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Nup358 integrates nuclear envelope breakdown with kinetochore assembly

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Nuclear envelope breakdown (NEBD) and release of condensed chromosomes into the cytoplasm are key events in the early stages of mitosis in metazoans. NEBD involves the disassembly of all major structural elements of the nuclear envelope, including nuclear pore complexes (NPCs), and the dispersal of nuclear membrane components. The breakdown process is facilitated by microtubules of the mitotic spindle. After NEBD, engagement of spindle microtubules with chromosome-associated kinetochores leads to chromatid segregation. Several NPC subunits relocate to kinetochores after NEBD.

siRNA-mediated depletion of one of these proteins, Nup358, reveals that it is essential for kinetochore function. In the absence of Nup358, chromosome congression and segregation are severely perturbed. At the same time, the assembly of other kinetochore components is strongly inhibited, leading to aberrant kinetochore structure. The implication is that Nup358 plays an essential role in integrating NEBD with kinetochore maturation and function. Mitotic arrest associated with Nup358 depletion further suggests that mitotic checkpoint complexes may remain active at nonkinetochore sites.

Introduction

Segregation of chromatids during mitosis is accomplished by the mitotic spindle in a highly orchestrated process that requires the interaction of each chromosome with spindle microtubules. Chromosomal attachment of microtubules is mediated by kinetochores. These are elaborate structures that are assembled during mitotic prophase and that contain multiple protein species, including cell cycle regulatory molecules and microtubule motor proteins. Formation of each kinetochore takes place at the centromere, a chromosomal site enriched in a group of centromere-specific proteins (CENPs), which includes CENP-A (a histone H3-like molecule) as well as CENP-B and CENP-C (Cleveland et al., 2003). Mature kinetochores appear as flat trilaminar plate-like structures that are able to capture the plus ends of spindle microtubules. In vertebrate cells, the mitotic spindle is an exclusively cytoplasmic structure. Consequently, for chromosomes enclosed within a nucleus to gain access to spindle microtubules, the nuclear envelope (NE) must be either partially or completely broken down.

In all eukaryotes, the NE features a pair of inner and outer nuclear membranes that are joined in places where they are spanned by nuclear pore complexes (NPCs) (Gerace and Burke, 1988; Gant and Wilson, 1997). Metazoan NEs contain an additional structural element, the nuclear lamina, which is associated with the nuclear face of the inner nuclear membrane. The lamina consists of a thin (20–50 nm) filamentous meshwork composed primarily of A- and B-type nuclear lamin proteins (Gerace and Burke, 1988; Gant and Wilson, 1997). During mitosis in mammalian somatic cells, the nuclear lamina and NPCs are disassembled. At the same time, nuclear membrane components disperse within the ER, which itself exhibits numerous connections with the outer nuclear membrane (Ellenberg et al., 1997; Yang et al., 1997; Ostlund et al., 1999). Disassembly of the lamina and NPCs occurs in response to phosphorylation of both lamina and NPC subunits (Gerace and Blobel, 1980; Heald and McKeon, 1990; Macaulay et al., 1995). Although the majority of these components become distributed throughout the mitotic cytoplasm, certain NPC proteins (nucleoporins or Nups) and associated molecules, including Rae1, Nup107, and Nup133, become preferentially associated with kinetochores (Belgareh et al., 2001; Wang et al., 2001; Babu et al., 2003). Another nucleoporin, Nup358, which is a component of the short (100 nm) filaments that extend from the cytoplasmic

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face of the NPC during interphase, relocates to both spindle microtubules and kinetochores (Joseph et al., 2002). This relocation occurs in association with Ran GTPase activating protein 1 (RanGAP1), a molecule with which Nup358 also interacts during interphase. Conversely, certain mitotic checkpoint proteins, such as Mad1 and Mad2, that are kinetochore associated during mitosis are found at the nuclear face of NPCs during interphase (Campbell et al., 2001). In yeast, this localization is mediated by Nup53p, part of a larger complex of NPC proteins that includes Nup157p and Nup170p (Iouk et al., 2002). Remarkably, yeast strains deficient in Mad1p exhibit a reduced rate of nuclear protein import as well as decreased stability of the Nup53p complex (Iouk et al., 2002). The implication is that there is a functional relationship between the mitotic apparatus and the NE. However, the significance of this has only recently become a focus of investigation.

The interplay between the NE and the mitotic spindle is further highlighted by findings that the spindle itself plays an active role in nuclear membrane dispersal during prometaphase (Beaudouin et al., 2002; Salina et al., 2002). The entire process is driven by cytoplasmic dynein, a microtubule minus end–directed motor protein, which concentrates on the NE during late G2/early prophase (Salina et al., 2002). By engaging with spindle microtubules, NE-linked dynein causes the deformation and rupture of the nuclear membranes, leading to release of the condensed chromosomes into the cytoplasm. This process, in effect, represents a mechanical checkpoint because it provides a means to delay NE breakdown (NEBD) until functional spindle microtubules have been assembled.

Although the identity of the dynein-binding partner on the NE remains unknown, cytoplasmically exposed NPC subunits have been suggested as possible candidates. One such protein, Nup358, is of particular interest because it is known to associate with both the mitotic spindle and kinetochores. To determine whether Nup358 does indeed play a role in early mitotic progression, we employed an siRNA approach to deplete cells of this particular nucleoporin. Although we were unable to find any evidence for an involvement in dynein binding and NEBD (unpublished data), we did observe a surprising effect on chromosome congression. Our data suggest that Nup358 plays an essential role in kinetochore function and chromatid segregation.

Results

Examination of HeLa cell cultures exposed to Nup358 siRNA reveals a series of striking changes that occur over a period of several days. During the first 24–48 h of siRNA treatment, an accumulation of prometaphase cells becomes apparent (Fig. 1 B). By 120 h, this reaches a peak of almost 50% of the total mitotic population, a threefold increase in the frequency of prometaphase cells over that observed in mock-treated cultures (Fig. 1 B). A second population of cells, characterized by the presence of multiple micronuclei (Fig. 1 A), emerges between 24 and 96 h. This very unusual morphology is rarely encountered in control cultures and is a characteristic feature of Nup358 siRNA treatment. It is not observed after depletion of other nucleoporins such as
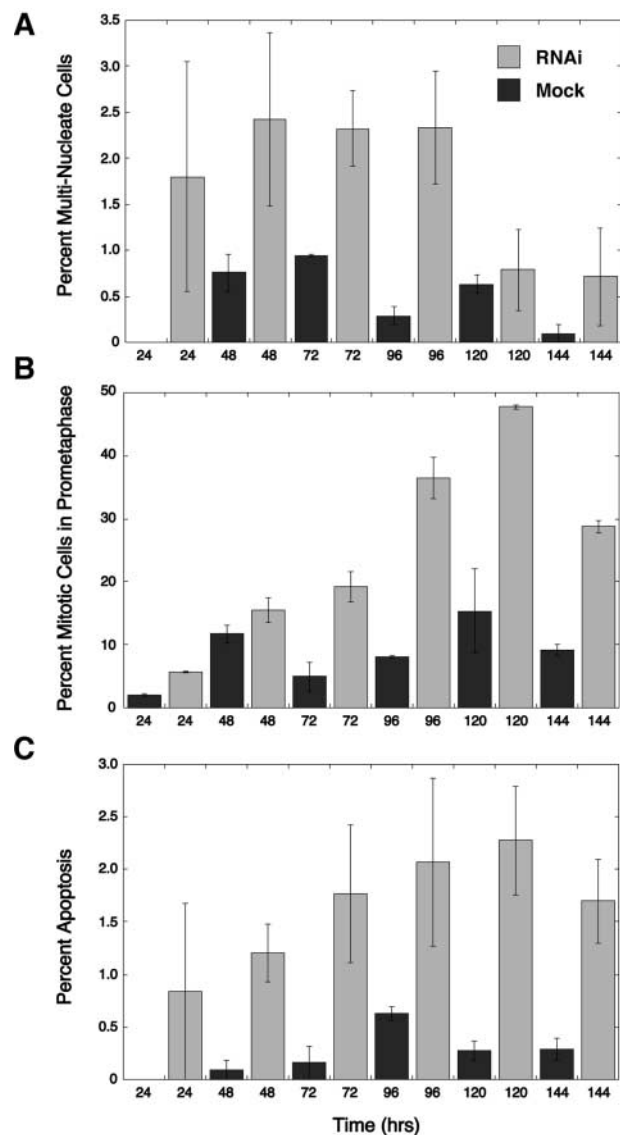

