Aurora B Kinase Regulates the Postmitotic Endoreduplication Checkpoint via Phosphorylation of the Retinoblastoma Protein at Serine 780

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The phenotypic change characteristic of Aurora B inhibition is the induction of polyploidy. Utilizing specific siRNA duplexes and a selective small molecule inhibitor (AZD1152) to inhibit Aurora B activity in tumor cells, we sought to elucidate the mechanism by which Aurora B inhibition results in polyploidy. Cells treated with AZD1152 progressed through mitosis with misaligned chromosomes and exited without cytokinesis and subsequently underwent endoreduplication of DNA despite activation of a p53-dependent pseudo G1 checkpoint. Concomitant with polyploid cell formation, we observed the appearance of Rb hypophosphorylation, an event that occurred independently of cyclin-dependent kinase inhibition. We went on to discover that Aurora B directly phosphorylates Rb at serine 780 both in vitro and in vivo. This novel interaction plays a critical role in regulating the postmitotic checkpoint to prevent endoreduplication after an aberrant mitosis. Thus, we propose for the first time that Aurora B determines cellular fate after an aberrant mitosis by directly regulating the Rb tumor suppressor protein.

INTRODUCTION

The Aurora kinases constitute a family of serine/threonine kinases that have been shown to play critical roles in mitosis. Three members of Aurora kinase family have been identified in mammals: Aurora A, B, and C (Nigg, 2001). All three Aurora kinases are overexpressed in a variety of human cancers. Aurora A amplification/overexpression has been detected in human breast, bladder, colon, ovarian, and pancreatic cancers (Sakakura *et al.*, 2001; Fraizer *et al.*, 2004; Tong *et al.*, 2004). Aurora B is overexpressed in cancer cells, and an increased level of Aurora B correlates with advanced stages of colorectal cancer (Katayama *et al.*, 1999; Giet *et al.*, 2005). It has been hypothesized that these elevated levels of Aurora kinase activity may promote tumor evolution either by providing a growth advantage or promoting genetic instability.

The expression levels, as well as the kinase activity, of all three Aurora kinases are regulated in a cell cycle-dependent manner and peak during mitosis. In addition, Aurora kinases A and B show differential subcellular localization. Aurora A is detected in proliferating cells at the centrosome, where it regulates centrosome function, and also along mitotic spindles. In contrast, Aurora B shows dynamic changes in localization during mitosis. It associates along chromosomes during prophase, inner centromeres until metaphase, and the central spindle at the cleavage furrow in anaphase and ultimately resides in the midbody during cytokinesis (Andrews *et al.*, 2003; Carmena and Earnshaw, 2003; Meraldi

et al., 2004). This localization pattern reflects the critical role Aurora B plays as a chromosome passenger protein to ensure accurate chromosome segregation and timely cytokinesis.

In fact, depletion of Aurora B by double-strand RNA interference (RNAi; oligo) leads to decreased histone H3 phosphorylation, defective chromosome condensation, failed cytokinesis, and polyploidy induction in *Drosophila* (Giet and Glover, 2001). Given their importance in mitosis and overexpression in human cancers, Aurora kinases have been identified as promising therapeutic targets, and considerable effort has been devoted to developing inhibitors of the kinases. ZM447439 (AstraZeneca, Alderley Park, Macclesfield, Cheshire, United Kingdom), Hesperadin (Boehringer Ingelheim, Ridgefield, CT), and VX-680 (Vertex Pharmaceuticals, Cambridge, MA) are a few of the small molecule Aurora family inhibitors that have been shown to induce an aberrant mitosis and polyploidy, depending upon the status of a p53-dependent postmitotic checkpoint (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Keen and Taylor, 2004; Gizatullin *et al.*, 2006).

The retinoblastoma (Rb) protein is a tumor suppressor that plays a pivotal role in the negative control of the cell cycle and in tumor progression. It has been shown that Rb is responsible for a major G1 checkpoint, blocking S-phase entry and cell growth. Loss of Rb functions may induce cell cycle deregulation, leading to a malignant phenotype (Giacinti and Giordano, 2006). Compromise of Rb and p53 functions leads to suppression of a postmitotic checkpoint that prevents endoreduplication after aberrant mitosis by eliciting a pseudo G1 arrest that depends on p53, p21, and Rb (Borel *et al.*, 2002). Previous studies using small molecule inhibitors of Aurora kinases also reported the induction of polyploidy or a pseudo G1 arrest is dependent on these same pathways (Keen and Taylor, 2004).

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On the basis of these findings, in this study we have made an effort to understand the roles of Rb and p53 in regulating the postmitotic checkpoint after Aurora B inhibition. We use specific small interfering RNA (siRNA) and the Aurora B small molecule inhibitor, AZD1152 HQPA, to selectively down-regulate Aurora B activity (Wilkinson *et al.*, 2007). AZD1152 HQPA is the active metabolite of AZD1152, a selective Aurora B inhibitor (Aurora A, $K_i = 1369$ nM; Aurora B, $K_i = 0.3$ nM) now in clinical trial (Carvajal *et al.*, 2006). AZD1152 HQPA will be herein referred to as simply AZD1152. In accord with previous studies of Aurora B inhibitors, AZD1152 induces polyploidy in tumor cells. However, in contrast to those results, AZD1152-mediated polyploidy occurs independent of p53 status. We also provide evidence that the inhibition of Aurora B results in the inhibition of Rb phosphorylation concomitant with polyploidy induction. Furthermore, we demonstrate that Aurora B regulates the postmitotic checkpoint by directly phosphorylating Rb at serine 780. Taken together, we propose that Aurora B protects the integrity of key mitotic processes, as well as prevents endoreduplication and polyploidy formation by directly regulating Rb phosphorylation status.

MATERIALS AND METHODS

Cell Culture

HCT-116 human colon carcinoma cell line, its p53 and p21 null variants, and Mad2 haploinsufficient were a generous gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Cultures were maintained at 37°C in the presence of 5% CO₂ in McCoy's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. SaOs2, a human osteosarcoma cell line, and MCF10A, a normal human breast epithelial cell line, were purchased from the American Type Culture Center (Manassas, VA) and were maintained in McCoy's media containing 100 U/ml penicillin, and 100 µg/ml streptomycin supplemented with 15% FBS and mammary epithelial cell basal medium (MEBM) containing bovine pituitary extract, 2 ml; human epidermal growth factor, 0.5 ml; hydrocortisone, 0.5, 0.5 ml; GA-1000, 0.5 ml; and insulin, 0.5 ml. Two leiomyosarcoma cell lines, SKUT1 and SKUT1B, were a gift from Dr. David Spriggs (Memorial Sloan-Kettering Cancer Center) and were maintained in DME containing 100 U/ml penicillin, and 100 µg/ml streptomycin supplemented with 10% FBS. To synchronize cells at G1/S phase, cells were treated with 2 mM thymidine (Sigma, St. Louis, MO) for 16 h followed by release into regular media for 8 h and subsequent treatment with 2 mM thymidine for another 16 h. The cells were released to either drug-containing or drug-free media for the indicated times. AZD1152 was obtained from AstraZeneca.

