Dynamic behavior of Nuf2-Hec1 complex that localizes to the centrosome and centromere and is essential for mitotic progression in vertebrate cells

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Summary

Nuf2 and Hec1 are evolutionarily conserved centromere proteins. To clarify the functions of these proteins in vertebrate cells, we characterized them in chicken DT40 cells. We generated GFP fusion constructs of Nuf2 and Hec1 to examine in detail the localization of these proteins during the cell cycle. We found that Nuf2 is associated with Hec1 throughout the cell cycle and that this complex is localized to the centrosomes during G1 and S phases and then moves through the nuclear membrane to the centromere in G2 phase. During mitosis, this complex is localized to the centromere.

We also created conditional loss-of-function mutants of Nuf2 and Hec1. In both mutants, the cell cycle arrested at prometaphase, suggesting that the Nuf2-Hec1 complex is essential for mitotic progression. The inner centromere proteins CENP-A, -C, and -H and checkpoint protein BubR1 were localized to chromosomes in the mutant cells arrested at prometaphase, but Mad2 localization was abolished. Furthermore, photobleaching experiments revealed that the Nuf2-Hec1 complex is stably associated with the centromere and that interaction of this complex with the centrosome is dynamic.

Movies available online

Key words: Nuf2, Hec1, Centrosome, Centromere, FRAP

Introduction

Mitosis is the period of the cell cycle during which chromosomes are segregated to daughter cells. The centromere is a component of the chromosome that is required for accurate segregation of chromosomes during mitosis in eukaryotes. Centromere functions include sister chromatid cohesion and separation, microtubule attachment, chromosome movement and mitotic checkpoint control (Choo, 1997; Pidoux and Allshire, 2000; Sullivan et al., 2001). Because problems in chromosome segregation can lead to aneuploidy, cancer and cell death, we must understand the mechanism by which centromeres interact with the microtubules of the spindle apparatus during cell division.

The vertebrate centromere is a trilaminar plate structure (Pluta et al., 1995; Craig et al., 1999). A number of centromere proteins have been identified in higher vertebrates. Proteins located in the inner plate, including CENP-A, -C, -H and -I, are required for formation of the kinetochore structure (Saitoh et al., 1992; Tomkiel et al., 1994; Warburton et al., 1997; Fukagawa et al., 1999a; Fukagawa et al., 2001; Howman et al., 2000; Sugata et al., 2000; Nishihashi et al., 2002). Motor proteins and spindle checkpoint proteins such as CENP-E and Mad/Bub family proteins are associated with the outer plate (Cooke et al., 1997; Jablonski et al., 1998). Genetic approaches have been taken to understand the molecular mechanism underlying centromere formation in vertebrates, and several molecular mechanisms for centromere formation in cells of higher vertebrates been reported (Howman et al., 2000; Fukagawa et al., 2001; Nishihashi et al., 2002). To further understand the mechanisms underlying centromere formation and function, it is necessary to characterize centromere components.

The Ndc80p complex in *Saccharomyces cerevisiae* has been identified as a spindle pole component (Wigge et al., 1998). Further analysis has shown that the Ndc80p complex, which contains Ndc80p, Spc24p, Spc25p and Nuf2p, is associated with the yeast centromere (Janke et al., 2001; Wigge and Kilmartin, 2001; He et al., 2001). Two of the components, Ndc80p and Nuf2p, have homologues in *S. pombe*, *C. elegans* and vertebrates (Nabetani et al., 2001; Howe et al., 2001; McCleland et al., 2003). The human homologue of Ndc80p, Hec1, was originally identified as a retinoblastoma protein (Rb)-associated protein. Microinjection of Hec1 antibodies into cultured cells disrupts mitotic progression (Chen et al., 1997). Recently, Martin-Lluesma et al. (Martin-Lluesma et al., 2002) reported that human Hec1 is required for recruitment of spindle checkpoint components, including Mps1 and Mad1/Mad2 complexes, to centromeres. It has also been reported that the human homologue of Nuf2p localizes to centromeres during M phase and is related to mitotic function

(Nabetani et al., 2001; Wigge and Kilmartin, 2001; DeLuca et al., 2002).

We hypothesized that both Hec1 and Nuf2 play important roles in mitotic progression in vertebrate cells. However, it is unclear whether these proteins form a complex in vertebrate cells or how such a complex functions in cell cycle regulation. How this complex is involved in assembly of the vertebrate centromere is also of interest. Although much of the detailed analysis of the Ndc80p complex has been in yeast, we used chicken DT40 cells because they have a high level of homologous recombination and condensed mitotic chromosomes that are easily seen under light microscopy (Fukagawa et al., 1999a; Fukagawa et al., 2001; Nishihashi et al., 2002). In addition, structures such as the mitotic spindle, centrosome, and centromere in DT40 cells are much larger and more elaborate than the analogous structures in yeast. Furthermore, the stages of mitosis (prometaphase chromosome congression, metaphase, and transition to anaphase) are easily distinguished under light microscopy in DT40 cells.

To complement previous studies in yeasts, we isolated the chicken homologues of Nuf2 and Ndc80 (Hec1) for further analysis. We found that Nuf2 binds tightly to Hec1 throughout the cell cycle and that the complex localizes to the centrosome in G1 and S phases, moves to the centromere during early G2 phase, and remains at the centromere until completion of mitosis. Furthermore, we describe the generation of conditional loss-of-function mutants of both Nuf2 and Hec1 in DT40 cells to evaluate the in vivo functions of these proteins in higher vertebrates. Phenotypic analysis of these mutants revealed that both Nuf2 and Hec1 play essential roles in the formation of functional centromeres and in the progression of mitosis. Photobleaching experiments showed that interaction of the complex with the centromere is relatively stable, whereas interaction with centrosomes is dynamic.

Materials and Methods

Molecular biology

The chicken *Nuf2* and *Hec1* cDNAs were cloned by screening a chicken testis cDNA library (Stratagene) with the RT-PCR products as a probe. We used the chicken *Nuf2* and *Hec1* cDNAs as a probe to isolate genomic clones specific for *Nuf2* and *Hec1* from a DT40 genomic DNA library.

The knockout constructs were created by a standard molecular biology technique. To generate the expression construct for the Nuf2-*GFP* and *Hec1-GFP* fusion genes, the full-length cDNAs were inserted into pEGFP-N1 plasmid (Clontech). To make the expression construct for the *Hec1-HcRed*, a full-length *Hec1* cDNA was inserted into pHcRed-N1 plasmid (Clontech). An expression construct for GFP-tubulin (pEGFP-Tub) was purchased from Clontech.

Cell culture and cell cycle analysis

DT40 cells were cultured and transfected as described previously (Fukagawa and Brown, 1997). Cell cycle analysis was carried out as described previously (Fukagawa et al., 2001). Flow-cytometry was performed with an Epics Altra cytometer (Beckman-Coulter).

Antibody production

A chicken *Nuf2* expression construct (amino acids 91-293) and a chicken *Hec1* expression construct (amino acids 465-640) were created in vector pET28a (Novagen). The histidine-tagged recombinant protein was expressed in *E. coli* BL21 (DE3) cells. Purified recombinant proteins were used to immunize a rabbit. Serums were affinity purified against recombinant proteins on a CNBr-activated Sepharose 4B column. Immunoprecipitation was performed by a standard method.

Immunocytochemistry and FISH analysis

Immunofluorescent staining of whole cells was performed as described previously (Fukagawa et al., 1999a). Anti-α-tubulin and γ-tubulin monoclonal antibody (Sigma), rabbit anti-chicken CENP-A antibody (Fukagawa et al., 2001) (Regnier et al., 2003), rabbit anti-chicken CENP-C antibody (Fukagawa et al., 1999a), rabbit anti-chicken CENP-H antibody (Fukagawa et al., 2001), rabbit anti-chicken Nuf2 antibody or rabbit anti-chicken Hec1 antibody were used.

Metaphase chromosome spreads for immunofluorescence staining were prepared by a method modified from Earnshaw et al. (Earnshaw et al., 1989). Chromosome spreads were stained with rabbit anti-chicken CENP-C antibody (Fukagawa et al., 1999a) or rabbit anti-chicken BubR1 (Nishihashi et al., 2002).

FISH was performed as described previously (Fukagawa et al., 1999b). All immunofluorescence and fluorescence in situ hybridization (FISH) images were collected with a cooled CCD camera (Photometrics Image Point) mounted on a Zeiss Axioscope microscope with a 63× objective (Zeiss) together with a filter wheel. Images were manipulated with Iplab software (Signal Analytics).

Fluorescence microscopy in living cells

Fluorescently stained living cells were observed with an Olympus inverted microscope IX70 with an oil immersion objective lens (PlanApo 60×, NA=1.40). The DeltaVision microscope system used in this study was purchased from Applied Precision, Inc. For temperature control during microscopic observations, the system was assembled in a custom-made, temperature-controlled room (Haraguchi et al., 1997; Haraguchi et al., 1999). Time-lapse images were recorded at 2 minutes intervals with an exposure time of 0.2-0.3 sesonds.

Fluorescence recovery after photobleaching (FRAP) assay

FRAP was performed using an Olympus Fluoview confocal microscope. Cells on glass-bottomed dishes were grown at 37°C on a heated stage. One sectioned area was photo-bleached four times. After 1 second, time-lapse images were collected. The average signal intensity relative to the pre-bleached image was analyzed from 20-30 live-cell sequences by measuring the net intensity of the bleached area minus background relative to that of the whole cells in each frame by using ImageJ version 1.28u (provided by W. Rasband, National Institute of Health; http://rsb.info.nih.gov./ij/).

Results

Chicken Nuf2 and Hec1 form a complex, and this complex localizes to the centrosome during G1 and S and moves to the centromere during early G2 and mitosis

We cloned and sequenced several chicken cDNAs encoding Nuf2 and Hec1. The full-length chicken *Nuf2* cDNA encodes a 469 amino acid (aa) polypeptide with a predicted molecular mass of 55.1 kDa, and the *Hec1* cDNA encodes a 640-aa polypeptide with a predicted molecular mass of 73.7 kDa. Chicken Nuf2 protein has 75% amino acid sequence similarity with human Nuf2 and 49% amino acid similarity with *S. cerevisiae* Nuf2p. Chicken Hec1 protein has 82% amino acid

sequence similarity with human Hec1 and 48% amino acid similarity with *S. cerevisiae* Ndc80p. Sequence information for the chicken *Nuf2* and *Hec1* genes is available in DDBJ/EMBL/NCBI under accession nos. AB098621 and AB098622, respectively.

