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Report

Aurora B Kinase-Dependent Recruitment of hZW10 and hROD to Tensionless Kinetochores

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Summary

The mitotic checkpoint ensures proper chromosome segregation by monitoring two critical events during mitosis. One is kinetochore attachment to the mitotic spindle, and the second is the alignment of chromosomes at the metaphase plate, resulting in tension across sister kinetochores (reviewed in [1, 2]). Mitotic-checkpoint proteins are known to accumulate at unaligned chromosomes that have not achieved proper kinetochore-microtubule attachments or established an adequate level of tension across sister kinetochores [3]. Here, we report that hZW10 and hROD, two components of the evolutionarily conserved RZZ complex [4, 5], accumulate at kinetochores in response to the loss of tension. By using live-cell imaging and FRAP, we showed that the accumulation of hZW10 at tensionless kinetochores stems from a 4-fold reduction of kinetochore turnover rate. We also found that cells lacking hZW10 escape loss-of-tension-induced mitotic-checkpoint arrest more rapidly than those arrested in response to the lack of kinetochore-microtubule attachments. Furthermore, we show that pharmacological inhibition of Aurora B kinase activity with ZM447439 in the absence of tension, but not in the absence of kinetochore-microtubule attachments, results in the loss of hZW10, hROD, and hBub1 from kinetochores. We therefore conclude that Aurora B kinase activity is required for the accumulation of tension-sensitive mitotic-checkpoint components, such as hZW10 and hROD, in order to maintain mitotic-checkpoint arrest.

Results and Discussion

Low-Dose Taxol Induces a Mitotic-Checkpoint Response Specific to the Loss of Kinetochore Tension

To study mitotic-checkpoint response to the loss of kinetochore tension, we devised an assay that could monitor the response of mitotic-checkpoint components in both fixed and living cells (Figure S1A available online). In short, HeLa cells are first arrested in metaphase with MG132 (a 26S proteosome inhibitor) for 1.5 hr. This ensures that all of the chromosomes have aligned and that full tension is exerted across sister kinetochores. One micromolar of taxol is added for the dampening of microtubule (MT) dynamics, thus reducing kinetochore tension. This concentration of taxol specifically induces the loss of kinetochore tension while retaining kinetochore-microtubule attachments, as outlined in Figure 1A and as shown previously [6]. The loss of tension is confirmed by the measurement of the interkinetochore distance with anti-centromere antibody (ACA) staining. The mean interkinetochore distance in MG132-treated cells was 1.9 ± 0.3 μm (n = 100), whereas in the cells treated with MG132 and taxol, the distance was reduced to 0.9 ± 0.1 μm (n = 101), thus indicating the loss of kinetochore tension. Previous work has shown that hBub1 accumulates at tensionless kinetochores [7]. We therefore quantitated the intensity of kinetochore hBub1 in our tension assay. As a control, we also stained the cells for hMad2, a mitotic-checkpoint protein whose kinetochore localization is sensitive to the loss of kinetochore-microtubule attachment but not directly responsive to the loss of tension [7, 8]. Our results showed that there was an approximately 2-fold increase in the kinetochore signal of hBub1 when tension was reduced, whereas the hMad2 signal remained unchanged (Figure 1B and Figure S1B). We confirmed the data by conducting our tension assay analysis in live cells expressing enhanced green fluorescent protein (EGFP)-hBub1 and mCherry-hMad2. Again we observed EGFP-hBub1 but not mCherry-hMad2 respond to the loss of kinetochore tension (Figures 1D–1F, Movies S1–S3). Although previous studies have shown that Mad2 remains at a few kinetochores in *Potorous tridactylis* (PTK) cells treated with taxol, we did not detect any hMad2 at tensionless kinetochores in HeLa cells [9]. Although we cannot exclude the possibility that some hMad2 that is beyond our detection limit remains at kinetochores, we conclude that our assay monitors mitotic-checkpoint protein recruitment to tensionless kinetochores without disturbing kinetochore-MT attachment.

hZW10 and hROD Respond to the Loss of Kinetochore Tension by Accumulating at Tensionless Kinetochores

Having established our loss-of-tension assay, we next analyzed the loss-of-tension response for hZW10 and hROD. Treatment with MG132 alone lead to low levels of kinetochore hZW10 and hROD when compared to prometaphase, whereas levels of hZwint-1, a structural kinetochore protein, remained constant (Figure 2A). However, in the presence of taxol, we observed a marked increase of kinetochore associated hZW10 and hROD but not of hZwint-1 or hMad2. Kinetochore intensity measurements indicated an approximately 2-fold increase in hZW10 kinetochore signal and an approximately 1.6-fold increase in hROD signal in response to the loss of kinetochore tension (Figure S1C).

