

Chromosome instability in tumor cells due to defects in Aurora B mediated error correction at kinetochores

Haomin Huang^a, Michael Lampson^b, Andrey Efimov^a, and Timothy J. Yen ^a

^aCancer Biology Program, Philadelphia, PA, USA; ^bDepartment of Biology, University of Pennsylvania, Philadelphia, PA, USA

ABSTRACT

We characterized a panel of cancer cells and found that they exhibited chromosome instability (CIN) that was associated with high frequencies of aberrant kinetochore: microtubule attachments. Failure to resolve these defective attachments before anaphase onset can lead to missegregation of chromosomes. Aurora B kinase is concentrated at the inner centromere where it contributes to multiple kinetochore functions, one of which is in error-correction. Analysis of several CIN cell lines showed that many aspects of Aurora B kinase functions were normal. Furthermore, the amount and activity of Aurora B kinase was not reduced at the kinetochores of CIN cells that were examined. However, phosphorylation of a centromeric biosensor for Aurora B in OVCAR10, MCF7 and U2OS cells was consistently reduced relative to non CIN cells. This suggested a localized problem with Aurora B's ability to phosphorylate substrates important for error correction. This possibility was supported by our ability to improve error correction and reduce the frequency of lagging chromosome in CIN cells by directing endogenous Aurora B to the region of centromere that was tested by the biosensor. Our studies suggest that the kinetochores of CIN cells have a defect that limits accessibility of Aurora B to substrates that are important for error-correction.

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Introduction

Chromosomal instability (CIN) is a hallmark of many tumors and is a condition whereby cells can missegregate their chromosomes at every division to generate aneuploid cells [1,2]. The CIN condition is highly complex as it is not merely the disruption of chromosome segregation but must include other alterations that allow the tumor cell to tolerate an unstable aneuploid state [3,4]. These properties allow tumor cells to rapidly change gene expression patterns on a global scale and rapidly adapt to adverse growth conditions and likely to survive chemo- and radiotherapy [5–7]. This prompted us to investigate the molecular basis of CIN as such information may reveal novel ways to improve treatment.

There are two types of defects that can cause chromosome missegregation. The mitotic checkpoint ensures that a cell does not prematurely enter anaphase before all of its chromosomes have established bipolar attachments and achieved metaphase alignment. Indeed, disruption of the

mitotic checkpoint will result in chromosome missegregation in vitro and in vivo [7,8]. The mitotic checkpoint is however essential as homozygous mutants are not viable presumably because the cells cannot tolerate massive levels of chromosome missegregation. Cells with reduced expression of mitotic checkpoint proteins (i.e. heterozygous mutants, or hypomorphs) are viable and can become aneuploid if chromosomes fail to properly attach to the spindle in a timely manner. Despite the importance of the mitotic checkpoint, mutations in these genes are infrequent in human cancers and thus may not be the predominant mechanism for chromosome instability [9,10]. Chromosome missegregation can also occur due to defects in the process that correct improper microtubule attachments to kinetochores [11]. A proper attachment is one where the plus end of the microtubule terminates perpendicularly into the face of the kinetochore. Because of the stochastic manner by which kinetochores search and capture microtubules, defective attachments can

CONTACT Timothy J. Yen  timothy.yen@fcc.edu; Haomin Huang  huanghaomin@3sbio.com
 supplementary data for this article can be accessed [here](#).

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sometimes occur. For example, merotelic attachments occur when one sister of a kinetochore pair is attached to microtubules from opposite poles. If unrepaired, merotelic attachments will give rise to lagging chromosomes [2,12–14]. Other defects such as monotelics and syntelics, where only one or both kinetochores are attached to the same pole, respectively, can also occur. The kinetochore therefore possesses an error correction mechanism to resolve these defective attachments, and disrupting this system will result in chromosome missegregation [2,15].

The kinetochore associated error-correction mechanism is centered on the conserved Aurora B/Ipl1 kinase whose function, amongst others, is to sever aberrantly attached microtubules [16–21]. Through an iterative process, bad attachments are ultimately converted to proper attachments. How Aurora B, which is localized at the inner centromere, distinguishes good versus bad is believed to rely on its spatial relationship with substrates that bind microtubules [22–24]. Proper attachments generate poleward forces that physically stretch the kinetochore and displace substrates away from Aurora B. Kinetochores that are not properly attached do not stretch and the microtubule binding proteins remain within proximity of Aurora B where they are phosphorylated and release the defectively attached microtubules. Defective attachments can be induced in normal or non-CIN tumor cells by disrupting microtubule dynamics with drugs or depletion of depolymerases, such as Kif2b and MCAK, which regulate microtubule turnover [2,25]. Indeed, the reduced dynamicity of kinetochore: microtubules in CIN cancer cells is a defect that stabilizes aberrantly attached microtubules and thus interferes with error correction [14]. Furthermore, these errors can be corrected by overexpressing Kif2b or MCAK. These studies suggest an elegant model that explains how kinetochore biorientation is temporally and spatially regulated. During prometaphase, Plk1 activates and recruits Kif2b to kinetochores where it prevents the accumulation of aberrant attachments during congression [26]. Once chromosomes are aligned, Kif2b is released, and MCAK takes over. More recent evidence suggests that a dysfunctional Rb tumor suppressor pathway alters centromeric cohesion [27,28] such that the geometries of the sister

kinetochores favor merotelic attachments [29]. Given that the majority of tumor cells are deficient in the Rb pathway [30], this along with defective repair mechanisms are likely promote chromosome instability.

In this study, we characterized a panel of CIN cancer cells for the integrity of their mitotic checkpoint, spindle organization and kinetochore: microtubule attachments, and found they all shared a problem with correcting defective attachments. We then focused on error correction as this is essential for resolving defective attachments. Given the central role of Aurora B in error correction, we used a previously described FRET biosensor that specifically monitors Aurora B kinase activity at the centromeres on a limited number of CIN cell lines. We detected a slight reduction in phosphorylation of the biosensor that suggested the selective impairment of error-correction and not other Aurora B dependent functions at the kinetochore. This reduction was not due to a lowered amounts or activity of Aurora B at centromeres of CIN cells. Using a construct that can recruit endogenous Aurora B to the region occupied by the biosensor, it was possible to improve error correction and reduce the frequency of lagging chromosomes in CIN cells. The cumulative data suggest that there is a localized kinetochore defect in CIN cells that limits access of Aurora B to substrates that are important for error-correction.

Results

Aneuploid cancer cell lines exhibit chromosome instability (CIN)

OVCAR 3, OVCAR 5 and OVCAR 10 are aneuploid ovarian cancer cells that were derived from patients with varied history and treatment status [31–34]. We first used timelapse microscopy to obtain evidence of chromosome missegregation. As can be seen (Figure 1(a) and B), chromosomes in all three lines were able to achieve metaphase alignment in a timeframe similar to that of HeLa cells. The average times from NEBD to metaphase, and the metaphase to anaphase transition did not differ by more than 10 minutes amongst the four cell lines. The ovarian cancer cells did not exhibit noticeable kinetochore attachment defects that would have significantly delayed mitotic

progression. However, a high percentage (55–80%) of the ovarian cancer cells exhibited what appeared to be lagging chromosomes in anaphase when compared to HeLa cells (5%) or HCT116 (Figure 1(c) and data not shown).