The yeast homologues of Hec1, Ndc80p and Nuf2p form a complex with Spc24p and Spc25p (Janke et al., 2001; Wigge and Kilmartin, 2001; He et al., 2001). Therefore, we examined the interaction between chicken Hec1 and Nuf2 in chicken DT40 cells. We generated a DT40 cell line in which Nuf2 was replaced with Nuf2-GFP (called Nuf39-15 cells) and a DT40 cell line in which Hec1 was replaced with Hec1-GFP (called Hec25-1/5 cells). Immunoprecipitation experiments with pre-immune IgG or anti-GFP antibody were performed on extracts from Nuf39-15 cells and Hec25-1/5 cells. Immune complexes were then analyzed by immunoblotting with anti-Hec1 and anti-Nuf2 antibodies. We found that these proteins coprecipitate (Fig. 1A).

To determine the subcellular localization of these proteins, GFP and HcRed were tagged on the C termini of Nuf2 and Hec1, respectively, and a DT40 cell line was generated in which the fused genes were expressed simultaneously (called NufG/HecR cells). The fusion proteins were functional and fully suppressed the phenotypes observed in knockout cells (data not shown). Nuf2-GFP and Hec1-HcRed signals throughout the cell cycle are shown in Fig. 1B. During interphase, Nuf2-GFP was colocalized with Hec1-HcRed at or near centrosomes (Fig. 1B, column 1). Colocalization of Nuf2-GFP with Hec1-HcRed was also observed as discrete signals during prometaphase (Fig. 1B, column 2), metaphase (Fig. 1B, column 3) and anaphase (Fig. 1B, column 4). Non-overlapping GFP and HcRed signals were not observed, indicating that Nuf2 is tightly associated with Hec1 throughout the cell cycle.

To examine localization of the Nuf2-Hec1 complex in detail in DT40 cells, cells expressing *Nuf2-GFP* (Nuf39-15 cells) were immunostained with anti-CENP-C or anti γ-tubulin antibody. We used CENP-C as a centromere marker (Saitoh et al., 1992) and γ-tubulin as a centrosome marker (Wiese and Zheng, 1999). During G1, Nuf2-GFP was colocalized with γ-tubulin and not CENP-C (Fig. 2A,B). We observed Nuf2-GFP colocalized with CENP-C and γ-tubulin during G2 after Nuf2-GFP signals were trapped at the nuclear membrane. Nuf2-GFP signals localized to the centromere during mitosis (Fig. 2A,B). Centrosomic Nuf2-GFP signals were strong and centromeric signals were relatively weak in early G2 when the centrosome begins to split (Fig. 2B). In contrast, the intensity of centromeric Nuf2-GFP signals increased in late G2 when the centrosome split was proceeding; and strong Nuf2-GFP signals were observed at centromeres during late G2 (Fig. 2A,B).

To confirm that localization of Nuf2-GFP changes, we examined the behavior of Nuf2-GFP signals in individual living cells (Fig. 2C; Movie 1, http://jcs.biologists.org/supplemental). We stained the chromosomes of living cells expressing *Nuf2-GFP* with Hoechst 33342 and observed the cells microscopically at 37°C. An example of the time-lapse data is shown in Fig. 2C. The dynamic changes in Nuf2

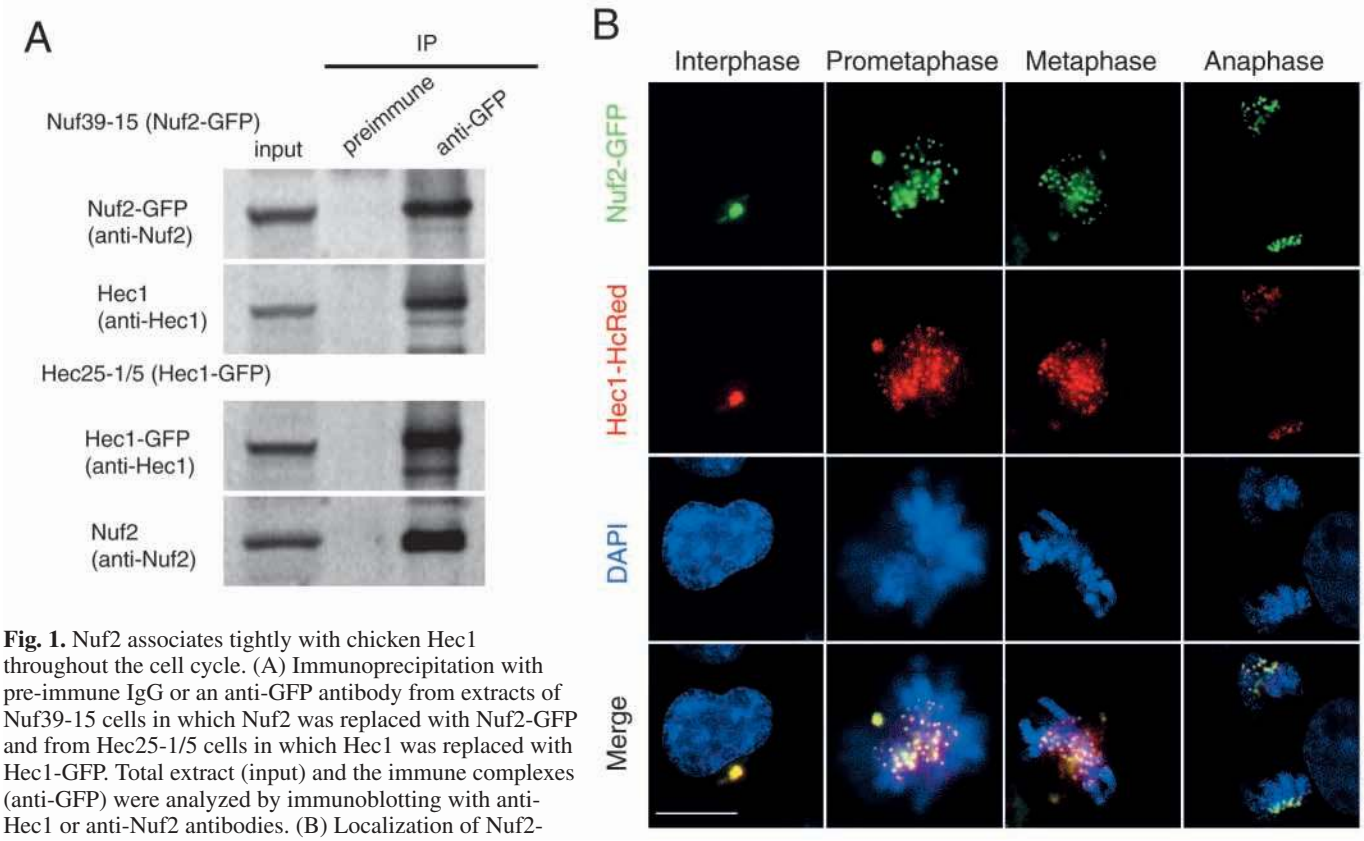

Fig. 1. Nuf2 associates tightly with chicken Hec1 throughout the cell cycle. (A) Immunoprecipitation with pre-immune IgG or an anti-GFP antibody from extracts of Nuf39-15 cells in which Nuf2 was replaced with Nuf2-GFP and from Hec25-1/5 cells in which Hec1 was replaced with Hec1-GFP. Total extract (input) and the immune complexes (anti-GFP) were analyzed by immunoblotting with anti-Hec1 or anti-Nuf2 antibodies. (B) Localization of Nuf2-GFP (green) and Hec1-HcRed (red) at progressive stages of the cell cycle in DT40 cells that expressed Nuf2-GFP and Hec1-HcRed simultaneously. Nuclei and chromosomes are counterstained with DAPI (blue). The scale bars correspond to 10 μm. As shown in the merged images, Nuf2-GFP signals are colocalized with Hec1-HcRed signals throughout the cell cycle.

localization observed during the cell cycle in living cells were similar to those identified in fixed cells.

Generation of conditional loss-of-function mutants of Nuf2 and Hec1 in DT40 cells

To investigate the roles of Nuf2 and Hec1 in higher vertebrate cells, we generated conditional loss-of-function mutants of Nuf2 and Hec1. In our system, expression of both *Nuf2* and *Hec1* was under the control of a tetracycline (tet)-repressible promoter. A *Nuf2* deletion construct was generated such that the 8.0 kb genomic fragment encoding amino acids 1-223 was replaced with one of several selection cassettes (Fig. 3A). We initially transfected a *Nuf2* disruption construct containing the