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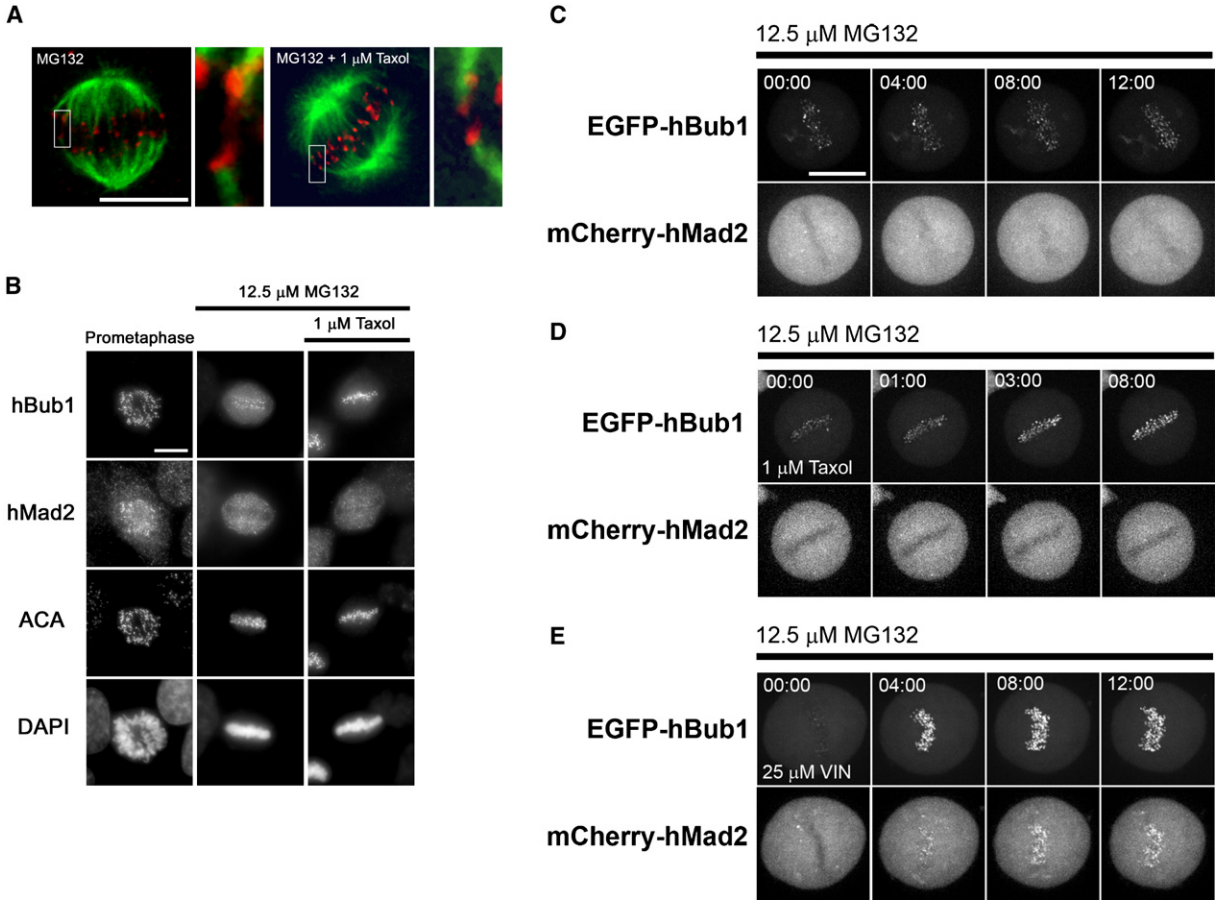

Figure 1. Low-Dose Taxol-Induced Loss of Tension Results in the Accumulation of hBub1 at Tensionless Kinetochores

(A) A representative immunofluorescence image of HeLa cells treated with MG132 or MG132 and taxol, stained with tubulin (green) and ACA (red) antibodies. The insets outline the differences in distance measurements between centromeres in the MG132-treated cells versus cells treated with MG132 and taxol. The scale bar represents 10 μm.