Clonogenic Assay

Log-phase cells were plated, in triplicate, onto 100-mm dishes at 1000 per dish and were allowed to attach for 24 h before treatment. At the end of treatment, cells were cultured in drug-free medium for 7–10 d. The resulting colonies were scored after staining with 0.01% crystal violet.

Fluorescent Microscopy

Cells were collected after drug treatment and fixed in 3% paraformaldehyde. The nuclear morphology of cells was examined under fluorescence microscope after staining with 4', 6-diamidino-2-phenylindole (DAPI) to a final concentration of 25 µg/ml. Cells were scored as apoptotic based on the presence of condensed fragmented chromatin. Multinucleated cells were defined as cells containing huge, multilobed nuclei. A minimum of 400 cells were counted for each sample and taken as a percentage of untreated cells.

Immunofluorescence Assay

HCT 116 cells were plated in four-well chamber slides and treated with or without 50 nM AZD1152, and cells were incubated for 10 min on ice followed by incubation with 100 mM K-PIPES at pH 6.9, 10 mM EGTA, 1 mM MgCl₂, and 0.2% Triton X-100 and 4% paraformaldehyde for another 10 min. The cells were then blocked in 2% goat serum followed by incubation in primary antibody as described. The following antibodies were used: monoclonal anti-tubulin (Sigma; 1:250), polyclonal anti-NuMA was a gift from Duane A. Compton (Dartmouth Medical School, Hanover, NH), rabbit polyclonal anti-phospho-ser 10 histone H3 (Cell Signaling Technology, Danvers, MA; 1:250), and anti-human CREST (1:10000) was a generous gift from B. R. Brinkley (Baylor College of Medicine, Houston, TX). DNA was labeled with DAPI at a final concentration of 25 µg/ml for 10 min.

Time-Lapse Fluorescent Microscopy

HCT116 cells expressing green fluorescent protein (GFP)-histone 2B and GFP-tubulin were developed as previously described (Tse and Schwartz, 2004). For these studies, cells were grown on 40-mm round coverslips and allowed to attach for 48 h before assembly onto a FCS2 close-system flow-observation chamber (Bioptechs, Butler, PA). The chamber was mounted onto the stage of a Zeiss Axiovert 200 M inverted microscope (Thornwood, NY) with the chamber and coverslip temperature maintained at 37°C. Drug containing media was introduced into the chamber via a peristaltic pump. Epifluorescent and phase-contrast images were acquired with a 40× objective lens, using a cooled CCD camera every 5 min, with exposure time limited to 4–5 s/image (MicroMax-1300YHS, Roper Scientific, Tucson, AZ). For each time point, images were taken with five to six different focal planes along the z-axis 2–3 µm apart. Imaging data were analyzed using MetaMorph (Universal Imaging, West Chester, PA).

For time-lapse experiments, cells were cultured in the same medium containing 20 mM HEPES without phenol red.

Flow Cytometry

Biparameter flow cytometry was performed as previously described (Motwani *et al.*, 1999). Cells were analyzed for DNA content after propidium iodide staining (PI) and for mitotic index after labeling with the MPM-2 mAb (Upstate Biotechnology, Lake Placid, NY). Samples were analyzed on a FAC-Scan (Becton Dickinson, Franklin Lakes, NJ) for cell cycle distribution and mitotic index (percentage of MPM-2-positive cells) using the Cell Quest software (Becton Dickinson).

siRNA Transfection

HCT 116 cells were plated on 60-mm plates and transfections using oligofectamine (Invitrogen, Carlsbad, CA) were carried out according to the manufacturer's protocol. The siRNA sequences used were Aurora B (5'-AACGCGGCACUUCACAAUUGA-3'; Lampson and Kapoor, 2005) and Aurora A (Aurora-A, 725AUGCCCCUGUCUACUGUC A₇₄₃; Kufer *et al.*, 2002) purchased from Dharmacon Research (Lafayette, CO), and control siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Extraction, Immunoprecipitation, and Immunoblotting

Cell lysates were prepared as previously described (Nair *et al.*, 2002). Fifty micrograms of protein were fractionated by SDS-PAGE and transferred onto Immobilon membranes (Millipore, Billerica, MA). Equal protein loading was confirmed by Amido black staining (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk, membranes were probed with primary antibodies. The following antibodies were used in this study: rabbit polyclonal and mouse monoclonal anti-Aurora B (1:1000), rabbit polyclonal Aurora A, rabbit polyclonal to p16, rabbit polyclonal to E2F1 from Abcam (Cambridge, United Kingdom), mouse monoclonal anti-Rb (1:1000) from BD Biosciences (San Jose, CA), mouse monoclonal anti-p53, cdk2, cdk4, cyclin B, cyclin E, rabbit polyclonal to Rb, mouse monoclonal to E2F1, and cyclin D from Santa Cruz Biotechnology, mouse monoclonal anti-p21 (1:1000) was from Oncogene (Cambridge, MA), mouse monoclonal anti-H2AX and anti-tubulin were from Upstate Biotechnology rabbit polyclonal anti-phospho-serine Histone 3 (1:1000) mouse monoclonal anti-hemagglutinin (HA; 1:1000), rabbit polyclonal to phospho-Rb (Ser 807/811), Ser 780 and Ser 795, and rabbit polyclonal to phosphor-histone H3 (Ser 10) were from Cell Signaling. Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (ICN/Jackson ImmunoResearch, West Grove, PA) and visualized by enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NJ).

Immunoprecipitation was performed by using 500 µg to 1 mg of soluble protein. First, lysates were incubated with 2 µg of the indicated primary antibody overnight at 4°C followed by incubation with 50 µl of protein A-agarose beads (Upstate Biotechnology). Immunocomplexes were washed five times in lysis buffer, suspended in 30 µl of 2× sample buffer, and fractionated by SDS-PAGE. Western blot analysis was performed as previously described.

Plasmid Transfections

Plasmid transfections were carried out as followed in respective cell lines. Cells were plated so as to gain 50% confluence after 24 h in 60-mm tissue culture plates. Transfections were carried out using Fugene 6 transfection reagent (Roche, Indianapolis, IN) in OPTI MEM 1 media (Invitrogen) and 2 µg of HA-tagged Rb plasmid (Addgene, Cambridge, MA), its mutant forms or HA-tagged p16 (a generous gift from Dr. Andrew Koff, Memorial Sloan Kettering Cancer Center) were used per plate. Treatments were carried out 16 h after transfection.