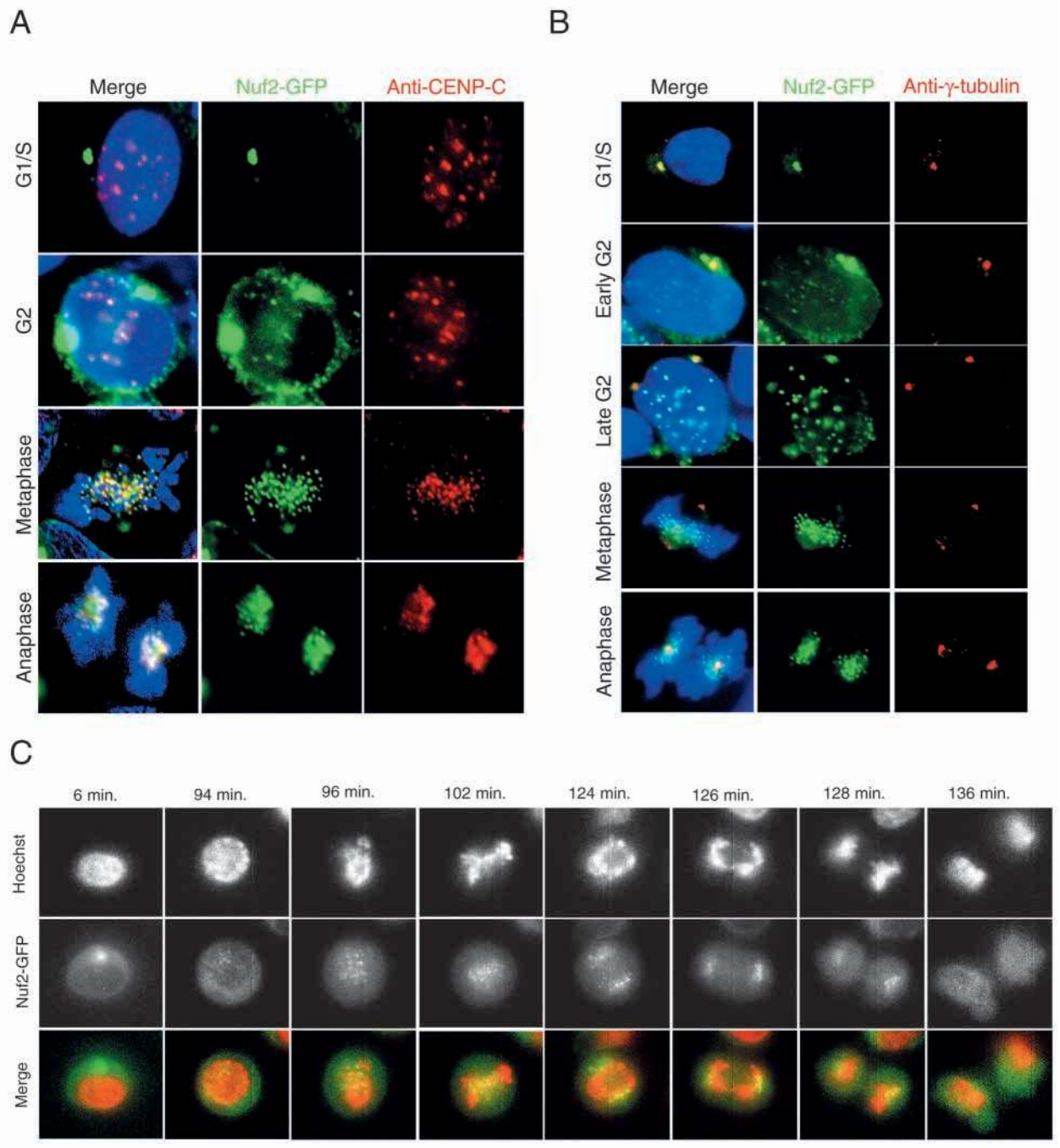

Fig. 2. Dynamic localization of the Nuf2-Hec1 complex during the cell cycle. (A) Localization of Nuf2-GFP at progressive stages of the cell cycle in DT40 cells. Cells were fixed and stained with anti-CENP-C antibody (red). Green signals are specific for Nuf2-GFP. Nuclei and chromosomes are counterstained with DAPI (blue). As shown in the merged images, Nuf2-GFP signals begin to colocalize with CENP-C signals in G2 and are colocalized with CENP-C throughout mitosis. (B) Cells that express Nuf2-GFP (green) were fixed and stained with anti-γ-tubulin (red). Nuclei and chromosomes are counterstained with DAPI (blue). (C) Selected images of chromosomes (Hoechst; top row) and Nuf2-GFP (middle row) in living cells. In the superimposed images (lower row), chromosomes and Nuf2-GFP are displayed in red and green, respectively.

histidinol-resistance cassette into DT40 cells (Fig. 3A) and isolated *Nuf2*$^{+/-}$ clones (Fig. 3B). One *Nuf2*$^{+/-}$ clone was cotransfected with a chicken *Nuf2* transgene under the control of a tet-repressible promoter and a tet-repressible transactivator containing a zeocin-resistance cassette. We selected zeocin-resistant colonies and identified several clones carrying these constructs integrated at random sites in the genome (*Nuf2*$^{+/-/Nuf2\ \text{transgene}}$). Six clones with the *Nuf2*$^{+/-/Nuf2\ \text{transgene}}$ genotype were transfected with the puromycin-*Nuf2* disruption construct to disrupt the remaining *Nuf2* allele (Fig. 3B). We obtained two clones with the *Nuf2*$^{-/-/Nuf2\ \text{transgene}}$ genotype, and one clone, Nuf23-63, was chosen for further analysis.

We used the same strategy to generate *Hec1* mutants. Three *Hec1* alleles are present in the chicken DT40 genome. A *Hec1* deletion construct was generated such that the 4.0 kb genomic fragment encoding amino acids 1-75 was replaced with one of several selection cassettes (Fig. 3A). We sequentially transfected *Hec1* disruption constructs containing either the histidinol- or puromycin-resistance cassette into DT40 cells (Fig. 3A). After generating clones with the *Hec1*$^{+/-/-/Hec1\ \text{transgene}}$ genotype, we transfected the neomycin-Hec1 disruption construct to delete the remaining Hec1 allele and obtained six clones with the *Hec1*$^{-/-/-/Hec1\ \text{transgene}}$ genotype (Fig. 3B). One clone, Hec25-1, was chosen for further analysis. Western blot analysis did not detect Nuf2 protein in Nuf23-63 cells 24 hours after tet addition, or Hec1 protein in Hec25-1 cells 12 hours after tet addition (Fig. 3C).

Deletion of Nuf2 and Hec1 results in accumulation of cells in prometaphase and subsequent cell death

The growth curves of Nuf23-63 (Nuf2+, tet–) and Hec25-1 (Hec1+, tet–) cells were indistinguishable from that of wild-type DT40 cells (Fig. 4A). We then examined cell proliferation and viability following the addition of tet to the medium. Nuf23-63 cells (Nuf2–, tet+) stopped proliferating approximately 1.5 cell cycles after addition of tet, and most cells died by 48 hours (Fig. 4A). Hec25-1 cells (Hec1–, tet+) stopped proliferating approximately 10 hours after addition of tet, and most cells died by 36 hours (Fig. 4A). These findings indicate that depletion of Nuf2 and Hec1 causes growth arrest and subsequent cell death.

For cell cycle analysis following gene depletion in both knockout cells, we measured both cellular DNA content and DNA synthesis by fluorescence-activated cell sorting (FACS) after pulse-labeling with BrdU (Fig. 4B). Nuf23-63 cells (tet+) started to accumulate in the G2/M phase 18 hours after addition of tet and 48% of cells were in the G2/M phase by 24 hours. Hec25-1 cells (tet+) also started to accumulate in the G2/M phase 12 hours after addition of tet and 54% of cells were in the G2/M phase by 18 hours (Fig. 4B). After a prolonged delay in the G2/M phase, cells of both clones died suddenly. Degradation of chromosomal DNA due to massive cell death was observed (Fig. 4B and Fig. 5A).

To determine the exact nature of the mitotic delay, we used DNA staining and immunocytochemical staining of microtubules to examine the time course of events following the depletion of Nuf2 and Hec1 (Fig. 5A,B). In Nuf2-deficient cells, the mitotic index began to increase at 12 hours and reached 44% at 24 hours after addition of tet (Fig. 5A,B). In Hec1-deficient cells, the mitotic index began to increase at 12 hours and reached 52% at 18 hours after addition of tet (Fig. 5A,B). We did not observe any anaphase cells after 24 hours in either mutant cell line (Fig. 5B). These data suggest that both Nuf2- and Hec1-deficient cells accumulate in the M phase rather than in the G2 phase. Although we observed the movement of these proteins to the centromere from the centrosome, both mutant cell types displayed a terminal phenotype during mitosis. We did not detect further uptake of BrdU after cell-cycle arrest. We did not observe many polyploid cells, in contrast to CENP-H- or CENP-I-deficient cells, which proceed to the next interphase after a long delay in mitosis (Fukagawa et al., 2001; Nishihashi et al., 2002). Considering the FACS data together with cytological data, we suggest that Nuf2- and Hec1-deficient cells died after cell-cycle arrest during mitosis.

Deletion of Nuf2 or Hec1 causes chromosome aberrations and leads to chromosome missegregation.

During the course of cytological analyses of Nuf2- and Hec1-deficient cells, we observed many abnormal mitotic cells that contained hypercondensed chromosomes that failed to congress normally to the metaphase plate (Fig. 5A). Eventually, the cells underwent apoptosis (Fig. 5A). In control cells, the chromosomes appeared ordered and aligned properly on the metaphase plate (Fig. 5A).

Control cells cultured in the absence of tet showed well-ordered bipolar spindles during both metaphase and anaphase (Fig. 5A, 0 hours). In the mutant cell lines, most cells cultured in the presence of tet had normal bipolar spindles, but some contained multi- or monopolar spindles. We found, by observing living cells, that normal bipolar spindles formed just after entrance into mitosis and the abnormal spindles appeared during mitotic arrest (Fig. 6; Movies 2 and 3, http://jcs.biologists.org/supplemental). In most mutant cells with bipolar spindles, chromosome alignment was disordered. We frequently observed arch-shaped spindles away from chromosomes (Fig. 5A). These results indicate that Nuf2 and Hec1 are required either directly or indirectly for congression and/or maintenance of stable chromosome alignment and, ultimately, for progression from metaphase to anaphase.

We then examined whether the mitotic defects caused by depletion of Nuf2 and Hec1 were associated with induction of aneuploidy. We performed FISH analysis of metaphase spreads with chromosome painting probes specific for chicken chromosomes 1 and 2. Because DT40 cells contain three copies of chromosome 2, five fluorescent chromosomes can be observed in each wild-type cell. As shown in Fig. 5C, we observed abnormal numbers of painted chromosomes in both Nuf2- and Hec1-deficient cells in the presence of tet. The proportion of these aneuploid cells increased gradually after addition of tet (Fig. 5C), suggesting that deficiency of Nuf2 and Hec1 cause chromosome missegregation. Although Nuf2- and Hec1-deficient cells died in M phase, the proportion of aneuploid cells increased. We explain this observation as follows. At an early stage after addition of tet, cells have a small amount of Hec1 or Nuf2 protein. In this stage, several populations of cells can exit M phase with a small amount of Hec1 or Nuf2. These cells may fail to complete normal mitosis, and cells with chromosome loss accumulate.