(B) Immunofluorescence images of HeLa cells subjected to the loss-of-tension assay and stained with hBub1, hMad2, and ACA antibodies. Chromosomes were stained with DAPI. hBub1 kinetochore signal is significantly stronger in cells treated with MG132 and taxol, whereas hMad2 signals remain unchanged. The scale bar represents 10 μm.

(C–E) HeLa cells cotransfected with EGFP-hBub1 and mCherry-hMad2 and arrested with MG132 are imaged live with a spinning-disk confocal microscope. Time is indicated in the top right-hand corner as minutes:seconds from the start of imaging. The scale bar represents 10 μm. In the presence of MG132 alone, no significant accumulation of either EGFP-hBub1 or mCherry-hMad2 is observed (C). The addition of taxol only induced EGFP-hBub1 kinetochore accumulation (D). The addition of vinblastine induced both EGFP-hBub1 and mCherry-hMad2 to accumulate at kinetochores (E).

Tensionless kinetochore accumulation of hZW10 and hROD but not hMad2 suggests that although hZW10 and hROD might be required for the recruitment of hMad2 to kinetochores [10, 11], they are not required for the retention of hMad2 at kinetochores that have achieved kinetochore-MT attachments. Although hMad2 is required for maintenance of checkpoint arrest in response to the loss of kinetochore tension [12], our study shows that accumulation of hMad2 at kinetochores is not. This suggests that tension-sensitive kinetochore components, such as the RZZ complex, can signal hMad2-mediated checkpoint arrest without having to recruit hMad2 to kinetochores.

We again confirmed the immunofluorescence data by conducting our tension assay analysis in live cells expressing EGFP-hZW10 and mCherry-hMad2. Similar to EGFP-hBub1, we observed that only EGFP-hZW10 responded to the loss of kinetochore tension, whereas both EGFP-hZW10 and mCherry-hMad2 readily responded to the loss of kinetochore-MT attachments (Figures 2B–2D, Movies S4–S6). On the basis of our findings, we propose that hZW10 and hROD are involved in the mitotic-checkpoint response to the loss of tension, a notion that has been previously suggested solely on the basis of observed hZW10 behavior [5, 13, 14]. We further investigated the role of hZW10 in the mitotic-checkpoint response to the loss of tension by small interfering RNA (siRNA) depletion. Depletion of hZW10 resulted in an approximately 2-fold faster escape from mitotic arrest in the absence of kinetochore tension (taxol arrest escape: 162.3 ± 61.0 min, n = 12) than in the absence of kinetochore-microtubule attachments and tension (vinblastine arrest escape: 292.0 ± 61.4 min, n = 17) (Figure S2 and Movies S7–S11). Mitotic-checkpoint escape was observed as decondensation of chromatin and re-entry into the cell cycle. These results suggest that the accumulation of hZW10 at tensionless kinetochores is essential for the maintenance