Luciferase Assay

Cells were plated on 24-well plates and TransLucient E2F1 reporter vector was used (Panomics, Fremont, CA). Cell extracts were analyzed for luciferase activity using the Dual Luciferase Reporter Assay (Promega, Madison, WI).

Aurora Kinase Assays

Purified active and inactive forms of Aurora B kinase were obtained from Upstate Biotechnology for *in vitro* kinase assays and histone H3 and full-length Rb (QED Biosciences, San Diego, CA) were used as substrates. Different Rb constructs were a gift from Dr. Nikola P. Pavletich (Memorial Sloan Kettering Cancer Center; Rubin *et al.*, 2005).

Reactions were carried out in 30 μ l kinase buffer (250 mM HEPES, 50 mM $MgCl_2$, 50 mM β -glycerophosphate, 5 mM DTT, 12.5 mM EGTA, 0.5 mM sodium vanadate, and 5 μ M sodium fluoride) containing 5 μ Ci of ^{32}P -ATP, 15 μ mol/L ATP at 30°C for 20 min. Products were resolved by SDS-PAGE and visualized by autoradiography. For *in vivo* kinase assays, Aurora B was immunoprecipitated from lysates prepared from control or AZD1152-treated HCT-116 cells. The kinase reaction was performed under

the same conditions as the *in vitro* assay using full-length human Rb as the substrate.

Mutagenesis, Overexpression, and Purification of RbC-99

6xHis-tagged RbC-99 was mutated using Quick Change Site Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) according to the manufacturer's protocol. Mutants and wild-type RbC-99 were expressed in BL-21 (Stratagene) and purified by Ni-NTA column chromatography (Qiagen, Hilden, Germany).

MALDI Q-TOF Mass Spectra and LC-MS/MS Sequencing

MALDI Q-TOF MS (matrix-assisted laser desorption/ionization-quadrupole time-of-flight mass spectrometry) and LC-MS/MS (liquid chromatography-

Figure 1. (A) Inhibition of histone H3 phosphorylation on serine 10 by AZD1152 and Aurora B kinase knockdown. HCT-116 cells were treated with drug-free medium (ND), 50 nM AZD1152 (AZD), or transfected with Aurora B-specific (BSi) or control siRNA (CSi) for 24 h before immunoblotting for phospho-histone H3 Ser-10 and Aurora B. (B) (i) Immunofluorescence assay of HCT-116 cells after treatment with 50 nM AZD1152HPQA for 24 h and the DAPI stain showing completely misaligned chromosome (blue, b, d, and f) when compared with untreated controls (a, c, and e). Normal bipolar spindle formation (green, α -tubulin, b) and its binding to kinetochore proteins (pink, CREST, b). Intact pole formation as determined by probing for pole associated protein NUMA (orange, NUMA, c and d) and normal number of centrosomes by γ -tubulin (red, e and f). Down-regulation of Aurora B by siRNA shows the same effect of a misaligned chromosome (h, blue) and a normal number of centrosomes, whereas down-regulation Aurora A by si RNA shows no centrosome but normal metaphase (g). (ii) HCT-116 cells were double thymidine blocked and released to either drug-free (ND) or in 20 nM AZD1152. Cells were harvested at different time points for FACS analysis and probed for MPM2. MPM2-positive cells show the kinetics of enter and exit from mitosis. HCT-116 cells treated with or without AZD1152 HPQA entered and exited mitosis with the same kinetics as determined by FACS analysis after MPM2 labeling. (C) (i) AZD1152 induces multinucleation in cells. HCT-116 cells were untreated with 50 nM AZD1152 for 24 h (AZD) or transfected with control (CSi) or Aurora B kinase targeting siRNA (BSi). Nuclear morphology was visualized after staining with DAPI. ND, untreated control. (ii) Cell cycle analysis of HCT-116 cells after Aurora B kinase inhibition by AZD1152 or gene knockdown using siRNA. (D) HCT116 cells undergo multinucleation and polyploidy upon treatment with AZD1152 by live time-lapse microscopy. (A) HCT116 cells stably transfected with GFP H2B treated with AZD1152 were followed. (B) HCT116 cells undergo multiple round of mitosis, as determined by time-lapse microscopy of HCT-116 cells stably transfected with GFP tubulin. Shown here are representative figures from three or more independent experiments.

tandem mass spectrometry) were performed as described previously (Xu *et al.*, 2005). Briefly, 1 μ g of purified Rb construct RbC-99 (771–928) was incubated with active or inactive Aurora B kinase in 250 mM HEPES, 50 mM $MgCl_2$, 50 mM β -glycerophosphate, 5 mM DTT, 12.5 mM EGTA, 0.5 mM sodium vanadate, 5 μ M sodium fluoride 15 μ mol/l ATP at 30°C for 20 min, and resolved on SDS page. The bands corresponding to RbC99 were excised and after tryptic digestion, a MALDI Q-TOF MS spectra of the tryptic peptides was acquired with a Micromass Q-TOF Ultima MALDI mass spectrometer (Waters, MA). Titanium dioxide chromatography was used to enrich the phosphopeptides in the tryptic digest and MALDI Q-TOF MS spectra was acquired on the enriched peptides and subtracted from background. The enriched peptide mixture was also sequenced using LC-ESI Q-TOF MS.

RESULTS

Selective Inhibition of Aurora B Results in Polyploidy

To validate selective inhibition of Aurora B by AZD1152, the effects of the drug were compared with those obtained by siRNA (BSi)-mediated down-regulation of Aurora B. First, we examined the phosphorylation status of histone H3 on the serine 10 residue, a known substrate of Aurora B (Hsu *et al.*, 2000; Crosio *et al.*, 2002; Sugiyama *et al.*, 2002). As indicated by the Western blot, 50 nM AZD1152 in HCT-116 cells completely inhibited histone H3 phosphorylation, similar to that achieved with BSi, hence validating target inhibition (Figure 1A). Immunofluorescent microscopy showed both modalities of Aurora B inhibition resulted in misaligned chromosomes (DAPI, blue) in the setting of an apparently normal bipolar spindle (α -tubulin, green) that was attached to kinetochore proteins (CREST, pink; Figure 1Bi, a and b). Figure 1Bi further shows that spindle poles and centrosomes identified using immunofluorescence probes for pole associated protein NUMA (orange, c and d) and centrosome marker, γ -tubulin (red, e and f) were intact and normal. Down-regulation of Aurora B by siRNA had no effect on the centrosome however, Aurora A down-regulation (ASi) is shown to deplete it (g and h). Despite these changes in mitosis, Aurora B inhibition did not alter the rate of mitotic entry or exit as determined by phospho MPM2 (a mitotic marker) positive cells (Figure 1Bii).

