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Cytoplasmic Dynein as a Facilitator of Nuclear Envelope Breakdown

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Summary

During prophase in higher cells, centrosomes localize to deep invaginations in the nuclear envelope in a microtubule-dependent process. Loss of nuclear membranes in prometaphase commences in regions of the nuclear envelope that lie outside of these invaginations. Dynein and dynactin complex components concentrate on the nuclear envelope prior to any changes in nuclear envelope organization. These observations suggest a model in which dynein facilitates nuclear envelope breakdown by pulling nuclear membranes and associated proteins poleward along astral microtubules leading to nuclear membrane detachment. Support for this model is provided by the finding that interference with dynein function drastically alters nuclear membrane dynamics in prophase and prometaphase.

Introduction

Progression through mitosis requires that chromosomes gain access to microtubules (MTs) of the mitotic spindle. In organisms such as yeast, the spindle poles are embedded in the nuclear envelope (NE) and spindle MTs form within the nucleus. This is a "closed" mitosis. In higher cells, the mitotic spindle is a cytoplasmic structure, and consequently, for mitotic chromosomes to align at the spindle equator, the NE must be either partially or completely dispersed.

The most prominent feature of the NE is a pair of inner and outer nuclear membranes (INM and ONM) (Gant and Wilson, 1997; Gerace and Burke, 1988). While the ONM displays frequent connections with the ER and features numerous ribosomes, the INM has a unique set of membrane proteins, is ribosome free, and maintains close contacts with chromatin (Wilson, 2000). Regardless of these differences, the INM and ONM are joined where they are spanned by nuclear pore complexes (NPCs), the channels that mediate trafficking between the nucleus and cytoplasm. In this way, the INM, ONM, and ER form a single continuous membrane system. Metazoans contain an additional NE structure, the nuclear lamina (Gerace and Burke, 1988). In mammalian somatic cells, this appears as a thin (20 nm) protein meshwork lining the INM and maintains interactions with both chromatin and INM specific proteins. The lamina is composed primarily of the A and B type lamin family of intermediate filament proteins and plays an essential role in the maintenance of NE integrity and nuclear organization (Gruenbaum et al., 2000; Wilson et al., 2001).

Prophase in higher cells is defined by condensation of chromatin, and initiation of events leading to NE breakdown (NEB). NEB involves the disassembly and dispersal of all major NE structural components (Lee et al., 2000), including the nuclear lamina (Heald and McKeon, 1990; Peter et al., 1990; Pfaller and Newport, 1995; Ward and Kirschner, 1990; Gerace and Blobel, 1980; Stick et al., 1988), NPCs (Macaulay et al., 1995; Snow et al., 1987), and membranes. The disruption of the nuclear membranes marks the end of prophase. At this time, integral proteins of the INM and NPCs are lost from the nuclear periphery and become distributed throughout the cell (Chaudhary and Courvalin, 1993; Ellenberg et al., 1997; Yang et al., 1997). By midprometaphase, the NE has largely dispersed and the nuclear contents are released into the cytoplasm.

The mechanisms of nuclear membrane breakdown are still unclear. Subcellular fractionation and studies on nuclear disassembly and reassembly in *Xenopus* egg extracts suggest that dividing cells contain unique populations of NE-derived vesicles (Newport and Spann, 1987; Vigers and Lohka, 1991). These findings provide a basis for models in which nuclear membrane breakdown is accomplished by a process of vesiculation. Other studies in mammalian systems suggest that NEB involves intermingling of ER and INM components (Ellenberg et al., 1997; Yang et al., 1997). Indeed, ultrastructural analyses in several mammalian cell-types, including thyroid epithelia (Zeligs and Wollman, 1979), PtK2 cells (Roos, 1973), and Hela cells (Robbins and Gonatas, 1964) consistently reveal the detachment of membrane cisternae, often described as ER like, from the nuclear periphery, without extensive vesiculation. While these two views of nuclear membrane breakdown are mechanistically quite distinct, the notion of intermixing of nuclear membrane components with bulk ER during prophase can nonetheless be reconciled with data supporting the vesicular model. For instance, if nuclear membrane components were to enter or to form microdomains within the ER, then subcellular fractionation would be anticipated to yield populations of microsomal vesicles enriched in NE components. These arguments are discussed in a recent review (Collas and Courvalin, 2000).

In the absence of vesiculation, the question arises as to what processes might promote dispersal of the nuclear membranes. Reports that centrosome-associated MTs are responsible for changes in nuclear shape during prophase (Georgatos et al., 1997; Robbins and Gonatas, 1964) have led to the suggestion that MTs actually initiate NEB. The most prevalent view is that this involves MTs piercing the nuclear membranes (Georgatos et al., 1997). However, evidence supporting such a model is lacking. Additional studies have indicated that the MT minus-end-directed motor, cyto-

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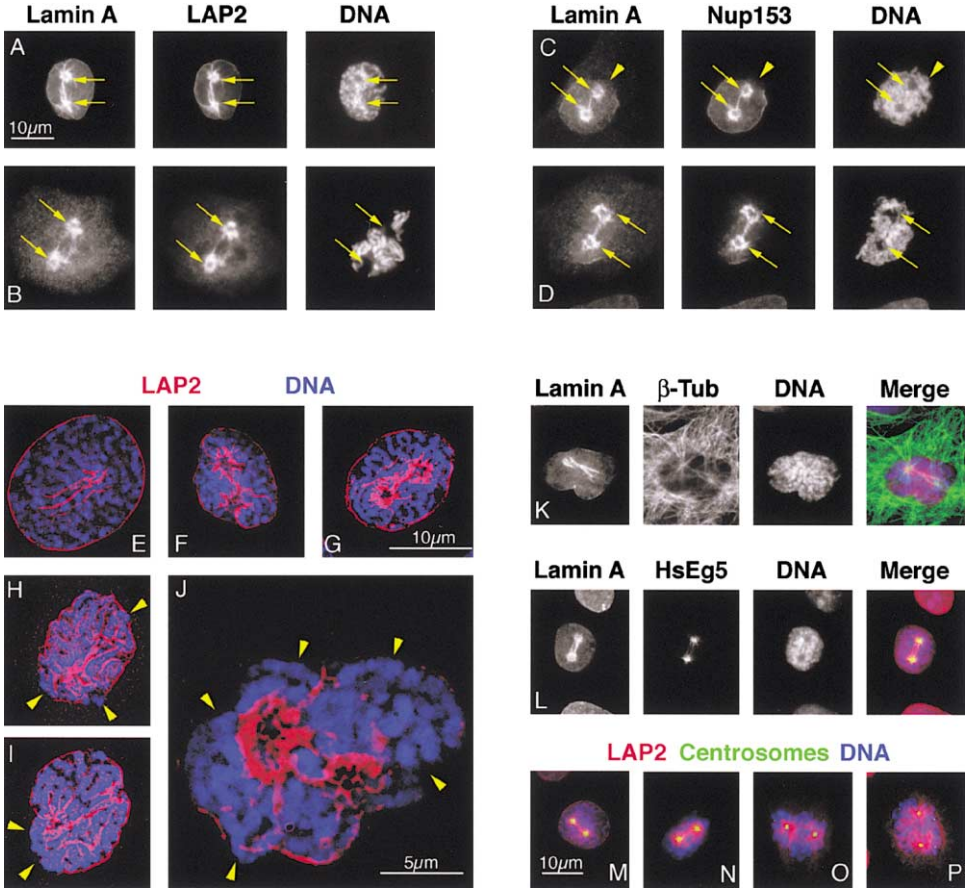