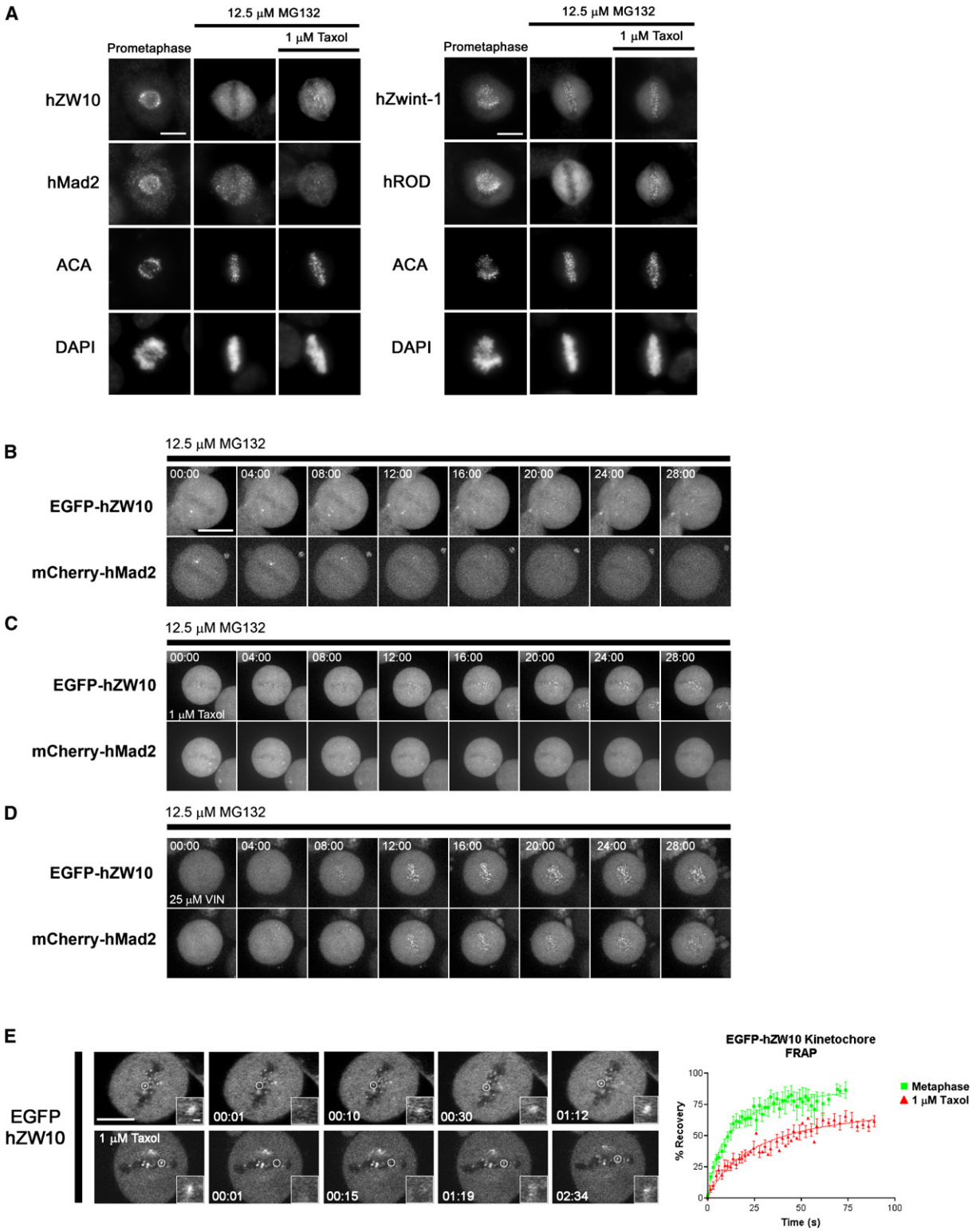

Figure 2. hZW10 and hROD Respond to the Loss of Tension by Accumulating at Tensionless Kinetochores

(A) Immunofluorescence images of HeLa cells subjected to the loss-of-tension assay and stained with hZW10, hMad2, hROD, hZwint-1, and ACA antibodies. Chromosomes were stained with DAPI. hZW10 and hROD are observed to accumulate at tensionless, taxol-treated kinetochores, whereas hZwint-1 and hMad2 kinetochore intensity remains unchanged. The scale bar represents 10 μm.

(B–D) HeLa cells stably expressing EGFP-hZW10 were transiently transfected with mCherry-hMad2, arrested with MG132 for 1.5 hr, and imaged live with a spinning-disk confocal microscope. Time is indicated in the top right-hand corner as minutes:seconds from the start of imaging. The scale bar represents 10 μm. In the presence of MG132 alone, no significant accumulation of either EGFP-hZW10 or mCherry-hMad2 is observed (B). The addition of taxol only induced EGFP-hZW10 kinetochore accumulation (C). The addition of vinblastine induced both EGFP-hZW10 and mCherry-hMad2 to accumulate at kinetochores (D).

of mitotic-checkpoint signaling. Because the depletion of other mitotic-checkpoint proteins has not been analyzed in this manner, we cannot rule out the possibility that the depletion of other mitotic-checkpoint proteins might lead to a faster escape from taxol than vinblastine-induced mitotic arrest. In agreement with previous work, we also observed that hZW10 knockdown only slightly affected the generation of kinetochore tension. The mean interkinetochore distance measured only dropped to 1.7 ± 0.4 μm (n = 42) in the siRNA-treated cells [15]. hZW10 is therefore not essential for the generation of kinetochore tension but is essential for maintenance of checkpoint arrest in its absence.