HCT-116 cells were next treated for 24 h with 50 nM AZD1152, and their nuclear morphology was evaluated by fluorescent microscopy after DAPI staining. One hundred percent of the cells were multinucleated after 24 h of drug exposure without any evidence of apoptosis, again mimicking the effects observed with BSi (Figure 1Ci). Cell cycle analysis revealed that both AZD1152 treatment and BSi transfection resulted in prominent 4N and >4N cell populations, consistent with aberrant mitosis and failed cytokinesis followed by endoreduplication and polyploidy formation (Figure 1Cii). Time-lapse fluorescent microscopy in HCT-116 cells stably transfected with either GFP-H2B or GFP- α tubulin also confirmed that AZD1152 disrupted chromosome alignment despite normal bipolar spindle formation (Figure 1D). Conversely, Aurora A siRNA down-regulation did not result in these mitotic changes or multinucleation, instead preferentially induced apoptosis (data not shown). These observations confirm that the phenotype of cells treated with AZD1152 are consistent with that of selective Aurora B inhibition as predicted by *in vitro* kinase assays, reasserting the critical role of Aurora B in regulating chromosome alignment, spindle checkpoint control and cytokinesis (Hannak *et al.*, 2001; Ducat and Zheng, 2004).

Aurora B Inhibition Leads to Polyploidy Despite Activation of a p53-dependent Postmitotic Checkpoint

It has been previously proposed that under conditions of DNA damage or aberrant mitotic exit, the p53-p21 axis is induced, resulting in cyclin-dependent kinase (cdk) inhibition and hypophosphorylation of Rb, the active form of the protein that elicits a pseudo G1 arrest presumably via repression of E2F transcription factors (Lanni and Jacks, 1998; Motwani *et al.*, 2000; Andreassen *et al.*, 2001b). We examined the response of this checkpoint to the aberrant mitosis induced by AZD1152 in HCT-116 cells, which possess an intact p53-p21 pathway. As shown in Figure 2Ai, p53 and p21 were induced at 8 and 18 h, respectively, after AZD1152

Figure 2. (A) Activation of post mitotic p53 pathway. (i) Western blot analysis in HCT 116 cells showing a time-dependent induction of p53 (8 h, lane 2) and p21 (18 h, lane 3) with no change in the cdk2 protein level upon AZD1152 treatment. (ii) Cdk2 was immunoprecipitated, and kinase assay was performed using recombinant Rb as the substrate. Autoradiograph showing inhibition of cdk2 activity at 24 h (top panel). Western blot for Rb showing the appearance of the hypophosphorylated form before the inhibition of cdk2 (middle panel). (iii) Cdk4 was immunoprecipitated and kinase assay was performed using recombinant Rb as the substrate. Autoradiograph showing inhibition of cdk4 activity at 24 h (top panel). Western blot for Rb showing the appearance of the hypophosphorylated form but no change in cdk4 activity. (iv) After transient transfection of HCT-116 cells with p16, the binding of Rb and E2F1 was assessed by coimmunoprecipitation assay. Autoradiograph showing no effect in the binding of Rb to E2F1 in the absence (–) or presence (+) of AZD1152 (top panel). (B) Polyploidy is independent of p53. HCT-116 parental (i), p53–/– (ii), and p21–/– (iii) cells were treated with (AZD) or without (ND) AZD1152 for 48 h. Flow cytometry analysis revealed polyploid cell formation (>4N DNA content) in all three cell lines after treatment. Immunoblot showed hypophosphorylation of Rb in all cell lines after AZD1152 treatment. Each figure is a representative of three or more experiments.

Table 1. List of cell lines showing polyploidy after treatment with 50 nM AZD1152 independent of p53 status

Cell line	Origin	P53 status	Induction of polyploidy upon AZD1152 HPQA treatment (% 8N)	
HCT-116	Colon	Wild type	Yes	87
HCT-116 (p53 ^{-/-})	Colon	Null	Yes	73
HCT-116 (p21 ^{-/-})	Colon	Wild type	Yes	80
SW-620	Colon	Mutant	Yes	63
SW-480	Colon	Mutant	Yes	67
H747	Colon	Mutant	Yes	36 ^a
HT-29	Colon	Mutant	Yes	66
MKN-74	Gastric	Mutant	Yes	30
MCF-7	Breast	Wild type	Yes	30
MCF-10A	Normal breast	Wild type	Yes	40
SKML19	Melanoma	Wild type	Yes	85
SKML28	Melanoma	Mutant	Yes	88
SKML103	Melanoma	Mutant	Yes	74
SKML173	Melanoma	Wild type	Yes	86
DDLS	Sarcoma	Wild type	Yes	60

n = 3 for each cell line.

^a 500 nM AZD1152 was used.

treatment. Although cyclin-dependent kinase 2 (cdk2) protein levels were unchanged, cdk2 kinase activity immunoprecipitated from drug treated cells was completely inhibited by 24 h (Figure 2Aii). Importantly, the fastest migrating form of Rb (representing un- and hypo-phosphorylated Rb) appeared at 18 h, well before cdk2 kinase inhibition was observed (Figure 2Aii). Similarly, cdk4 immunoprecipitation-kinase assays in drug-treated cells confirmed that cdk4 kinase activity was maintained while the hypophosphorylated form of Rb appeared (Figure 2Aiii). To further rule out any role played by cdk4 in the AZD-induced polyploidy, HCT 116 cells transiently overexpressing p16 were treated with AZD1152, and the binding of Rb and E2F1 was assessed by coimmunoprecipitation assay. As shown in Figure 2Aiv p16 overexpression by itself or with treatment showed no effect in the binding of Rb to E2F1, indicating the release of E2F1 upon Aurora B inhibition is independent of p21/p16 and may be independent of p53/cdk2 pathway. These results strongly suggest that the hypophosphorylation of Rb in the context of Aurora B inhibition may occur independently of cdk inhibition.

