate NudC during mitosis (T. H. Zhou, J. P. Aumais, X. Liu, L.-y. Yu-Lee and R. L. Erikson, unpublished). We determined the effect of *NudC* downregulation on the level and subcellular distribution of Plk1. HeLa cells were transfected with either *Luc* or *NudC* siRNA for 72 hours and the cell lysates were immunoblotted with anti-NudC, anti-Plk1 or anti-β-actin antibodies. In *NudC*-siRNA-treated cells, NudC levels were downregulated (Fig. 5A, top), as also observed in Fig. 2A, but Plk1 levels remained essentially unchanged (Fig. 5A, middle). β-Actin was used as a loading control (Fig. 5A, bottom).

We next examined Plk1 localization during M phase in control and *NudC*-downregulated cells. Plk1 staining was clearly localized to the centrosomes (Fig. 5Ba,c) and midbody (Fig. 5Be) in control cells, consistent with the multiple well-described roles for Plk1 during M-phase progression (Glover et al., 1998; Donaldson et al., 2001). By contrast, Plk1 was mislocalized in 40-45% of cells progressing through various stages of M phase in the *NudC*-downregulated cells. In *NudC*-siRNA-transfected cells, Plk1 staining was diffuse and not localized to the centrosomes (Fig. 5Bg,i) or midzone (Fig. 5Bk). Lagging chromosomes were observed in the midzone region in *NudC*-downregulated cells (Fig. 5Bl, arrow), suggesting a defect in DNA segregation. These results show for the first time that, in *NudC*-downregulated cells, the mitotic kinase Plk1 is not properly localized to the mitotic structures in dividing mammalian cells.

NudC overexpression by adenovirus-NudC transduction inhibited cell proliferation

We next determined the effect of overexpressing NudC on cell proliferation. Recombinant adenovirus containing the *NudC* gene (Ad-NudC) was used for high efficiency expression of NudC in mammalian cells. Recombinant adenovirus containing the firefly luciferase gene (Ad-Luc) was used as a control. At 24 hours, 48 hours, 72 hours and 96 hours post transduction, total cell lysates were immunoblotted with anti-NudC antibodies. Cells transduced with Ad-NudC showed a reproducible increase in NudC protein levels 24-96 hours post transduction (Fig. 6A, lanes 5-8, top). By contrast, NudC levels remained unchanged in cells transduced with the Ad-Luc control (Fig. 6A, lanes 1-4). Reblotting with anti-β-actin antibodies showed equal loading of proteins (Fig. 6A, bottom). Ad-NudC-transduced HeLa cells grew at the same rate as the Ad-Luc-transduced control cells for the first 48 hours (Fig. 6B). However, by 72-96 hours post transduction, overexpression of NudC led to an inhibition of cell proliferation in the Ad-NudC-transduced cells as compared with the control cells (Fig. 6B). These results show that overexpression of NudC also led to an inhibition of cell proliferation in mammalian cells and is not the result of viral transduction.