Dynamitin but Not Dynein Accumulates at Tensionless Kinetochores

hZW10 is known to interact with a subunit of the dynactin complex, dynamitin (hp50), and is responsible for the recruitment of dynein to the kinetochore [16]. We therefore analyzed whether hp50 and dynein also accumulated at tensionless kinetochores. Similar to EGFP-hZW10, we found that YFP-hp50 accumulated at tensionless kinetochores, thus indicating that hZW10 remains in complex with dynactin (Figure S3A and Movie S12). On the other hand, we found that the dynein intermediate chain does not accumulate at tensionless kinetochores generated by either taxol or low-dose nocodazole (Figure S3B). These results indicate that although the localization of dynein to kinetochores might be independent of kinetochore tension, dynactin kinetochore localization clearly increases in response to the loss of tension. The inability of dynein to accumulate at tensionless kinetochores suggests the interaction between dynein and the RZZ complex is inhibited. The accumulation of hZW10 and hROD at tensionless kinetochores might therefore result from the inability of dynein to transport the RZZ complex off kinetochores.

Lack of Kinetochore Tension Reduces the Kinetochore Turnover Rate of hZW10

Having established that hZW10 and hROD localization is sensitive to kinetochore tension, we next analyzed the mechanism responsible for this response. We have recently shown that hZW10 is a stable component of the kinetochore during prometaphase, and a dynamic one during metaphase, depending on bipolar kinetochore-microtubule attachment and chromosome alignment [17]. However, in our tension assay, we observe an accumulation of hZW10 at tensionless kinetochores even though bipolar kinetochore-MT attachment has been achieved. To test whether the accumulation of hZW10 at tensionless kinetochores results from a reduced rate of turnover, we performed fluorescence recovery after photobleaching (FRAP) analysis on kinetochore EGFP-hZW10 in cells treated with MG132 and taxol. Interestingly, we observed a marked decrease in the rate of EGFP-hZW10 kinetochore turnover, with the time required for 50% fluorescence recovery ($T^{1/2}$) of EGFP-hZW10 increasing to 46.0 ± 22.1 s in the taxol-treated cells (n = 13) from that of 12.2 ± 5.3 s in control metaphase cells (n = 13) (Figure 2E). In addition, only 74.9% ± 12.5% of the fluorescence signal was recovered during the 2–3 min of imaging the taxol-treated cells, whereas the normal metaphase recovery was 88.4% ± 9.2%. These findings indicate that although hZW10 still turns over at tensionless kinetochores, the rate of its turnover is reduced nearly 4-fold. Our results are in contrast to that of GFP-tagged dmROD, which is completely transported off kinetochores in the presence of taxol [13]. We attribute this difference in behavior to the fundamental differences between human somatic cell and fly embryo mitosis. Cell division in fly embryos is syncytial and very rapid. The mechanism of the mitotic checkpoint might therefore be substantially different from that in somatic cells. hZW10 is the first example of a mitotic-checkpoint protein whose accumulation at tensionless kinetochores is regulated by its turnover rate.

When analyzing EGFP-hBub1 kinetochore dynamics, we found that the loss of tension had no effect on EGFP-hBub1 kinetochore turnover rate (MG132 $T^{1/2}$ of 14.0 ± 5.3 s, n = 7 versus MG132 + taxol $T^{1/2}$ of 14.8 ± 5.7 s, n = 16) or its percent recovery (MG132% recovery of 94.9 ± 6.3 versus MG132 + taxol % recovery of 90.3 ± 8.8) (Figure S4). This indicates that the mechanism driving the accumulation of hBub1 does not rely on a reduced turnover rate at tensionless kinetochores and therefore differs from that of hZW10. We propose that hBub1 might accumulate at tensionless kinetochores either through oligomerization or through an increase in the number of hBub1 kinetochore binding sites.

hZW10, hROD, and hBub1 Accumulation at Tensionless Kinetochores Requires Aurora B Kinase Activity

Aurora B is believed to be a key component of the mitotic-checkpoint response to the loss of kinetochore tension ([18, 19] and reviewed in [20]). Previous studies have shown that inhibition of Aurora B kinase activity or disruption of the chromosome passenger complex results in the escape from mitotic-checkpoint arrest induced by the lack of kinetochore tension but not by the lack of kinetochore-MT attachments [18, 19, 21, 22]. Aurora B kinase activity has also been shown to be required for the kinetochore recruitment of hBubR1, hCENP-E, and hMad2 [18, 19]. We therefore tested whether there was a connection between Aurora B kinase activity and hZW10 and hROD recruitment to tensionless kinetochores. Aurora B kinetochore localization and dynamics were not affected by the loss of tension, loss of kinetochore MTs, or inhibition of its kinase activity by 2 μM ZM447439, a concentration which has been