The presence of lagging chromosomes suggests the failure to resolve aberrant kinetochore attachments prior to anaphase onset. We therefore used deconvolution microscopy to examine the kinetochore-microtubule attachments of metaphase aligned chromosomes in the ovarian and in other cancer cells. The proteasome inhibitor MG132 was used to prevent cells from exiting mitosis and thus ensure cells had sufficient time to reach metaphase. Examination of HeLa and HCT116 cells showed that 95% of the kinetochores were bioriented as they exhibited proper “end-on” microtubule attachments (Figure 2(a), inset and B). By contrast, multiple types of defective attachments were commonly observed in cancer cells from ovary (OVCAR3, 5, 10, A1847, SKOV3, PEO1), breast (MCF7), colon (HT29 and Caco2) and bone (U2OS). The defects included merotelic (one sister attached to both poles), monotelic (attached to one pole), syntelic (both sisters attached to the same pole), and connections with the lateral surface of the microtubule were observed (Figure 2(a), insets). The frequencies of aberrant attachment ranged from 20% to over 40% in these cells (Figure 2(b)).

As not all lagging chromosomes result in their missegregation [35], we used fluorescence *in situ* hybridization (FISH) to directly monitor chromosome segregation in newly divided cells (Figure 3). To accomplish this, we seeded mitotic shakeoffs (no drug treatments) onto slides and allowed them to divide before fixing. This approach allowed us to directly visualize segregation events in individual dividing cells. Only 0.5% of the HeLa cells, which are not classified as CIN (Macville et al., 1999), and 0.3% of the diploid HCT116 colorectal cancer cells missegregated the chromosomes that were examined. Nearly 10-fold higher numbers of OVCAR10 (3%) and MCF7 (2%) cells exhibited missegregation. These missegregation frequencies were comparable to U2OS osteosarcoma cells that are classified as CIN. OVCAR3 and 5 exhibited similar frequencies of between 2–3% (data not shown). The missegregation frequency is likely

higher as we only tracked only one or two specific chromosomes.

We tested the integrity of the mitotic checkpoint in the OVCAR 3, 5, and 10 cells with spindle poisons, nocodazole and taxol (Figs. S1 and S2). Timelapse studies showed that under normal growing conditions, cells completed mitosis in ~50 minutes. In the presence of drugs, over 90% of the cells were delayed for over 50 minutes, with over 50% of the cells delayed for > 500 minutes. In all cases, the cells either died while arrested in mitosis or exited mitosis. The duration of the mitotic delay was similar to that of the checkpoint proficient HeLa cells. Thus, OVCAR 3, 5, and 10 cells exhibit a proficient mitotic checkpoint.

A possible source for CIN is multipolar spindles derived from multiple centrosomes [36]. Multipolar spindles establish many aberrant kinetochore attachments that have been argued to exceed the capacity of the error correction mechanism. These aberrant attachments persist after the multipolar spindle coalesces into a bipolar spindle. We stained OVCAR3, 5, 10, MCF7 and normal RPE1 cells with γ -tubulin antibodies and counted the number of centrosomes in cells that were in mitosis (images not shown). 1% of RPE cells had more than 2 centrosomes. Between 5–6% of mitotic OVCAR3, 5, 10 and MCF7 cells had greater than 2 centrosomes. The 5–6-fold increase however, cannot account for the high number of cells that exhibited aberrant attachments as described above.

Aurora B kinase functions are largely intact during mitosis

The stochastic nature by which kinetochores encounter microtubules can occasionally result in non-productive attachments. AuroraB/Ipl1 kinase plays a central role in error correction by promoting the release of microtubules that are not properly attached to kinetochores [16–20]. We examined the expression of Aurora B and its associated subunits in the chromosomal passage complex (CPC) in OVCAR3, 5, 10 and MCF7 cells. Western blots of mitotic lysates showed that CPC components (Aurora B, INCENP and survivin) were expressed to comparable levels in all the cells examined (Fig. S3A). In addition, the presence of the activating phospho-T232 within the