Figure 1.   **Mitotic defects associated with Nup358 siRNA treatment of HeLa cells.** Nup358 siRNA treatment of HeLa cells leads to the appearance of cells containing multiple micronuclei over a period of 24–96 h (A) as well as the accumulation of cells arrested in prometaphase (B). The latter reach a peak at 120 h, at which time they account for almost 50% of the total mitotic population (B). A gradual increase in apoptosis, peaking at 120 h, is also observed (C). This increase in apoptosis may account for the decline in the number of multinucleate cells observed after 96 h of Nup358 siRNA treatment. In each graph, siRNA-treated populations are represented by light gray bars. Dark gray bars represent control populations.

Nup153 (unpublished data) or when ineffective RNA oligonucleotides are used. As there is a progressive increase in apoptosis leading to a decline in the number of cells containing micronuclei after ~120 h of Nup358 siRNA treatment (Fig. 1 C), the majority of our subsequent experiments were performed on cells exposed to Nup358 siRNA for a maximum of 96 h.

Immunoblot analysis of HeLa cultures subjected to Nup358 siRNA treatment for a period of 4 d reveals a decline in the level of Nup358 of ~50% when compared with mock-treated cells (Fig. 2, A and B). As an independent
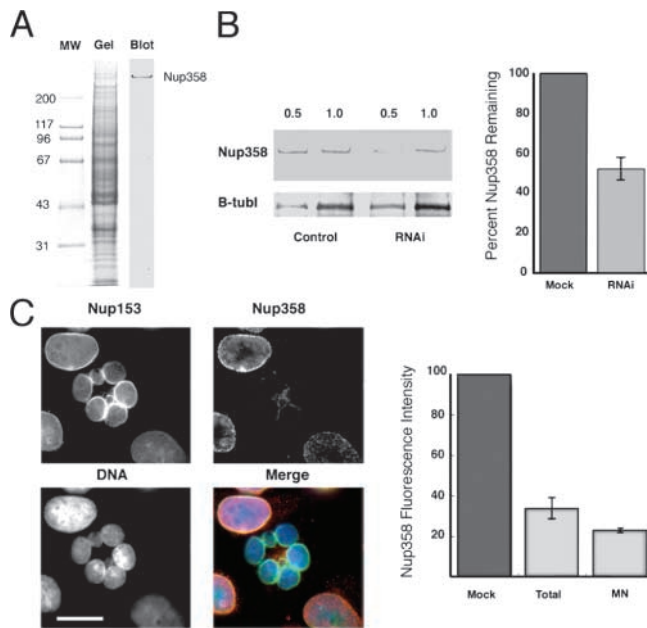
Figure 2.   **Cells containing multiple micronuclei are depleted of Nup358.** A single high molecular weight band is recognized on a Western blot of a HeLa whole cell extract by the anti-Nup358 antibodies that are employed in this study (A). Total protein content of the cell extract is shown in the Coomassie blue–stained lane (gel). Molecular weight markers (MW, kD) are indicated on the left. A 50% reduction in total Nup358 levels in Nup358 siRNA–treated (RNAi) versus control cultures (Mock) is shown by quantitative Western blot analysis (B). For this experiment, twofold dilution series of HeLa cell extracts were analyzed by Western blot using antibodies against Nup358 (A) as well as β-tubulin as an internal standard. Two sample pairs (differing in concentration by a factor of two) from both control and siRNA-treated cultures are shown. Changes in Nup358 levels were determined from the ratios of the Nup358/β-tubulin band intensities. These experiments were performed in quadruplicate over a 16-fold sample concentration range. Immunofluorescence microscopy of Nup358 siRNA–treated cultures reveals a loss of NE-associated Nup358 (C). This is particularly evident in cells containing multiple micronuclei. A second nucleoporin, Nup153, shows no such decline. Fluorescence intensity measurements performed on randomly selected interphase cells (Total) indicate a 66% reduction in Nup358 levels (±5%; $P < 0.001$) in siRNA-treated versus control (Mock) cells. A larger average reduction in anti-Nup358 fluorescence intensity of 77% (±1%; $P < 0.001$) is observed when measurements are restricted to cells containing multiple micronuclei (MN). Bar, 10 μm.

means of verification of these immunoblot results, we performed fluorescence intensity measurements on cells labeled with affinity-purified anti-Nup358 antibodies. In both siRNA-treated and control cultures, measurements were performed on all cells in multiple randomly selected fields. These measurements revealed an average decline in Nup358-associated fluorescence of ∼60%, a value that is consistent with the immunoblot data (Fig. 2 C). At the single cell level, fluorescence intensity reductions of 80% or more could be observed. Cells containing multiple micronuclei exhibited an average reduction in anti-Nup358 fluorescence intensity of ∼77% when compared with mock-treated cells (Fig. 2 C).

Loss of Nup358 had little, if any, effect on NPC number, as estimated in immunofluorescence experiments using a va-

riety of antinucleoporin antibodies. Nup153 (Fig. 2 C and Fig. 3 A) and other FG-repeat nucleoporins, including the NPC membrane protein POM121, remained NE associated and retained their usual punctate distribution (unpublished data). Puncta per unit NE surface remained essentially unchanged after siRNA treatment (unpublished data). Distribution of another nucleoporin, Nup214, which resides on the cytoplasmic face of the NPC, appears largely unaffected in cells, including multinucleate cells, depleted of Nup358 (Fig. 2). Similar results were obtained for Nup98, a mobile nucleoporin that, like Nup153, is localized to the nuclear face of the NPC (unpublished data). EM analysis of multinucleate cells from siRNA-treated cultures (such cells are always depleted of Nup358; Fig. 2 C and Fig. 3 D) revealed numerous NPCs (Fig. 3 B). However, the presence or absence of NPC-associated cytoplasmic filaments could not be reliably ascertained in this type of thin section analysis. Taken together, these data indicate that NPCs remain substantially intact after Nup358 depletion in vivo.