(E) Time-lapse series showing EGFP-hZW10 fluorescence recovery after photobleaching at single kinetochores in metaphase (top panels) and when treated with MG132 and taxol (bottom panels). The time scale indicated is in minutes:seconds. The photobleached kinetochores are outlined with white circles and enlarged in the bottom right-hand corner. EGFP-hZW10 is observed to recover 50% at metaphase kinetochores within approximately 10 s, whereas in the presence of MG132 and taxol, 50% EGFP-hZW10 recovery takes significantly longer, approximately 45 s. The large scale bar represents 10 μm, and the small scale bar represents 1 μm. On the right-hand are nonlinear regression curves representing percent recovery of EGFP-hZW10 kinetochore signals shown as averages from several experiments. Recovery in metaphase is shown in green, and recovery in the presence of MG132 and taxol is shown in red.

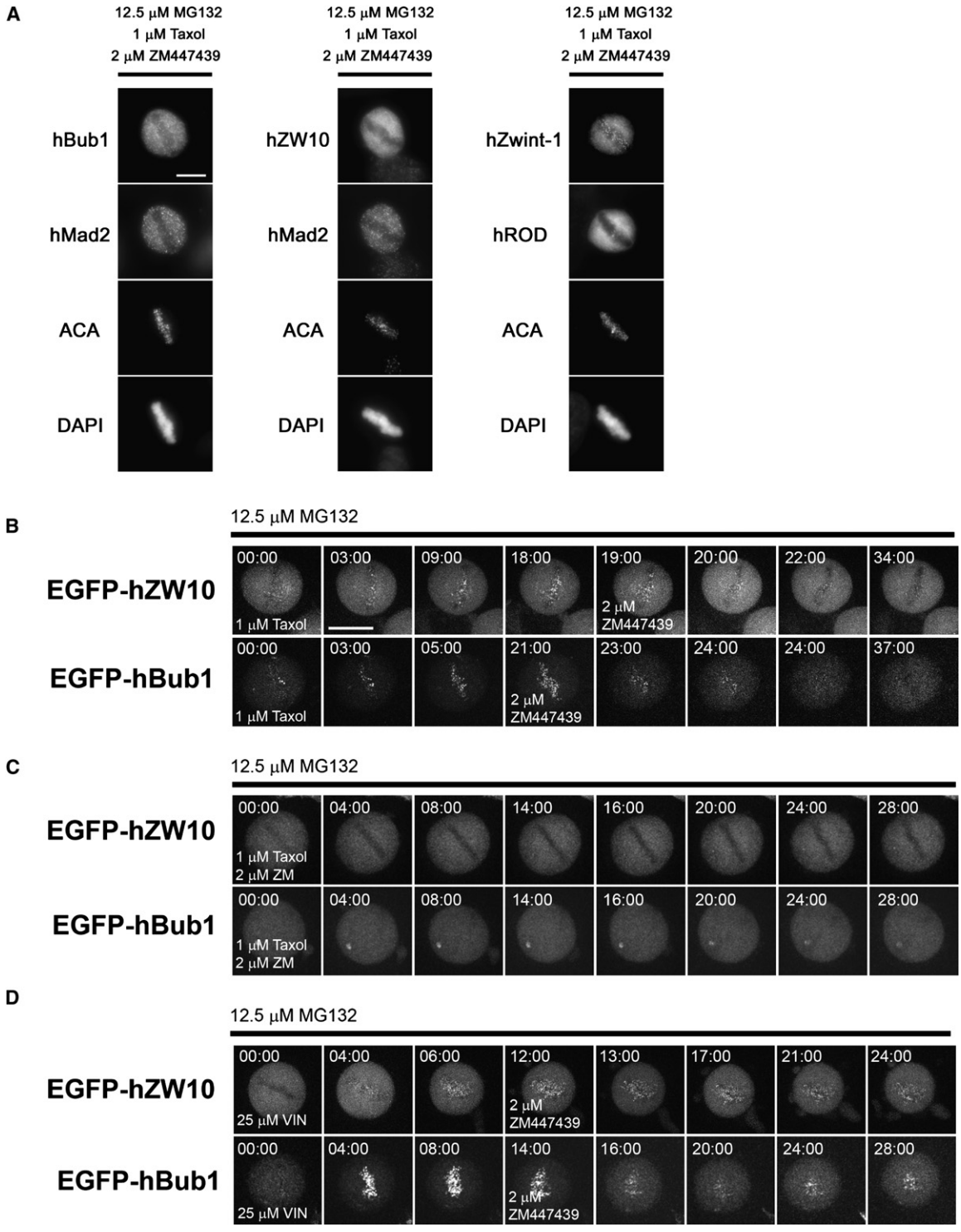

Figure 3. Aurora B Kinase Activity Regulates hBub1, hZW10, and hROD Kinetochore Accumulation during Mitotic-Checkpoint Response to the Loss of Tension