Figure 1. The NE Develops Centrosome-Containing Pockets or Invaginations during Prophase

Indirect immunofluorescence microscopy of prophase and prometaphase NRK cells. In (A) and (B), the cells are labeled with antibodies against lamin A and the INM protein, lamina associated protein 2 (LAP2). In (C) and (D), labeling is with antibodies against lamin A and the NPC protein, Nup153. In all cases, cells were labeled with Höchst dye to reveal the DNA. The pockets or invaginations within the NE (arrows) persist through prometaphase (B, D, and N–P). In (C), the arrowhead indicates a region of the nuclear periphery, outside of the invaginations, from which the NE has been lost. (E)–(J) are deconvolved images of prophase (E–G) and prometaphase (H–J) NRK cells. Labeling is with anti-LAP2 (red) and Höchst dye (blue). These panels document the development of deep NE invaginations as cells progress from early (E) to later stages of prophase/prometaphase. The images represent confocal slices at roughly the equatorial region of each nucleus. The arrowheads in (H)–(J) indicate regions of NE loss which are outside of the NE invaginations. In series (K), the cells are labeled with antibodies against lamin A (red) and β-tubulin (β-Tub, green). In (L), labeling is with antibodies against lamin A (red) and HsEg5 (green), a spindle associated kinesin. Also shown in (K) and (L) are merged images with chromosomes colored blue. In (M)–(P), three color merged images of anti-LAP2 (red), anti-centrosome (green), and chromosomes (blue) are shown.

plasmic dynein, and components of its associated regulatory complex, dynactin, may localize to the NE (Busson et al., 1998; Gönczy et al., 1999; Reinsch and Karsenti, 1997). However, no functional relationship between dynein and NEB has ever been made. In this manuscript, we demonstrate that deformation of the NE during prophase is indeed dependent upon dynein/dynactin. We propose a model in which the gross changes in NE morphology that occur during prophase/prometaphase, including disruption of the nuclear membranes, can be accounted for entirely by the action of NE-associated dynein and centrosome-associated MTs. Evidence supporting this model for NEB is presented.

Results

Centrosomes Become Localized within NE Invaginations in Prophase Cells

Mitotic prophase begins with the appearance of condensed chromosomes within the nucleus. Chromatin condensation is a gradual process that is not completed until after NEB, an event that marks the transition to prometaphase. During this same period of prophase/prometaphase, paired centrosomes separate to form the poles of what will become the mitotic spindle. Immunofluorescence microscopy reveals that shortly after the start of prophase, the NE develops a pair of pockets or invaginations. This phenomenon may be observed employing antibodies against a variety of NE components including lamin A (Figures 1A–1D, 1K, and 1L), NPC proteins such as Nup153 (Figures 1C and 1D), and the INM protein lamina associated protein 2 (LAP2, Figures 1A, 1B, 1E–1J, and 1M–1P) (Foisner and Gerace, 1993; Furukawa et al., 1995). Prophase NE invaginations are seen in all mammalian cell-types that we have examined to date (NRK, BHK, HeLa, L929, 3T3, and Indian Muntjac). Double-label experiments employing either a human anticentrosome antibody (Figures 1M–1P) or an antibody against HsEg5, a spindle-associated kinesin (Figure 1L), in combination with antibodies against either

lamin A or LAP2, reveal that each of the two invaginations contains a centrosome (Figures 1K–1P).

Analyses of prometaphase cells show that NEB and loss of NE constituents starts in regions that are at some distance (up to 5 μm) from the centrosomes. This is particularly obvious in Figure 1C where NEB has commenced at one pole of the nucleus where there is a clear loss of rim staining for both lamin A and Nup153. Confocal microscopy combined with anti-LAP2 labeling (Figures 1E–1J) provides a similar picture of NEB occurring at regions of the NE that do not actually form part of the invaginations (arrowheads in Figures 1H–1J). Identical conclusions have been arrived at by Beaudouin et al. (2002 [this issue of *Cell*]). The overall impression is that as prophase progresses, the NE invaginations deepen and become more extensive at the expense of the remainder of the NE. Indeed, NE components, including lamin A, Nup153, and LAP2 are retained in the vicinity of the centrosomes until late in prometaphase (Figures 1B, 1D, 1J, and 1N–1P). By metaphase, however, these NE remnants completely disperse. These observations suggest that the NE establishes intimate interactions with the centrosomes and/or centrosome-associated MTs during the early stages of mitosis. In fact, double-label experiments reveal MTs in what appear to be NE furrows connecting the pair of invaginations (Figure 1K). The implication is that prophase NE dynamics are linked to the MT cytoskeleton.

Disruption of Microtubules Delays NEB

It is well established that NEB can take place in cells treated with MT-disrupting agents such as nocodazole. Nevertheless, MTs do play a facilitative role in NEB. To demonstrate this, we examined NEB in cells that had been synchronized using a thymidine block. As a wave of cells entered mitosis, nocodazole was added to the medium for 10 min. The cells were then fixed and processed for microscopy employing antibodies against lamin A and LAP2 (Figures 2A–2E). Both control and nocodazole-treated cells, with chromatin morphology characteristic of prophase or prometaphase, were scored for NEB. This was defined as the presence of visible gaps in LAP2 labeling at the NE and failure to retain lamin A in the nucleoplasm (evident within the interstices between the condensed chromosomes). As shown in Figure 2F, only about 24% of prophase/prometaphase cells in the nocodazole-treated culture exhibited NEB, whereas the percentage in control cells was significantly higher at 66%. In control cells in which the chromatin was maximally condensed, the NE was always dispersed (Figure 2D). In nocodazole-treated cells, however, examples of intact NEs could still be observed (Figure 2E). In these cells, lamin A was largely dissociated from the NE but still concentrated within the nucleus. At the same time, a continuous rim of LAP2 labeling was still evident, indicating that the nuclear membranes had yet to be substantially compromised. Such images are rarely seen in control cultures. Notable also for their absence in nocodazole-treated cells were NE invaginations containing centrosomes. Taken together, these data indicate that in the absence of MTs, NEB is delayed relative to both chromatin condensation and lamina disassembly.