The role of p53 in response to Aurora B inhibition was further examined in isogenic HCT-116 clones: parental (wild type for p53 and p21), p53 null (p53^{-/-}) and p21 null (p21^{-/-}). Flow cytometry studies demonstrated that AZD1152 induced the same degree of polyploidy (>4N) irrespective of the p53 or p21 status of the cells (Figure 2B). As shown in Supplemental Figure S1, we observed no differences in the kinetics of polyploid cell formation between the two isogenic HCT-116 cell lines. Thus, we conclude p53 induction does not contribute to any differences in the kinetics or absolute amount of polyploidy induced in these cell lines. Furthermore, Rb was relegated to the hypophosphorylated form despite the absence of p53 or p21, strongly indicating that inhibition of Rb phosphorylation occurs independently of p53/p21-mediated G1 cdk inhibition (Figure 2B). Because these results were not consistent with a previous study demonstrating a p53-dependent postmitotic arrest in response to Aurora kinase inhibition (Keen and Taylor, 2004), we elected to test the effect of AZD1152 in 17 different cell lines of varying p53 status (eight for wild type, two nulls, and seven mutants), including MCF-10A, a normal

breast cell line. As shown in Table 1, AZD1152 induced polyploidy and hypophosphorylation of Rb (data not shown) in all the cell lines, independent of p53 status. Thus, although Aurora B inhibition does induce p53 and p21, these proteins are ineffective at preventing polyploidy in the context of selective Aurora B inhibition.

Rb Plays a Critical Role in the Induction of Polyploidy by Aurora B Inhibition

The impact of Aurora B inhibition upon Rb phosphorylation status was explored further. As shown in Figure 3Ai, the induction of hypophosphorylated Rb by AZD1152, beginning at around 8 h of treatment and steadily increasing over time and with increasing concentrations of drug. Concomitant treatment with the phosphatase inhibitor okadaic acid blocked the emergence of hypophosphorylated Rb and resulted in total inhibition of polyploidy, indicating that this change in Rb phosphorylation status may play a critical role in promoting polyploidy upon Aurora B inhibition (Figure 3Aii). Furthermore, BSi induced Rb hypophosphorylation (Figure 3Aiii), under conditions in which polyploidy is induced (Figure 1C), confirming that this biochemical event is specific to Aurora B inhibition. This was confirmed in two other cell lines, SK-UT1B and MCF-10A (data not shown). The importance of Aurora B kinase activity in inhibiting cells from entering endoreduplication is further confirmed in Mad2 haploinsufficient HCT-116 cells (Figure 3B). Overexpression of Aurora B substantially inhibited Mad2^{-/+} cells from undergoing nocodazole-induced polyploidy when compared with the vector-transfected cells without affecting the mitotic population (MPM2 positive). These results suggest the importance of Aurora B in inhibiting cells from undergoing endoreduplication after incomplete cytokinesis.

To further investigate the role of Rb in polyploidy induced by Aurora B inhibition, Rb expression was down-regulated in HCT-116 cells with siRNA (RbSi), and AZD1152-induced polyploidy was then evaluated. Partial down-regulation of Rb resulted in a 20–25% reduction in AZD1152-induced polyploidy relative to control siRNA, indicating that Rb expression promotes polyploidy in the context of Aurora B

Figure 3. (A) Aurora B kinase inhibition results in Rb hypophosphorylation. (i) Time- and concentration-dependent emergence of fast-migrating form of Rb after AZD1152 treatment. HCT-116 cells were harvested at the indicated times after treatment with AZD1152, and Western blot analyses were performed for Rb. (ii) Reversal of AZD1152-induced fast-migrating form of Rb by phosphatase inhibitor. HCT-116 cells were treated with 50 nM AZD1152 (AZD), 20 nM okadaic acid (OK), or the combination (AZD+OK) for 24 h, and immunoblots were performed for Rb. FACScan analysis showing inhibition of polyploidy (8N) when OK was treated concomitantly with AZD. (iii) Aurora B kinase knockdown induces Rb hypophosphorylation. HCT-116 cells were transfected with either control (CSi) or Aurora B kinase -targeting (BSi) siRNA for 48 h and analyzed for Rb protein levels by Western blot. (B) Overexpression of Aurora B kinase inhibits cells from undergoing polyploidy. HCT 116 Mad2-haplosufficient cells transiently overexpressing Aurora B (Aurora B) or vector (vector) were treated with nocodazole (Aurora B-Noc and Vector-Noc), and polyploidy was measured by FACScan analysis after staining with propidium iodide (PI). (i) Nocodazole-induced polyploidy showing a reduction from 40% in vector-transfected cells to 20% in Aurora B-overexpressing cells. (ii) Western Blot showing Aurora B overexpression. (C) Induction of polyploidy by Aurora B kinase inhibition is attenuated by Rb gene knockdown. HCT-116 cells were transfected with either control (CSi) or Rb-specific (RbSi) siRNA for 24 h and treated with drug-free media (ND) or 50 nM AZD1152 (AZD) for 24 h. Cell cycle analysis shows a 25% decrease in 8N peak in Rb knocked-down cells (SiRb-AZD) compared with control siRNA cells (CSi-AZD) after AZD treatment. Immunoblots showing efficient Rb knockdown by siRNA. (D) Ectopic expression of Rb in null cells promotes polyploidy upon Aurora B kinase inhibition. Rb-null SKUT1 cells were transfected with empty or Rb-expressing vector and treated with AZD1152 for 48 h. Western blot analysis shows hypophosphorylation of Rb (lane 4) and increased polyploidy (from 25 to 45%) upon AZD1152 treatment in cells transfected with Rb-expressing vector compared with control vector. Each figure is a representative of three or more experiments.

inhibition (Figure 3C). This relationship between Rb expression and polyploidy was further explored in an Rb null leiomyosarcoma cell line, SKUT1. Ectopic expression of Rb in SKUT1 cells increased the 8N population from 20 to 40% upon treatment with AZD1152 when compared with vector-transfected cells (Figure 3D). This Rb-mediated increase in polyploidy was also observed in AZD1152 treatment of Rb null SaOs2 cells ectopically expressing Rb (data not shown). At first glance, these results seem to be at odds with the perceived "gate-keeper" role of Rb in regulating G1/S transition. Our data suggest that Rb actually functions to promote polyploidy in the context of Aurora B inhibition. The induction of some polyploidy in Rb null cells also suggests that there may

also be Rb-independent mechanisms of polyploidy induction with Aurora B inhibition (see *Discussion*).

