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# Nuf2 and Hec1 Are Required for Retention of the Checkpoint Proteins Mad1 and Mad2 to Kinetochores

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## Summary

**Members of the Ndc80/Nuf2 complex have been shown in several systems to be important in formation of stable kinetochore-microtubule attachments and chromosome alignment in mitosis [1–9]. In HeLa cells, we have shown that depletion of Nuf2 by RNA interference (RNAi) results in a strong prometaphase block with an active spindle checkpoint, which correlates with low but detectable Mad2 at kinetochores that have no or few stable kinetochore microtubules [5]. Another RNAi study in HeLa cells reported that Hec1 (the human Ndc80 homolog) is required for Mad1 and Mad2 binding to kinetochores and that kinetochore bound Mad2 does not play a role in generating and maintaining the spindle assembly checkpoint [6]. Here, we show that depletion of either Nuf2 or Hec1 by RNAi in HeLa cells results in reduction of both proteins at kinetochores and in the cytoplasm. Mad1 and Mad2 concentrate at kinetochores in late prophase/early prometaphase but become depleted by 5-fold or more over the course of the prometaphase block, which is Mad2 dependent. The reduction of Mad1 and Mad2 is reversible upon spindle depolymerization. Our observations support a model in which Nuf2 and Hec1 function to prevent microtubule-dependent stripping of Mad1 and Mad2 from kinetochores that have not yet formed stable kinetochore-microtubule attachments.**

## Results and Discussion

We first set out to determine if the mitotic block produced by Nuf2 depletion in HeLa cells is Mad2 dependent. Previously, we demonstrated by Western blot analysis a >85% decrease in Nuf2 levels within a cell population after a 48 hr treatment with Nuf2 siRNA [5]. Some cells exhibited metaphase alignment and retained normal levels of Nuf2 at kinetochores, indicating poor transfection. However, the majority exhibited little or no chromosome alignment, and in these cells, Nuf2 fluorescence at kinetochores was 7% or less of normal levels, and the cells were blocked in prometaphase on average for 7.5 hr before undergoing cell death [5]. Microinjection of prometaphase-blocked cells with a dominant-negative inhibitor of Mad2, Mad2ΔC [10], induced cells to exit mitosis with mis-aligned chromosomes and complete cytokinesis (Figure S1 in the Supplemental Data available with this article online). Thus, as shown for depletion of Hec1 by siRNA [6], the mitotic block resulting from Nuf2 depletion is Mad2 dependent in HeLa cells.

We previously reported that Nuf2 depletion in HeLa cells resulted in reduced but detectable Mad2 at kinetochores [5]. We did not, however, distinguish whether Mad2 initially binds kinetochores at mitotic onset at low levels and remains low or, alternatively, whether Mad2 levels are initially elevated as cells enter mitosis and become reduced throughout the course of the mitotic block. To distinguish between these two possibilities, we performed live cell time-lapse imaging of HeLa cells expressing a GFP-hMad2 fusion protein [11] and transfected with Nuf2 siRNA. Using a spinning-disk confocal system [12], we obtained a detection limit above background fluorescence for kinetochores positive for GFP-hMad2 that was near 1/20$^{th}$ the fluorescence intensity at an unattached kinetochore in a control prometaphase cell (Figure S2). As shown in Figures 1A and 1B, we found that early prometaphase kinetochores of Nuf2 siRNA-transfected cells displayed prominent Mad2 fluorescence but that levels diminished as cells remained blocked in mitosis. After an average of 100 min, Mad2 levels decreased approximately 5-fold and then remained relatively constant throughout the prometaphase block. For comparison, we recorded time-lapse images of control, mock-transfected HeLa cells expressing GFP-hMad2 (Figure 1C). In control cells, Mad2 concentrated at unattached kinetochores during late prophase/early prometaphase and then diminished below our detection limit as chromosomes obtained kinetochore-microtubule attachments and alignment on the spindle equator (Figures 1C and 1D, left), consistent with previous reports [11, 13]. The rate of initial loss of Mad2 was similar between control and Nuf2 siRNA-transfected cells; however, the overall pattern of loss of Mad2 in the kinetochore populations differed. After approximately 50 min, control cells typically had 4–5 bright Mad2-positive kinetochores, whereas Nuf2-depleted cells contained an average of 32 kinetochores with reduced, but detectable, Mad2. After approximately 85 min, control cells contained no Mad2-positive kinetochores, whereas the average number of Mad2-positive kinetochores (clearly above our detection limit) in Nuf2 siRNA-transfected cells was approximately 15 from 80 min until the end of filming or just prior to cell death (Figures 1B and 1D, right). In control cells, the few kinetochores retaining Mad2 prior to completing metaphase alignment exhibited high levels of Mad2, typical of unattached kinetochores (Figure 1D, center). In contrast, loss of Mad2 from kinetochores in Nuf2-depleted cells was more uniform, following the general pattern of total Mad2 loss (Figure 1B, center), with no kinetochores exhibiting bright Mad2, regardless of position in the spindle. Thus, in cells transfected with Nuf2 siRNA, kinetochores can bind Mad2, but they lack the ability to retain