Nup358-depleted nuclei, including micronuclei, retained the capacity to import both A-type lamins (Fig. 3 D) and an inducible (by dexamethasone) nuclear transport substrate, a glucocorticoid receptor–β-galactosidase fusion protein (GRβ; Fig. 4, A and B). In the case of the latter, import was followed over time in both mock-treated cells and in cells depleted of Nup358. For this experiment, cells were double labeled with antibodies against both Nup358 and β-galactosidase. In this way, cells specifically depleted of Nup358 could be scored for nuclear accumulation of GRβ. As shown in Fig. 4 B, Nup358 depletion had little or no effect on the nuclear uptake of GRβ. In addition, there were no obvious changes in the gross distribution of the import receptor importin/karyopherin-β (Fig. 4 C) in mock-treated versus Nup358-depleted multinucleate cells. These observations are consistent with recent findings that nuclei assembled in vitro in the absence of Nup358 contain functional NPCs and are import competent (Walther et al., 2002).

The accumulation of "prometaphase" cells after 96 h of siRNA treatment is associated with abnormal or ineffective congression of chromosomes at the metaphase plate. Although centrosome separation appears to occur on schedule, some perturbation of spindle morphology is evident. Often, spindles associated with Nup358 siRNA treatment appeared elongated or somewhat irregular. On average, pole-to-pole distance was increased by ∼16% in Nup358 siRNA–treated versus control cells. The chromosome congression defect, observed in the majority of prometaphase cells at the 96-h time point, is manifest as a trimodal distribution of chromosomes with irregular, largely nonaligned clusters at the spindle equator as well as smaller clusters over either pole (Fig. 4 A). These cells showed a mean reduction in anti-Nup358–associated fluorescence intensity of ∼60% (Fig. 4 B). This very unusual chromosome distribution is rarely observed in untreated cultures or in cultures depleted of another nucleoporin, Nup153 (unpublished data). However, such a phenotype is observed after depletion of several spindle-, kinetochore-, and centromere-associated proteins, including *Drosophila* MAST/Orbit, CENP-A, CENP-E, and hMis12 (Yao et al., 2000; McEwen et al., 2001; Maiato et al., 2002; Putkey et al., 2002; Goshima et al., 2003).
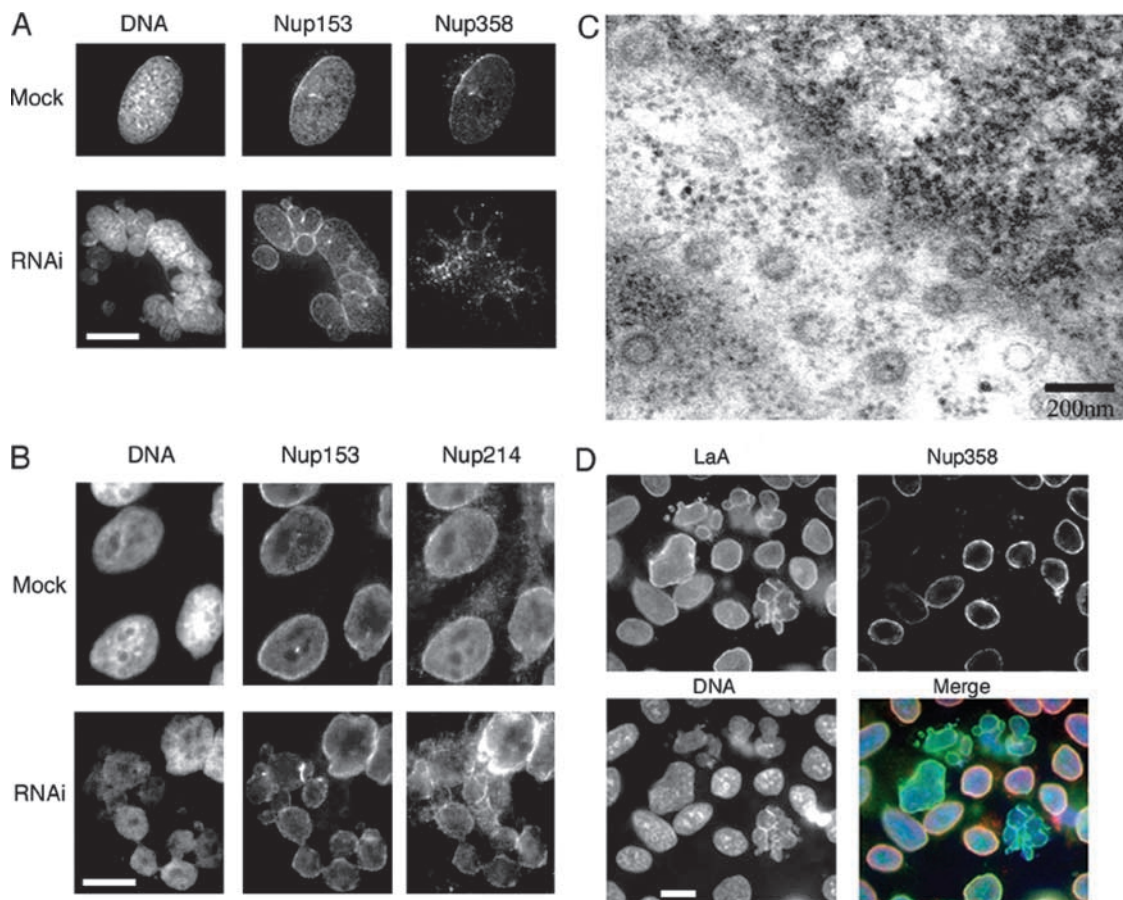
Figure 3. **Cells depleted of Nup358 contain identifiable NPCs.** Indirect immunofluorescence microscopy reveals little or no change in the levels or localizations of multiple nucleoporins, including Nup153 and Nup214, in multinucleate cells depleted of Nup358 (A and B). EM reveals the presence of abundant NPCs associated with micronuclei in cells subjected to Nup358 siRNA treatment (C). Immunofluorescence microscopy reveals that such cells are always depleted of Nup358 (D). Cells depleted of Nup358, including multinucleate cells, continue to incorporate A-type lamins (D) into the nuclear lamina over a period of 96 h. Bars: (A, B, and D) 10 μm; (C) 200 nm.

Until at least the fourth day of Nup358 siRNA treatment, some members of the unusual prometaphase population display the ability to escape mitotic arrest. In these cells, an NE reforms around individual chromosomes and groups of chromosomes, giving rise to the multiple micronuclei described above. These cells invariably show reduced labeling with anti-Nup358 antibodies (Fig. 2 C and Fig. 3 D). Surprisingly, many of these cells form an intracellular bridge and undergo cytokinesis (Fig. 5 C). Indeed at the 96-h time point, ∼34% of "telophase" or early G1 cells (defined by the presence of an intracellular bridge) were found to contain multiple micronuclei. Few such cells were observed in corresponding mock-treated populations. The ultimate fate of these unusual cells seems to be death, because, as pointed out above, the frequency of apoptosis increases steadily up to 5 d after siRNA treatment. After this time point, the occurrence of cells containing multiple micronuclei generally declines (Fig. 1 B). Remarkably, a virtually identical effect, including micronuclear formation and aberrant cytokinesis, has recently been reported in cells depleted of CENP-A and hMis12, a human kinetochore protein (Goshima et al., 2003).