To determine the consequences of Nuf2 deficiency in

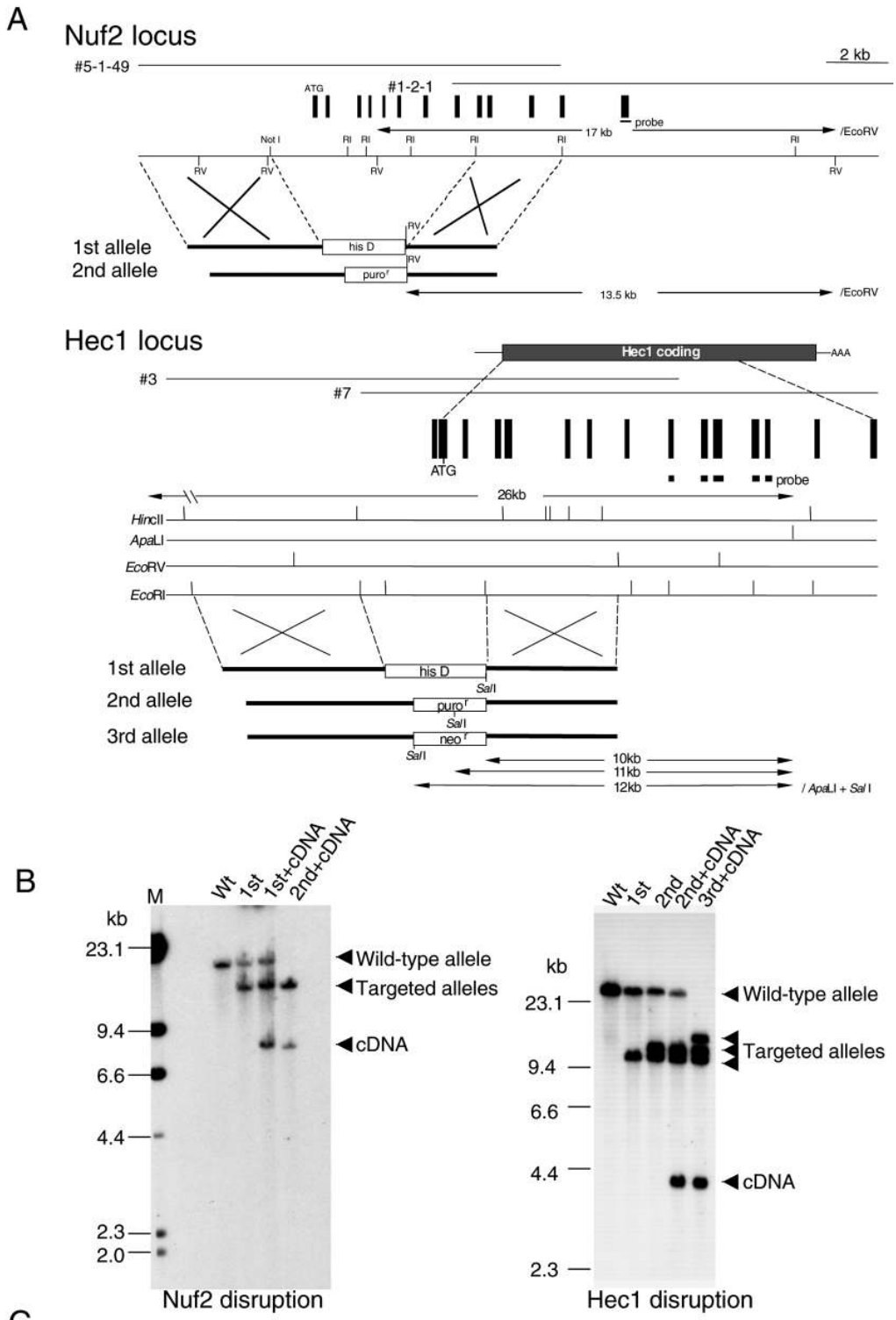

Fig. 3. Generation of ΔNuf2 and ΔHec1 clones carrying transgenes under the control of a tet-repressible promoter.
(A) Restriction maps of the Nuf2 and Hec1 loci, gene disruption constructs, and targeted loci. Black boxes indicate the positions of exons. Several restriction enzyme sites are shown. The position of the probe used for Southern hybridization is indicated.
(B) Restriction analysis of targeted integration of the Nuf2 and Hec1 disruption constructs. In Nuf2 disruption, genomic DNAs from wild-type DT40 cells (Wt), a clone after first round targeting (+/–, 1st), a clone after first round targeting and random integration of the Nuf2 transgene (+/– Nuf2+, 1st +cDNA), and a clone after second round targeting (–/– Nuf2+, 2nd +cDNA) were analyzed by Southern hybridization with the probe indicated in (A). In Hec1 disruption, genomic DNAs from wild-type DT40 cells (Wt), a clone after first round targeting (+/+/–, 1st), a clone after second round targeting (+/–/–, 2nd), a clone after second round targeting and random integration of the Hec1 transgene (+/–/– Hec1+, 2nd+cDNA), and a clone after third round targeting (–/–/– Hec1+, 3rd+cDNA) were analyzed by Southern hybridization with the probe indicated in A. (C) Western blot analysis of whole cell extracts of Nuf23-63 and Hec25-1 cells with anti-Nuf2 and anti-Hec1 antibodies at the indicated times following addition of tet. Equal amounts of extracts were separated by SDS-PAGE and analyzed by western blotting. Anti-α-tubulin antibody was used as a loading control.

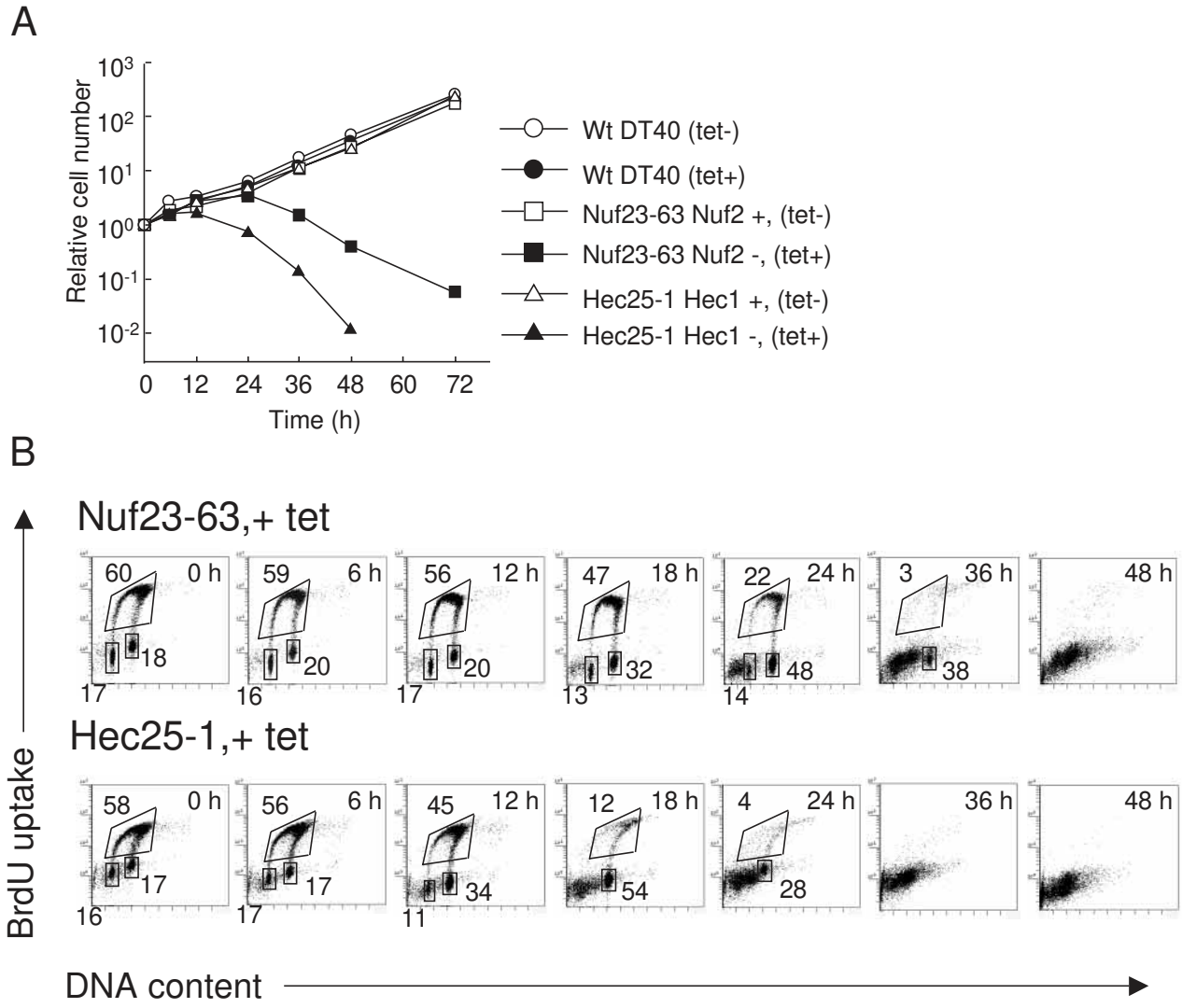

Fig. 4. Both Nuf2 and Hec1 are essential for normal progression of the cell cycle. (A) Representative growth curves for the indicated cell cultures. Tet was added at time 0 to the Nuf2- or Hec1-deficient cell tet (+) cultures, and the number of cells not stained with trypan blue was counted. Each experiment was performed twice, and each time point was examined in duplicate. (B) Cell-cycle distribution of Nuf23-63 and Hec25-1 in cells following inhibition of transgene expression due to addition of tet at time 0. Cells were stained with FITC-anti-BrdU (y-axis, log scale) to detect BrdU incorporation (DNA replication), and with propidium iodide to detect total DNA (x axis, linear scale). The lower-left box represents G1-phase cells, the upper box represents S-phase cells, and the lower-right box represents G2/M-phase cells. The numbers given in the boxes specify the percentage of gated events.

greater detail, we observed the dynamic behavior of individual living cells after suppression of *Nuf2* expression (Fig. 6; Movies 2 and 3). To visualize microtubules in living cells, the human α-tubulin gene fused with GFP was integrated into the genome of Nuf23-63 cells. We then stained the live Nuf23-63 cells expressing *GFP-α-tubulin* with Hoechst 33342 and observed the cells microscopically at 37°C. Representative time-lapse data for Nuf23-63 cells in the absence of tet (control) are shown in Fig. 6A. Control cells (n=10) required approximately 30 minutes to progress from prophase to telophase. After addition of tet to Nuf23-63 cells, we observed abnormal mitotic behavior (Fig. 6B). We observed normal progression during interphase and a normal split of centrosomes (data not shown). Time point 0 in Fig. 6B corresponds to 18 hours after addition of tet to cultures of Nuf23-63 cells. The cell shown in Fig. 6B entered mitosis (20 minutes) as condensed individual chromosomes appeared and the mitotic spindle formed. Chromosome congression to the metaphase plate and subsequent anaphase were not observed. Instead, the cell remained arrested in prometaphase for approximately 400 minutes with hyper-condensed chromosomes. During this prolonged mitotic arrest, the spindle structure underwent a series of abnormal changes. Early after mitotic entry (Fig. 6B, 16-20 minutes) a normal-looking bipolar mitotic spindle had formed, but subsequently an abnormal spindle formed (Fig. 6B, 126-304 minutes). After prolonged mitotic arrest, the cell underwent apoptosis. Similar events were observed in Hec1 mutant cells (data not shown). The time of mitotic arrest in Nuf2 and Hec1 mutants (approximately 400 minutes) was shorter than that observed