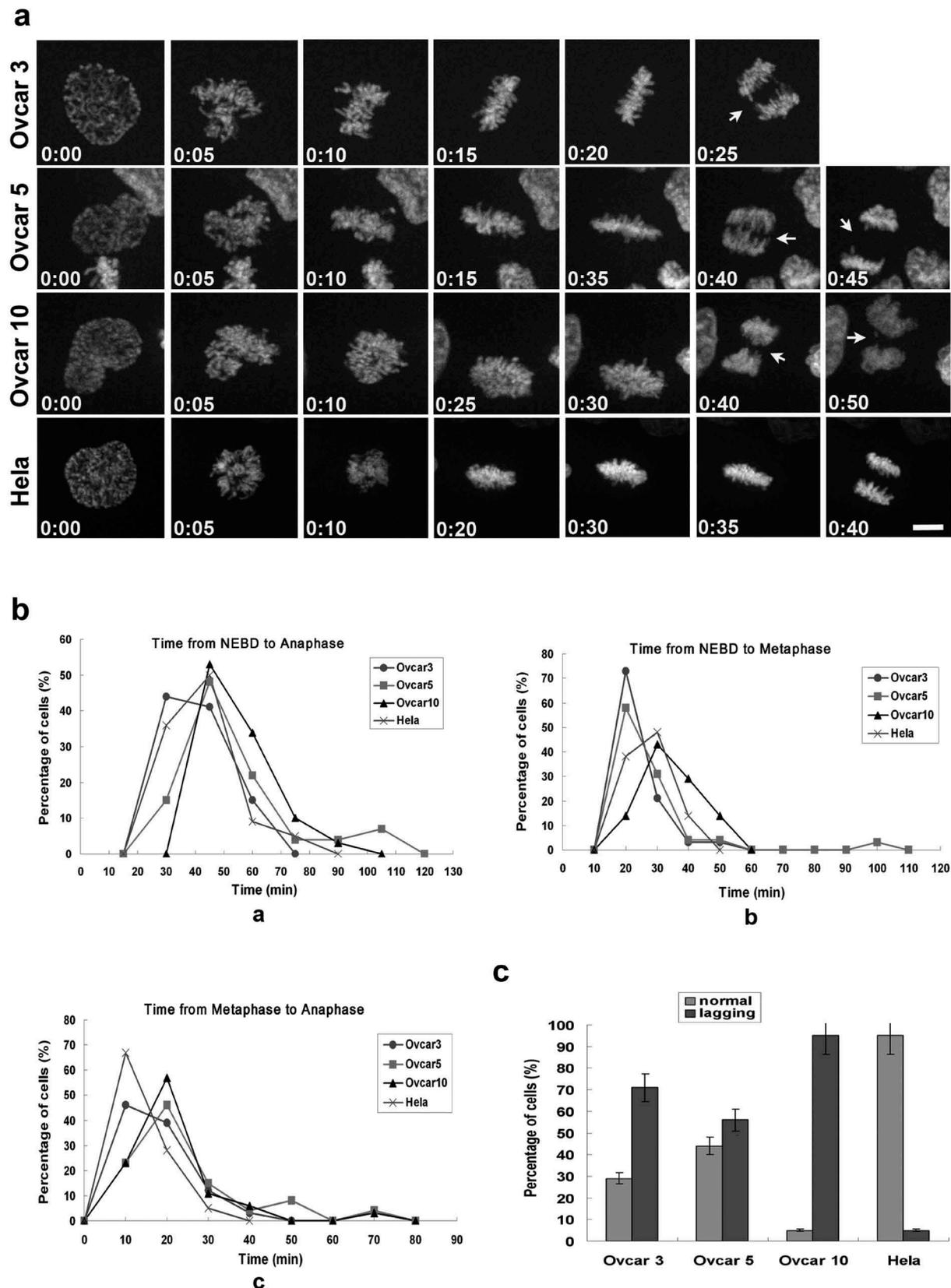


Figure 1. Ovarian cancer cells exhibit high incidences of lagging chromosomes. (A) Select confocal images from timelapse studies of HeLa, OVCAR3, 5 and 10 cells stably expressing H2B:GFP. Times in hours:minutes are shown. Arrows denote lagging chromosomes. Bar. 10 μ m. **(B)** Kinetics of mitotic progression was determined from time-lapse data. Data points were collected from >20 cells per experiment and the experiment was performed 3 times for each cell line. The percentages of cells undergoing the different stages of mitosis were plotted as a function of time. a) The time of anaphase onset was from NEBD to chromatid separation. b) The time from NEBD to metaphase was determined when all the chromosomes aligned at the cell equator. c) The time from metaphase to anaphase was determined as in "a". Purple, HeLa; Blue, OVCAR3; Red, OVCAR5; Black, OVCAR10. Error bars represent the SEM from three independent experiments. **(C)** Fraction of cells from time-lapse studies that exhibited lagging chromosomes in anaphase.

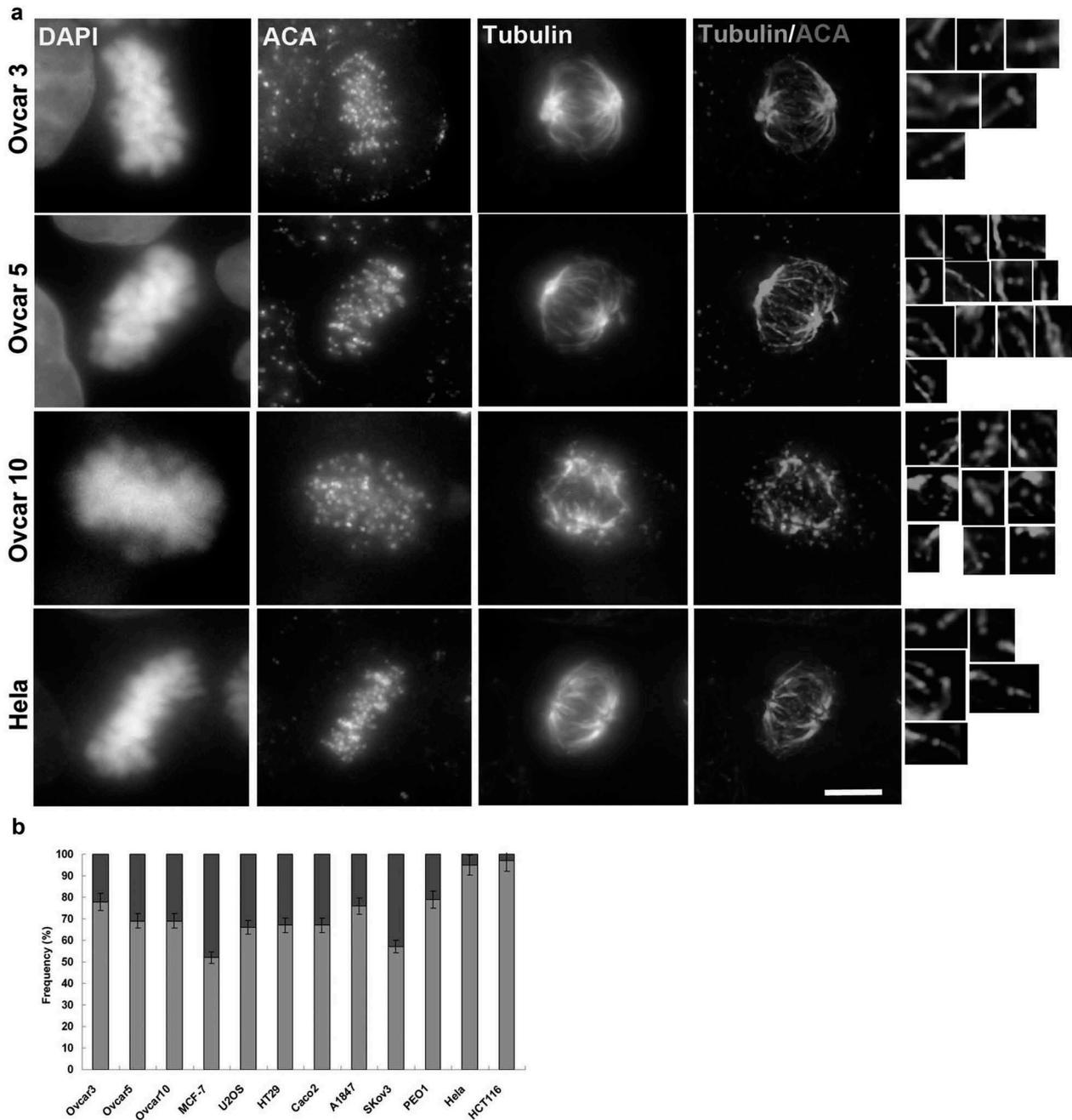


Figure 2. Kinetochores of CIN cancer cells accumulate defective attachments. (A) Cells were arrested at metaphase with MG132, cold-treated to enrich for kinetochore microtubules, and fixed and stained with ACA (red) and tubulin (green) antibodies. Images are from maximum projections of z-stacks (0.5um slices). Bar. 10um. Insets are deconvolved images from a single optical slice. **(B)** Comparison of the fraction of metaphase cells for each of 12 cell lines that exhibit defective attachments (red bars) and normal attachments (blue bars). N = 60–100 kinetochores examined.

T-loop of Aurora B's catalytic domain indicated that it is an active kinase during mitosis. This is supported by the presence of phospho-S10 in histone H3, a major *in vivo* substrate of Aurora B (Figs. S3A and C). MCAK, a microtubule depolymerase that contributes to error correction by promoting the release of

aberrantly attached microtubules [14], is also expressed in the cell lines examined (Fig. S3A).