Further analyses of arrested cells after 96 h of siRNA treatment suggest that congression failure is due, at least partially, to defects at the kinetochore. A role for Nup358 in kineto-chore function is suggested by work from Joseph et al. (2002), who have shown that a population of Nup358 is localized at kinetochores during mitosis. We have been able to confirm this observation, as well as show that Nup358 is associated, at least in part, with the outer portion of the kinetochore (Fig. 6). This localization was concluded from double label experiments using anti-Nup358 in combination with either an anticentromere human autoimmune serum (ACA; Fig. 6 A) or an antibody against CENP-F, a protein of the kinetochore fibrous corona (Fig. 6 B). Anti-Nup358 kinetochore labeling is clearly reduced in siRNA-treated cells (Fig. 6 C). We therefore examined the distribution, in arrested cells, of a number of components that normally associate transiently with mitotic kinetochores. These include dynein, CENP-E, CENP-F, the mitotic checkpoint proteins Mad1 and Mad2, and Zw10. None of these proteins show their normal mitotic distribution. Instead, they exhibit aberrant kinetochore targeting and many are partially or completely mislocalized to the cytoplasm (Fig. 7, A and B). Fluorescence intensity measurements indicate a reduction in kinetochore-associated CENP-E, CENP-F, Mad-1, and dynein of ∼60% using ACA labeling as an internal reference (Fig. 7 B). When these transient proteins do associate with the kinetochore in the Nup358-depleted prometaphase cells, they are almost in-
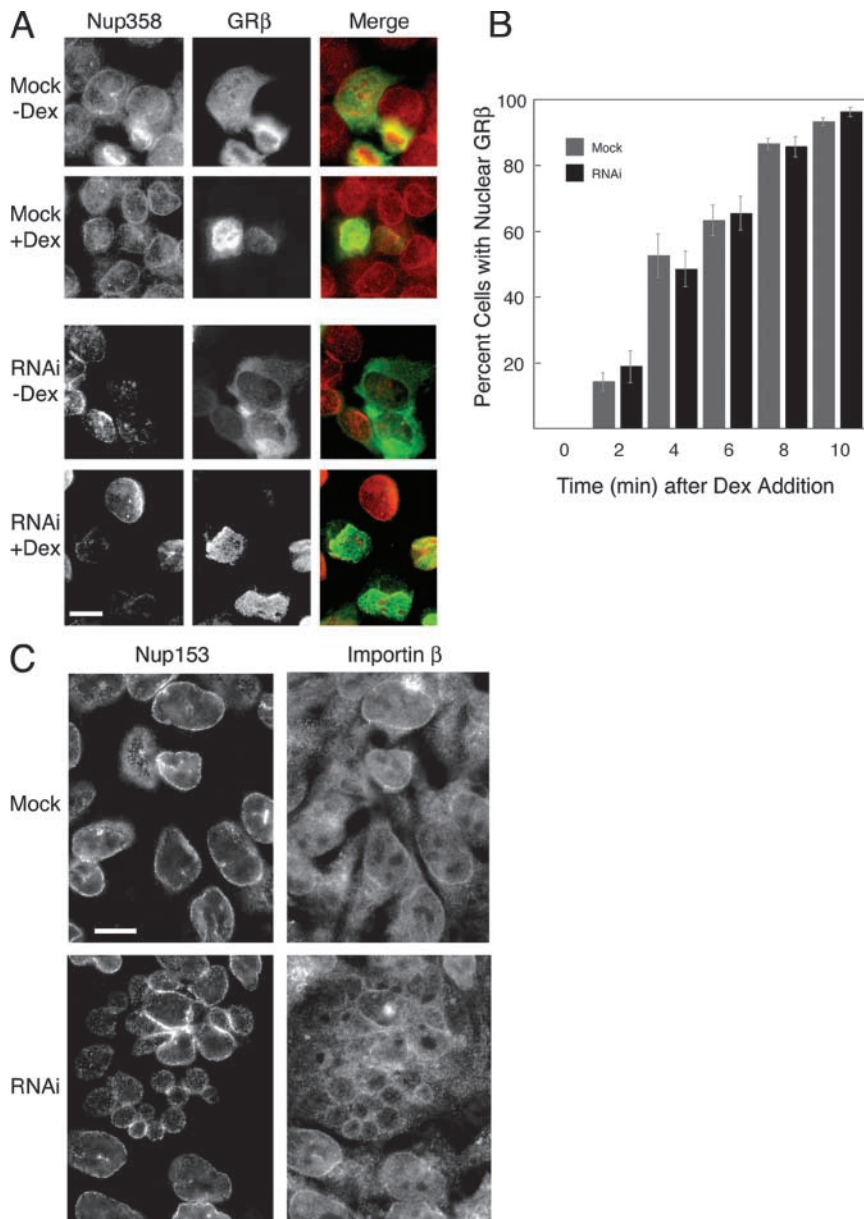
Figure 4. **Nuclei in cells depleted of Nup358 are import competent.** Nuclear import of GRβ is unaffected in Nup358 siRNA–treated (RNAi) versus mock-treated cells. In the absence of dexamethasone (−Dex), GRβ remains almost exclusively cytoplasmic (A). After exposure to dexamethasone for 10 min (+Dex), GRβ relocates to the nucleus (or micronuclei). To establish a time course of GRβ import (B), cells were scored for nuclear accumulation of GRβ. This was defined as average nuclear fluorescence intensity greater than average cytoplasmic intensity. The graph (B) represents the mean of four experiments in which 200 cells were scored at each time point. For the RNAi samples, only cells clearly depleted of Nup358 were included in the counts. The overall distribution of the nuclear import receptor importin-β is also largely unaffected by depletion of Nup358 (C). Compare the large multinucleate cell in the RNAi panels with the mock-treated cells. Bars, 10 μm.

variably associated with chromosome clusters at the spindle poles and not with the clusters near the spindle equator (Fig. 7 A). Cell cycle invariant kinetochore components, including those that are recognized by the ACA serum, do show an appropriate localization (Fig. 7 A) and are found on both polar and equatorial clusters. The implication of these observations is that Nup358 is required for the normal assembly of proteins at the kinetochore and hence for kinetochore function. This view is reinforced and expanded by ultrastructural analysis of kinetochores in prometaphase cells that accumulate during 4 d of siRNA treatment.

In contrast to the normal plate-like trilaminar kinetochore structure that is observed in control or mock-treated cultures (Fig. 7 C, a), siRNA-treated cells exhibit a variety of very unusual kinetochore morphologies (Fig. 7 C, b–d). In particular, the kinetochores in these cells may form a plate that is everted into a "C" shape (in thin section) and that fails to attain a compact trilaminar structure (Fig. 7 C, d). Alterna-