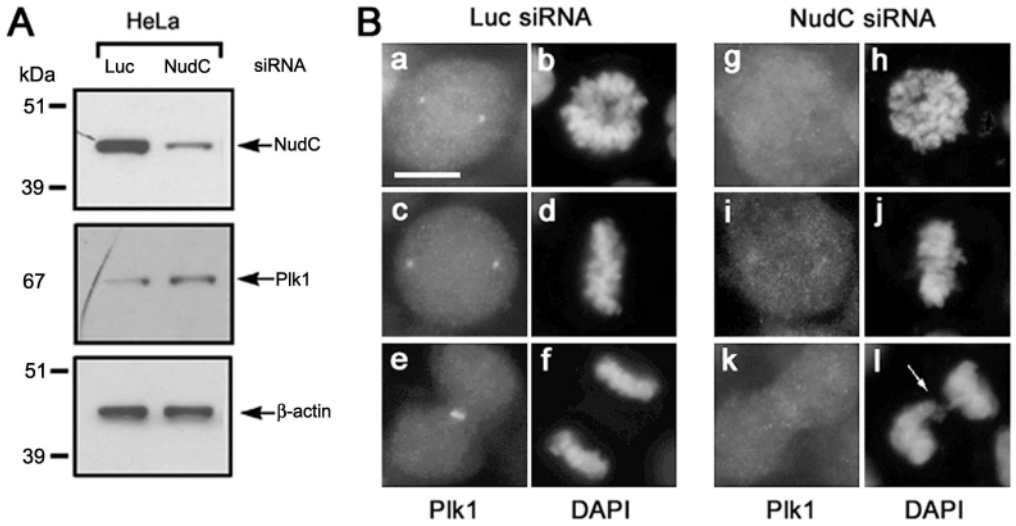

Fig. 5. Plk1 is mislocalized in NudC downregulated cells. HeLa cells were transfected with *Luc* siRNA or *NudC* siRNA for 72 hours. (A) Total cell lysates (10 μg) were immunoblotted with anti-NudC C peptide (top), anti-Plk1 (middle) or anti-β-actin (bottom) antibodies. (B) Subcellular localization of Plk1. Cells were stained with anti-Plk1 antibodies and counterstained with DAPI as indicated. In *Luc*-siRNA-treated control cells, Plk1 staining was found on the centrosomes during prometaphase (a) and metaphase (c), and at the midbody during cytokinesis (e). By contrast, in *NudC*-siRNA-treated cells, Plk1 staining was diffuse and not localized to the centrosomes (g,i) or midbody (k). Notice the lagging DNA in the midzone during telophase in *NudC*-siRNA-treated cells (l, arrow). Stages in M phase were determined by DAPI stains of DNA (b,d,f,h,j,l). Bar, 10 μm for all panels.

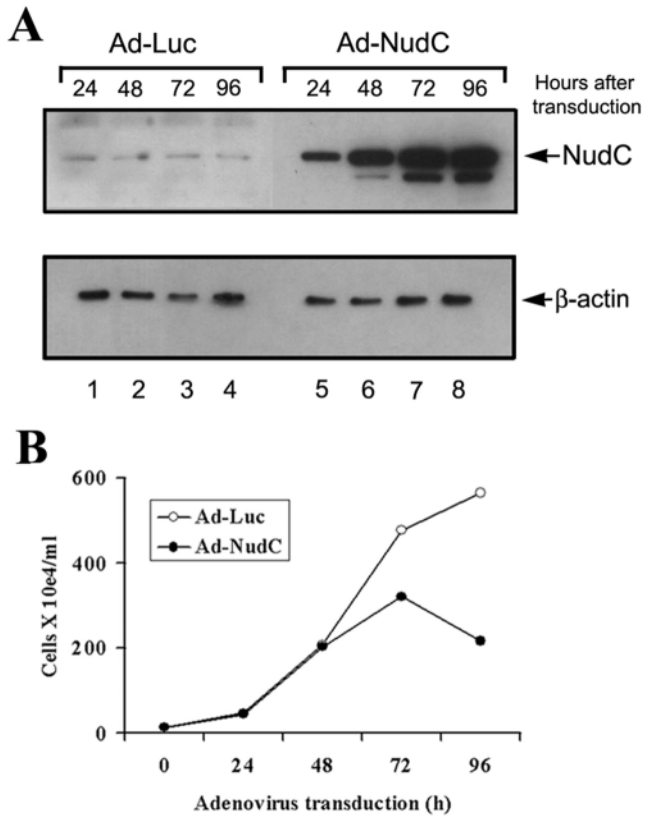

Fig. 6. NudC overexpression by adenovirus-mediated gene transfer. HeLa cells were transduced with Ad-Luc or Ad-NudC and analyzed from 24 hours to 96 hours. (A) Total cell lysates (5 μg) were immunoblotted with anti-MBP-NudC fusion protein antibodies (top) or anti-β-actin antibodies (bottom). In the NudC blot, lanes 1-4 were exposed five times longer than lanes 5-8 in order to detect endogenous NudC expression in the Ad-Luc control cells. The faster-migrating band below NudC in lanes 6-8 might be due to degradation products that were only observed in the NudC-overexpressing cells. (B) The proliferation of HeLa cells transduced with either Ad-Luc or Ad-NudC was determined over 96 hours. Total cell number $\times 10^4$ in 1 ml of growth medium was determined. Identical results were obtained counting triplicate samples in two independent experiments.

NudC overexpression induces multinucleation and a failure in cytokinesis

To determine the basis of Ad-NudC mediated growth inhibition, HeLa cells were transduced with Ad-NudC or Ad-Luc for 96 hours and examined by immunofluorescence microscopy. Overexpression of NudC led to an increase in multinucleated cells as early as 24 hours after Ad-NudC transduction (Fig. 7D, arrows). At 72 hours post Ad-NudC transduction, most of the cells showed significant increases in both cell size and nuclear size (Fig. 7F), and appeared spread out and flat. Some of the large cells contained micronuclei (Fig. 7F, arrow and inset). Indeed, by 96 hours post Ad-NudC transduction, greater than 90% of the cells have become very large and multinucleated. Typically, these large cells contained multiple nuclei (Fig. 7G) or a few enlarged nuclei (Fig. 7H), which suggests a failure in cytokinesis. Micronuclei were observed in these large multinucleated cells (Fig. 7G,H, arrow). Multinucleated cells with persistent cytoplasmic connections were also observed using transient transfection of NudC expression vectors (data not shown), thus suggesting that the observed multinucleation phenotype is unlikely to be a result of viral transduction. By contrast, in the Ad-Luc transduced control cells nuclear size remained constant (Fig. 7A-C) and the cell number increased between 24 hours and 72 hours, in agreement with the cell proliferation data (Fig. 6B). These results show that overexpression of NudC also caused inhibition in cell proliferation and led to an increase in multinucleation.