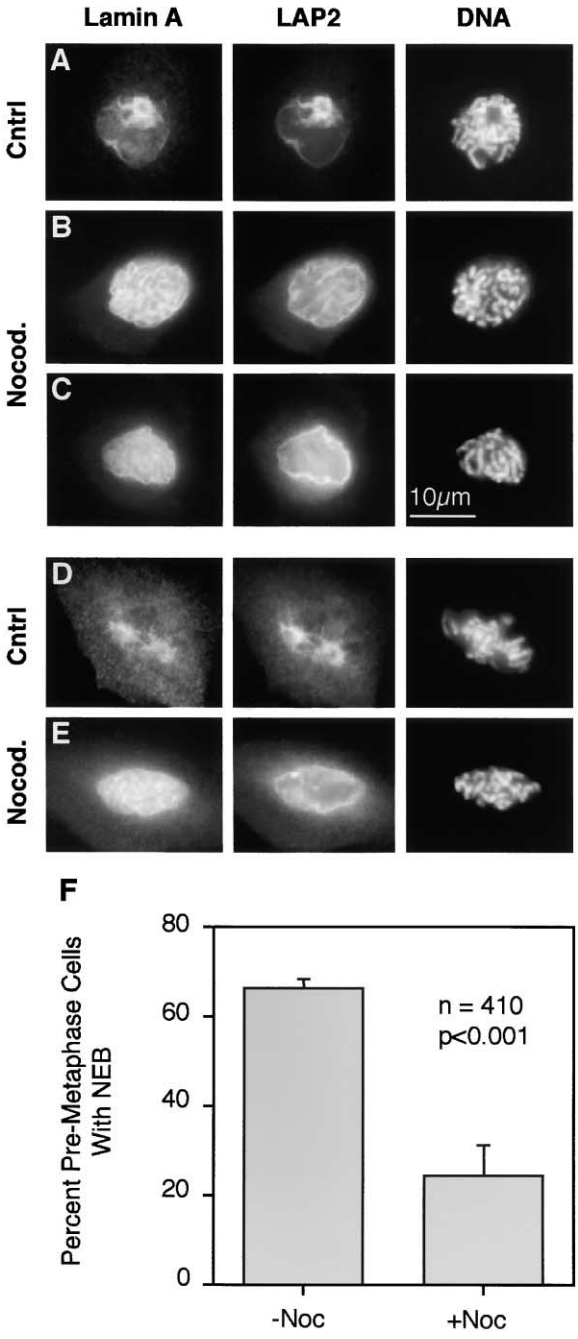

Figure 2. Nocodazole Delays NEB in NRK Cells

Six hours forty-five minutes after release from a twelve hour thymidine block, NRK cells were treated with nocodazole (3 μg/ml) for 10 min and then processed for microscopy (A–E), employing antibodies against lamin A, LAP2, and Höchst dye. Prometaphase cells from both nocodazole-treated (Nocod.) and control (Cntrl) cultures were scored for NE breakdown (F). Nocodazole-treated cultures (+Noc) contain 24 ± 6.8% of premetaphase cells in which NEB has occurred. This compares with 66 ± 1.7% ($p < 0.001$) in untreated cultures (−Noc). The values represent the mean (± SD) of three data sets where a total of 410 cells were scored in each category.

NEB Commences in Regions of the NE that Are Remote from the Centrosomes

Ultrastructural analyses of prophase cells indicate that the centrosome-containing invaginations in the NE de-

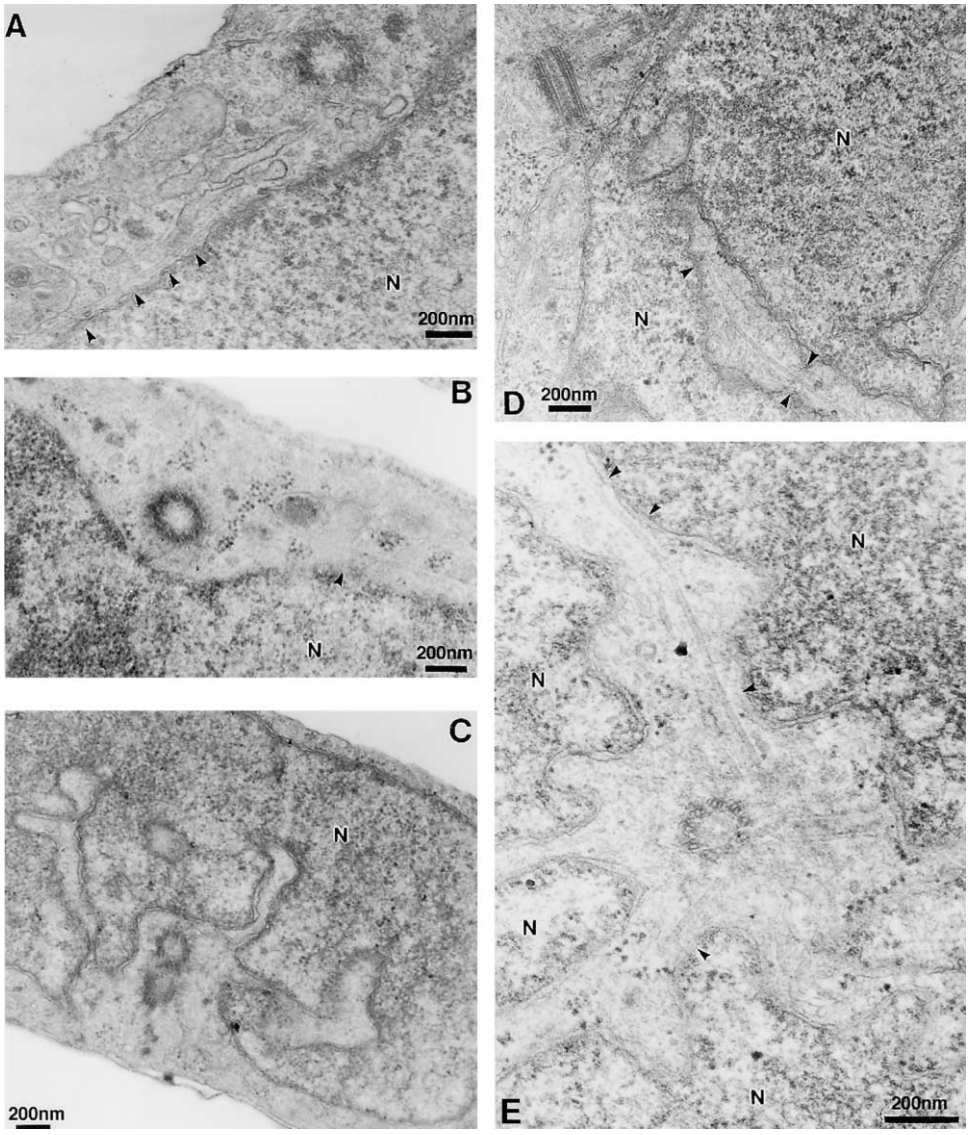

Figure 3. Astral MTs Are Closely Associated with the NE in Prophase Cells

Electron micrographs of the NE of prophase and prometaphase cells in the vicinity of a centrosome. From a small depression in the NE (A and B), each centrosome becomes situated in a deep NE pocket or invagination containing multiple finger-like projections (C–E). Each projection contains numerous MTs and extends up to 2 μm into the nucleus. Grazing contacts between the NE and MTs are frequently observed (arrowheads). In (D), a pair of centrioles (top left) has been sectioned longitudinally. In each of the panels, N indicates the nuclear interior.