tively the plate may be absent entirely so that the kinetochore appears as a diffuse mass of material similar to that described for prekinetochores in untreated cells (Fig. 7 C, b and c). Both of these kinetochore morphologies are often associated with centromeric hetrochromatin that shows anomalous condensation. It seems likely that these two morphologies result from the partial or complete disruption of normal kinetochore formation. Thus far, we have been unable to make any correlation between the appearance of these aberrant kinetochore morphologies and chromosome location within the cell. Few microtubules impinge upon the aberrant kinetochores, indicating that they are defective with respect to microtubule capture (Fig. 7 C, b–d). This loss of kinetochore organization can account for the trimodal distribution of chromosomes that is characteristic of Nup358 siRNA–treated prometaphase cells. The majority of chromosomes, containing defective kinetochores, remains clustered and nonaligned (Fig. 6 C) at the cell center flanked by a pair of
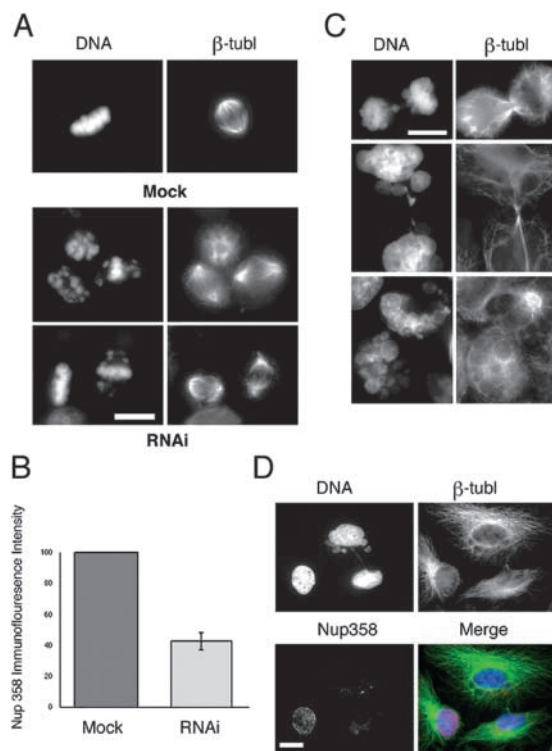
Figure 5. **Abnormal chromosome congression and defective mitosis after Nup358 siRNA treatment.** Indirect immunofluorescence microscopy of Nup358 siRNA–treated (RNAi) versus mock-treated HeLa cells. Prometaphase and metaphase cells labeled with anti–β-tubulin and Hoechst dye to reveal the condensed chromosomes (A). Up to 75% of premetaphase cells in siRNA-treated cultures exhibit abnormal or elongated spindle morphology associated with abnormal chromosome congression (A) featuring irregular chromosome clusters at the spindle equator as well as smaller clusters over each pole. This unusual morphology is rarely observed in control cells. Fluorescence intensity measurements on prometaphase cells labeled with an anti-Nup358 antibody (B) reveal an average reduction of 61% when compared with mock-treated cells (±6%; P < 0.001). Telophase and early G1 siRNA-treated cells exhibit a high frequency of multiple micronuclei and abnormal cytokinesis (C) in which lagging chromosomes and chromatin bridges connecting the daughters are observed (D).

spindle poles. Only the few chromosomes that do assemble kinetochores, and are capable of capturing microtubules, are able to migrate to either pole. These gross aberrations in kinetochore morphology and function clearly indicate that Nup358 plays an essential role in early mitotic progression.

A final issue that arises is whether Nup358 itself might have a role as a spindle assembly checkpoint protein. To address this, we examined the effects of simultaneous depletion of Nup358 and the bona fide checkpoint protein Mad1 (Fig. 8). Depletion of Mad1 has previously been shown to result in premature anaphase and the appearance of lagging chromosomes (Luo et al., 2002; Martin-Lluesma et al., 2002). The prediction is that if the spindle assembly checkpoint remains functional in Nup358-depleted cells, then loss of Mad1 should result in a decline in the number of prometaphase/metaphase cells. At the same time, given the Nup358-associated congression defect, there should be an increase in the number of cells containing multiple micronuclei. As shown in Fig. 8, this is precisely what occurs. In cultures depleted

only of Nup358, there is a 70% increase in the number metaphase and premetaphase cells over mock-treated cultures. It must be emphasized that this figure represents a minimum value, given the heterogeneity of the Nup358 siRNA–treated cells. If we had only counted cells overtly depleted of Nup358, this increase would be on the order of 150–300%. Simultaneous depletion of both Nup358 and Mad1 yielded a sevenfold decline in the number of metaphase and premetaphase cells (compared with Nup358 depletion alone). Taken together, these results indicate that Nup358 is unlikely to possess a checkpoint function.

Discussion

Nup358, also known as Ran binding protein 2 (RanBP2), is a very large protein that is a core component of the 100-nm filaments that extend from the cytoplasmic face of NPCs (Yokoyama et al., 1995; Walther et al., 2002). The Nup358 molecule contains numerous FG repeats that have been shown to form binding sites for transport receptors of the importin/karyopherin-β family (Delphin et al., 1997). Early studies on the mechanisms of signal-mediated nuclear protein import revealed that import substrates bearing NLSs initially docked at the cytoplasmic filaments before translocation across the NPC (Richardson et al., 1988; Panté and Aebi, 1996). Taken together, these results imply that Nup358 represents an early docking site for NLS-bearing proteins associated with their cognate receptors. Nup358 also binds RanGAP1, the activating protein for Ran, a small Ras-related GTPase that is an important regulator of nucleocytoplasmic transport (Saitoh et al., 1997). Only RanGAP1 molecules that have been modified with the small ubiquitin-like protein SUMO associate with Nup358 at the nuclear periphery (Mahajan et al., 1997; Matunis et al., 1998). Intriguingly, Nup358 itself has SUMO E3 ligase activity (Pichler et al., 2002). In this way, Nup358 might act not just as a docking protein but also as a regulator, albeit indirect, of the cytoplasmic levels of Ran-GDP versus Ran-GTP. It therefore comes as some surprise that Nup358 is apparently dispensable with respect to nucleocytoplasmic transport. Nuclei assembled in vitro in *Xenopus* egg extracts depleted of Nup358 are transport competent, although the NPCs within these nuclei lack cytoplasmic filaments (Walther et al., 2002). Whether the kinetics of import in vitro are affected by elimination of Nup358 is not clear. However, these results are consistent with our own findings that depletion, albeit incomplete, of Nup358 in HeLa cells has no profound affect on the uptake of proteins into the nucleus.