Overexpression of NudC resulted in cytokinetic abnormalities at 96 hours post Ad-NudC transduction. The most striking phenotype was that many cells showed abnormal midbody and cytoskeletal structures (Fig. 8A,B, compare with Fig. 4C,D). Lagging chromosomes were observed in cells exhibiting unusual midbody structures (Fig. 8A, inset), suggesting a defect in DNA segregation. Many cells were connected by an intercellular bridge that contained disorganized microtubules (Fig. 8C, arrow). Large multinucleated cells were connected by an abnormally dense midbody-like structure (Fig. 8D, DNA and micronuclei in inset). These abnormal cellular connections and cytoskeletal morphologies, which persisted after the cells have re-entered interphase, probably resulted from abortive cytokinesis. There was also an increase in cells displaying numerous MTOCs (Fig. 8E) or multipolar spindles with unattached metaphase chromosomes (Fig. 8F, inset). Unattached, apolar, fragmented and sometimes curving microtubules at the cell cortex were found in NudC-overexpressing cells (Fig. 8C,G, arrows). Thus, overexpression of NudC disrupted cytoskeletal structures and microtubule organization in the cytoplasm and at the midbody.

NudC overexpression results in the mislocalization of the mitotic kinase Plk1

Because reduction of NudC levels by RNAi resulted in Plk1 mislocalization during M phase (Fig. 5B), we next determined whether NudC overexpression has a similar effect on the localization of Plk1. HeLa cells were transduced with Ad-NudC or Ad-Luc for 96 hours and examined by immunofluorescence microscopy. In Ad-Luc-transduced control cells, normal Plk1 staining was observed at the centrosomes during metaphase (Fig. 9A) and the midbody during cytokinesis (Fig. 9C). By contrast, Plk1 was mislocalized in 30% of Ad-NudC-transduced cells. In NudC-overexpressing cells, Plk1 exhibited punctate and irregular staining, as shown in cells connected by an intercellular bridge (Fig. 9E) and in large multinucleated cells (Fig. 9G). These results show that Plk1 exhibits aberrant localization in NudC-overexpressing cells.

NUD-1 depletion in *C. elegans* causes cleavage-furrow regression

We further used a different model genetic system, the nematode *C. elegans*, to investigate the functional consequence of depletion of NUD-1, the *C. elegans* ortholog of mammalian NudC (Dawe et al., 2001), on mitosis. Using injection of *nud-1* dsRNA, Dawe et al. (Dawe et al., 2001) previously showed that the putative zygotic function of NUD-1 involves the development of the germ line and nervous