Aurora B Phosphorylates Rb In Vitro and In Vivo

Given that we observed Rb hypophosphorylation after Aurora B inhibition to occur independent of cdk inhibition, we next explored the possibility that Aurora B directly phosphorylates Rb to prevent endoreduplication after an aberrant mitosis. As shown in Figure 4A, Rb and Aurora B could be coimmunoprecipitated from HCT-116 cells. The ability of Aurora B to phosphorylate Rb was tested in an in vitro kinase assay utilizing Aurora B immunoprecipitated from control or AZD1152-treated HCT-116 cells and purified Rb as a substrate. Although

Figure 4. Aurora B kinase binds and phosphorylates Rb in vitro and in vivo. (A) HCT-116 cell lysates were immunoprecipitated with Aurora B antibody and Western blotted for Rb (top panel). Reciprocal coimmunoprecipitation experiment was carried out for Rb (bottom panel). (B) In vitro kinase assay was performed using Aurora B immunoprecipitated from lysates prepared from untreated or AZD1152-treated HCT-116 cells using purified full-length human Rb as a substrate (top panel). Coomassie-stained gel showing equal amount of Rb substrate present (bottom panel). (C) In vitro kinase assay using inactive (lanes 1 on both blots) or active Aurora B (lanes 2 on both blots). Histone H3 (left blot) or full-length human Rb (right blot) were used as substrates. (D) In vitro kinase assay using purified Aurora B kinase and different Rb constructs as substrates. Coomassie-stained gel showing the sizes of various Rb constructs, histone H3, and Aurora B itself after SDS-PAGE (top panel). Autoradiograph of 32 P-labeled polypeptides with their sizes predicted from the Coomassie-stained gel marker (*). An Rb construct containing amino acids 771–928 shows a phosphorylation level (RbC99) comparable to that seen with histone H3 (histone 3) and Aurora B autophosphorylation (Aurora B).

immunoprecipitated Aurora B from untreated cells successfully incorporated radiolabeled 32 P onto the Rb substrate, kinase activity directed at Rb was significantly decreased in drug-treated cells (Figure 4B). Further, recombinant human Aurora B directly phosphorylated full-length Rb in vitro (Figure 4C, right panel, lane 2) at levels commensurate to that observed with histone H3 (Figure 4C, left panel, lane 2). Conversely, purified inactive Aurora B failed to phosphorylate both Rb and histone H3 (Figure 4C, lane 1 in right and left panels, respectively).

To identify the specific Aurora B phosphorylation site(s) on Rb, several Rb constructs were created and tested as substrates in vitro using purified Aurora B kinase. Shown in Figure 4D are the Coomassie-stained gel (top panel) and autoradiograph of the corresponding 32 P-labeled Rb constructs (bottom panel). Histone H3 (lane 4) and Aurora B autophosphorylation (lane 6) were used as positive controls. Among the six different constructs used, the construct containing amino acids 771–928 (lane 3) was the most heavily phosphorylated (Figure 4D), a level comparable to that observed with Histone H3 (lane 4).

Aurora B Phosphorylates Rb on Serine 780

To identify the specific residue(s) of Rb that are phosphorylated by Aurora B kinase, the 771–928 C-terminus construct of Rb (RbC-99) was incubated with purified inactive (lane 1) or active (lane 2, Figure 5A) Aurora B in an in vitro kinase assay. After fractionation by SDS-PAGE, the protein bands of interest were excised from the gel and digested with trypsin. The digested mixture was enriched for phosphopeptides by TiO_2 method (Larsen *et al.*, 2005) and were

analyzed by MALDI-Q-TOF and by LC-MS/MS. Three peptides were identified by MALDI Q-TOF after subtracting background from control (Figure 5Aii). Six potential Aurora B phosphorylation sites (in bold) were identified in three peptides with amino acid sequences, YASTRPPTLSPIPHIPR (771–787), IYISPLKSPYKISEGLPTPTK (804–824), and MT-PRSRILVSIGESFGTSEK (825–844). All putative phosphorylation sites on Rb-C99 (771–928) were mutated to alanine individually or in combination, and Aurora B in vitro kinase assays were carried out using these mutants as substrates. As shown in Figure 5B, the only mutation which consistently decreased the level of phosphorylation alone or in combination with other sites was Serine 780, a known cdk4 site. The efficiency and timing of Rb dephosphorylation at Serine 780 was examined in vivo utilizing a phosphospecific antibody directed at serine 780 (Figure 5C). A decrease in phosphorylation on serine 780 was observed at 12–18 h after treatment with AZD1152, correlating to the time course observed with the emergence of hypophosphorylated Rb. Of note, no inhibition of Rb phosphorylation at serine 807/811 was observed. Thus, serine 780 is a physiological Aurora B specific phosphorylation site.

Phosphorylation on Rb at Serine 780 by Aurora B is an Important Determinant of Polyploidy Induced by Aurora B Inhibition

To further explore the functional significance of Aurora B-mediated phosphorylation of Rb at serine 780, three HA-tagged Rb constructs [wild type, phospho-mimetic mutant Rb (S780D), and Rb (S780A)] were ectopically overexpressed in

Figure 5. Mapping of Aurora B kinase phosphorylation site on Rb by mass spectrometry and site-directed mutagenesis. (A) (i) Autoradiograph showing incorporation of ^{32}P into the Rb-C99 fragment when kinase assay was performed using active but not kinase-dead Aurora B (top panel). Coomassie-stained gel showing equal amount of Rb-C99 present in the reaction containing active and inactive kinase (bottom panel). (ii) MALDI Q-TOF mass spectrum of the tryptic digest of Rb-C99 after phosphopeptide enrichment by TiO_2 chromatography. Four putative peptides phosphorylated by Aurora B kinase were identified after background subtraction (*). Amino acid sequence of His-tagged Rb construct (Rb-C99, 721–928) with the suggested phosphorylation sites highlighted. (B) Autoradiograph showing incorporation of ^{32}P into wild-type and phosphorylation site mutants (S→D) of Rb-C99. Only those constructs containing a 780 S→A showed (red) a decrease in ^{32}P incorporation. (C) Western blot analysis for Rb and phospho-Rb showing a decrease in phosphorylation at Ser 780 but not Ser 807/811 at the time of emergence of hypophosphorylated Rb after AZD1152 treatment in HCT116 cells. Western blots and kinase assay results are representative of at least three experiments.

Rb null SaOs2 cells. Cells were then treated with AZD1152 and analyzed for polyploidy. As predicted, expression of wild-type Rb increased polyploidy induced by Aurora B inhibition from 30% in vector-transfected cells to 55% in Rb-transfected cells (Figure 6A). Changing the serine to an alanine (S780A) did not affect the ability of Rb to promote polyploidy in the context of Aurora B inhibition (Figure 6A). Hypothesizing that Rb phosphorylation by Aurora B blocks endoreduplication, we predicted that cells expressing the S780D mutant Rb, which mimics irreversible phosphorylation at the putative Aurora B phosphorylation site, would block polyploidy induction by AZD1152. Indeed, expression of the mutant Rb (S780D) failed to increase levels of polyploidy over that observed in the Rb null, vector-transfected cells (Figure 6A). These observations provide direct evidence that Aurora B phosphorylation of Rb at serine 780 is functionally significant for preventing endoreduplication after an aberrant mitosis.