Quantitation of Aurora B, and one of its substrates, pS7 CENP-A, showed that their levels at kinetochores, after normalization with ACA, were actually higher in the CIN cell lines than normal

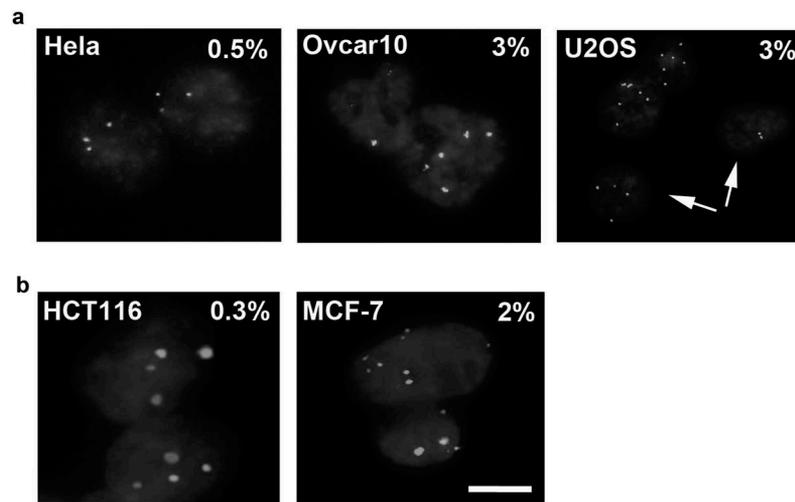


Figure 3. Frequency of chromosome missegregation as determined by FISH. Mitotic cells were collected, replated and allowed to divide before fixing and processing for FISH. Bar. 10 μ m. FISH was performed with a-satellite probes specific for chromosome 7 (green) and chromosome 3 (red). Progeny cells that shared the same number (1:1 segregation) of FISH signals were compared to those that showed unequal FISH signals (deviations from 1:1). % of all divided cells that exhibited deviations from 1:1 for each cell line was calculated from counting >300 pairs of divided cells. HeLa and HCT116 are non-CIN cells. U2OS, OVCAR10 and MCF7 are CIN cells.

RPE1 cells (Fig. S3B, D). Thus, the error-correction defects of OVCAR3, 5, 10 and MCF7 cells cannot be simply explained by loss of Aurora B kinase activity.

Beyond its role in error correction, Aurora B is required for other essential kinetochore activities that include centromere cohesion, microtubule binding and the spindle checkpoint [37,38]. All of these functions appear intact in these CIN cells because their chromosomes were able to achieve metaphase alignment within a normal timeframe. Furthermore, their ability to complete cytokinesis also indicated that Aurora B functions were not grossly perturbed during mitotic exit. This is further supported by the fact that proteins such as Hec1/Ndc80 (Fig. S4A) together with MCAK, BubR1 and Sgo2 (data not shown) whose localization at kinetochores depends on Aurora B [39–45], were all present in the CIN cell lines examined. In addition, phosphorylation of histone H3T3 by the Haspin kinase [46] that is critical for the recruitment of CPC to kinetochores [47] was also present at kinetochores of these CIN cells (Figs. S4A). This is consistent with the presence of Aurora B (see above) as well as TD60, which is another CPC subunit, at the kinetochores of the CIN cells that were examined (Fig. S4B). Beyond this, other essential kinetochore proteins such as CENP-F, Bub1, Plk1, were also present at the kinetochores of these CIN cells (Figs.

S4B and C). We then conducted a functional test for Aurora B by treating the OVCAR 3, 5 and 10 cells with a kinase inhibitor (Hesperadin) or with a siRNA. In all cases, the treated cells exhibited mitotic defects consistent with loss of Aurora B functions [37,38] (Figure 4(a,b)). The cumulative data show that many Aurora B functions are intact in CIN cells. This left open the possibility that the defect may be restricted to a subpopulation of Aurora B that is responsible for error correction.

Reduced phosphorylation of an aurora b biosensor at the kinetochores of OVCAR10, MCF7 and U2OS cells

We next examined the ability of Aurora B that is present at the centromeres of OVCAR10, U2OS, MCF7 and HeLa cells to phosphorylate a previously described FRET biosensor used to demonstrate tension sensitive phosphorylation of kinetochore substrates by Aurora B [23,48]. The biosensor consists of CFP donor and YFP acceptor, the centromere targeting domain of CENP-B, and an Aurora B substrate peptide that is connected to a FHA2 phospho-binding domain via a flexible linker (Figure 5(a)). Maximal FRET (emission ratio of YFP:CFP is high) occurs when the sensor is unphosphorylated. Phosphorylation of the