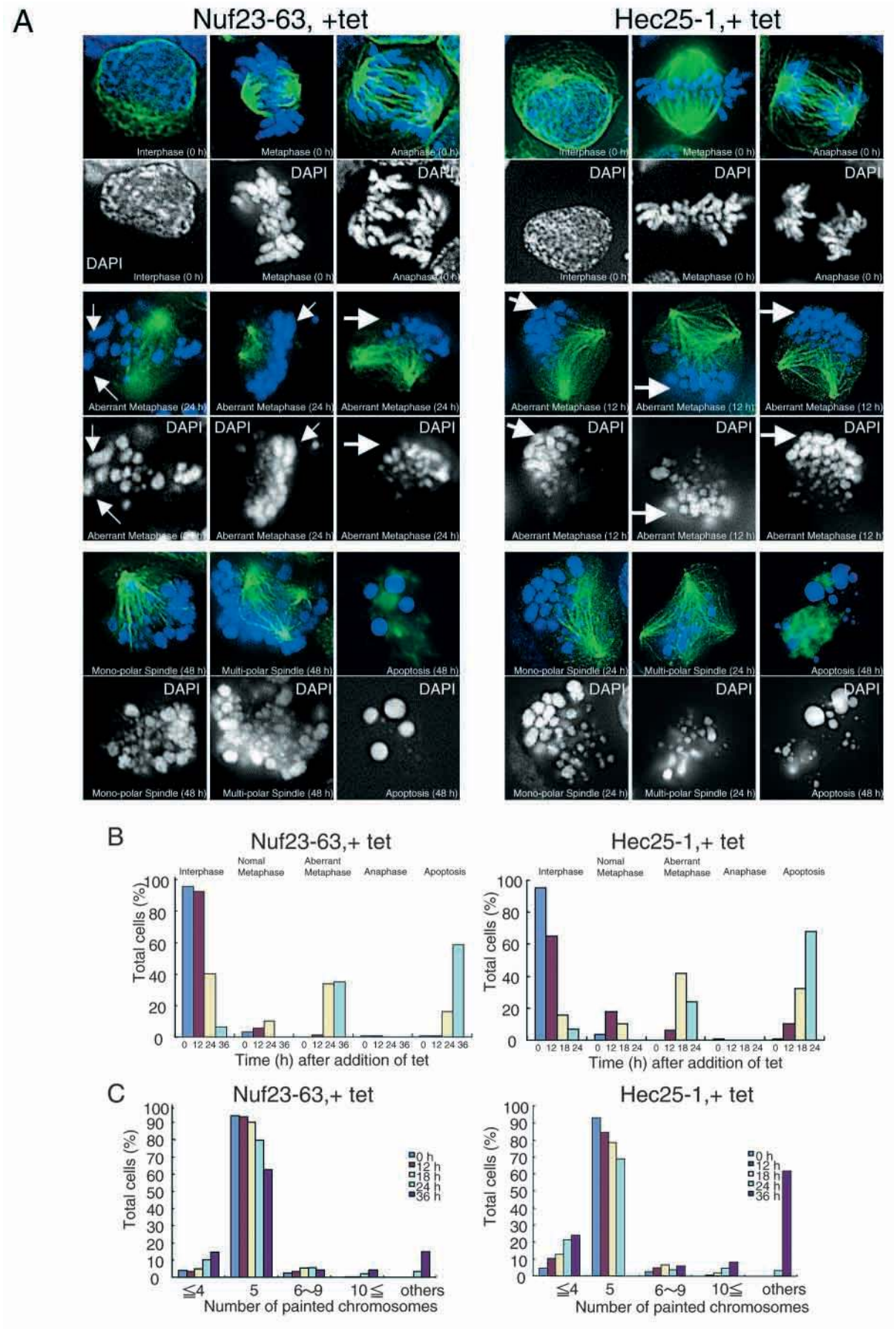
A
Nuf23-63, +tet
Hec25-1,+ tet
Interphase (0 h)
Metaphase (0 h)
Anaphase (0 h)
DAPI
Aberrant Metaphase (24 h)
Aberrant Metaphase (12 h)
Mono-polar Spindle (48 h)
Multi-polar Spindle (48 h)
Apoptosis (48 h)
Mono-polar Spindle (24 h)
Multi-polar Spindle (24 h)
B
Nuf23-63,+ tet
Hec25-1,+ tet
Interphase
Nomal Metaphase
Aberrant Metaphase
Anaphase
Apoptosis
Total cells (%)
Time (h) after addition of tet
C
Nuf23-63,+ tet
Hec25-1,+ tet
Total cells (%)
0 h
12 h
18 h
24 h
36 h
≦4
5
6~9
10≦
others
Number of painted chromosomes

Fig. 5. Nuf2- and Hec1-deficient cells show prometaphase arrest associated with aberrant chromosomes and spindles that lead to chromosome missegregation. (A) Chromosome morphology and α-tubulin staining (green) of Nuf23-63 and Hec25-1 cells in the absence or presence of tet. DNA was counterstained with DAPI (blue). In the absence of tet, cells show the normal staining pattern for α-tubulin (upper panels in both cell lines). In the presence of tet, chromosomes are not aligned at the metaphase plate. Arrows indicate misaligned chromosomes at the metaphase plate. Apoptotic cells were observed in both mutants 48 hours after addition of tet. We also detected cells with monopolar and multipolar spindles. (B) Quantitation of aberrant Nuf23-63 and Hec25-1 cells after inhibition of transgene expression following addition of tet at time 0. We scored the number of interphase cells, normal metaphase cells, aberrant metaphase cells, anaphase cells and apoptotic cells. Apoptotic cells were detected by TUNEL assay. We scored approximately 3000 cells for each time point. (C) To examine chromosome loss, we used FISH analysis with chromosome-specific painting probes. We used painting probes for chicken chromosomes 1 and 2. Because DT40 cells have three copies of chromosome 2, five painted chromosomes were observed in normal cells. Nuf23-63 and Hec25-1 cells were cultured after addition of tet. At the indicated times cells were treated with colcemid for 1.5 hours, and the number of painted chromosomes per cell was determined. The number of painted chromosomes was scored in approximately 1000 metaphase cells.

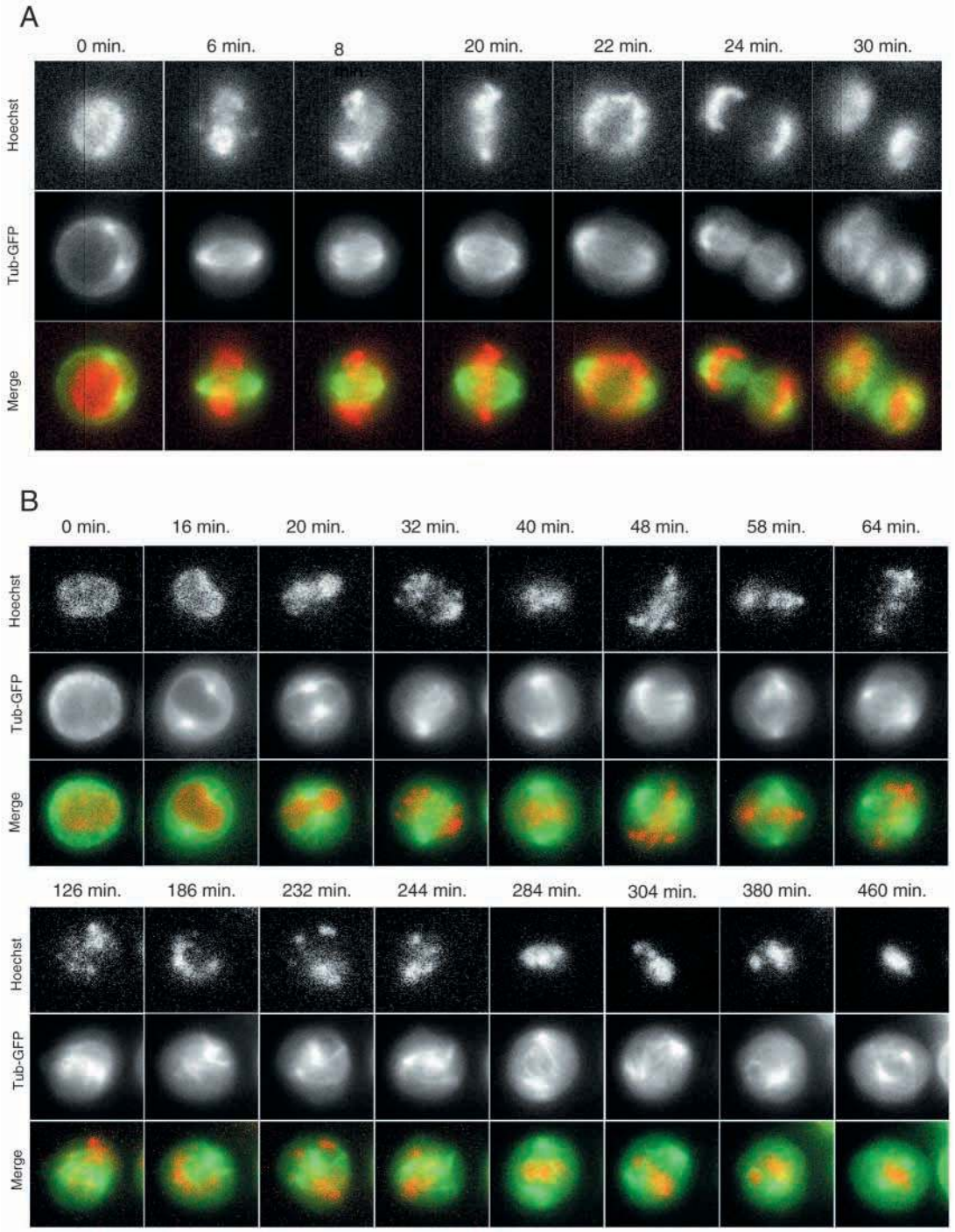

Fig. 6. Chromosome and microtubule dynamics in Nuf2-deficient cells. (A) Selected images of chromosomes (upper) and microtubules (middle) in Nuf23-63 cells (–tet) from prophase to telophase. In the merged images (lower), chromosomes and microtubules are displayed in red and green, respectively. The numbers at the top of each image represent the time in minutes. (B) Observation of a single cell beginning 18 hours after addition of tet. Selected frames are shown. Once the cell entered mitosis, it arrested at prometaphase for approximately 400 minutes. The cell died during the mitotic stage between 380 and 460 minutes.