velop many finger-like projections that extend at least 1–2 μm into the nucleus. Each projection contains multiple MTs (Figure 3). NRK nuclei are ellipsoidal with a diameter of 10–15 μm and a maximum thickness in fixed specimens that rarely exceeds 3–4 μm (i.e., they are quite flat). Given these dimensions, the prophase invaginations represent major distortions of the NE (roughly 30% of the nuclear surface, D. S. and B.B., unpublished results). This is particularly evident in Figure 3C. Numerous grazing contacts (within 40–50 nm) between the lateral margins of MTs and the outer nuclear membrane can be seen within the projections that form each invagination during late prophase. However, in examining approximately 100 prophase/prometaphase cells, we have found no convincing evidence that any of these MTs ever pierce the nuclear membranes. Earlier serial section analyses have also failed to reveal examples of MTs piercing the nuclear membranes (Rattner and Berns, 1976a, 1976b) (J.B.R., unpublished observations). Instead, discontinuities within the nuclear membranes and detachment of large membrane cisternae from condensed chromosomes are observed in regions of the NE that are not obviously associated with centrosomes or MTs (Figure 4). This finding is consistent with our immunofluorescence results (Figure 1), as well as with the results of Beaudouin et al. (2002 [this issue of *Cell*]).

What role might MTs play in NEB? The prophase/prometaphase dynamics of the nuclear membranes as we see them could be most reasonably accounted for were there a MT minus-end-directed motor associated

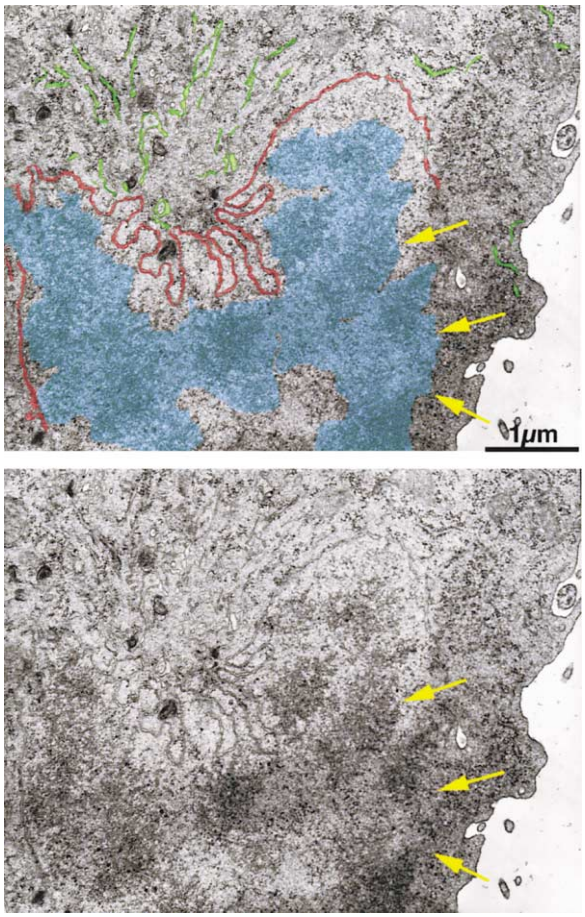

Figure 4. Nuclear Membrane Breakdown Commences outside of the NE Invaginations

Low-power electron micrographs of a prometaphase cell in the vicinity of one centrosome. The same field is shown in both images. To aid identification in the upper image, chromatin has been colored blue, the nuclear membranes are in red, and other rough and smooth membranes have been colored green. Arrows indicate regions of condensed chromatin from which the nuclear membranes have been lost. Many membrane cisternae (green) are oriented with their long axes pointing in toward the very obvious NE invagination.

with the cytoplasmic face of the NE. Activation of such a motor during prophase would have the effect of pulling the nuclear membranes toward the centrosomes along astral MTs. This would cause the formation of progressively deeper and more extensive NE invaginations around each centrosome as prophase advances (Figures 1, 3, and 4). Withdrawal of nuclear membranes toward the centrosome would also account for the disappearance of NE components from regions outside of the invaginations (Figures 1 and 4) as well as for the retention of NE components in the vicinity of the centrosomes during prometaphase (Figures 1 and 2).

Dynein/Dynactin Associates with the NE at the End of G2

Several groups have reported that cytoplasmic dynein, a MT minus-end-directed motor protein, can associate with the NE (Busson et al., 1998; Gönczy et al., 1999; Reinsch and Karsenti, 1997; Robinson et al., 1999). As shown in Figures 5A and 5B and in Figures 6A, 6B, and 6J, we have found that dynein is actually recruited to the NE during late G2 or early prophase in both NRK and BHK cells, as well as in L929 and HeLa cells (D.S. and B.B., unpublished observations). Busson et al. (1998) have observed a similar phenomenon in MDCK cells. In late prophase and prometaphase, dynein can be seen in the NE invaginations (Figure 5C) as well as associated with the NE remnants that persist in the vicinity of the centrosomes (Figure 5D). The dynactin complex component, p62 (Eckley et al., 1999), also concentrates on the NE in early prophase cells (Figures 5E–5G) as does an additional dynactin subunit, Arp1 (Busson et al., 1998). Taken together, these results indicate that dynein, along with its regulatory complex, dynactin, becomes associated with the NE at the end of G2. Significantly, this recruitment of dynein/dynactin occurs *prior* to any changes in NE morphology (Figure 5B). Thus, dynein is present on the NE at the appropriate time to drive the movement of NE components toward the centrosomes leading to the formation of the NE invaginations and ultimately to NEB.

The association of dynein with the NE can be reproduced in vitro. Figure 5H reveals that dynein concentrates at the periphery of rat liver nuclei incubated for 30 min at 34°C in mitotic but not interphase CHO cytosol. This observation was backed up by immunoblot analysis of nuclei and cytosol both before and after incubation (Figure 5I). These findings reinforce the notion that dynein undergoes a cell-cycle-dependent recruitment to the NE and further implicate dynein as a potential mediator of the NE rearrangements that are a prelude to NEB in prometaphase. At the same time, the NE could potentially contribute to centrosome movement by providing a dynein-coated surface, relative to which the centrosomes can migrate. This has been suggested as a mechanism for centrosome separation in *C. elegans* early embryos (Gönczy et al., 1999).

Interference with Dynein Function Impedes NEB

To test the hypothesis that dynein contributes to NE dynamics and breakdown, we have interfered with dynein function in BHK cells by overexpressing the p62 dynactin subunit. We have used wild-type-, GFP- and HA-tagged-p62 with comparable results. Quintyne et al. (1999) have shown that p62 overexpression induces the dissociation of dynein from membrane cargo but without significantly impairing the MT organizing function of centrosomes. Other studies in *N. crassa* indicate that p62 regulates membrane cargo binding (Lee et al., 2001). As revealed by immunofluorescence microscopy, overexpression of p62 was found to cause an obvious decline in NE-associated dynein in prophase BHK cells (Figures 6A–6E). On average, this decline is reflected in a 3-fold reduction in NE-specific fluorescence intensity between transfected and nontransfected prophase cells (Figure 6J). This compares with a roughly 10-fold difference in NE fluorescence intensity between nontransfected prophase versus interphase cells (Figure 6J). Consistent with the report of Quintyne et al. (1999), focusing of MTs at the centrosome(s), both in interphase and mitotic cells (Figures 6F–6I), was relatively insensitive to p62 overexpression.