(A) Immunofluorescence images of HeLa cells subjected to the loss-of-tension assay with 2 μM ZM447439 treatment 15 min after the addition of taxol. hBub1, hZW10, and hROD are lost from kinetochores even in the presence of taxol. hZwint-1, Mad2, and ACA, on the other hand, retain their kinetochore localization intensities. The scale bar represents 10 μm.

(B–D) HeLa cells stably expressing EGFP-hZW10 or transiently transfected with EGFP-hBub1 were subjected to the loss-of-tension assay and imaged live. Time is indicated in the top right hand corner as minutes:seconds from the start of imaging. The scale bar represents 10 μm. Two micromolars of ZM447439 was added to the media approximately 20 min after taxol addition (B). Both EGFP-hZW10 and EGFP-hBub1 are observed to initially accumulate in response to the loss of tension; however, upon addition of ZM447439, they are both rapidly lost from kinetochores. When Taxol and ZM447439 are added simultaneously, neither EGFP-hZW10 nor EGFP-hBub1 accumulated at the tensionless kinetochores (C). Vinblastine was added to MG132 arrested cells for approximately

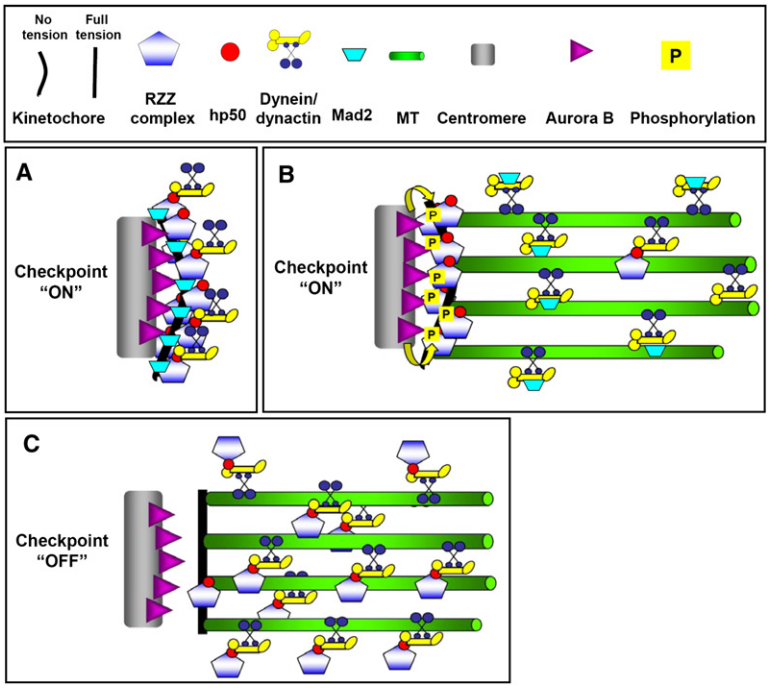

Figure 4. A Model of Aurora B Regulation of the Transport of the RZZ Complex off Kinetochores in Response to the Loss of Kinetochore Tension

(A) The RZZ complex and hp50 accumulate at prometaphase (or vinblastine-treated) kinetochores that lack kinetochore-MT attachments. Dynein is in turn recruited to these kinetochores through the interaction of hp50 and the dynactin complex. The RZZ complex is a stable component of the kinetochore in prometaphase, thus maintaining mitotic-checkpoint signaling.