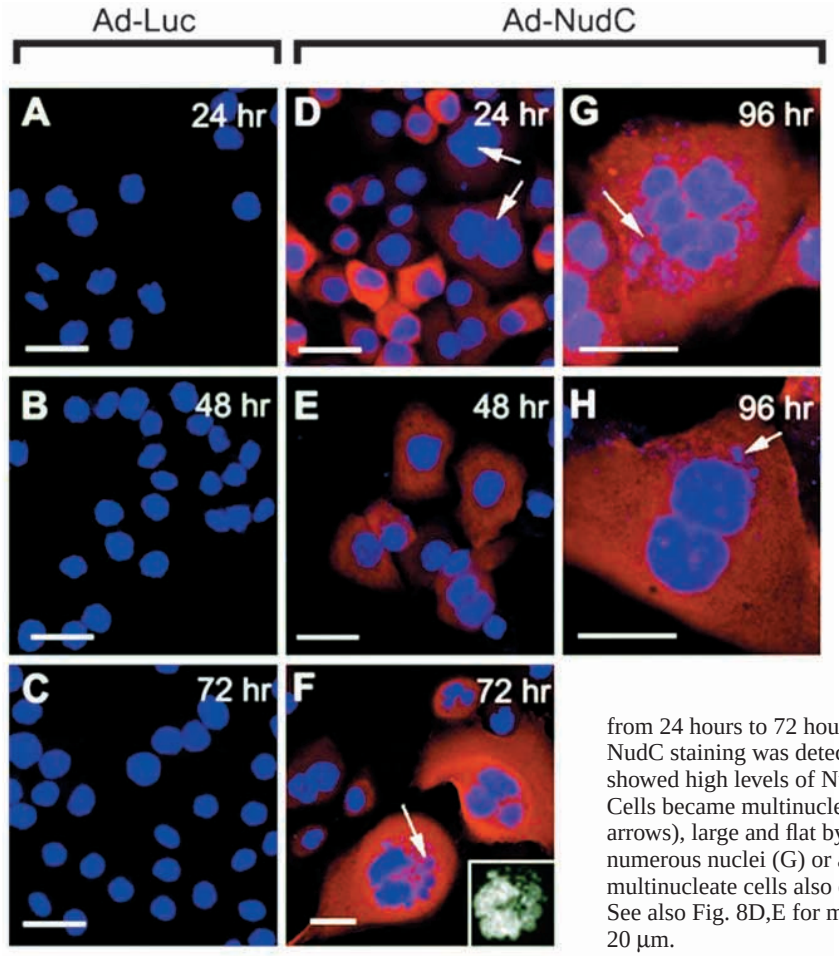

Fig. 7. NudC overexpression induces multinucleation. HeLa cells transduced with either Ad-Luc or Ad-NudC were examined by immunofluorescence microscopy. Cells were stained with anti-NudC C peptide antibodies (red) and counterstained with DAPI (blue), and the same fluorescence intensity setting was used to capture NudC staining for control and Ad-NudC transduced cells. (A-C) Control Ad-Luc cells showing normal increases in cell numbers from 24 hours to 72 hours. At the selected fluorescence setting, no endogenous NudC staining was detectable in control cells. (D-H) Ad-NudC transduced cells showed high levels of NudC staining from 24 hours to 96 hours of infection. Cells became multinucleate as early as 24 hours after Ad-NudC transduction (D, arrows), large and flat by 72 hours (F), and over 90% of the cells contained numerous nuclei (G) or a few enlarged nuclei (H) by 96 hours. Some of these multinucleate cells also contained micronuclei (F, arrow and inset, G,H, arrows). See also Fig. 8D,E for multinucleate cells in the Ad-NudC population. Bar, 20 μm.

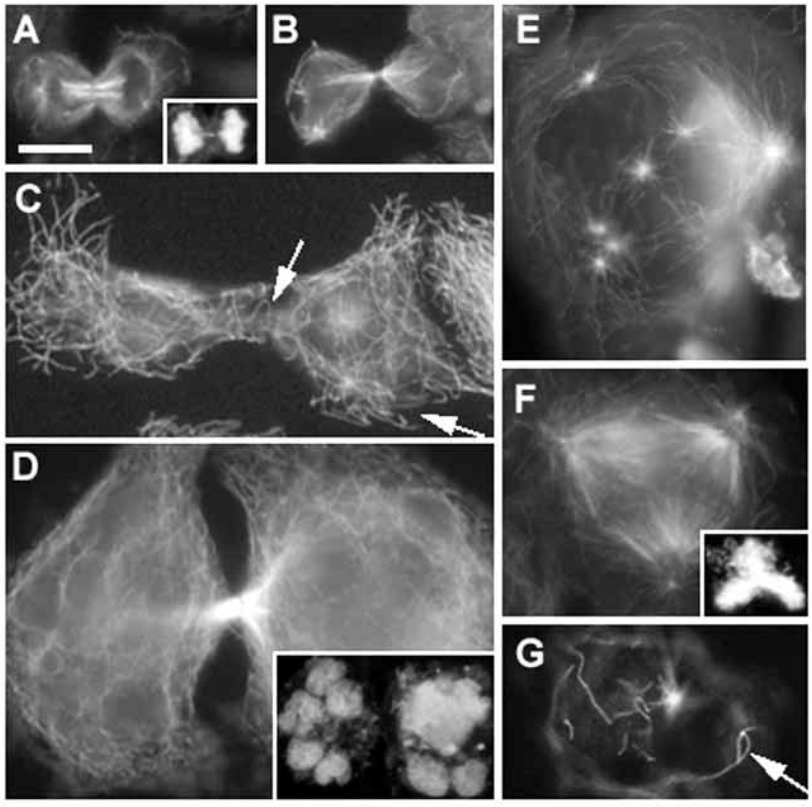

Fig. 8. NudC overexpression induces cytokinesis failure and multiple centrosomes. HeLa cells transduced with Ad-NudC for 96 hours were stained with anti-β-tubulin and counterstained with DAPI. (A,B) Abnormal midbody structures and unusual microtubule matrix are found in NudC-overexpressing cells. (A, inset) Lagging chromosomes are observed in the abnormal midzone/midbody structure. (C) Interphase cells are connected by intercellular bridge with disorganized, curving microtubules (arrows). (D) Large multinucleate cells are connected by an unusually dense midbody-like structure (inset, multiple nuclei and micronuclei in both cells). (E) Many MTOCs are detected in large multinucleate cells. (F) Multipolar spindles are observed (inset, DNA with unattached chromosomes). (G) Elongated, apolar, fragmented and curving microtubules are detected at the cell cortex (arrow). Bar, 10 μm for all panels.