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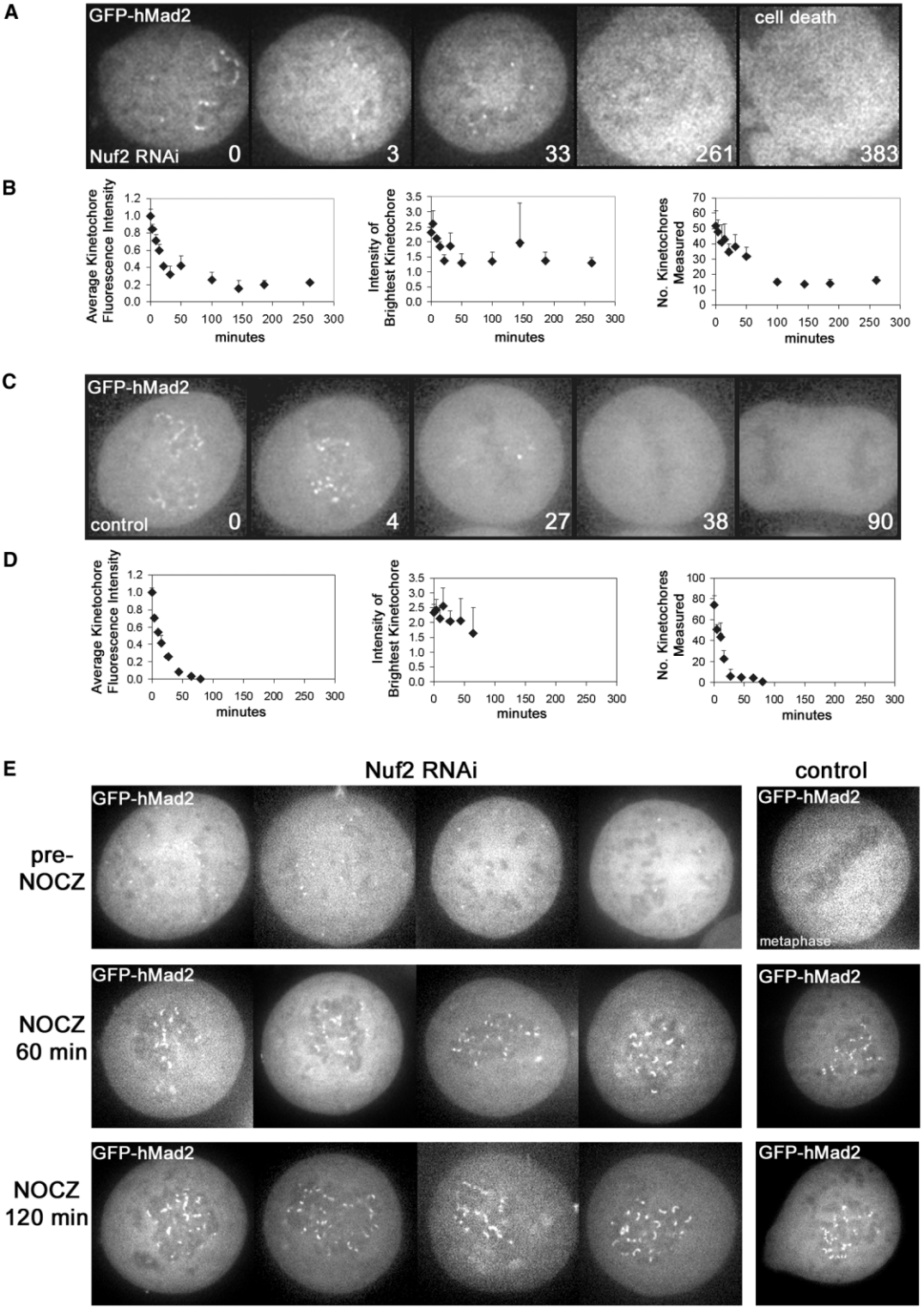