Although we observed little effect on nuclear protein import in Nup358 siRNA–treated HeLa cells, the effects on mitotic progression were quite dramatic. We noted the emergence of two unusual cell populations: prometaphase cells in which there was a failure in chromosome congression and interphase cells containing multiple micronuclei. The most reasonable explanation for the appearance of these cell populations is a failure of spindle microtubules to capture chromosomes followed eventually by mitotic exit and NE reformation around dispersed chromosomes or groups of chromosomes. In this way, the defective prometaphase cells would represent the precursors of the multinucleate cells.
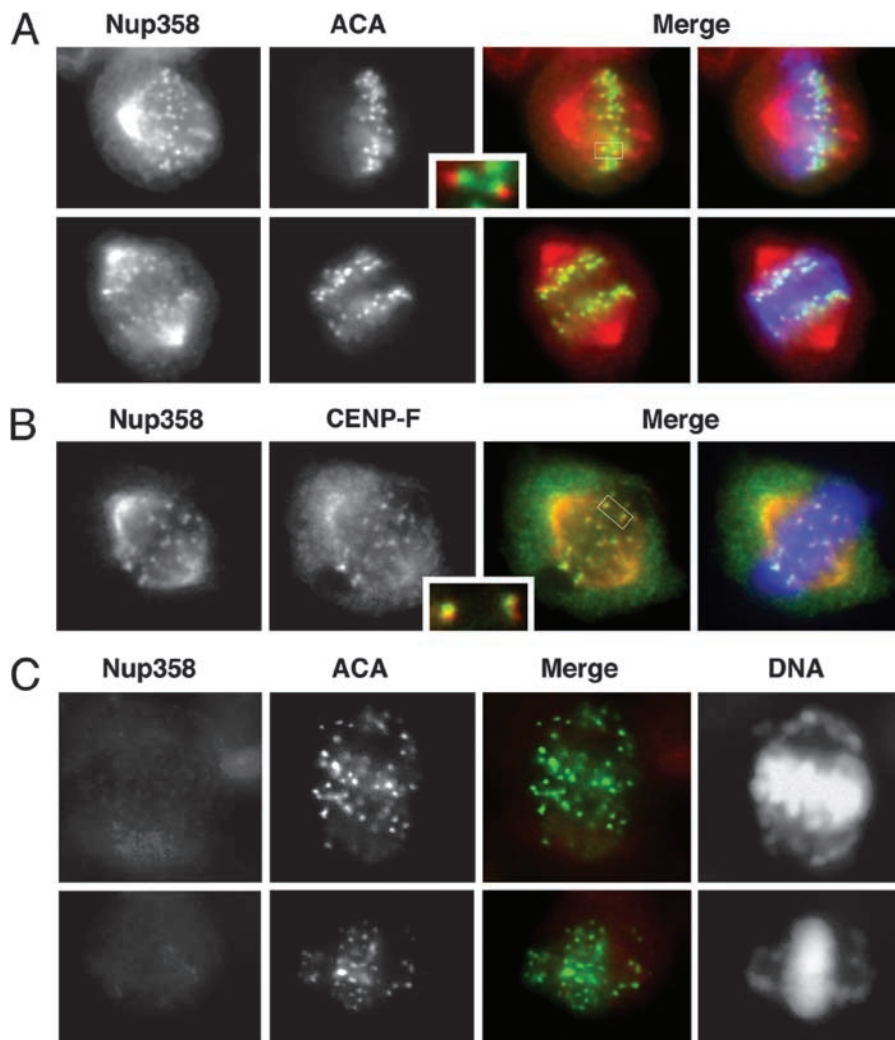
Figure 6.   **Loss of kinetochore-associated Nup358 after siRNA treatment.** Double indirect immunofluorescence microscopy of mitotic HeLa cells using antibodies against Nup358 in combination either with a human anticentromere auto-immune serum (ACA) or an antibody against CENP-F. Cells were also labeled with Hoechst dye (shown in blue in A and B) to reveal the mitotic chromosomes. Untreated HeLa cells are shown in A and B. The insets reveal that Nup358 lies distal to the centromere and instead largely colocalizes with CENP-F, an outer kinetochore protein. Nup358 siRNA–treated cells are shown in C and reveal a loss of both kinetochore and spindle-associated Nup358, although ACA labeling is unaffected. Chromosome congression is clearly abnormal in these cells. In A and B, merged images are shown both with (right) and without (left) superimposition of the chromosomes. In C, the merged images show only the antibody labeling. In each set, Nup358 labeling is shown in red.

That this is indeed the case is suggested by the increasing numbers of multinucleate telophase and early G1 cells that can be seen in the siRNA-treated cultures. In many anaphase and telophase cells in 4-d siRNA-treated cultures, the presence of lagging chromosomes and chromatin strands spanning the intracellular bridge is yet further indication of congression failure and eventual mitotic exit.

The findings that Nup358 is associated with both spindle microtubules and kinetochores in mitotic cells are consistent with a number of recent studies that have revealed a direct role for components of the Ran system in mitotic spindle assembly. Members of the importin/karyopherin family are also implicated in these processes. A role for Nup358 in chromatid segregation has been highlighted in a recent study on *Caenorhabditis elegans* early embryos (Askjaer et al., 2002). An RNA interference approach has revealed that depletion of Nup358/RanBP2 leads to inhibition of chromosome congression associated with aberrant spindle morphology, very similar to the situation described here in HeLa cells. Although asters do form, chromosome capture does not occur and bipolar spindles are not seen. Identical effects have also been observed in *C. elegans* embryos that have been depleted of CENP-A, a protein required for normal kinetochore formation (Oegema et al., 2001). These observations confirm a

role for kinetochores in spindle organization and indicate that Nup358 depletion could be interfering with either kinetochore or spindle microtubule function, or indeed both. Clearly, defects in either of these structures could in principle give rise to the types of aberrations in chromatid segregation that both we and Askjaer et al. (2002) have observed.

Taken together, our data suggest that the primary effects of Nup358 depletion on spindle assembly and function are operating at the level of kinetochore formation and maturation. EM studies of Nup358-depleted prometaphase cells reveal aberrant kinetochore morphology that features partial or complete loss of the trilaminar plate structure as well as incomplete condensation of subjacent centromeric heterochromatin. The C-shaped kinetochore morphology has also been reported after premature chromatin condensation in cell fusion experiments (Rattner and Wang, 1992), as well as after exposure to caffeine (Brinkley et al., 1988), whereas the expanded morphology is characteristic of prekinetochores (He and Brinkley, 1996). Of particular significance is our finding that certain kinetochore components, including the checkpoint proteins Mad1, Mad2, and Zw10, are mislocalized in prometaphase cells depleted of Nup358. Studies by Chan et al. (2000) using an antibody microinjection strategy have demonstrated quite convincingly that interference with
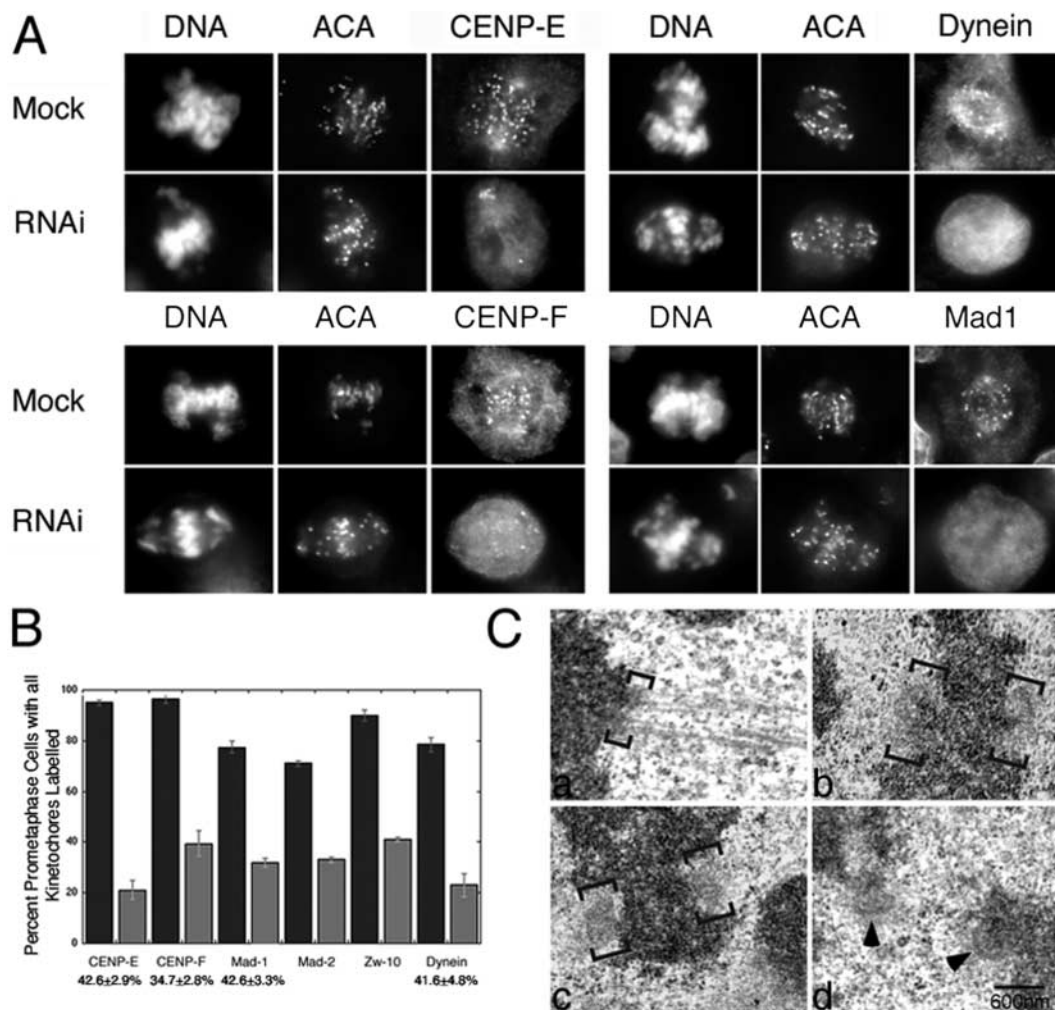
Figure 7. **Nup358 siRNAi treatment causes changes in both kinetochore composition and morphology in prometaphase cells.** Double indirect immunofluorescence microscopy of mitotic HeLa cells reveals that multiple proteins are lost from kinetochores in Nup358 siRNA–versus mock-treated cells (A). These include CENP-E, CENP-F, dynein, and Mad1 (A) as well as Mad2 and Zw10 (B; siRNA-treated cell populations are represented by light gray bars, whereas dark gray indicates control populations). Occasional kinetochore labeling is observed with antibodies against these various components (A). This is invariably associated with polar chromosome clusters and not with the larger equatorial clusters. This is particularly evident in the CENP-E and CENP-F panels (A). Electron micrographs (C) of the kinetochores of control (a) versus siRNA-treated cells (b–d). The square brackets indicate a normal kinetochore with associated microtubules in panel a and expanded, diffuse kinetochores in b and c. C-shaped everted kinetochores are also a feature of Nup358 siRNA treatment (d, arrowheads). Bar, 600 nm.