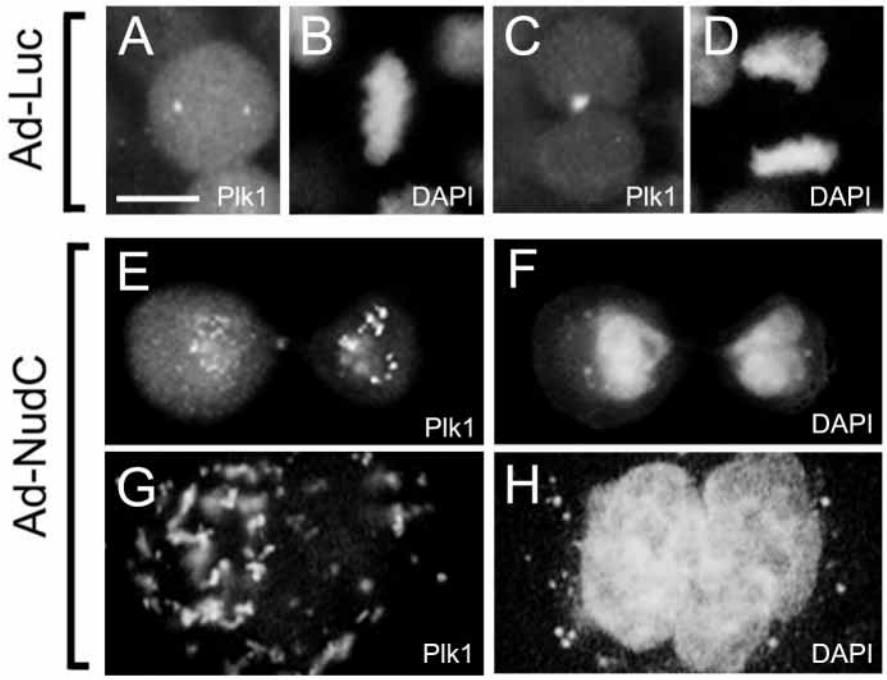

Fig. 9. Plk1 is mislocalized in NudC-overexpressing cells. HeLa cells were transduced with Ad-Luc or Ad-NudC for 96 hours and cells were stained with anti-Plk1 antibodies and counterstained with DAPI as indicated. In Ad-Luc-transduced control cells, Plk1 staining is found on the centrosomes during metaphase (A) and at the midbody during cytokinesis (C). In Ad-NudC-transduced cells, Plk1 staining is punctate and irregular throughout the cell, as observed in cells that are connected by an intercellular bridge (E) and in multinucleate cells (G). (H) Multiple nuclei and micronuclei are observed by DAPI staining of DNA. Bar, 10 μm for all panels.

system. Embryonic cell division defects were observed using this method. However, oogenesis fails rapidly after injection of *nud-1* dsRNA, precluding a detailed analysis of embryonic phenotypes by this method. We used the new technique of RNAi feeding to expand upon these prior analyses by examining cell division defects associated with NUD-1 knockdown during development (Kamath et al., 2000; Timmons et al., 2001). In addition to a defect in pronuclear rotation in the one-celled embryos, as previously described (Dawe et al., 2001), *nud-1* RNAi feeding yields a more severe, highly reproducible defect in late cytokinesis during the first cell cycle following fertilization (Fig. 10) (see also movies online: http://jcs.biologists.org/supplemental). In both wild-type and NUD-1-depleted embryos, a spindle