Figure 1. GFP-hMad2 Dynamics at Kinetochores in Control and Nuf2 siRNA-Transfected Cells

Cells expressing GFP-hMad2 were transfected with Nuf2 siRNA (A and B) or mock transfected with transfection reagent alone (C and D). Cells were imaged on a spinning-disk confocal microscope 48 hr after transfection. At each time point, 5–8 images were collected at different focal planes within the cells, and for each image displayed (A and C), one focal plane is shown. For each cell, at each time point, the fluorescence intensity of GFP-hMad2 was measured for each kinetochore and averaged [11] (five cells each for Nuf2 siRNA and mock transfection; B and D graphs, left). At each time point, the brightest kinetochore was also measured for each cell and averaged (B and D graphs, center), and the number of kinetochores with detectable Mad2 was determined and averaged (B and D graphs, right). Graphs at left and center: the average kinetochore intensity value just after nuclear envelope breakdown for each cell was normalized to 1. Bars indicate

high levels at kinetochores of unaligned chromosomes during the prometaphase block [5].

The high levels of Mad2 at kinetochores in early prometaphase of Nuf2 siRNA-transfected cells suggested that Mad2 may be depleted from kinetochores by interactions with spindle microtubules even though kinetochores do not make stable microtubule attachments [5]. To test this possibility, we used time-lapse fluorescence microscopy to image Nuf2-depleted cells expressing GFP-hMad2 and subsequently treated them with 20 μM nocodazole to depolymerize all spindle microtubules. The intensity of Mad2 fluorescence at kinetochores increased dramatically after spindle disassembly in a Nuf2-depleted cell population, in a manner similar to that of control cells (Figure 1E). Therefore, kinetochores in Nuf2 siRNA-transfected cells are able to bind high levels of Mad2 in the absence of microtubules, and the reduction of Mad2 observed in Nuf2-depleted cells reversibly depends on microtubules.

Next, we quantified the levels of Mad2 at kinetochores in nocodazole-treated Nuf2-depleted and control cells by using standard immunofluorescence techniques. Relative to controls, Nuf2-depleted cells recover approximately 83% of Mad2 at kinetochores (Figure 2A, left). We could not be certain, however, that our values of Mad2 fluorescence intensity were not contaminated by kinetochores from cells that were not substantially depleted of Nuf2; both antibodies were generated in rabbits, and the RNAi procedure results in incomplete depletion of Nuf2 protein in some cells [5]. To confirm that our reported values are in the correct range, we repeated the assay with a monoclonal antibody to Mad1, which is required for Mad2 binding to kinetochores [14, 15]. In this case, we identified individual cells containing very low levels of Nuf2 and determined the corresponding level of Mad1 at kinetochores. Cells with kinetochores depleted of Nuf2 exhibited high Mad1 fluorescence at all kinetochores in the presence of nocodazole (Figure 2B). This fluorescence was approximately 89% the level for Mad1 bound to kinetochores in control cells (Figure 2A, center). Nuf2 levels at kinetochores in Nuf2 siRNA-transfected, nocodazole-treated cells were on average 16% of levels in control cells (Figure 2A, right). These values are higher than those measured on kinetochores of Nuf2 siRNA-transfected cells in the absence of nocodazole, the latter being less than 7% of control values [5]. This is likely due to the increased recruitment of kinetochore outer-domain proteins that is observed in cells treated with microtubule depolymerizing reagents [13, 16].