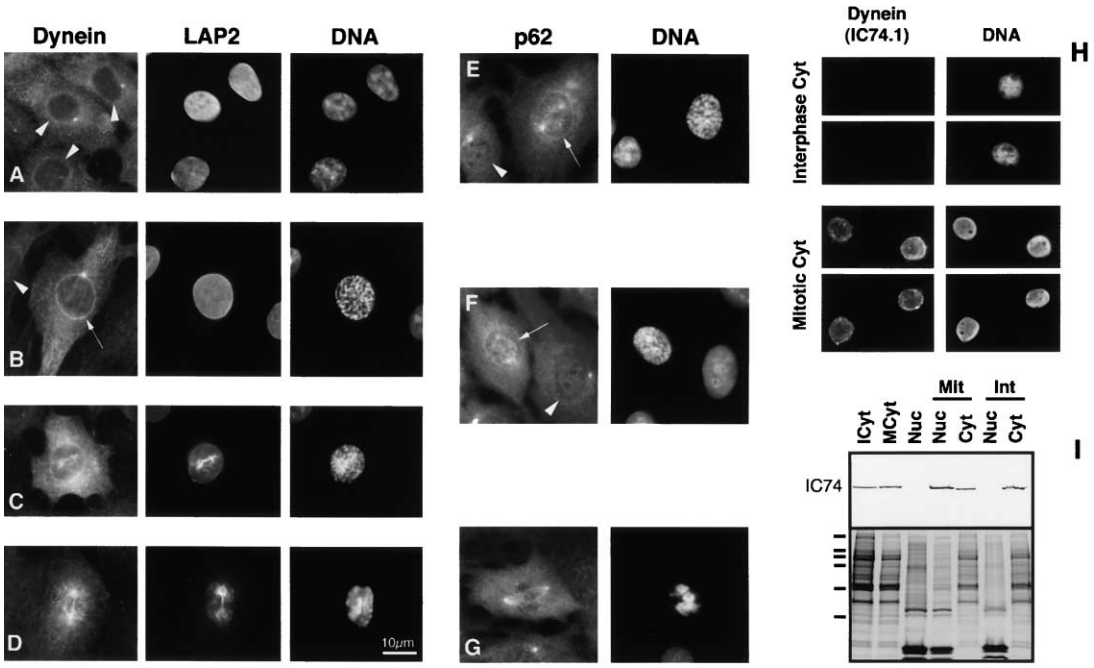

Figure 5. Dynein and Dynactin p62 Associate with the NE in Prophase Cells

Double indirect immunofluorescence microscopy of NRK cells labeled with antibodies against dynein and LAP2 (A–D), or p62 (E–G). Arrows indicate prophase nuclei, while interphase nuclei are indicated by arrowheads. A metaphase cell is shown in (G) where p62 is associated with the mitotic apparatus. Rat liver nuclei incubated for 30 min at 34°C in mitotic, but not interphase, cytosol are labeled by an antibody against dynein (IC74.1) (H). The immunoblot (I) employing the anti-dynein antibody (IC74.1) confirms the association of dynein from mitotic cytosol with rat liver nuclei. The starting material is interphase and mitotic cytosol (ICyt and MCyt) and nuclei (Nuc). Following incubation, the mitotic (Mit) and interphase (Int) reactions were separated into nuclear (Nuc) and cytosol (Cyt) fractions. The Coomassie blue stained gel indicates that there are no large-scale changes in the protein composition of nuclei and cytosol following the incubation. Dashes indicate molecular weight markers (from the top, 200, 117, 96, 67, 45, and 31 kDa).

Analysis of prophase BHK cells employing antibodies against lamin A and LAP2 revealed that overexpression of p62 caused an 8-fold decline in the appearance of centrosome-containing NE invaginations (Figure 7M). We defined these operationally as NE pockets with a length to width ratio greater than one. Such quantitation is simplified in BHK cells since the invaginations tend to form on the side of the nucleus (Figures 7A–7D) rather than on the bottom (as occurs in NRK cells, Figures 1 and 3). We found that nuclei in prophase BHK cells overexpressing p62 adopted at best, a dimpled or "kidney bean" shape (Figures 7E–7G). This clearly suggests a role for dynein/dynactin in NE dynamics during prophase. The frequent appearance of nucleoplasmic lamin A (e.g., Figure 7F) also indicates that NEB was delayed relative to lamina disassembly. In fact, the percentage of premetaphase cells in which NEB has clearly occurred declines by a factor of about two following p62 overexpression (Figure 7M). The fact that we can see such an effect is further consistent with the view that p62 overexpression does not dramatically affect spindle function. Were this to be the case, then we would predict that p62 overexpression should cause an arrest in prometaphase, ultimately leading to an increase in the percentage of premetaphase cells featuring NEB. Of equal significance was the observation that there was little or no clustering of NE remnants around centrosomes once NEB did occur (Figures 7H and 7I). This indicates that the interactions between the NE and centrosome-associated MTs involves the participation of dynein/dynactin. All told, the effect of p62 overexpression on nuclear morphology was qualitatively similar to that of nocodazole treatment (Figure 2).

In a series of related experiments, overexpression of a second dynactin subunit, p50/dynamitin (Echeverri et al., 1996) was also found to give rise to prophase BHK cells in which there was virtual elimination of NE invaginations (20-fold, Figures 7J–7M). At the same time, we observed only a marginal effect of p50/dynamitin overexpression on NEB (Figure 7M). However, the interpretation of these results is complicated by the fact that in contrast to p62, overexpression of p50/dynamitin strongly interferes with centrosome assembly (Young et al., 2000), resulting in loss of MT focusing (Quintyne et al., 1999). Thus, the failure to form NE-invaginations in this case might simply reflect the absence of functional centrosomes. Loss of MT focusing in cells overexpressing p50/dynamitin is also associated with an inhibition of mitotic progression, meaning that any p50/dynamitin-mediated delay in NEB would be overshadowed by an accumulation of prometaphase cells during the time course of the experiment (Figure 7M). Nevertheless, these p50/dynamitin overexpression results do reinforce the view that dynein- and/or centrosome-focused MTs must play a central role in NE dynamics during prophase. Clearly, we can not unequivocally rule out the