Despite profound differences in polyploidy induction, expression of these Rb constructs did not impact the consequences of Aurora B inhibition upon other mitotic events: AZD1152 treatment still resulted in defective cytokinesis and production of micro- and multinucleated cells with at least 4N DNA content (Figure 6). Thus, Aurora B regulation of endoreduplication through Rb is distinct from the role Aurora B plays in regulating key mitotic events (chromosome alignment, spindle checkpoint control, and cytokinesis).

In G1, Rb is pivotal for regulating the cellular commitment to entering S-phase and undergoing DNA synthesis by directly associating with members of the E2F family transcription factors. Binding of hypophosphorylated Rb to the E2F1 blocks expression of the E2F target genes required for the G1-S transition; hyperphosphorylation of Rb by G1 cyclin:cdks leads to

dissociation of Rb from E2F, expression of G1-S genes, and DNA replication. Similarly, the Rb:E2F interaction also has been shown to be a critical regulator of the cellular commitment to endoreduplication. Therefore, we went on to examine the potential significance of this interaction for regulating DNA synthesis after Aurora B inhibition by measuring Rb:E2F1 complexes in SaOs-2 cells expressing the three Rb mutants described above. In cells expressing wild-type Rb or the S780A mutant, AZD1152 treatment resulted in decreased Rb:E2F1 complexes, consistent with a release of E2F1 and promotion of DNA synthesis necessary for polyploid cell formation. Conversely, in cells expressing the S780D mutant, AZD1152 treatment did not result in a reduction of detected Rb:E2F1 complexes, suggesting that phosphorylation at this site by Aurora B promotes association with E2F1. Overexpression of p16 had no effect on the binding of Rb:E2F1 further rules out any role for cdk in AZD induced E2F1 release from Rb (Figure 2Aiv).

To further delineate the functional significance of these findings, E2F1 promoter activity was measured via luciferase assay (cells were cotransfected with vector containing E2F1 consensus sequence fused to firefly luciferase gene and Rb). In cells expressing the wild type or S780A mutant, AZD1152 treatment resulted in increased E2F1 promoter activity, a requisite event for DNA synthesis (Figure 6C). In contrast, the S780D phospho-mimetic Rb mutant blocked AZD1152-mediated enhancement of E2F1 promoter activity (Figure 6C). This result is further confirmed in the expression level of cyclin E, an E2F1 target gene (Figure 6B). Thus, we conclude that Aurora B phosphorylation of Rb at serine 780 after an aberrant mitosis negatively regulates endoreduplication by promoting Rb binding to E2F1, thereby preventing E2F1 promoter activation and subsequent polyploid cell formation.

Figure 6. Phosphorylation of Rb on Serine 780 by Aurora B is an important determinant of polyploidy checkpoint. Serine 780 of HA-tagged full-length Rb was mutated to alanine (unphosphorylatable) or aspartate (phospho-mimetic) and was ectopically over expressed in Rb-null SaOs2 cells. (A) Induction of polyploidy was compared in the cells transfected with vector, wild type, S780A, or S780D by FACS analysis. Representative images are shown of the PI-stained nuclei. Large cells with multiple giant nuclei are specified (MN). (B) E2F1 was immunoprecipitated and the binding of HA-tagged Rb (WT, 780A and 780D) after treatment with AZD1152 was determined by Western blot for HA. Level of HA-tagged Rb expression is shown in the bottom panel. Also, the protein level of cyclin E, an E2F1 target gene, is shown. (C) E2F1 promoter activity was determined in SaOs2 cells over expressing Rb (WT, 780A, and 780D) by luciferase assay after treatment with AZD1152.

DISCUSSION

The Aurora kinases (A, B, and C) are required for the orderly execution of several critical events in mitosis, including spindle assembly (Aurora A), histone H3 phosphorylation (Aurora B), chromosome segregation (Aurora B), and cytokinesis (Aurora B). A number of kinase inhibitors of varying selectivity for each Aurora family member have been developed. These compounds include ZM447439 (Aurora A, $IC_{50} = 0.11 \mu M$; Aurora B, $GI_{50} = 0.13 \mu M$; Ditchfield *et al.*, 2003) and VX-680 (Aurora A, $K_i = 0.6 nM$; Aurora B, $K_i = 18 nM$; Aurora C, $K_i = 4.6 nM$; Harrington *et al.*, 2004; Carvajal *et al.*, 2006). Whereas the phenotype of Aurora B inhibition predominates in cells treated with these drugs, the impact upon other Aurora family members cannot be completely discounted. Conversely, AZD1152 possesses profound selectivity for Aurora B (Aurora A, $K_i = 1369 nM$; Aurora B, $K_i = 0.3 nM$). The pharmacological effects of AZD1152 on cells closely mimics those observed with Aurora B knockdown via siRNA: decreased histone H3 phosphorylation at serine 10 and override of the mitotic checkpoint, leading to aberrant mitosis characterized by chromosomal mis-segregation, failed cytokinesis, mitotic exit, and endoreduplication/polyploidy (Figure 1). Hence, AZD1152 is a uniquely selec-

tive tool that can be used to study the biological role of Aurora B in human tumor cells.

Utilizing both this reagent and an siRNA approach, we addressed the question of how Aurora B inhibition results in polyploidy. The p53 tumor suppressor has been implicated as the regulator of a "postmitotic checkpoint" that functions to block endoreduplication after exit from failed mitoses caused by mitotic spindle poisons (Lanni and Jacks, 1998; Motwani *et al.*, 2000; Andreassen *et al.*, 2001a). It has been suggested that p53 also prevents endoreduplication after Aurora inhibition. Studies with combined Aurora A/B inhibitors ZM44739 and VX-680 reported that mutant p53 promoted increased percentages of polyploidy relative to cells with intact p53 function (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Harrington *et al.*, 2004; Gizatullin *et al.*, 2006). However, selective Aurora B inhibition with AZD1152 demonstrated no such dependence on the p53 pathway: comparable levels of polyploidy were observed in HCT-116 cells (p53 wild type, 87% polyploidy) relative to isogenic HCT-116 p53 null (73% polyploidy) and HCT-116 p21 null (80% polyploidy) cells (Figure 2B and Supplemental Movie 1). An extensive survey involving AZD1152 treatment of cell lines varying in p53 status ($n = 8$, wild type; $n = 9$, mutant/null

for p53; Table 1) confirmed that although a range of polyploidy was observed (30–87%), rates of polyploidy induction did not correlate with p53 status. Taken together, the data demonstrates that p53 status does not predict the cellular propensity to undergo polyploidy induction with selective Aurora B inhibition.

p53 is thought to prevent endoreduplication and elicit cell cycle arrest through p21 induction, leading to inhibition of G₁ cdk activity and Rb hypophosphorylation (Lanni and Jacks, 1998; Motwani *et al.*, 2000; Andreassen *et al.*, 2001b). Indeed, down-regulation of cdk 2 activity and the appearance of the fastest migrating form of Rb on immunoblot (representing both un- and hypo-phosphorylated Rb; Ezhevsky *et al.*, 2001) has been correlated with exit from an aberrant mitosis into a pseudo G1 phase (Lanni and Jacks, 1998; Motwani *et al.*, 1999, 2000; Chan *et al.*, 2007). Rb-negative cells are more susceptible to polyploidy induction in response to a variety of stimuli, including treatment with mitotic spindle poisons (Di Leonardo *et al.*, 1997), exogenous p21 expression (Niculescu *et al.*, 1998), and ionizing radiation (Avni *et al.*, 2003), consistent with a model in which hypophosphorylated Rb inhibits endoreduplication and polyploidy.