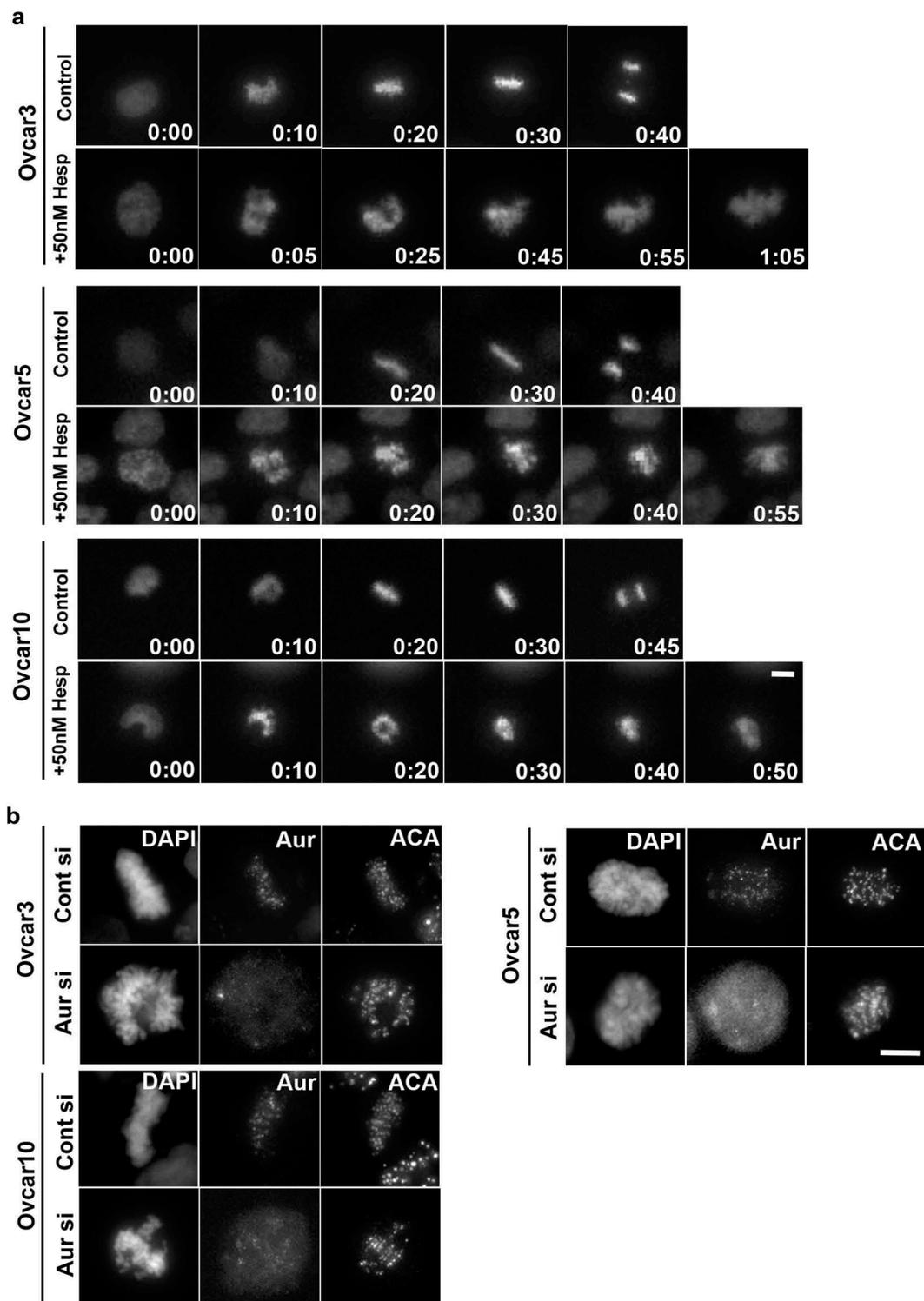


Figure 4. CIN tumor cells are sensitive to Aurora B inhibitors. (A) Select frames from timelapse movies of OVCAR3, 5 and 10 cells expressing H2B:GFP with or without 50nM Hesperadin. **(B)** OVCAR3, 5 and 10 cells transfected with control and Aurora B siRNA were fixed and stained for AuroraB and ACA to reveal defects in chromosome alignment. Bar. 10μm.

sensor by Aurora B, as in the case when kinetochores lack attachments, induces a conformational change that reduces FRET (emission ratio of YFP:CFP is low). This biosensor was previously used to

monitor Aurora B kinase activity at kinetochores and to show that its ability to phosphorylate its substrates was spatially regulated by microtubule attachment status [23]. OVCAR10, U2OS, MCF7

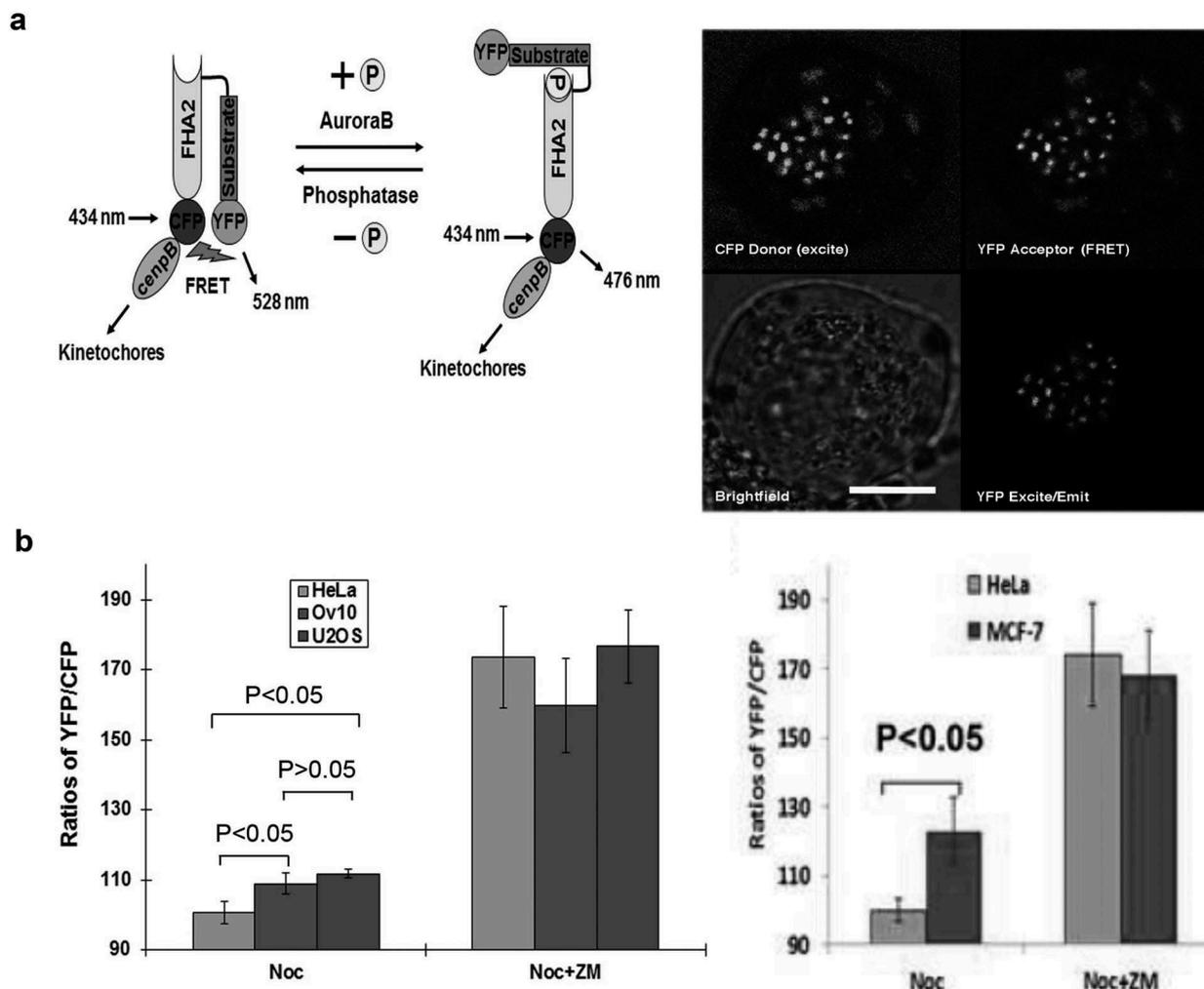


Figure 5. Assessing Aurora B kinase activity at centromeres of CIN cells. (A) Schematic of the biosensor undergoing conformational changes upon phosphorylation by Aurora B. The unphosphorylated biosensor assumes a conformation that produces maximum FRET. **(B)** A live mitotic HeLa cell transfected with the biosensor and CFP was excited (blue) and FRET was detected by emission of the YFP (yellow) at kinetochores. Bar, 10 μ m. **(C)** HeLa, OVCAR10, U2OS (left) and HeLa, MCF7 cells (right) transfected with the biosensor were treated with nocodazole (Noc), or nocodazole + ZM447439 (Noc+ZM), and the YFP/CFP emission ratio was calculated. Increased emission ratio indicates unphosphorylated sensor. The average emission ratio was obtained after measuring the emissions of >300 kinetochores from 10–15 cells (~20–30 kinetochores measured/cell). Error bar, Mean \pm SEM. P values were determined by Student's t-test.

and HeLa cells were transfected with the sensor and blocked in mitosis with nocodazole to prevent microtubule assembly (this should lead to phosphorylation of the biosensor and low FRET). To confirm that the biosensor was phosphorylated by Aurora B, a parallel sample was treated with an Aurora kinase inhibitor (ZM447439). **Figure 5(b)** shows a representative image of the FRET signal from the biosensor (YFP emission) after excitation of the CFP in nocodazole arrested mitotic cells that were treated with ZM447439. As expected, the FRET signals for the different cell types were

highest in the presence of ZM447439 when compared to the nocodazole alone samples (**Figure 5(c)**). The reduced FRET seen in the nocodazole treated samples is therefore due to phosphorylation of the biosensor by Aurora B kinase. Amongst the nocodazole treated samples, the FRET signals for the OVCAR10, U2OS and MCF7 cells were always slightly higher than HeLa cells. Statistical analysis (Student T-test) showed that the difference between HeLa cells and each of the CIN cell lines was significant ($p < 0.05$), while the difference between the CIN cell lines was not ($p > 0.05$).