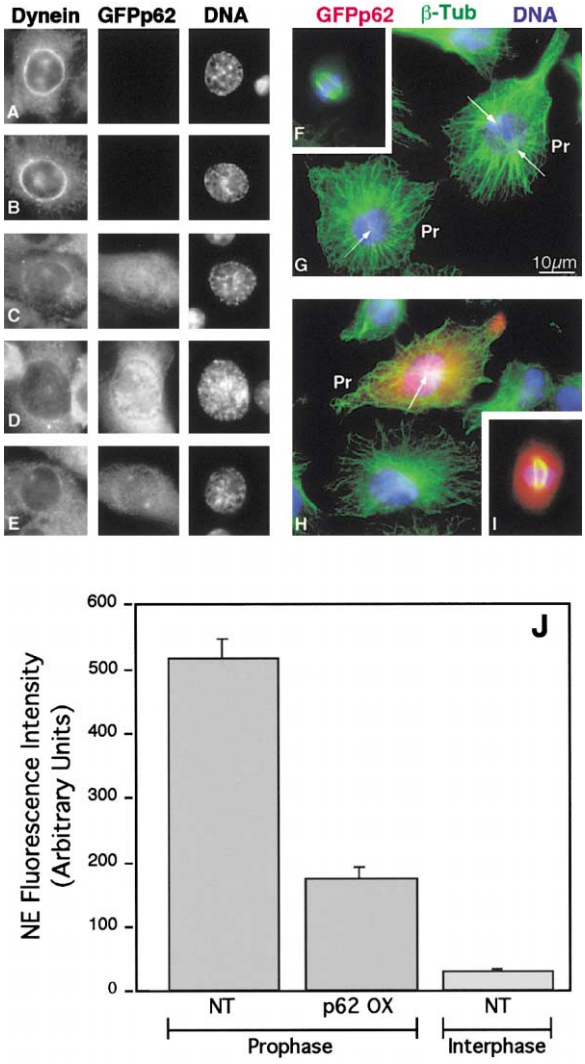

Figure 6. Overexpression of Dynactin p62-GFP Inhibits the Association of Dynein with Prophase NEs

BHK cells 24 hr posttransfection were labeled with monoclonal anti-dynein (IC74.1). Mock-transfected early prophase cells are shown in (A) and (B). Early prophase cells overexpressing p62-GFP are shown in (C)–(E). The intranuclear GFP signal in (D) confirms a previous report that p62, when overexpressed, will enter the nucleus (Karki et al., 2000). (F)–(I) demonstrate that GFP-p62 overexpression (H and I) causes little perturbation of MT focusing at centrosomes (arrows) in prophase (Pr) or prometaphase/metaphase (H–I) cells. Nontransfected cells are shown in (F) and (G). For clarity, the GFP-p62 has been false-colored in red while β-tubulin and DNA are colored green and blue, respectively. Quantitation of the anti-dynein fluorescence intensity at the NE of nontransfected cells (NT) and cells overexpressing p62-GFP (p62 OX) is plotted in (J). A total of 150–200 independent measurements was made for each category. The values represent the net NE fluorescence intensity (± SE). The differences between all three categories is considered statistically significant (X^2 $p < 0.001$).

possibility that overexpression of p62 or p50 has some unanticipated dynein-independent effects on prophase nuclear morphology. However, the simplest interpretation of all of the findings presented here suggest a model in which NEB is driven by poleward-directed movement of NE components (Figure 7N), and that this movement is mediated by NE-associated dynein. In this way, rather than piercing the nuclear membranes, MTs are employed to literally pull them apart (Figure 7N).

Discussion

MT-Dependent Tearing as a Mechanism for NEB

The results described here highlight the role that MTs play in the rearrangements of the nuclear membranes during prophase. The key observations are as follows: (1) separating centrosomes become localized within deep invaginations within the nuclear membranes during early prophase in an MT-dependent manner; (2) NEB is delayed by the MT disrupting drug nocodazole; (3) cytoplasmic dynein and dynactin complex components concentrate on the NE prior to the formation of the centrosome-containing invaginations; (4) initial loss of nuclear membranes occurs in regions of the NE that lie beyond the invaginations; and (5) overexpression of the dynactin complex component p62 interferes with the association of dynein with the NE, prevents the formation of NE invaginations, and retards NEB. Taken together, these observations suggest a model (Figure 7N) in which NE-associated dynein and centrosome-focused MTs facilitate the disruption of the nuclear membranes. In this scheme (Figure 7N), dynein, attached to the cytoplasmic face of the prophase NE, serves to pull nuclear membranes and other NE components toward the minus end of astral MTs. This has two consequences: (1) NE components concentrate around the centrosomes as the cell progresses through prophase; this results in the formation of an invagination (or "hof" as described by Robbins and Gonatas [1964]) with a centrosome at its center. (2) Minus-end-directed movement of NE components toward the centrosome results in the withdrawal of nuclear membranes from other regions of the nuclear periphery, leading to NEB.

Our model predicts that as a cell progresses through prophase, the NE should be placed under tension due to the dynein-dependent movement of NE components toward the centrosomes. Photobleaching experiments described by Beaudouin et al. (2002 [this issue of *Cell*]) demonstrate that this is exactly what occurs. As NE invaginations form around each centrosome, other regions of the NE become distorted or stretched. The next question concerns how the initial opening of the nuclear membranes takes place. Disassembly and dispersal of both lamins and NPCs are gradual processes that are not complete until the end of prometaphase (Figure 1) (Georgatos et al., 1997; Miake-Lye and Kirschner, 1985). Consequently, as cells advance through the early stages of mitosis, progressive loss of NPCs will result in the appearance of increasing numbers of fenestrae within the nuclear membranes. At some point, an individual fenestra or group of fenestrae may be induced to expand to form a much larger gap in the nuclear membrane due to tension across the nuclear surface combined with loss of structural support as the lamina depolymerizes. It follows then that nuclear membrane breakdown should be a catastrophic process that is initiated perhaps at a single point on the nuclear surface. Indeed, Beaudouin et al. (2002) have demonstrated in live cells that nuclear membrane breakdown involves the rapid