Based on our live-cell GFP-hMad2 data, we suspected Mad1 would also be highly concentrated at kinetochores at the onset of mitosis in Nuf2-depleted cells. Thus, we identified cells that had low levels of Nuf2 and appeared to have just entered mitosis, as judged by nuclear-envelope breakdown and chromosome positioning, and we examined Mad1 localization to kinetochores. Kinetochores in both control and Nuf2-depleted early prometaphase cells exhibited prominent Mad1 levels, regardless of the level of Nuf2 at kinetochores (Figure 2C). Thus, we conclude that in cells with depleted levels of Nuf2, Mad1 and Mad2 are recruited to kinetochores, but after a prolonged mitotic block, substantial but incomplete depletion of these checkpoint proteins occurs in a microtubule-dependent manner.

We next tested if Hec1 could bind kinetochores in Nuf2 siRNA-transfected cells. As shown in Figure 3A, Hec1 levels at kinetochores were reduced in Nuf2-depleted cells, and levels did not increase upon the addition of nocodazole. Furthermore, depletion of Nuf2 protein by siRNA transfection resulted in depletion of cellular Hec1 protein (Figure 3B), a result similar to a recent report for Nuf2 and Hec1 in chicken DT40 cells [8]. The low levels of Hec1 in Nuf2 siRNA-transfected cell extracts suggested that normal levels of Hec1, like Nuf2, are not required for Mad1 or Mad2 binding to kinetochores. To confirm this, we treated cells with a Hec1 siRNA [6], which decreased Hec1 cellular protein levels by at least 90% by Western blotting (and also decreased cellular Nuf2 protein levels, Figure 3B) and performed live-cell imaging in cells expressing GFP-hMad2. As we reported above for Nuf2, kinetochores in Hec1 siRNA-transfected, prometaphase-blocked cells exhibited low levels of Mad2 (Figure 3C). Furthermore, the reduction in kinetochore Mad2 levels was microtubule dependent; addition of nocodazole significantly increased the level of Mad2 (Figure 3C). Finally, we coimmunolocalized Mad1 and Hec1 in cells transfected with Hec1 siRNA for 48 hr and found that when prometaphase-blocked cells were treated with nocodazole, kinetochores exhibited high levels of Mad1 (Figure S3).

Nuf2 and Ndc80/Hec1 are highly conserved proteins essential for proper chromosome congression in many cell types [1–9]. In human and chicken cells, depletion of Nuf2 and Hec1 results in a prolonged mitotic block with an activated spindle checkpoint [5, 6, 8] that is dependent on functional Mad2 ([6] and this paper, Figure S1]. We show here that Mad1 and Mad2 bind kinetochores at the onset of mitosis in Hec1 or Nuf2 siRNA-transfected cells but become reduced as cells remain arrested in mitosis. This suggests that the depletion of Nuf2 and Hec1 results in a defect in which the checkpoint proteins Mad1 and Mad2 are prematurely released from unattached kinetochores. A defect of this type has previously been reported for cells expressing a dominant-negative form of Aurora B kinase [17], in which unattached kinetochores prematurely release Mad2 (and also the motors CENP-E and dynein), but upon spindle disassembly, these proteins are able to rebind kinetochores. Our results are not produced by loss of Aurora B kinase binding at centromeres (our unpublished data).

In our Nuf2 and Hec1-depleted cells, checkpoint activation is correlated with persistent low levels of Mad2

standard error. (E) GFP-hMad2 levels increase upon spindle microtubule disassembly. Prior to nocodazole incubation, images were collected of individual cells expressing GFP-hMad2 (top row). The chamber was removed from the microscope stage, and the medium was replaced with medium containing 20 μM nocodazole and returned for image collection at 60 and 120 min (examples of four cells for each condition are shown for Nuf2 siRNA transfection; one example is shown for the control).