This suggested that the biosensor is less efficiently phosphorylated by Aurora B in the CIN cells versus non-CIN cells. The higher amounts of unphosphorylated biosensor at the kinetochores of CIN cells would produce the higher FRET signal relative to a more extensive phosphorylation of the biosensor in HeLa cells.

Endogenous Aurora B can restore the attachment defects and reduce lagging chromosomes of the CIN cells

The reduced phosphorylation of the biosensor in CIN cells might reflect a local problem whereby Aurora B was less able to access substrates that are critical for error correction. We therefore attempted to rectify this deficit by recruiting Aurora B closer to its targets by using a CENP-B:INCENP:mCherry fusion construct [23]. This construct is targeted to centromeres through CENP-B, and uses INCENP (lacking its own centromere targeting domain) to recruit endogenous Aurora B. As the CENP-B targeting domain is identical to what was used for the biosensor, we expected Aurora B to localize to areas that should improve access to defective attachments. OVCAR10 and MCF7 cells were transfected with CENP-B:INCENP:mCherry construct or one lacking INCENP, and their kinetochore: microtubule attachments were examined after arresting cells at metaphase with MG132. Comparison of the mcherry positive kinetochores between OVCAR10 cells that were transfected with the two constructs showed that the frequency of biorientation was improved with the CENP-B:INCENP:mCherry construct (Figure 6(a,b)). In some OVCAR10 cells, nearly all the kinetochores expressing CENP-B:INCENP:mCherry appeared to be properly bioriented. OVCAR10 cells expressing CENP-B:INCENP:mCherry showed a near 5-fold reduction (25% vs. 5%) in the number of defective attachments than the cells transfected with just the CENP-B:mCherry construct (Figure 6(b)). A 2–3 fold reduction in defective attachments was seen in MCF7 cells that expressed CENP-B:INCENP:mCherry (Figure 6(b)). We note that not all cells expressing CENP-B:INCENP:mCherry had normal attachments. Cells expressing high levels of CENP-B:INCENP:mCherry at the kinetochores invariably had fewer attached kinetochores (data not shown). This was expected if Aurora B is chronically phosphorylating

and severing microtubule attachments as has been shown in HeLa cells [23]. These results demonstrated that endogenous Aurora B is fully functional in OVCAR10 and MCF7 cells, and the defect in error correction is consistent with its inability to access its targets.

Next, we wanted to confirm that directing functional endogenous AuroraB to the kinetochores of CIN cells could increase the phosphorylation of the FRET biosensor. The CENP-B:INCENP:mCherry or CENP-B:mcherry constructs were co-transfected with the FRET biosensor into HeLa, OVCAR10 and MCF7 cells. FRET was performed exactly the same as described above on kinetochores that were positive for mcherry and CFP. Cells were blocked in mitosis with nocodazole and in the presence and absence ZM447439. All of the ZM447439 treated samples exhibited strong FRET because Aurora B was inhibited and thus the biosensor was unphosphorylated (Figure 6(c), purple and green bars). In the nocodazole alone treatment, HeLa, OVCAR10 and MCF7 cells co-transfected with the biosensor and the CENP-B:mcherry (Figure 6(c), blue bars) exhibited FRET signals comparable to what was reported in Figure 5 for biosensor alone. HeLa cells showed the strongest phosphorylation (low FRET) of the biosensor relative to OVCAR10 and MCF7 cells (Figure 6(c), blue bars). Importantly, for cells co-transfected with the CENP-B:INCENP:mCherry, the biosensor, in all cases, showed increased phosphorylation (lower FRET) when compared to the CENP-B:mcherry control (Figure 6(c), compare red vs blue bars). Student T-test showed that the differences in HeLa ($P < 0.05$), OVCAR 10 and MCF7 were significant ($P < 0.01$). This data directly demonstrates that endogenous Aurora B can increase phosphorylation of the biosensor in OVCAR10 and MCF7 cells if it is relocated closer to the biosensor. By extension, the relocalization of Aurora B by the CENP-B:INCENP:mCherry construct can explain the improved attachment status of kinetochores in these two CIN cell lines.

The ability to restore the integrity of kinetochore microtubule attachments prompted us to test whether this strategy could be used to reduce the incidence of lagging chromosomes of CIN cells. CB:INCENP:mcherry and CB:mcherry were transiently transfected into OVCAR10 cells that stably expressed H2B:GFP so that chromosome segregation could be monitored in real-time (Figure 6(d)).