one of these, Zw10, leads to bypass of the spindle assembly checkpoint, appearance of lagging chromatids, and aneuploidy (Chan et al., 2000). More recently, Yao et al. (2000) have shown that depletion of CENP-E in mammalian cells gives rise to a spectrum of anomalies that is virtually identical to what we have reported in this paper. This is consistent with our own finding that CENP-E is mislocalized in Nup358-depleted cells. Very similar defects have also been reported in studies involving depletion of several other kinetochore proteins, including Hec1, hMis12, and *Drosophila* Mast/Orbit (Maiato et al., 2002; Martin-Lluesma et al., 2002; Goshima et al., 2003).

The finding that depletion of Nup358 perturbs kinetochore structure and interferes with microtubule binding suggests that Nup358 has an important function in the assembly of the kinetochore. Such a role for Nup358 might be related to its ability to attract and bind other proteins of the Ran system. Indeed, RanGap1 and SUMO-I have both

been found at the kinetochore (Joseph et al., 2002). Given that SUMO-modified RanGAP1 binds Nup358 at the NPC, it is tempting to imagine that Nup358 performs a similar function at the kinetochore during mitosis. In fact, a population of Ran is found at the kinetochore during mitosis while its nucleotide exchange factor, RCC1, remains chromatin associated (Moore et al., 2002). In *C. elegans*, depletion of RCC1 by RNA interference produces effects similar, although less severe, to those observed after Nup358 depletion (Askjaer et al., 2002). As Ran has been shown to be essential for kinetochore–microtubule interaction, these various Ran system components could well function to modulate the cycling of proteins on and off the kinetochore. Interference with one branch of the Ran system might then result in the structural and compositional defects detected in our study. Such a view is lent considerable support by the recent findings of Arnaoutov and Dasso (2003) on the critical role of the Ran GTPase in
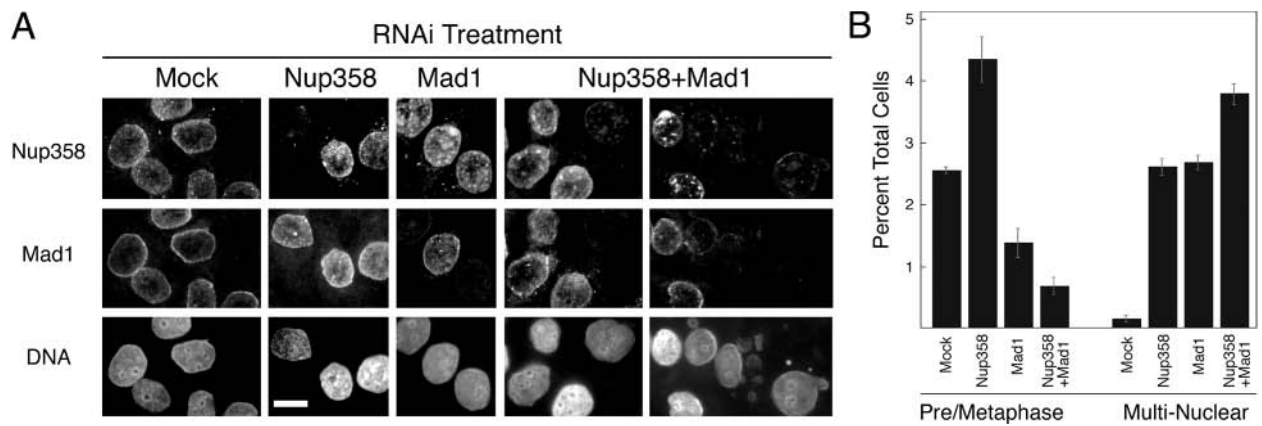
Figure 8.   **Depletion of Mad1 reverses the Nup358 siRNA–induced prometaphase arrest.** Double indirect immunofluorescence microscopy of HeLa cell cultures treated with Nup358 and/or Mad1 siRNAs and labeled with antibodies against both Nup358 and Mad1 (A). In interphase cells, Mad1, like Nup358, localizes almost exclusively to the nuclear periphery. Depletion of either protein in interphase cells has little or no effect on the distribution of the other. During mitosis, Nup358 depletion results in a significant (70%) increase in the number of premetaphase and metaphase cells over mock-treated cultures (B). This effect is reversed by codepletion of Mad1. Conversely, depletion of both proteins causes an increase in the number of multinucleate cells. 2–4,000 cells were scored in each category in four independent experiments. It should be noted that these counts represent percentages derived from total cell populations. To eliminate bias, no attempt was made to discriminate between transfected versus nontransfected cells. Bar, 10 μm.

kinetochore function. A second possibility is that since Nup358 is a SUMO ligase (Pichler et al., 2002), this activity might be required for proper kinetochore organization and function. In this regard, it is intriguing that SUMO-1 can act as a suppressor of certain CENP-C mutations in vertebrate cells (Fukagawa et al., 2001).