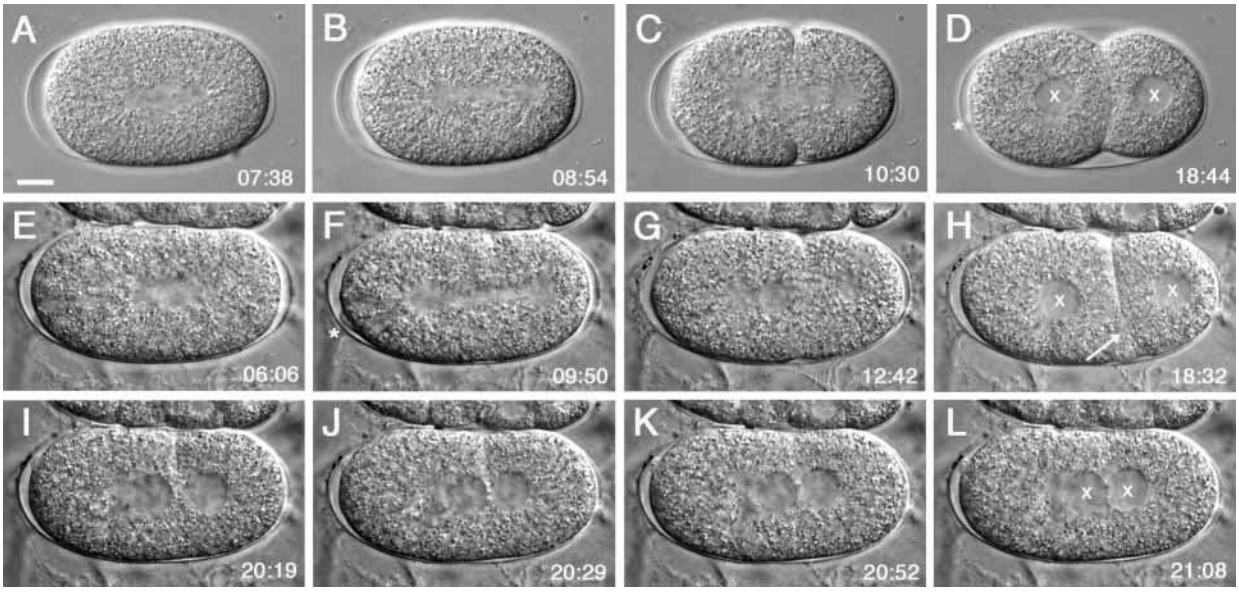

Fig. 10. *nud-1* RNAi in *C. elegans*: cleavage-furrow regression. Selected Nomarski images from digital time-lapse video recordings of live *C. elegans* embryos progressing through the two-cell stage in a wild-type (A-D) and a *nud-1* RNAi embryo (E-L). Each image displays the time elapsed (minute:second) since initiating sequence capture, approximately 20 minutes after fertilization. Embryos are aligned with the anterior to the left, as determined by the presence of the polar body and denoted by asterisks (some polar bodies are out of the focal plane). (A,E) In wild-type and *nud-1* RNAi embryos, the pronuclei have fused and the spindle is beginning to elongate. (B,F) Spindle elongation is not affected in *nud-1* RNAi embryos. (C,G) The first cleavage furrow has been initiated. (D,H) Telophase is completed in the wild-type embryo. However, the cleavage furrow is incomplete in the *nud-1* RNAi embryo (arrow). An 'x' is used to label each nucleus. (I-L) The furrow in the *nud-1* RNAi embryo quickly regresses and the nuclei aggregate in the center of the embryo. Bar, 5 μm in all panels. Digital time-lapse videos of these embryos are available online (http://jcs.biologists.org/supplemental).

developed and elongated along the longitudinal axis (Fig. 10A,B,E,F). The first cleavage furrow appeared at the appropriate time and position in both types of embryos (Fig. 10C,G). The wild-type embryo underwent cleavage-furrow ingression, which resulted in the generation of two differently sized cells (Fig. 10D). By contrast, in the *nud-1* RNAi embryo, the cleavage furrow stalled (Fig. 9H, arrow indicates the furthest point of ingression of the furrow) and then quickly regressed (Fig. 10I-K), resulting in a multinucleated one-celled embryo (Fig. 10L).

To examine further the late cytokinesis failure in the *nud-1* RNAi embryos, we determined whether midzone microtubules were present by staining for α-tubulin (green) when NUD-1 was depleted. Midzone microtubules are typically robust in anaphase wild-type embryos (Fig. 11A-C). However, in *nud-1* RNAi embryos, midzone microtubules were absent in 26% (10/39) of one-cell staged embryos (Fig. 11D-F). The remaining 74% of one-cell staged embryos (29/39) contained midzone microtubules that were less well defined (Fig. 11G-I). Frequently, *nud-1* RNAi embryos (15/29) containing weak midzone microtubules exhibited chromatin bridges, which are indicative of DNA mis-segregation (Fig. 11H,I). After the first cell cycle, cytokinetic furrows in NUD-1-depleted embryos continued to form and regress dynamically without complete stabilization of the furrows, as observed in live embryos (data not shown). To confirm this, older embryos were analysed by α-tubulin staining (Fig. 11L). Multipolar spindles and additional DNA were detected in these embryos, suggesting that embryos depleted for NUD-1 underwent multiple rounds of the cell cycle without completing cytokinesis. Together, the combined mammalian and *C. elegans* studies show that NudC/NUD-1 is crucial for cleavage-furrow ingression, midzone microtubule organization and the completion of cytokinesis.

Discussion

NudC encodes a protein that is highly conserved from fungus to human, suggesting that it has an evolutionarily conserved function. In this study, we show for the first time that NudC is involved in cleavage-furrow ingression and microtubule organization at the midbody, and thus plays an important role in cytokinesis. We first show that depleting endogenous NudC (Fig. 2) or ectopically overexpressing NudC (Fig. 6) inhibited proliferation in mammalian cells. The proliferation defect could be a result of increased incidences of both mitotic and cytokinetic abnormalities. In NudC-altered cells, we observed misaligned chromosomes during metaphase (Fig. 4) and multipolar spindles (Fig. 8), which was also observed by others in cells in which *NudC* mRNA was downregulated (Zhang et al., 2002). The most striking phenotype when NudC levels were altered was the increase in multinucleated cells (Figs 3, 7). Multinucleation can arise from centrosome aberrations (Brinkley, 2001; Nigg, 2002) or defects in cytokinesis (Meraldi et al., 2002; Nigg, 2002). Our data are consistent with the interpretation that the multinucleated cells resulted from a failure to complete cytokinesis. Our studies show that NudC is localized on midzone microtubules and at the midbody in epithelial cells (Fig. 1) and fibroblasts (data not shown). Altering NudC levels led to a disorganization of midzone/midbody microtubule matrices during anaphase/telophase and cytokinesis (Figs 4, 8), supporting a role for NudC in midzone microtubule dynamics. Midzone microtubules have an important role in promoting the ingression of the cleavage furrow and a continued presence of midzone microtubules is required for the completion of cytokinesis (Wheatley and Wang, 1996; Field et al., 1999; Straight and Field, 2000; Murata-Hori and Wang, 2002). Furthermore, in time-lapse recordings of developing one-cell