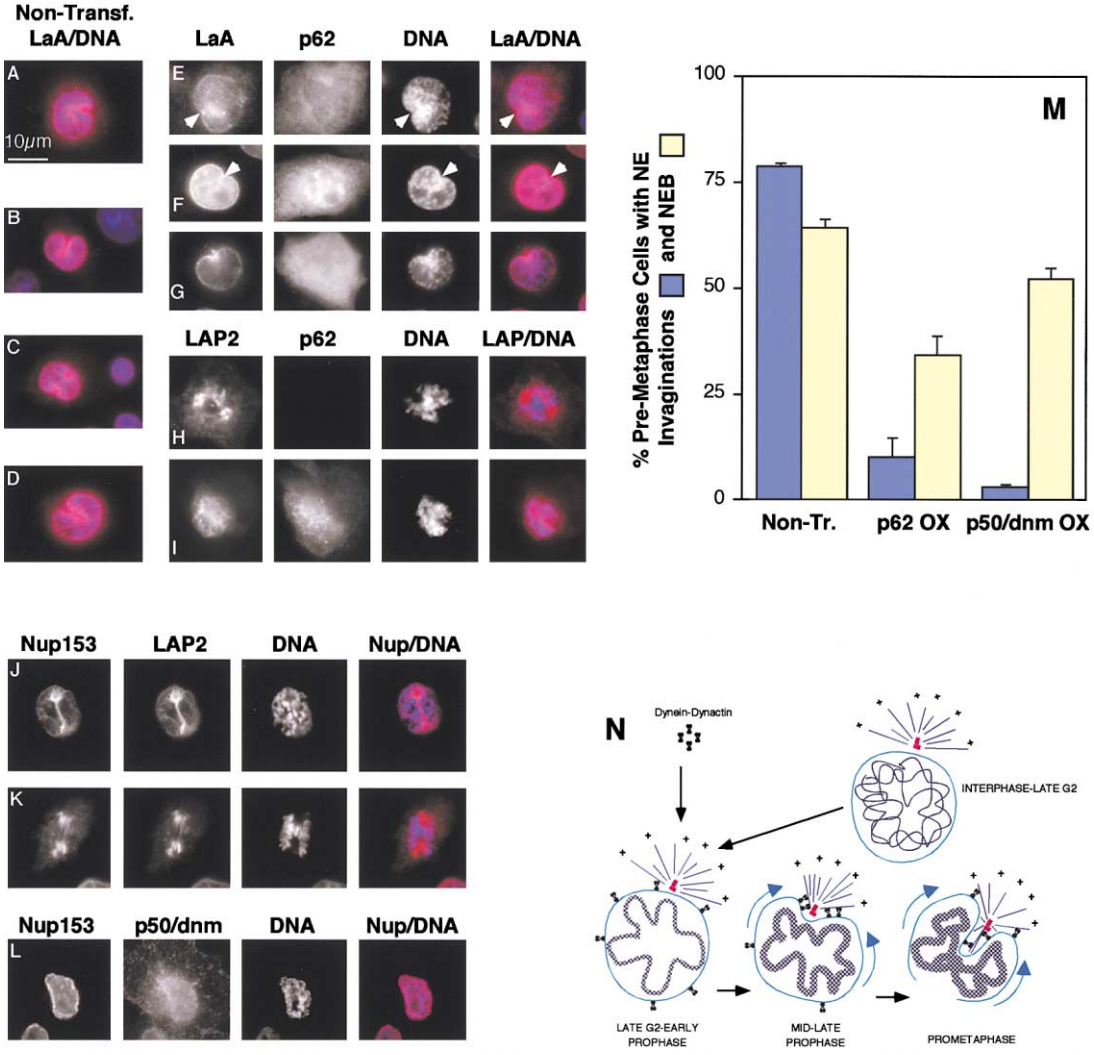

Figure 7. GFP-p62 Overexpression Inhibits the Formation of NE Invaginations in Prophase BHK Cells and Retards NEB

Nontransfected prophase BHK cells (A–D, J, and K) develop obvious NE invaginations revealed by labeling with an antibody against lamin A (red) and Höchst dye (blue) or with antibodies against Nup153 and LAP2. Overexpression of GFP-p62 (E–G) results in an 8-fold reduction in the number of prophase cells with NE invaginations (M). The majority of nuclei adopt no more than a "kidney bean" shape (E–G, arrowheads). At the same time, the number of premetaphase cells undergoing NEB is reduced by a factor of two (M). When NE breakdown does occur, there is little or no LAP2 (red) clustering in the vicinity of centrosomes (compare H and I). Overexpression of p50/dynamitin (p50/dnm) effectively abolishes the formation of NE invaginations (L and M). In (M), a total of approximately 200 cells, originating from four independent experiments (three in the case of p50/dnm), were scored in each category (± SD). The differences between nontransfected (Non-Tr) and p62-overexpressing (p62 OX) cells are considered statistically significant (X^2 $p < 0.001$). Similarly, the low frequency of NE invaginations in cells overexpressing p50/dynamitin (p50/dnm OX) is also significant relative to Non-Tr. The role of dynein, MTs, and centrosomes in nuclear membrane dynamics in early mitosis is outlined in (N). We propose that NE-associated dynein interacts with astral MTs, pulling NE components toward the centrosome, gradually forming a deep pocket or invagination. Withdrawal of NE components into this pocket results in disruption of the NE, potentially by causing the catastrophic expansion of nuclear membrane fenestrae created by NPC disassembly. For simplicity, only one of the two centrosomes is shown in the model.

(catastrophic) expansion of a single hole. Terasaki and colleagues have shown that in star fish embryos the NE becomes permeable to macromolecules before overt gaps in the nuclear membranes are evident (Terasaki et al., 2001). This abrupt increase in permeability may be accounted for by loss of only a few NPCs. If this is the case, then one or more of these vacated NPCs could form the epicenter for nuclear membrane disruption.

The proposed role for dynein and centrosome-associated MTs in NEB provides a unifying theme for results from several laboratories. There have been numerous reports of MT-dependent deformation of the NE during prophase (Georgatos et al., 1997; Robbins and Gonatas, 1964), although possible mechanisms have been largely lacking. It has been suggested that the deformations form as a result of centrosome-associated MTs pushing against the NE which they ultimately pierce (Georgatos et al., 1997), thereby initiating NEB. However, we have been unable to find any convincing evidence that such piercing occurs (Rattner and Berns, 1976b)(D.S., J.B.R.,

and B.B., unpublished observations). Indeed, Roos has remarked (Roos, 1973) that during prophase in PtK2 cells "....no trace of MTs is found in the nucleus, and the MTs do not penetrate the intact NE". Robbins and Gonatas (1964) described sharp membrane projections extending from the NE toward the centrosomes in prophase HeLa cells. The formation of these projections can be most reasonably accounted for by the action of an NE-associated-minus-end-directed motor. In contrast, it is hard to envisage how MTs simply pushing against the NE could induce such a phenomenon. In related studies, Zeligs and Wollman (1979) described NE-derived cisternae in prometaphase cells concentrated in the vicinity of the centrosomes as well as aligned along astral MTs (see Figures 3 and 4). Similar observations have been made on PtK2 and LLCPK2 cells labeled with a fluorescent lipophilic dye (Waterman-Storer et al., 1993). All of these findings are consistent with our own data, and with the suggestion that NE deformation and breakdown is facilitated by an NE-associated motor protein.

Dynein on the NE

There is a growing body of evidence that dynein associates with the NE. Reinsch and Karsenti (1997) demonstrated that nuclei, assembled in vitro in *Xenopus* egg extracts, will move along MTs in a dynein-dependent manner. They suggest that this may represent a mechanism for the positioning of pronuclei in vivo. *Ropy-2*, a *Neurospora* gene involved in nuclear migration (Vierula and Mais, 1997), encodes the ortholog of the dynactin complex protein p62 (Garces et al., 1999). Gönczy et al. (1999) have reported cytoplasmic dynein associated with both male and female pronuclei in *C. elegans* embryos, and that it is required for nuclear migration. Furthermore, they suggest that NE-associated dynein may interact with astral MTs to drive the movement of centrosomes relative to the surface of the nucleus. In this way, NE-associated dynein could function in centrosome separation. A similar scheme has been proposed in *Drosophila* early embryos (Robinson et al., 1999). In mammalian systems, there has been a single report of dynein and the dynactin subunit Arp1 localizing to the NE of prophase MDCK cells (Busson et al., 1998). While the functional significance of this was not addressed, it exactly mirrors our own results.