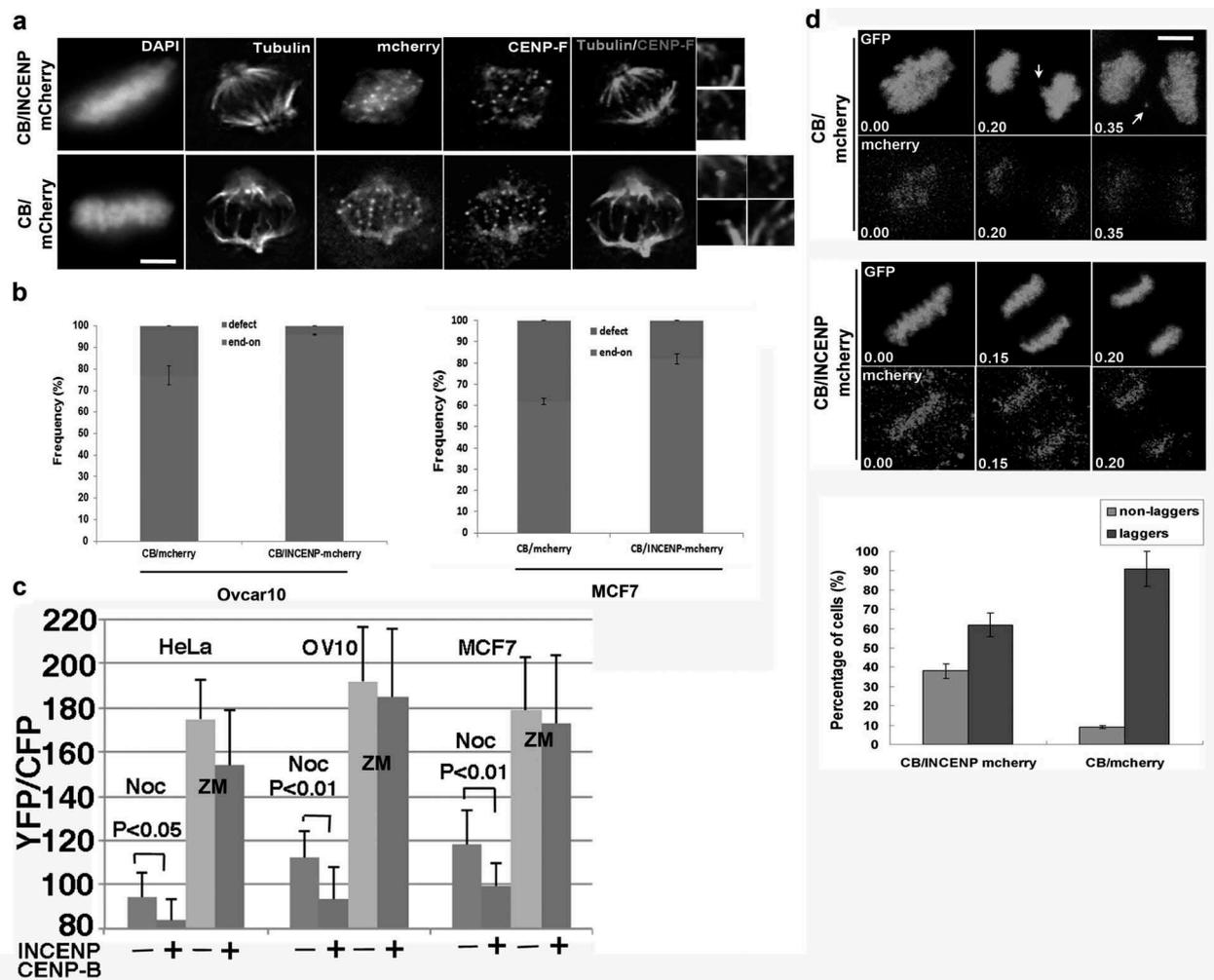


Figure 6. Improving error-correction in Ovarcar10 and MCF7 cells. (A) OVCAR10 cells transfected with CB:INCENP:mcherry or CB:mcherry were stained for tubulin (green), CENP-F (red). DNA was stained with DAPI. Images from a single optical slice are shown. Bar, 10 μ m. Insets show microtubule attachment geometries at individual kinetochores. (B) Comparison of the frequency of defective attachments in OVCAR10 (left panel) and MCF7 cells (right panel) transfected with the indicated constructs. ~10 cells, each with >30 well defined kinetochore pairs in each cell were analyzed. (C) CB:INCENP:mcherry improves Aurora B phosphorylation of FRET sensor. Analysis of nocodazole arrested mitotic HeLa, OVCAR10 and MCF7 cells were co-transfected with CB:INCENP:mcherry (+) or CB:mcherry (-) and the biosensor, and FRET (YFP/CFP emission ratio) analysis was performed and compared. Inhibiting Aurora kinase with ZM447439 (ZM) treatment gave the highest FRET for cells transfected with either CB:INCENP:mcherry (purple) and CB:mcherry (green). In nocodazole alone samples, when Aurora B kinase at kinetochores should be high, cells transfected with CB:INCENP:mcherry (red) showed reduced (stronger phosphorylation of biosensor) FRET relative to CB:mcherry (blue). The average YFP/CFP emission ratio was obtained from the emissions collected from 150–250 kinetochores from 10–20 mitotic cells. P values were determined by Student's t-test. (D) Select frames from videos of H2B:GFP expressing OVCAR10 cells transfected with CB:INCENP:mcherry (n = 11) or CB:mcherry (n = 12). Arrows indicate lagging chromosomes. Bar, 10 μ m. Frequency of the transfected mitotic cells that showed lagging chromosomes. Blue, normal; Purple, lagging chromosomes. All error bars, Mean \pm SEM.

Only 9% of the mitotic cells that expressed CB:mcherry at their kinetochores divided without evidence of lagging chromosomes. In contrast, 38% of the cells transfected with CB:INCENP:mcherry divided without lagging chromosomes (Figure 6 (d)). This result suggests that with the improvement in the frequency of biorientation by the CB:INCENP:mcherry construct, the frequency of lagging

chromosomes and by extension, chromosome mis-segregation was reduced.

Discussion

We have characterized a panel of tumor cell lines and presented evidence that show that they exhibit chromosome instability. Chromosome instability in our

panel of CIN cells was not due to defects in the mitotic checkpoint as they are able to delay mitosis (12--18 hours) when treated with the spindle poisons, nocodazole and taxol. Although experimental disruption of the mitotic checkpoint functions will lead to aneuploidy, this pathway is not commonly targeted by tumor cells to achieve CIN [9,10]. Kinetochore attachment defects have also been proposed to result from an extended mitotic delay as a result of overexpression of key mitotic checkpoint proteins [7]. In the CIN cell lines that we studied, the timing of mitosis was not delayed when compared to HeLa cells. The CIN cells were able to reach metaphase and enter anaphase within the same timeframe as HeLa or HCT116 cells. This is consistent with studies of many other CIN cell lines where mitotic timing and spindle checkpoint functions were intact [49,50].

Chromosome instability is largely the result of defective microtubule attachments [14,25,36,51]. Those studies showed that CIN cancer cells routinely accumulate defective attachments that can result from reduced kinetochore: microtubule dynamics or from multipolar spindles. In both cases, the failure to resolve the defective attachments before anaphase onset will cause chromosome missegregation. In the CIN cells that were examined in this study, all of them accumulated a variety of defective attachments that failed to be resolved before anaphase onset. These defects can explain the increase in frequency of lagging chromosomes that were observed. The defective attachments are not monitored by the spindle assembly checkpoint as this failsafe system only recognizes kinetochores that have unoccupied microtubule binding sites. The mechanism that distinguishes good versus bad attachments is mediated by a pool of Aurora B kinase that is concentrated at the inner centromere. Defective microtubule attachments do not generate tension that is capable of spatially separating the microtubule binding proteins away from Aurora B. Subsequent phosphorylation of these proteins by Aurora B promotes the release of the microtubules. In the case of a productive attachment that generates kinetochore tension, the microtubule binding proteins become physically separated from the negative influences of Aurora B.

Western blots and immunofluorescence staining showed that Aurora B along with its subunits in the Chromosome Passenger Complex (CPC) were expressed and localized at centromeres of the CIN

cells examined in this study. In addition, the levels of phosphoS7 CENP-A, a substrate of Aurora B, was also not noticeably reduced in CIN cells relative to non CIN cells. While it is difficult to accurately compare the staining intensities of Aurora B and other proteins across cell lines, there must be sufficient amounts of these proteins to provide critical kinetochore functions. Aurora B provides functions essential for chromosome congression and alignment, mitotic checkpoint and cytokinesis [37,38]. Our timelapse studies did not reveal noticeable defects in these activities that would indicate such Aurora B deficiencies. Indeed, the cells we tested were still sensitive to Aurora inhibitors as they exhibited all of the defects associated with loss of Aurora B functions. That Aurora B was functional was also supported by the observations that proteins such as Tripin/Sgo2 [45], BubR1 [42,43] and MCAK [40,41], whose localization at kinetochores depend on Aurora B, were present in the cell lines that were examined. Finally, FRET biosensor experiments detected Aurora B kinase activity at the kinetochores of OVCAR10, MCF7 and U2OS cells, albeit slightly reduced from HeLa cells. The cumulative data suggest that Aurora B kinase is largely functional in the CIN cells that were examined, and defective kinase activity cannot be the basis for CIN. This is perhaps expected given the essential roles that are played by Aurora B at the various stages of mitosis.