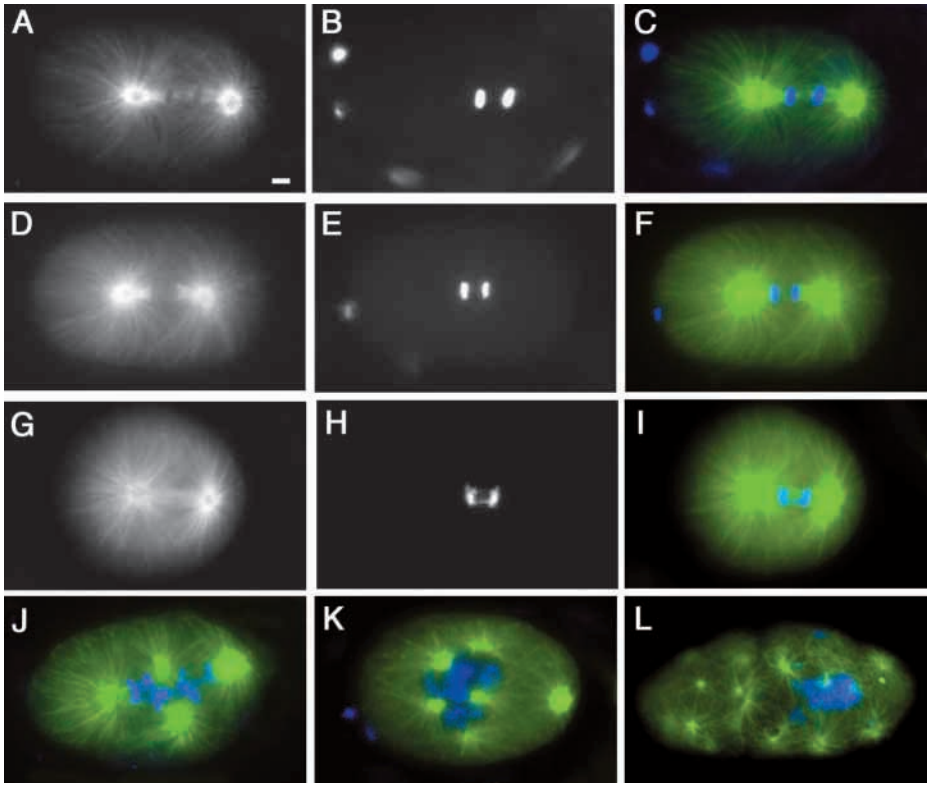

Fig. 11. *nud-1* RNAi in *C. elegans*: absence of midzone microtubules and abortive cytokinesis. Fluorescent images from embryos displaying anaphase-stage microtubules in a one-cell-stage wild-type embryo (A-C) and *nud-1* RNAi embryos (D-L). The embryos were stained with anti-tubulin antibodies (green) (A,D,G,J-L) and counter-stained with DAPI (blue) (B,E,H,J-L). Merged images are shown in C,F,I-L. Embryos are aligned as described in Fig. 9. (A-C) Anaphase-stage wild-type embryo with midzone microtubules. (D-I) *nud-1* RNAi one-cell-stage embryos during anaphase. Midzone microtubules are absent (D,F) or weak (G,I). Weak midzone microtubules usually correspond with the presence of chromatin bridges during anaphase (H). NUD-1-depleted embryos continue the cell cycle without cytokinesis (J-L). Bar, 5 μm in all panels.

C. elegans embryos in which *nud-1* has been silenced by RNAi, cleavage furrows formed but quickly regressed (Fig. 10), which probably resulted from absent or weak midzone microtubules found in these telophase-staged one-cell embryos (Fig. 11). Previous studies have also localized NudC to both the animal and vegetal poles of the cleavage furrow in two-celled embryos of the amphibian *Pleurodeles waltl* (Moreau et al., 2001). Taken together, these observations support potential NudC functions in cleavage-furrow ingression, midbody microtubule organization and the completion of cytokinesis, thus suggesting an evolutionarily conserved role for NudC in midzone/midbody microtubule stability and matrix organization.