We have observed a significant recruitment of dynein, along with the dynactin complex subunits p62 and Arp1 to prophase NEs of NRK, BHK, HeLa, and L929 cells. In addition, we have found that dynein in mitotic, but not interphase cytoplasmic extracts, will bind to the periphery of rat liver nuclei, pointing to cell-cycle-dependent regulation of the dynein-NE association. What is not clear is the identity of the dynein binding sites on the NE. Dynein is known to mediate the extension of ER membranes along MTs (Allan, 1995). Given that the ONM forms part of the ER, it is possible that the same binding partners could mediate dynein attachment to both membrane domains. However, Reinsch and Gönczy (1998) make the argument, based on in vitro motility studies, that NEs must possess a separate and unique class of dynein binding sites that can transmit force to both the INM and ONM. The only NE components that we know of that could accomplish this are NPCs. All other proteins or protein complexes that are known to be exposed on the cytoplasmic face of the NE are common to the ER. A priority will be to perform immuno-EM analyses of prophase cells to determine whether there is in fact an association between dynein and NPCs in situ.

Our data favor the view that both dynein-dynactin and astral MTs play a facilitative role in NEB. However, they are clearly not essential. NEB still occurs in nocodazole-treated cells (Nusse and Egner, 1984), although the process is delayed relative to nuclear lamina disassembly and chromatin condensation. Similarly, microsurgical ablation of centrosomes fails to prevent NEB and mitotic progression (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Are there, then, different processes involved in NE breakdown in the presence versus the absence of MTs and centrosomes? We would argue that there are not, and would suggest that nuclear membrane disruption still occurs via expansion of fenestrae created as a result of NPC disassembly. In the absence of MTs, this might be stochastically triggered by other ongoing mechano-chemical processes as the infrastructure of the NE (e.g., the lamina) is disassembled. In fact, we know from other studies that NEs deficient in lamins are mechanically very fragile (Newport et al., 1990; Sullivan et al., 1999). The role of MTs and dynein therefore is to accelerate a process that would eventually occur spontaneously and simply ensures that NEB is temporally coupled to other ongoing mitotic processes. While not addressed in this study, it is possible that following NEB, further dispersal of NE components could be mediated by the activation of an additional plus-end-directed motor protein.

Conclusion

Our suggestion that NEB is facilitated by NE-associated dynein-dynactin will account for all of the documented changes that are observed in prophase and prometaphase. We have been able to demonstrate that dynein is present on the NE during the critical time period and that interference with dynein function causes clear changes in NE dynamics. This role for dynein obviates any requirement for vesicle budding in NE breakdown and is fully compatible with the view that nuclear membrane components disperse within the bulk ER during mitosis in mammalian somatic cells.

Experimental Procedures

Cell Culture

BHK and NRK cells were maintained at 7.5% CO_2 and 37°C in DMEM (GIBCO BRL Gaithersburg, MD) plus 10% fetal bovine serum (HyClone, UT), 100 μg/ml penicillin/streptomycin (GIBCO BRL) and 2 mM glutamine. CHO cells were maintained in MEM-α containing the same supplements

Antibodies

The following antibodies have been described in previous publications: Monoclonal anti-Nup153 (SA1) (Bodoor et al., 1999), rabbit anti-lamin A (Burke, 1990), rabbit anti-HsEg5 (Whitehead et al., 1996), and human anti-centrosome (Mack et al., 1998). Dr. Larry Gerace (Scripps Research Foundation) provided antibodies against LAP2. The monoclonal antibody 74.1 against the dynein intermediate chain was from BAbCo (Richmond, CA). Rabbit anti-myc was a

gift from Jacomine Krijnse-Locker (EMBL, Heidelberg). Secondary antibodies were from Biosource International (Camarillo, CA).

Transfections

Plasmids (pcDNA based) were propagated in *E. coli* XL-1 Blue (Stratagene, Inc., La Jolla, CA) and purified using Qiagen kits (Qiagen, Santa Clarita, CA). DNA was introduced into BHK cells using the Superfect reagent as described by the manufacturer (Qiagen) or using calcium phosphate. Cells were fixed and processed for microscopy 20–24 hr posttransfection. For some experiments, thymidine (2 mM) was added to the medium at 6–8 hr posttransfection. After a further 12 hr at 37°C, the cells were washed free of thymidine and returned to normal medium. Six to seven hours later as a wave of cells entered prophase, they were fixed and processed for microscopy.

Immunofluorescence Microscopy

Cells on glass coverslips were fixed in methanol at −20°C for 10 min and labeled with appropriate antibodies plus DNA-specific Höchst dye #33258 as previously described (Ash et al., 1977). In some experiments, a thymidine block (above) was used to increase the number of prophase cells. Specimens were observed using a Leica DMRB microscope. Images were collected using a Princeton Instruments (Princeton, NJ) MicroMax CCD camera linked to an Apple Macintosh G4 computer running IP Lab Spectrum software (Signal Analytics, Inc.). Fluorescence intensity (FI) measurements at the NE were performed using IP Lab. To accomplish this, the NE was traced by hand to yield a region of interest (ROI) in which the average FI was determined. The average FI from an immediately adjacent ROI in the cytoplasm was subtracted from this value to yield the net NE-associated FI. For deconvolution microscopy, images acquired at 0.2–0.5 μm focal intervals were processed using Micro-Tome version 3.1 (VayTek, Inc.) to yield stacks of confocal slices.

Electron Microscopy

NRK cells, grown in 35 mm petri dishes were fixed in 3% glutaraldehyde and 0.2% tannic acid in 200 mM sodium cacodylate buffer for 1 hr at room temperature. Postfixation was in 2% OsO_4, for 20 min. The cells were dehydrated in ethanol, lifted from the culture dish using propylene oxide, and then infiltrated with Polybed 812 resin. Polymerization was carried out at 60° for 24 hr. Silver-gray sections were cut using a Leica ultramicrotome equipped with a diamond knife. The sections were stained with uranyl acetate and lead citrate and examined in a Hitachi 7000 electron microscope.

In Vitro Incubations

CHO cells were synchronized as previously described (Burke and Gerace, 1986). Cytosol in KHM buffer (78 mM KCl, 50 mM HEPES [pH 7.4] , 4.0 $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT, and 20 μM cytochalasin B) (Burke and Gerace, 1986) was prepared from both mitotic and interphase cells using published procedures (Burke, 1990). Rat liver nuclei were prepared according to the methods of Blobel and Potter (1966). For in vitro reactions, nuclei were incubated (30 min at 34°C) in mitotic or interphase cytosol that was adjusted to a final protein concentration of 10 mg/ml. For immunofluorescence analyses, nuclei were resuspended in 400 μl of KHM, centrifuged for 30 s at 400 × g onto glass coverslips and immediately fixed in methanol at −20°C for 5 min. Immunoblot analyses (Burnette, 1981) employing the anti-dynein antibody (IC74.1) were carried out on nuclei and cytosol recovered by centrifugation as previously described (Burke and Gerace, 1986).

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