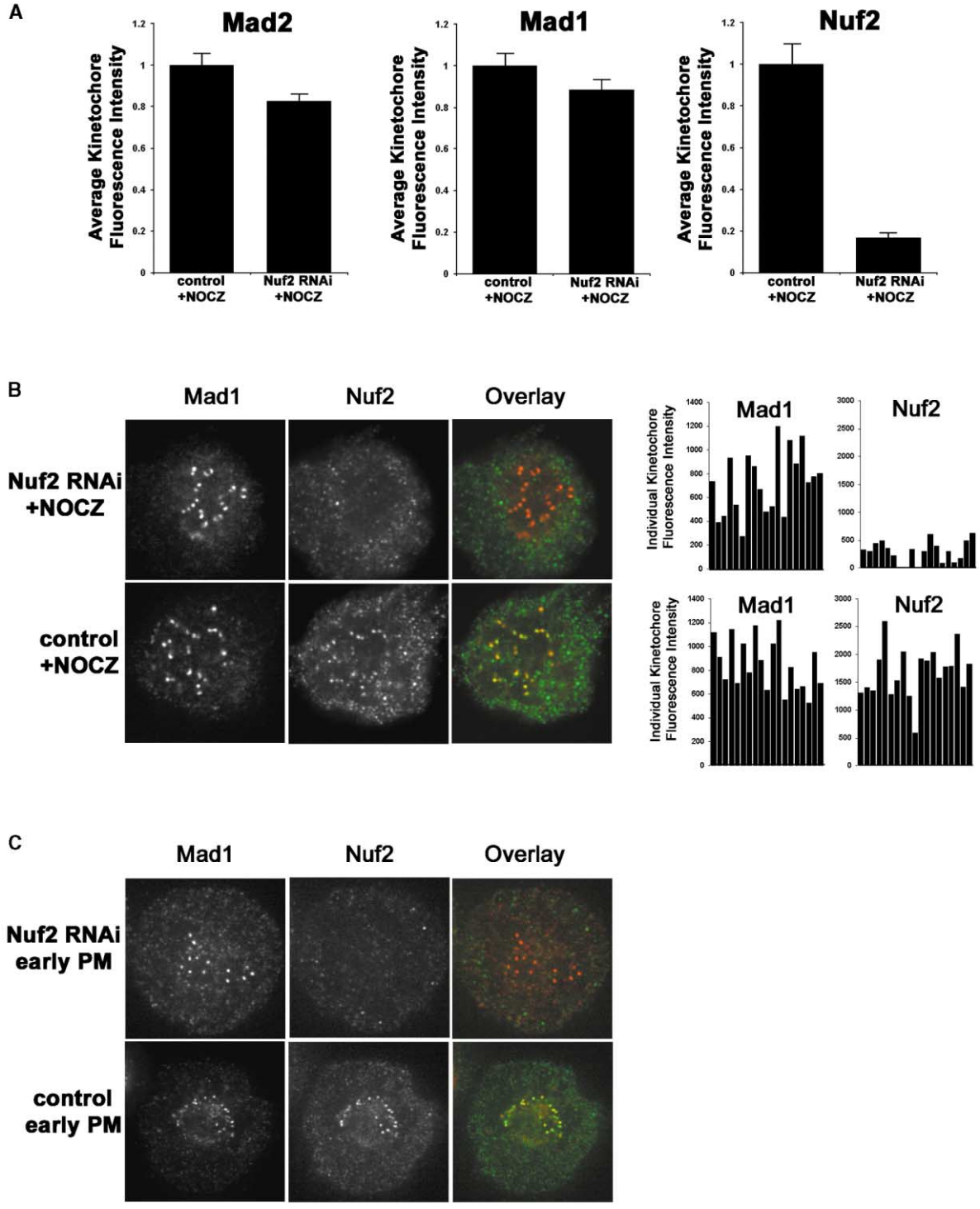


Figure 2. Nuf2 siRNA-Transfected Cells Recruit High Levels of Mad1 and Mad2 in the Absence of Microtubules and in Early Mitosis

(A) The average kinetochore fluorescence intensities of Mad2 (left), Mad1 (center), and Nuf2 (right) were measured in nocodazole-treated, Nuf2 siRNA-, and mock-transfected cells. For each condition, approximately 150 kinetochores from ten cells were measured and averaged [13]. For Mad2 and Nuf2 analysis, cells were costained with CREST and either Mad2 or Nuf2, and ten mitotic cells were chosen at random. For the Mad1 experiment, cells were costained with Mad1 and Nuf2, and only cells depleted of Nuf2 were analyzed. For each graph, the control levels were normalized to one. Scale bars indicate standard error.

(B) Cells with low levels of Nuf2 bind near control levels of Mad1 in the presence of nocodazole. Graphs indicate the individual Mad1 and Nuf2 kinetochore fluorescence intensities of the corresponding cells shown. Values for intensity were determined separately for Mad1 and Nuf2.