The molecular defect that prevents CIN cells from recognizing and repairing defective attachments remain to be identified. The small but statistically significant reduction in the phosphorylation of the biosensor in the CIN cells that were examined, relative to the signal observed for HeLa cells suggests a localized defect. As nocodazole was used to block microtubule assembly, the difference in FRET is unlikely due to differences in microtubule attachments at the kinetochores amongst these cells. When we used a CENP-B:INCENP construct to recruit Aurora B to the same location that was occupied by the biosensor, the frequency of biorientation in OVCAR10 and MCF7 cells was improved. The CENP-B:INCENP construct likely recruited Aurora B to sites that were critical for error correction. Relocalization of Aurora B towards the kinetochore in HeLa cells has been shown to prevent stable attachments owing to the persistence of kinase activity near the attachment sites [23]. For OVCAR10 and MCF7 cells, the

change in the amount or location of Aurora B probably enhanced the destabilization of microtubules which would then provide new opportunities to achieve biorientation. It is noteworthy that overexpression of either MCAK or Kif2b in MCF7 cells reduced the incidence of lagging chromosomes. Given that Aurora B acts upstream of these depolymerases, our strategy may in fact be mediated through the actions of endogenous Kif2b or MCAK. Nevertheless, there are other targets within the kinetochore that are substrates for Aurora B's error correction activity.

A critical point of our study is that the defect in error correction must be subtle. Otherwise, the error rate would be so high that the resulting massive missegregation rates would be lethal. We currently do not know how often aberrant attachments occur during the course of establishing bipolar attachments in normal cells. Thus, we cannot say if there is an increase rate of aberrant attachments in CIN cells due to abnormal spindle geometries as has been proposed [36]. Regardless of the cause of the defective attachments, it is clear that the cells we examined had a defect in their Aurora B dependent error correction. We are currently seeking to understand the nature of the inaccessibility issue that we propose to be an explanation for why Aurora B kinase cannot resolve defective attachments in CIN cells. Based on the original spatial model for how physical stretching of the centromere/kinetochore complex by proper end-on microtubule attachments displaces the microtubule binding factors away from Aurora B kinase, we hypothesize that Aurora B is physically located too far away to efficiently perform its error correction functions. There could be a subtle architectural problem with the centromere/kinetochore complex that is shared amongst CIN cells.

The fact that we were able to improve proper bipolar attachments that reduced the frequency of lagging chromosomes suggest that it is possible to reduce missegregation in CIN cells. This then opens the opportunity to test whether limiting chromosome instability in cancer cells will reduce their ability to adapt. The exciting prospect is whether it is possible to block the evolutionary capacity of cancer cells and thus limit their ability to adapt to changing growth environments, and also overcoming drug treatments.

Methods and materials

Cell culture, transfections and antibodies

All cells were grown in a humidified incubator at 37°C with 5% CO₂. HeLa, and U2OS, were grown in DMEM + 10% FBS, while all OVCAR, and other cells were grown in RPMI +10% FBS. All the established cell lines originated from ATCC (Manassas, VA). Cells were synchronized by thymidine block and release. Nocodazole was used at 60nM, MG132 was used at 20µM final concentration and ZM447439 at 50nM.

SMARTpool siRNAs (Dharmacon) were diluted in serum-free OptiMEM and HiPerfect (Qiagen) and used at a final concentration of 20nM. DNA constructs were transfected with Fugene 6 (Roche) according to the manufacturer's instructions. Cells were fixed and stained 48hrs. after transfection. Commercial antibodies were used to detect μ -tubulin (Sigma-Aldrich), MCAK (Cytoskeleton), CyclinB1 (BD Pharmingen), pT232AuroraB (Rockland), Aurora B (Millipore), INCENP (Cell Signaling Technology), Survivin (Cell signaling Technology), Hec1 (BD Sciences), PH3 (Millipore) and Plk1 (Santa Cruz biotechnology). Human anticentromere antibody (ACA) serum was kindly provided by Dr. J.B. Rattner (University of Alberta, Calgary, Canada). TD60 antibody was kindly provided by Dr. D. Palmer (deceased, University of Washington, Seattle, WA). Phospho-H3T3 antibody was a gift from Dr. M.G. Higgins (Harvard Medical School, Boston, MA). Spc24 and Spc25 antibodies were a gift from Dr. P.T. Stukenberg (University of Virginia, Charlottesville, VA). CENP-F antibodies were generated by our laboratory [45,52]. Antibodies were used at a final concentration of 0.5–1.0 µg/ml. Secondary antibodies conjugated to Alexa Fluor 488, 555, and 647 (Invitrogen) were used at 1 µg/ml. Alkaline-phosphatase conjugated secondaries were used for chemiluminescent detection of blots.

Microscopy and FISH

Cells collected by mitotic shakeoff were replated onto slides and allowed to complete division before fixing. FISH was performed using α -satellite probes specific for chromosomes 7 (Abbott) according to

the manufacture's protocol. For immunofluorescence staining, cells were pre-extracted in KB (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% BSA) with 0.2% Triton X-100 for 90s and then fixed for 7 min in freshly prepared 3.5% paraformaldehyde/PBS, pH 6.9, and rinsed in KB. Primary and secondary antibodies were sequentially added to coverslips for 30 min at 37°C in a humidified chamber. DNA was stained with DAPI. Images were visualized with a 100 ×/1.4 NA objective attached to a Nikon Eclipse TE2000S microscope. Optical sections between 0.25–1 µm were captured with a Photometrics Cascade 512F camera (Roper Scientific) and the data analyzed with Meta-Morph (MDS Analytical Technologies) after deconvolution with AutoQuant (Media Cybernetics). All image files were reformatted as TIFF files, and Photoshop (Adobe) was used to assemble the figures.

For time-lapse studies, cells were seeded into 6 or 12 well dishes (Falcon) in HEPES buffered medium and imaged with either an Nikon Eclipse TE2000S inverted microscope (20X objective) or plated onto glassbottomed 35-mm dishes (MakTek) and imaged (60X 1.4NA objective) with an UltraView spinning disc confocal microscope (Perkin Elmer). Images were captured every 5 min at 37°C and processed with ImagePro Plus software (Media Cybernetics).

FRET

For imaging of the FRET biosensors, cells were plated on 22 × 22 mm no. 1.5 glass coverslips (Fisher Scientific) coated with Poly-D-lysine (Sigma). 24 hrs post-transfection of DNA constructs, cells were subjected to a double thymidine block and then released in the presence of nocodazole ± ZM447439 (Sigma) to enrich for mitotic cells. Images were acquired on a Leica DM4000 microscope with a 100 × 1.4 NA objective, an XY-piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal (Yokogawa), an electron multiplier CCD camera (Hamamatsu ImageEM), and an LMM5 laser merge module (Spectral Applied Research), controlled by IP Lab software (BD Biosciences). CFP was excited at 440 nm. CFP and YFP emissions were acquired simultaneously with a beamsplitter (Dual-View, Optical Insights). Custom software written in Matlab (Mathworks)

was used for image analysis [23]. The program identified individual kinetochores from the confocal image stacks, and the YFP/TFP emission ratio was calculated at each centromere/kinetochore as previously described [23].

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Disclosure statement

The authors have no conflicts to disclose

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ORCID

Timothy J. Yen  <http://orcid.org/0000-0003-2159-0997>

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