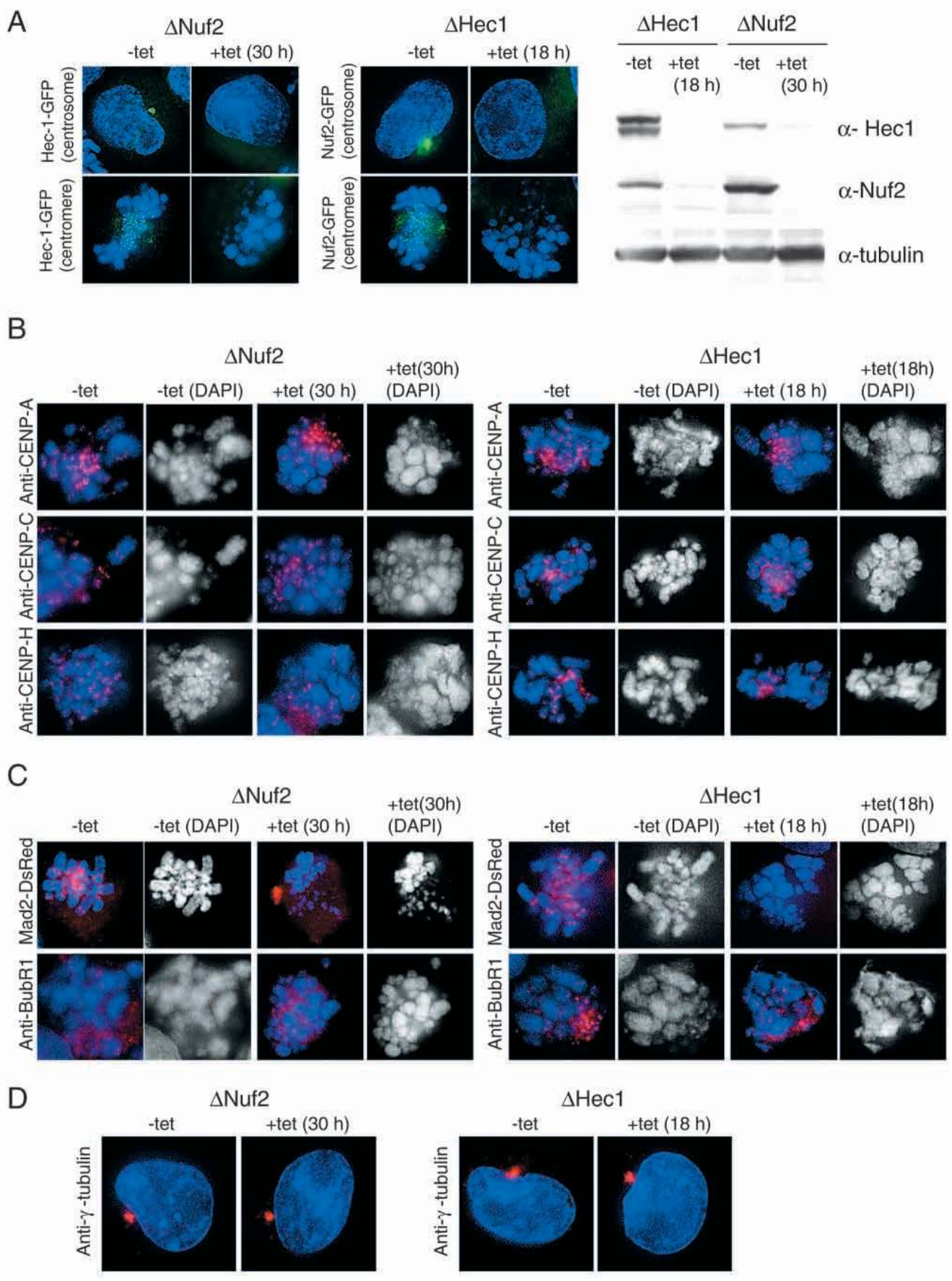
A
ΔNuf2
-tet
+tet (30 h)
Hec-1-GFP (centrosome)
Hec-1-GFP (centromere)
ΔHec1
-tet
+tet (18 h)
Nuf2-GFP (centrosome)
Nuf2-GFP (centromere)
ΔHec1
ΔNuf2
-tet
+tet (18 h)
-tet
+tet (30 h)
α- Hec1
α-Nuf2
α-tubulin
B
ΔNuf2
-tet
-tet (DAPI)
+tet (30 h)
+tet(30h) (DAPI)
ΔHec1
-tet
-tet (DAPI)
+tet (18 h)
+tet(18h) (DAPI)
Anti-CENP-A
Anti-CENP-C
Anti-CENP-H
C
ΔNuf2
-tet
-tet (DAPI)
+tet (30 h)
+tet(30h) (DAPI)
ΔHec1
-tet
-tet (DAPI)
+tet (18 h)
+tet(18h) (DAPI)
Mad2-DsRed
Anti-BubR1
D
ΔNuf2
-tet
+tet (30 h)
Anti-γ -tubulin
ΔHec1
-tet
+tet (18 h)
Anti-γ -tubulin

Fig. 7. Localization of centromere- and checkpoint-related proteins in Nuf2- and Hec1-deficient cells. (A) Localization analysis of Hec1-GFP in Nuf2-deficient cells and Nuf2-GFP in Hec1-deficient cells. In control cells (–tet), Hec1-GFP (green) and Nuf2-GFP (green) signals are localized at the centrosome (G1/S phase) and centromere (mitosis). After addition of tet, both signals are reduced. DNA is counterstained with DAPI (blue). The panel on the right shows a western blot analysis with a mixture of anti-Nuf2 and anti-Hec1 antibodies of Nuf2- and Hec1-deficient cells. The levels of Hec1 in Nuf2-deficient cells and of Nuf2 in Hec1-deficient cells are reduced. (B) Immunofluorescence analysis with antibodies against CENP-A, -C and -H of prometaphase chromosomes in Nuf2- and Hec1-deficient cells after addition of tet. (C) Localization of checkpoint proteins in Nuf2- and Hec1-deficient cells. Both control cells (-tet) have strong Mad2-DsRed signals. After addition of tet, the Mad2-DsRed signals are diffuse in both mutants. Both mutants were also stained with anti-BubR1 (red) in the presence or absence of tet. BubR1 signals are present on the centromeres of cells in mitotic arrest caused by Nuf2 or Hec1 depletion. (D) Immunofluorescence analysis with antibody against γ-tubulin of Nuf2- and Hec1-deficient cells.

in CENP-I-deficient cells (approximately 800 minutes) (Nishihashi et al., 2002).

Localization of centromere- and checkpoint-related proteins in Nuf2- and Hec1-deficient cells

We observed that Nuf2 and Hec1 formed a complex that was maintained throughout the cell cycle (Fig. 1). Therefore, we investigated Hec1 localization in Nuf2-deficient cells and vice versa. We used a Nuf2-deficient cell line that stably expresses *Hec1-GFP* and a Hec1-deficient cell line that stably expresses *Nuf2-GFP*. We found that in Nuf2-deficient cells the Hec1-GFP signals were diffuse at both centrosomes and centromeres. Nuf2-GFP signals were diffuse in Hec1-deficient cells (Fig. 7A). We also found by western blot analysis that the levels of Hec1 in Nuf2-deficient cells and of Nuf2 in Hec1-deficient cells were reduced (Fig. 7A). These findings support our conclusion that the Nuf2-Hec1 complex is tight during the cell cycle.

In the present study, we found that the Nuf2-Hec1 complex localizes to the centromere during mitosis. We reported previously that centromere components were not assembled in a simple linear fashion (Nishihashi et al., 2002). Therefore, we examined whether inner centromere proteins localize to the centromere in Nuf2- and Hec1-deficient cells. Metaphase spreads of Nuf2- and Hec1-deficient cells were analyzed with antibodies against chicken CENP-A, -C, and -H, which are typical inner centromere proteins. Representative results are shown in Fig. 7B. In Nuf2- and Hec1-deficient cells, strong centromeric signals for CENP-A -C, and -H were observed, and the signal intensities were similar to those in control cells (Fig. 7B). These results indicate that Nuf2 and Hec1 do not influence localization of CENP-A, -C, and -H in mitotic chromosomes.

We observed mitotic arrest in Nuf2- and Hec1-deficient cells (Figs 4, 5 and 6). This phenotype could be due to activation of a mitotic checkpoint (Wassmann and Benezra, 2001). We analyzed localization of a checkpoint protein, Mad2, in Nuf2- and Hec1-deficient cells (Fig. 7C). We used a Nuf2-deficient cell line that stably expressed *Mad2-DsRed* (Sonoda et al., 2001) and a Hec1-deficient cell line that stably expressed *Mad2-DsRed*. In both Nuf2- and Hec1-deficient cells we did not detect Mad2-DsRed signals at centromeres that we can detect easily in control cells (Fig. 7C). Because both mutants are clearly arrested in mitosis for 400 minutes before dying, we analyzed the localization of BubR1, which is another checkpoint protein (Chan et al., 1999). We found strong BubR1 signals in mitotically arrested Nuf2- and Hec1-deficient cells (Fig. 7C). These results suggest that the mitotic arrest in both mutants is due to activation of the BubR1 pathway.

We have shown that the Nuf2-Hec1 complex is localized at centrosomes during the G1 and S phases (Fig. 2). Thus, we investigated localization of γ-tubulin in Nuf2- and Hec1-deficient cells during these phases, because the ring complex with γ-tubulin is a major component of centrosomes (Wiese and Zheng, 1999). We stained Nuf2- and Hec1-deficient cells with an antibody against γ-tubulin. We detected typical centrosome signals for γ-tubulin in both control and mutant cells (Fig. 7D), suggesting that the Nuf2-Hec1 complex is localized in the pericentriolar material away from the γ-tubulin complex.