(C) Mad1 binds kinetochores in Nuf2 siRNA-transfected cells at early prometaphase in the absence of nocodazole.

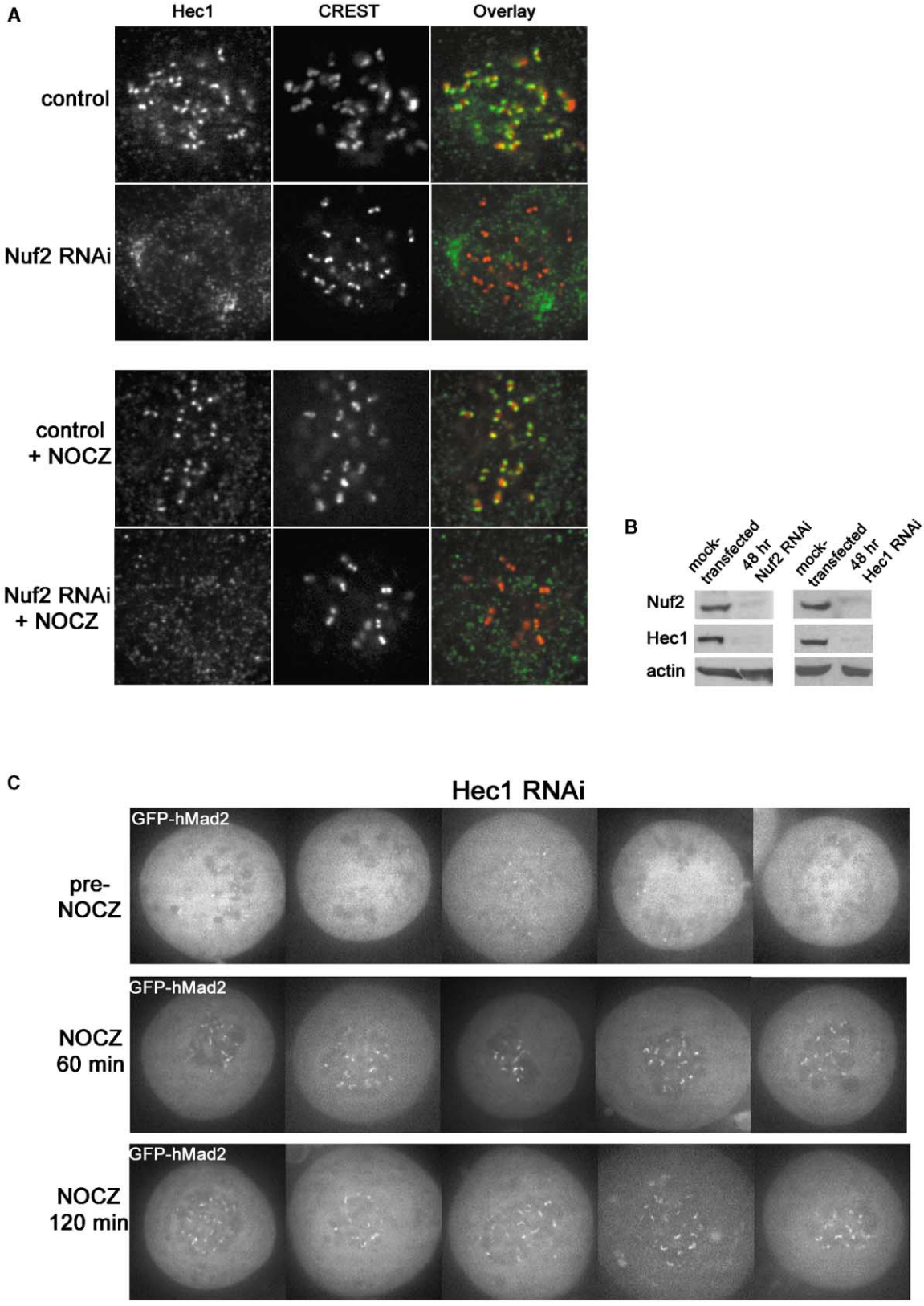


Figure 3. Nuf2 RNAi Results in Hec1 Depletion at Kinetochores and in the Cytoplasm

(A) Hec1 binding to kinetochores is reduced in Nuf2 siRNA-transfected cells in the presence and absence of nocodazole. Cells were transfected with Nuf2 siRNA or mock-transfected and fixed for immunofluorescence at 48 hr.