Importantly, however, we observed that polyploidy was induced in HCT-116 cells despite increases in p53 and p21 protein levels (Figure 2A), implying that Aurora B inhibition overcomes a potential p53-mediated arrest. In fact, polyploidy was induced in the presence of hypophosphorylated Rb (Figure 2B). Furthermore, decreased polyploidy was observed with Aurora B inhibition in cells that lack Rb compared with cells with intact Rb (Figure 3C), suggesting that Rb may functionally promote polyploidy in this cellular context. Introduction of wild-type Rb into Rb null cells enhanced AZD1152-induced polyploidy concomitant with the appearance of hypophosphorylated protein, whereas transfection of siRNA targeting Rb led to decreased polyploidy in Rb wild-type cells (Figure 3D). The importance of active Aurora B in inhibiting cells from entering endoreduplication is obvious from the reduction of nocodazole-induced polyploidy in Mad2-haplosufficient HCT-116 cells overexpressing Aurora B (Figure 3B). Taken together, these initial observations suggest that in the context of Aurora B inhibition Rb promotes DNA endoreduplication and polyploidy after an aberrant mitosis.

We went on to investigate how this unique Rb function may be regulated. Rb phosphorylation status and function has been classically attributed to cyclin:cdk activity (Weinberg, 1995). Although both cdk 2 and cdk 4 activity did decrease with Aurora B inhibition, Rb hypophosphorylation preceded this inhibition of kinase activity (Figure 2Ai). We hypothesized that Aurora B itself was the endogenous kinase specifically responsible for regulating Rb in this context. In fact, we found that Aurora B directly phosphorylates the C terminus of Rb *in vitro*, and utilizing mass spectrometry, we identified serine 780 as an Aurora B phosphorylation site. We confirmed that phosphorylation at serine 780 also decreases *in vivo* with the same kinetics as the appearance of hypophosphorylated Rb in response to AZD1152 treatment (Figure 5C). Although introduction of an Rb wild-type construct increased AZD1152-induced polyploidy in Rb-negative SaOs2 cells, an Rb mutant with serine 780 changed to a phospho-mimetic aspartate (S780D) prevented Rb-mediated polyploidy in response to Aurora B inhibition (Figure 6A). This effect was further confirmed by the increased binding of the S780D to E2F1 and a decreased E2F1 promoter activity in S780D-overexpressed cells upon Aurora B inhibition (Figure 6, B and C). Thus, we conclude that

serine 780 is an endogenous Aurora B phosphorylation site on Rb that impacts regulation of endoreduplication after exit from an aberrant mitosis.

These findings collectively suggest that the functional role of Rb in regulating the commitment to DNA replication differs depending upon the cellular context. It is well established that hypophosphorylated Rb is the functional form of the tumor suppressor that inhibits E2F transactivation, resulting in G1 arrest (Weinberg, 1995; Lam and La Thangue, 1994). However, we found that, although expression of wild-type Rb enhanced the G1 peak in asynchronous cells, in the context of Aurora B inhibition Rb actually promoted cell cycle progression with polyploidy induction (Figure 6). These results suggest that in the context of unperturbed cell cycles and normal mitoses, ectopic Rb expression delays G1 progression, but after aberrant mitoses in the setting of Aurora B inhibition, Rb plays a functionally distinct role by promoting endoreduplication. In fact, endoreduplication in the context of active Rb has been previously reported: cells expressing the phosphorylation resistant murine Δ cdk Rb mutant (mutated at 10 of the 16 potential cdk phosphorylation sites), which constitutively inhibits E2F transactivation, surprisingly undergo endoreduplication after a prolonged G1 arrest (Lukas *et al.*, 1999). Additionally, Rb expression has been reported to be required for cell cycle progression as well. The proliferative advantage of the constitutively active H-Ras^{V12} mutant is dependent on the presence of functional Rb; cells harboring the Ras mutant proliferate more slowly when Rb is down-regulated via RNAi (Williams *et al.*, 2006). Although the mechanism(s) by which Rb may execute these functions has yet to be elucidated, one hypothesis that has been posed is that Rb-mediated suppression of other pocket protein family members' expression is required for cell cycle progression (Williams *et al.*, 2006).

Although this question clearly is worthy of further investigation, our data does indicate that when Rb is present, Aurora B phosphorylation leads to stabilization of Rb:E2F1 complexes and inhibition of endoreduplication, consistent with an Aurora B role in negatively regulating DNA synthesis after an aberrant mitosis with failed cytokinesis (Figure 6C). Though the addition of phosphates on Rb in the G1-S phase transition is generally associated with cell cycle progression, it has been previously demonstrated that the activation of cyclin D-associated kinase activity in the G0-G1 transition also leads to phosphorylation of Rb that stabilizes the Rb:E2F association, leading to repression of E2F target genes (Ezhevsky *et al.*, 2001). Our data reaffirms that the functional significance of Rb phosphorylation at a specific phospho-acceptor site has to be interpreted in the cellular context in which it occurs. After failed mitosis with aberrant mitotic exit, persistent Aurora B activity in pseudo G1 acts as an intrinsic cellular checkpoint to prevent inappropriate DNA replication and polyploid cell formation by directly phosphorylating Rb to repress expression of E2F1 target genes. This is a novel role for Aurora B outside of mitosis to prevent cellular progression toward aberrant cell cycles that may be relevant to other physiological scenarios of mitotic failure.

Lastly, our findings also hold therapeutic implications for the future development of Aurora B targeted therapy as tumors with differing Rb status would be predicted to have distinct responses. Preliminary work suggests that Rb negative tumor cells undergo increased apoptosis in the context of decreased polyploidy (data not shown). Such findings will be critical for eventually delineating susceptibility and resistance mechanism(s) of Aurora B targeted therapy that would need to be incorporated in future clinical testing.

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