Consistent with midzone microtubule disruption, the mitotic kinase Plk1, which normally localizes to centrosomes and midbody microtubule matrix of mitotic cells, is mislocalized in cells in which NudC levels were altered (Figs 5, 9). Plk1 is known to play several roles during M phase progression, including mitotic entry, mitotic exit and cytokinesis (Glover et al., 1998; Nigg, 2001; Donaldson et al., 2001) and is suggested to affect spindle organization and microtubule dynamics (Tong et al., 2002). Plk1 depletion by RNAi resulted in an increase in binucleate cells and cells connected by a long cytoplasmic bridge (Liu and Erikson, 2002), whereas overexpression of Plk1 resulted in large cells containing multiple, fragmented nuclei (Mundt et al., 1997). These phenotypes are reminiscent of both depletion (Fig. 3, Fig. 4J,K) and overexpression (Figs 7-9) of NudC, respectively. Time-lapse microscopy studies revealed that Plk1 is involved in cleavage furrow ingression, which supports a role of Plk1 in cytokinesis (Seong et al., 2002; Chase et al., 2000). Notably, we recently demonstrated that NudC is phosphorylated by Plk1 in vitro and that NudC phosphorylation increases during M phase and correlates with increased Plk1 kinase activity in vivo (T. H. Zhou, J. P. Aumais, X. Liu, L.-y. Yu-Lee and R. L. Erikson, unpublished). Furthermore, phosphorylation of NudC by Plk1 on the highly conserved C-terminal S274 and S326 residues seems to be important during cytokinesis. These results suggest that, although NudC activity might be regulated by Plk1 phosphorylation, NudC might, in turn, be involved in the temporal and spatial localization of Plk1 during M phase. Although it is possible that Plk1 mislocalization from the midbody in NudC altered cells is a consequence of the effects of NudC on microtubule stability and/or organization, we suggest that Plk1 mislocalization contributes to cytokinesis failure and the increased incidence of multinucleation and aneuploidy in cells with altered NudC expression.

Studies in the fungus *A. nidulans* have shown that NudC is genetically and functionally linked to other *nud* loci that encode components of the dynein/dynactin complex (Morris, 2000). Biochemical evidence in mammalian cells show that NudC is associated with the microtubule minus-end-directed dynein/dynactin motor complex (Aumais et al., 2001). NudC also forms a complex with Lis1 (*A. nidulans* NUDF) and the NudC/Lis1 complex is localized to the MTOC and the microtubule network in neurons and fibroblasts (Morris and Yu-Lee, 1998; Aumais et al., 2001). We previously suggested that the NudC/Lis1 complex plays a role in mediating nuclear movement and transport in neuronal progenitors as well as in migrating neurons (Aumais et al., 2001). In dividing cells, Lis1 potentiates dynein function and is suggested to regulate dynein/dynactin interactions with the cell cortex (Faulkner et al., 2000; Vallee et al., 2000) and with the kinetochore (Tai et al., 2002; Coquelle et al., 2002), through association with microtubule plus ends and CLIP-170 (Tai et al., 2002; Coquelle et al., 2002). Lis1 also regulates microtubule dynamics (Sapir et al., 1997; Smith et al., 2000). Given the observations that NudC/Lis1 interacts with the dynein/dynactin complex (Morris et al., 1998; Aumais et al., 2001), that dynein/dynactin subunits are localized to the midbody (Karki et al., 1998; Campbell et al., 1998; Karki and Holzbaur, 1999) and that NudC is localized to the cleavage furrow (Moreau et al., 2001) and midbody (this study), we speculate that the NudC/Lis1/dynein/dynactin complex functions in regulating cytokinesis either by regulating midbody matrix organization or by targeting regulatory and effector molecules to the midzone and midbody during cytokinesis. Our studies also raise the interesting possibility that Plk1 might affect the formation and/or function the NudC/Lis1/dynein/dynactin complex. In support of this notion, we recently observed that Plk1 associates with the dynein/dynactin complex upon entry into mitosis (T. H. Zhou, J. P. Aumais, X. Liu, L.-y. Yu-Lee and R. L. Erikson, unpublished), the significance of which awaits further experimentation.

Many proteins are localized to the midzone and play a role during cytokinesis. These include chromosomal passenger proteins, mitotic kinases such as Plk1 and aurora kinases, GTPases and kinesin-like motor proteins CHO1/MKLP1, which are needed for microtubule stability and microtubule-dependent protein and vesicular transport to support furrow ingression and cytokinesis (Straight and Field, 2000). Inappropriate expression of some of these proteins also results in cleavage furrow regression and cytokinesis failure (Matuliene and Kuriyama, 2002; Raich et al., 1998; Liu and Erikson, 2002; Seong et al., 2002; Chase et al., 2000). It would be interesting to determine whether these diverse groups of proteins, which are needed for the successful completion of cytokinesis, are also missing or mislocalized in NudC altered cells. Our findings that NudC is involved in Plk1 localization during M phase, cleavage-furrow ingression and midzone/midbody microtubule organization suggest novel functions for this highly conserved protein in mitosis and in cytokinesis.

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