Our findings clearly indicate that Nup358 has an important role in the recruitment of kinetochore proteins, including those involved in the spindle assembly checkpoint. However, Nup358 itself appears unlikely to be a checkpoint protein per se. Rather, our data suggest that the spindle assembly checkpoint remains substantially intact in cells depleted of Nup358, and that such cells display only a relatively slow escape from the mitotic arrest. This escape could, however, be accelerated by codepletion of Mad1. Conversely, nocodazole treatment of both mock- and Nup358-depleted cells yielded little difference in the numbers of cells arrested in prometaphase (unpublished data). Given the substantial, albeit incomplete, mislocalization of kinetochore-associated proteins in cells depleted of Nup358, these results involving Mad1 codepletion are consistent with the notion that checkpoint complexes may remain functional at other cytoplasmic sites.

Could Nup358 depletion be having an indirect effect on kinetochore function? We know that Nup358 is part of the nucleocytoplasmic transport machinery. It is formally possible, therefore, that the mitotic defects we have observed could be a consequence of failure to import crucial kinetochore components into the nucleus in late G2. This scenario, however, seems very unlikely. All the evidence that we and others have available suggests that nuclear protein import is not seriously perturbed by Nup358 depletion (Walther et al., 2002). Indeed, if it were, we would expect cells to arrest in interphase and not to enter mitosis. Given its localization during mitosis, the most reasonable model remains that Nup358 is actually functional at the kinetochore.

Why should NPC or NE components play any role at all during mitosis? Joseph et al. (2002) have made the interesting proposal that the reciprocal relationship between the NPC and the mitotic spindle, represented by the cycling of proteins between these two structures, provides a fail-safe signal that defines the interphase versus mitotic status of the cell. Our data on Nup358 would suggest that this idea can also be extended to the kinetochore, which can only become functional once NEBD, disassembly of NPCs, and transfer of some NPC components to the kinetochores has commenced. In this way, an orderly and stepwise progression of mitotic events is ensured. This relationship between the NPC (or at least the NE) and the kinetochore may have its roots in the evolutionary history of these two structures. Many primitive cell types that undergo a closed mitosis, dinoflagellates for example, have well-differentiated kinetochores that remain closely associated with the nuclear surface of the NE (Kubai, 1975; Ris, 1975). In *Trichonympha agilis*, spindle microtubules, which are exclusively cytoplasmic, make contact not with the kinetochore itself, but with the patch of nuclear membrane that overlies the kinetochore (Kubai, 1975). In this way, chromosome segregation, although driven by the mitotic spindle, is actually mediated by NE components. It is possible that this mechanism has been conserved in organisms that have evolved an open mitosis such that disassembled NE components still maintain their ancient role.

Materials and methods

Cell culture
HeLa cells were maintained at 7.5% CO_2 and 37°C in DME (GIBCO BRL) plus 10% FBS (Hyclone), 10% penicillin/streptomycin (GIBCO BRL), and 2 mM glutamine.

Antibodies
The following antibodies were used in this study. Antibodies against LAP 2β and Nup358 were obtained from L. Gerace (Scripps Research Institute,

La Jolla, CA). An additional antibody against Nup358 was also obtained from T. Nishimoto (Kyushu University, Fukuoka-shi, Japan). Anti–CENP-E, -Zw10, and -Mad1/2 antibodies were obtained from G. Chan (University of Alberta, Alberta, Canada). Antibodies against Mad1 were also provided by T. Yen (Fox Chase Cancer Center, Philadelphia, PA). The antibody against importin β was obtained from D. Gorlich (ZMBH, Heidelberg, Germany). Antibodies against various nucleoporins (QE5), including Nup153 (SA1) and Nup214, have been described elsewhere (Panté et al., 1994; Bodoor et al., 1999). The antibody against CENP-F as well as the human autoimmune anticentromere antibody (ACA) have also been described previously (Kingwell and Rattner, 1987; Liao et al., 1995). The anti–β-tubulin antibody was obtained from Sigma-Aldrich. The monoclonal antibody 74.1 against dynein intermediate chain was obtained from BabCo. Anti–β-galactocidase antibody was obtained from Promega. Secondary antibodies were from Biosource International.

Immunofluorescence microscopy
HeLa cells were grown on glass coverslips and fixed in either 100% methanol at −20°C or 3% paraformaldehyde for 10 min followed by a 5-min permeabilization with 0.5% Triton X-100. The cells were then labeled with the appropriate antibodies plus the DNA-specific Hoechst dye 33258. For the experiment in Fig. 6, the cells were preextracted with 0.005% digitonin and later fixed in −20°C methanol exactly as previously described (Joseph et al., 2002). Specimens were observed using a Carl Zeiss MicroImaging, Inc. Axiophot microscope. Images were collected using a Photometrics CoolSnap HQ CCD camera linked to an Apple Macintosh G4 computer running IP Lab Spectrum software (Spectrum Analytics, Inc.). Fluorescence intensity measurements were performed using IP Lab (Salina et al., 2002).

EM
Cells grown and treated in 35-mm Petri dishes were fixed in 3% glutaraldehyde and 0.2% tannic acid in 200 mM sodium cacodylate buffer for 1 h 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 performed at 60°C for 24 h. 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 JEOL JEM-100CXII electron microscope.

In vivo nuclear import assay
To examine the effects of Nup358 depletion on nuclear protein import, HeLa cells, grown on glass coverslips, were exposed to Nup358 siRNA for 3 d. At this time, the cells were transfected with an expression plasmid encoding GRβ (Picard and Yamamoto, 1987). Transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. 24 h after transfection, dexamethasone was added to the medium to a final concentration of 10 μg/ml. The cells were returned to the incubator for a period of up to 30 min and then fixed with 3% formaldehyde in PBS. Finally, The cells were processed for immunofluorescence microscopy using antibodies against both β-galactosidase and Nup358.

siRNA methods
HeLa cells were depleted of Nup358 using siRNA corresponding to nucleotides 7632–7654 of human Nup358 (Dharmacon). The cells were exposed to the Nup358 siRNA in the presence of Oligofectamine (Invitrogen) precisely as described by Harborth et al. (2001). As a control, cells were exposed either to Oligofectamine alone, to Nup153 siRNA, or to an ineffective RNA duplex. For Mad1 depletion, the procedures and oligonucleotide sequences described by Martin-Lluesma et al. (2002) were followed precisely. In codepletion experiments, cells were first exposed to Nup358 siRNA. After 48 h, a combination of both Mad1 and Nup358 siRNAs was introduced to the cells. After an additional 48 h incubation, cells were fixed and processed for immunofluorescence microscopy.

Immunoblotting and gel electrophoresis
Cells (siRNA or mock treated) grown in 35-mm tissue culture dishes were washed once in PBS and then lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1:1,000 CLAP (10 mg/ml in DMSO of each of the following: chymostatin, leupeptin, antipain, and pepstatin). The lysate was centrifuged for 5 min in an Eppendorf centrifuge at 4°C. Proteins in the supernatant were precipitated by the addition of TCA to a final concentration of 10%. The precipitate was washed with ethanol/ether and then solubilized in sodium dodecyl sulfate–PAGE sample buffer. Protein samples were fractionated on

8% polyacrylamide gels and then transferred onto nitrocellulose filters, usually BA85 from Schleicher & Schuell (Burnette, 1981), using a semi-dry blotting apparatus manufactured by Hoeffer Scientific Instruments, Inc. Filters were blocked, labeled with primary antibodies, and then developed with peroxidase-conjugated secondary antibodies exactly as previously described (Burke et al., 1982).

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