(B) Hec1 and Nuf2 protein levels are both reduced after transfection with either siRNA. HeLa cells were mock-transfected or transfected with

at many kinetochores. This finding does not prove that kinetochore bound Mad2 is essential for the checkpoint, but it is consistent with such a role. Because it has been shown that a single normal unattached kinetochore with high levels of Mad2 can generate a strong enough signal to prevent APC activation and subsequent anaphase [18–20], it is reasonable to conclude that many kinetochores with low levels of Mad2 can accomplish the same task [21].

It is possible that both checkpoint activity and the low levels of Mad1 and Mad2 at unattached kinetochores in the presence of spindle microtubules depend on the very low levels of Nuf2 and Hec1 at kinetochores in our siRNA-transfected HeLa cells. However, this issue is not resolved. A recent study using genetic methods to disable genes for Nuf2 or Hec1 reported that chicken cells without detectable Nuf2 or Hec1 become blocked in prometaphase with Mad2 at undetectable levels at their kinetochores, but these levels of detectability were not quantified [8]. The checkpoint in fission yeast requires Nuf2 [3], and in budding yeast, *nuf2* and *ndc80* double mutants abrogate the checkpoint but not the single mutants [7]. Because depletion of either Nuf2 or Hec1 by siRNA in HeLa cells results in depletion of both, the strong prometaphase block after their depletion to low levels can't be explained by independent binding of Nuf2 and Hec1 to kinetochores. Using a different approach, McCleland et al. found that microinjection of antibodies to either Nuf2 or Ndc80 into early mitotic *Xenopus* tissue cells not only prevents metaphase chromosome alignment but also inactivates the spindle checkpoint. However, these antibodies could, by binding to Nuf2/Hec1 at kinetochores, affect the function of Mad1 and Mad2 or other kinetochore proteins in *Xenopus* tissue cells that are required for maintaining spindle checkpoint activity [7].

Loss of Mad2 at kinetochores has been commonly used as an indication of kinetochore microtubule formation in mammalian cells [22]. We have previously reported that Nuf2 depletion results in cells that are unable to maintain stable kinetochore microtubules and kinetochore tension [5], and few or no kinetochore microtubules are seen by electron microscopy in Nuf2-depleted cells blocked in prometaphase (B. McEwen, J.G. DeLuca, and E.D. Salmon, unpublished observations). This suggests that in Nuf2 and Hec1-depleted cells, lateral and/or transient, unstable kinetochore microtubules facilitate Mad1 and Mad2 depletion, perhaps mediated by dynein/dynactin function [23, 24]. These interactions are likely;the motor proteins dynein and CENP-E are not inhibited from binding kinetochores in Nuf2 and Hec1 siRNA-transfected cells [5, 6]. In addition, Nuf2-depleted cells induced into anaphase via Mad2ΔC exhibited separation of the chromosome mass (Figure S1), suggesting that at anaphase onset there are interactions between the chromosomes or kinetochores with microtubules. Our results suggest that normal levels of kinetochore Nuf2 and Hec1 prevent Mad1 and Mad2 depletion by lateral interactions with microtubules or by transient kinetochore microtubule formation so that stable kinetochore fiber formation is required for complete dissociation and cessation of the wait anaphase signal.

### Supplemental Data

Supplemental Experimental Procedures and three additional figures are available with this article online at http://www.current-biology.com/cgi/content/full/13/22/2103/DC1/.

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either Nuf2 siRNA (left) or Hec1 siRNA (right), and extracts were prepared at 48 hr and subjected to immunoblot analysis with Nuf2, Hec1, and actin antibodies (to control for gel loading).

(C) Kinetochores in Hec1 siRNA-transfected cells bind increased levels of Mad2 in the absence of microtubules. Cells expressing GFP-hMad2 were transfected with a Hec1 siRNA and imaged at 48 hr. Prior to nocodazole incubation, images of Hec1 siRNA-transfected cells expressing GFP-hMad2 were collected (top row). Images were then collected 60 and 120 min after nocodazole addition; five examples are shown of each.

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