Nuf2 and Hec1 localization in cells with disruption of the inner centromere structure

Immunocytochemical analysis of Nuf2- and Hec1-deficient cells with inner centromere proteins CENP-A, -C and -H revealed that Nuf2 and Hec1 are not necessary for localization of the inner centromere proteins to the centromere (Fig. 7B). Therefore, we examined whether Nuf2 and Hec1 are targeted to centromeres lacking the inner centromere proteins. We reported previously that BubR1, a checkpoint protein in the outer centromere, is targeted to disrupted centromeres in CENP-I knockout cells (Nishihashi et al., 2002). We used CENP-H-deficient cells that stably expressed *Nuf2-GFP* or *Hec1-GFP*. We also used CENP-I-deficient cells that stably expressed *Nuf2-GFP* or *Hec1-GFP*. We did not detect CENP-H or CENP-I signals in CENP-H- or CENP-I-deficient cell lines, respectively, 48 hours after addition of tet. Typical results are shown in Fig. 8. Nuf2-GFP and Hec1-GFP signals are significantly lower in both CENP-H- and CENP-I-deficient cells in comparison to control cells. However, we can still observe signals for Nuf2 and Hec1. These results suggest that association of Nuf2 or Hec1 with centromeres was weak in CENP-H- and CENP-I-deficient cells.

Nuf2 and Hec1 turn over rapidly at centrosomes and are stably associated with centromeres.

In the present study, we observed the dynamic behavior of the Nuf2-Hec1 complex during the cell cycle. To gain insight into the dynamics of Nuf2 and Hec1 in living cells, we used Nuf39-15 cells in which expression of *Nuf2* is completely replaced with *Nuf2-GFP* and Hec25-1/5 cells in which expression of *Hec1* is completely replaced with *Hec1-GFP* (described in Fig. 1A). Growth of these cell lines was identical to that of wild-type DT40 cells, which indicates that Nuf2-GFP and Hec1-GFP were functional. We used fluorescence recovery after photobleaching (FRAP) of these cells to assay the turnover of these proteins at centrosomes (during G1 or S phase) and at centromeres (during mitosis). We found that Nuf2-GFP and Hec1-GFP fluorescence at centrosomes recovered rapidly (Fig.

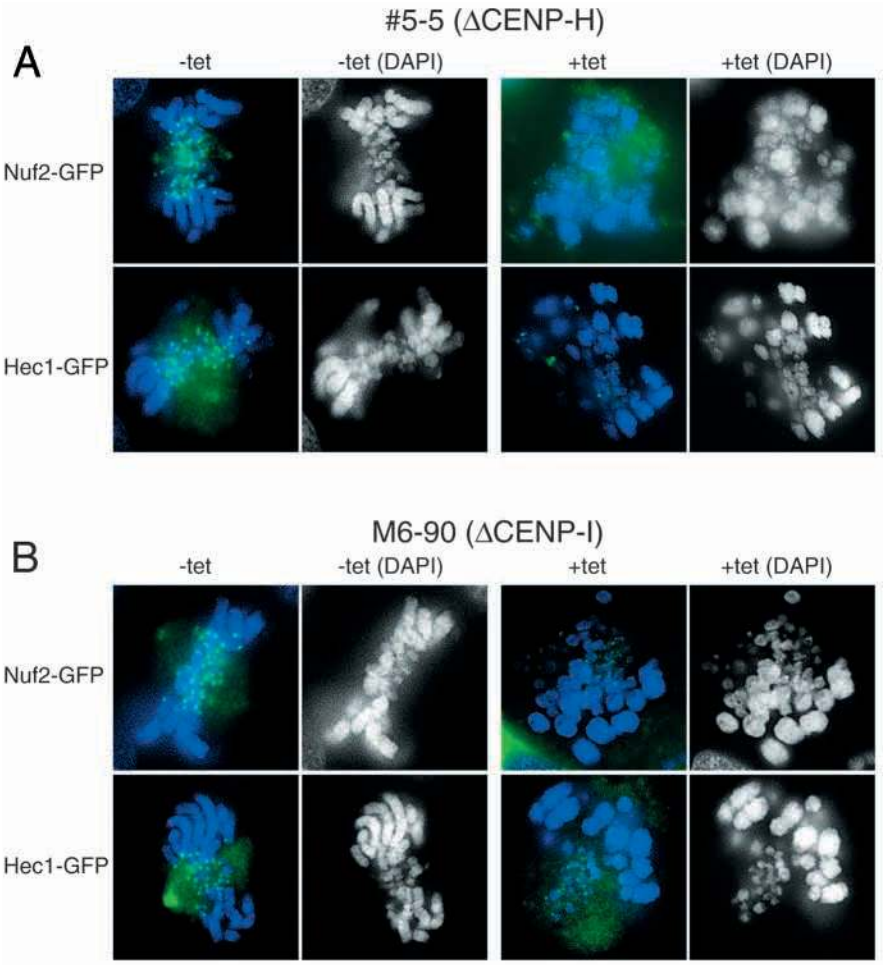

Fig. 8. Nuf2 and Hec1 localization in CENP-H- and CENP-I-deficient cells. (A) Localization of Nuf2-GFP and Hec1-GFP in #5-5 (Δ/CENP-H, CENP-H transgene) cells grown in the absence (–tet) or presence (+tet) of tet for 48 hours. Hec1-GFP (green) or Nuf2-GFP (green) signals are reduced after addition of tet (+tet). (B) Localization analysis of Nuf2-GFP and Hec1-GFP in M6-90 (Δ/CENP-I, CENP-I transgene) cells grown in the absence (–tet) or presence (+tet) of tet for 48 hours. Hec1-GFP (green) or Nuf2-GFP (green) signals are reduced after addition of tet (+tet).

9; Movies 4 and 5, http://jcs.biologists.org/supplemental) with half-times of 11.9 seconds (n=19) and 13.2 seconds (n=19), respectively. In contrast, fluorescent signals for both Nuf2-GFP and Hec1-GFP at centromeres did not recover rapidly (Fig. 9; Movies 6 and 7); it took more than 30 minutes to recover these signals. We did not observe substantial recovery of either GFP signal when the centromere fractions of both cell lines were photobleached during early mitosis. These findings indicate that the associations of Nuf2 and Hec1 with centrosomes during G1 and S phases are dynamic, and Nuf2 and Hec1 are stable components of centromeres during mitosis (Fig. 10).

Discussion

Dynamic localization of conserved Nuf2-Hec1 complex during the cell cycle

In the present study, we characterized the conserved Nuf2-Hec1 protein complex in chicken DT40 cells. Several groups reported independently that Nuf2p associates with Ndc80p, Spc24p and Spc25p at centromeres in *S. cerevisiae* (Janke et al., 2001; Wigge and Kilmartin, 2001; He et al., 2001). Other groups have characterized this complex in *S. pombe* or *C. elegans* (Nabetani et al., 2001; Howe et al., 2001). Recently, the human counterpart of Ndc80p, Hec1, and human Nuf2 were analyzed by RNAi experiments (Martin-Lluesma et al., 2002; DeLuca et al., 2002). In the present study, we showed that Nuf2 forms a complex with Hec1 in DT40 cells, and we detailed localization of this complex during the cell cycle. We studied a cell line that expressed *Nuf2-GFP* and *Hec1-HcRed* simultaneously and found that these proteins always colocalize. The changing localization of this complex during the cell cycle is interesting. During G1 and S phases, the complex associates with centrosomes. During G2 phase, the complex moves through the nuclear membrane and localized with interphase centromeres. During mitosis the complex is localized primarily with centromeres, although we observed small amounts of this complex at the spindle poles. There have been reports of other proteins whose localization changes during the cell cycle. Many of the mitotic checkpoint proteins and regulators of the anaphase promoting complex/cyclosome (APC/C), including Cdc20 and Bub and Mad family proteins, move to kinetochores from other locations during mitosis (Chen et al., 1996; Li et al., 1997; Kallio et al., 1998; Taylor et al., 1998). Kinetochore localization of these proteins is correlated with progression of mitosis. When checkpoint proteins leave the kinetochores, mitosis proceeds normally. It is possible that the dynamic localization of the Nuf2-Hec1 complex is associated with regulation of cell cycle progression. The time of Nuf2-Hec1 complex movement to centromeres is earlier than that of the Bub and Mad family, and the Nuf2-Hec1 complex localizes to the nuclear membrane before targeting to interphase centromeres. This changing pattern of localization