(B) At kinetochores that have achieved kinetochore-MT attachments but have not established kinetochore tension (curved kinetochore), the kinetochore RZZ complex, in conjunction with hp50, becomes stabilized and thus accumulates at kinetochores. Dynein, however, does not accumulate or stabilize at tensionless kinetochores and continues to cycle, and transport hMad2, off kinetochores by traveling along kinetochore MTs. Dynein/dynactin does not remove the RZZ complex from tensionless kinetochores because at tensionless kinetochores, the Aurora B kinase phosphorylates the RZZ complex. Phosphorylation of the RZZ complex might function to inhibit the interaction between the RZZ complex and dynein. Aurora B phosphorylation thus leads to a reduced turnover rate of the RZZ complex at tensionless kinetochores. The retention of kinetochore RZZ complexes might lead to the retention of other tension-sensitive kinetochore components such as hBubR1 (data not shown), which are also transported by dynein. The retained kinetochore checkpoint components signal hMad2-mediated checkpoint arrest, thus maintaining checkpoint activation.

(C) At kinetochores that are under tension (straight kinetochore), Aurora B is physically removed from phosphorylating the RZZ complex. Dynein/dynactin can now interact with and remove the RZZ complex, and other checkpoint proteins, from kinetochores that have established tension, thus leading to silencing of the checkpoint.

shown not to affect chromatin condensation [19, 23] (Figures S5A–S5C, and S6A and Movies S13–S15). Although 2 μM ZM447439 had no effect on Aurora B kinetochore localization, it did induce mitotic-checkpoint escape from taxol- but not vinblastine-arrested cells (Figure S6A and Movies S16–S18), which is in agreement with previous reports [18, 19]. We next repeated our loss-of-tension assay and inhibited Aurora B kinase by adding 2 μM ZM447439 15 min after taxol treatment. Cells treated with ZM447439 failed to accumulate hZW10, hROD, hp50, or hBub1 (Figure 3A, Figures S3B, S3C, and S6B, and Movie S19). In fact, hZW10 and hROD appeared to be totally absent from kinetochores, whereas hZwint-1 remained largely unaffected. In order to confirm our results we subjected live cells expressing EGFP-hZW10, or EGFP-hBub1, to our tension and ZM447439 assay. As expected, upon the addition of ZM447439, we observed a rapid loss of EGFP-hZW10 and EGFP-hBub1 from the tensionless kinetochores (Figure 3B and Movies S20–S21). In order to investigate whether Aurora B kinase activity is also required for the initial recruitment of EGFP-hZW10 and EGFP-hBub1 to tensionless kinetochores, we added taxol and ZM447439 to the cells at the same time, thus inhibiting Aurora B kinase activity at the time of tension loss. In these cells, we observed no accumulation of either EGFP-hZW10 or EGFP-hBub1 (Figure 3C and Movies S22–S23). Lastly, we found that the addition of 2 μM ZM447439 to cells treated with vinblastine reduced but did not totally eliminate kinetochore EGFP-hZW10 and EGFP-hBub1 (Figure 3D and Movies S24–S25). We therefore presume that Aurora B kinase activity is essential for the initial as well as the continuous kinetochore recruitment of hZW10, hROD, hp50, and hBub1 in response to the loss of tension (taxol treated) but not to the loss of kinetochore-MT attachments (vinblastine treated). The retention of hZW10 and hBub1 at kinetochores lacking tension and kinetochore-MT attachments might therefore explain the inability of ZM447439 to induce mitotic-checkpoint escape from vinblastine-treated cells.

In conclusion, we find that hZW10 and hROD are tension-sensitive components of the mitotic checkpoint and that their accumulation at tensionless kinetochores is regulated by their turnover dynamics in an Aurora B kinase-dependent manner. We propose that Aurora B phosphorylation of the RZZ complex might reduce its kinetochore turnover rate, therefore leading to the accumulation of hp50 and the RZZ complex at tensionless kinetochores (Figure 4). Lowering the kinetochore turnover rate of the RZZ complex might involve modification of the interaction between the RZZ complex and dynein. This could prevent dynein-mediated transport of the RZZ complex, and other essential mitotic-checkpoint components, off kinetochores. Mitotic-checkpoint arrest in

12 min, during which EGFP-hZW10 and EGFP-hBub1 accumulated at kinetochores (D). Once 2 μM ZM447439 was added, a significant amount of EGFP-hZW10 and EGFP-hBub1 was lost from kinetochores upon the addition of 2 μM ZM447439; however, a significant pool still remained.

response to the loss of kinetochore tension would thus be maintained by the prevention of the "shedding" of essential checkpoint proteins from kinetochores, even though bipolar attachment of microtubules has been achieved (Figure 4).

Supplemental Data

Experimental Procedures, six figures, and twenty-five movies are available at http://www.current-biology.com/cgi/content/full/17/24/2143/DC1/.

Acknowledgments

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