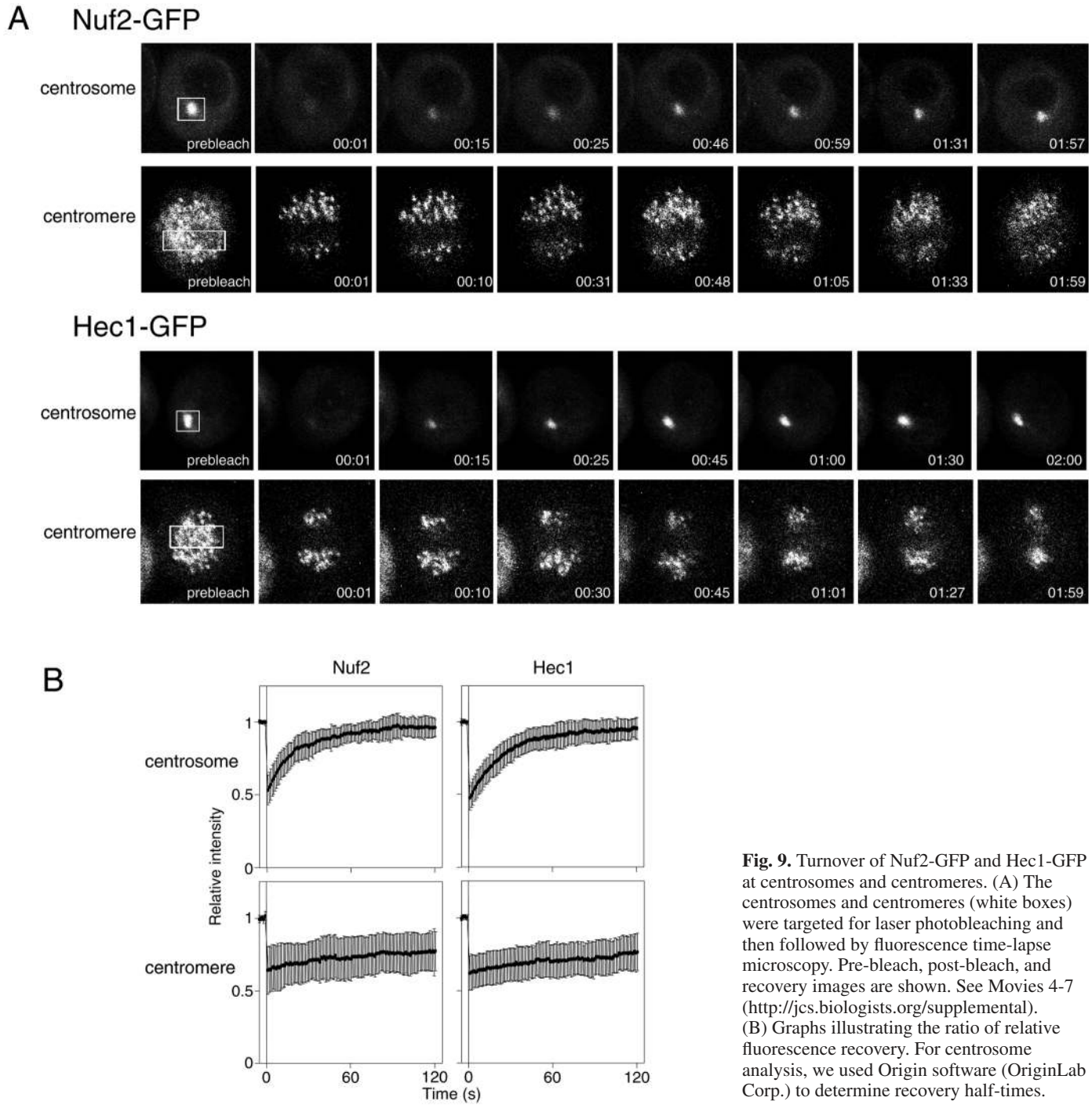

Fig. 9. Turnover of Nuf2-GFP and Hec1-GFP at centrosomes and centromeres. (A) The centrosomes and centromeres (white boxes) were targeted for laser photobleaching and then followed by fluorescence time-lapse microscopy. Pre-bleach, post-bleach, and recovery images are shown. See Movies 4-7 (http://jcs.biologists.org/supplemental). (B) Graphs illustrating the ratio of relative fluorescence recovery. For centrosome analysis, we used Origin software (OriginLab Corp.) to determine recovery half-times.

of the Nuf2-Hec1 complex differs from that observed for checkpoint proteins, which do not localize to interphase centromeres but instead localize to kinetochores after the nuclear envelope break down. The different localization patterns of the Nuf2-Hec1 complex and checkpoint proteins suggest that the Nuf2-Hec1 complex may not be directly involved in the spindle checkpoint machinery. As will be seen below, the knockout phenotype and FRAP analysis of the Nuf2-Hec1 complex suggest that this complex has a novel role in regulation of the cell cycle.

The Nuf2-Hec1 complex is required for centromere function during mitosis

To further our understanding of the Nuf2-Hec1 complex, we generated conditional loss-of-function mutants of Nuf2 and Hec1 in the hyper-recombinogenic chicken DT40 cell line. We previously created several conditional mutants of the inner centromere components CENP-C, -H and -I (Fukagawa and Brown, 1997; Fukagawa et al., 1999a; Fukagawa et al., 2001; Nishihashi et al., 2002). We then compared the phenotypes of the Nuf2 and Hec1 mutants with those of the CENP mutants.

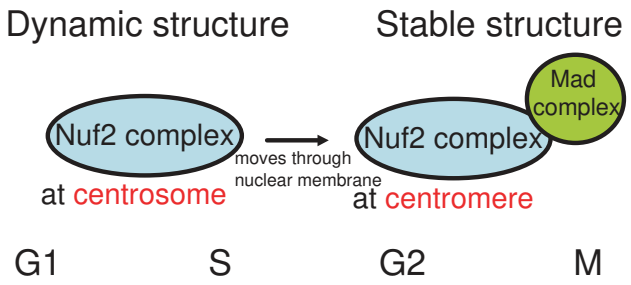

Fig. 10. Dynamic behavior of the Nuf2-Hec1 complex during the cell cycle. Illustration of the dynamic behavior of the Nuf2-Hec1 complex during the cell cycle. Association of the Nuf2-Hec1 complex with centrosomes is dynamic during G1 and S phases. The complex moves through the nuclear membrane to centromeres during G2 and mitosis. Association of the complex with centromeres is stable. In the centromere, the complex provides a place for the Mad2 complex to attach to the kinetochore.

Despite the dynamic localization of the Nuf2-Hec1 complex during the cell cycle, both Nuf2- and Hec1-deficient cells arrested specifically in prometaphase. Both mutants have hypercondensed chromosomes and abnormal spindle structures associated with prometaphase arrest. This phenotype with the accompanying abnormal mitosis is similar to those observed in CENP-C, -H, and -I mutants. Like CENP-C-, -H- and -I-deficient cells, Nuf2- and Hec1-deficient cells fail to align their chromosomes at the spindle equator. We observed by FRAP analysis that the Nuf2-Hec1 complex associates stably with the centromere (Fig. 9). We also used this technique to analyze turnover of CENP-H-GFP. The kinetics of turnover CENP-H-GFP and Hec1-GFP at the centromere were similar (T.F., unpublished). FRAP analysis of Mad2-GFP and Cdc20-GFP at the centromere revealed that turnover of these transient kinetochore proteins is rapid (Howell et al., 2000; Kallio et al., 2002). The association of Nuf2 and Hec1 with the centromere is more stable than that of checkpoint regulators such as Mad2 and Cdc20. Thus, we suggest that the Nuf2-Hec1 complex is a structural component of functional centromeres and essential for full centromere function.

Although we found similarities between the phenotypes of Nuf2- and Hec1-deficent cells and inner centromere protein knockouts, we also found differences. The living-cell analysis revealed that the length of time that Nuf2-deficient cells remained in prometaphase arrest is different from that observed in CENP-H- or CENP-I-deficient cells. Nuf2-deficient cells arrested for 400 minutes in prometaphase before undergoing apoptosis. In contrast, after an 800-minute delay in mitosis, CENP-I-deficient cells proceeded to the next cell cycle without normal cell division and then died. We reported previously that the lengthy delays in prometaphase observed in CENP-I-deficient cells were caused by disruption of the inner kinetochore structure (Nishihashi et al., 2002). In the present study, we showed that localization of inner centromere proteins was not altered in Nuf2- and Hec1-deficient cells (Fig. 7), suggesting that the Nuf2-Hec1 complex is not involved in the inner kinetochore structure. Nevertheless, we found that Nuf2 or Hec1 depletion yielded more severe mitotic phenotypes than did depletion of CENP-C, -H, or -I. One explanation for this phenotype difference is that the Nuf2-Hec1 complex functions not only as a simple structural component of the centromeres but also as a regulator for progression of mitosis.

The mitotic delay phenotype could be due to activation of mitotic checkpoints; however, we did not observe Mad2 localization in Nuf2- and Hec1-deficient cells. Martin-Lluesma et al. (Martin-Lluesma et al., 2002) showed by yeast two-hybrid analysis that human Hec1 interacts with Mad1, and they also showed that Mad2 and Mps1 signals were reduced by Hec1 RNAi in HeLa cells. DeLuca et al. (DeLuca et al., 2002) observed mitotic arrest in human Nuf2 RNAi experiments, and they detected Mad2 signals on mitotically arrested chromosomes. In our system, we did not detect Mad2 signals on arrested chromosomes in either Hec1- or Nuf2-deficient cells. However, we detected strong BubR1 signals at centromeres in both Nuf2- and Hec1-deficient cells, which suggests that mitotic arrest in both Nuf2- and Hec1-deficient cells is caused by activation of the BubR1 pathway. Hec1-RNAi cells still underwent a Mad2-dependent checkpoint arrest, even though they lacked Mad2 at kinetochores (Martin-Lluesma et al., 2002). Therefore, it is possible that the Mad2 pathway is still active in our Nuf2- and Hec1-deficient cells even though we cannot detect Mad2 signals.

We reported previously that the outer centromere protein BubR1 recognizes the centromere even if the inner centromere structure is disrupted due to CENP-H or CENP-I depletion, and we proposed that the kinetochore is not assembled through a simple linear pathway (Nishihashi et al., 2002). In CENP-H- and -I-deficient cells, Nuf2 and Hec1 signal intensities are reduced, although signals are still visible. These results indicate that centromere localization of Nuf2 and Hec1 is in part dependent on inner centromere structure.

What is the role of the Nuf2-Hec1 complex at centrosomes?

Although we clarified the important role of the Nuf2-Hec1 complex during mitosis, the role of this complex during interphase remained unclear. The Nuf2 complex is localized primarily at centrosomes during G1 and S phases, and we found by FRAP analysis that association of the complex with centrosomes is dynamic. This dynamic behavior suggests that this complex may not be a simple structural component of centrosomes and may function as a signal transmitter to components localized at centrosomes. It is also possible that the Nuf2-Hec1 complex is modified at centrosomes and that the localization changes depending on progression of the cell cycle. One possible modification is phosphorylation. Cheeseman et al. (Cheeseman et al., 2002) demonstrated that the yeast homologue of Hec1, Ndc80p, is phosphorylated by the aurora kinase Ipl1p. There are several counterparts of aurora kinase in vertebrate cells (Adams et al., 2001). Aurora A localizes to centrosomes, and aurora B is targeted to centromeres during mitosis. It is important to determine whether the Nuf2-Hec1 complex in vertebrate cells is phosphorylated by aurora kinases. We do not have any direct evidence for the functions of Nuf2 and Hec1 at centrosomes, and there is a possibility that neither protein has a significant function at centrosomes.

Before moving to the centromere from the centrosome, the Nuf2-Hec1 complex is trapped at the nuclear membrane (Fig.

2). It has been reported that some components of the nuclear pore redistribute in part to the kinetochore during mitosis in mammalian cells (Belgareh et al., 2001). The significance of this observation is unclear; however, it is possible that the Nuf2-Hec1 complex is involved in recruitment of nuclear pore components to the centromere.

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