Aurora A Regulates Prometaphase Progression by Inhibiting the Ability of RASSF1A to Suppress APC-Cdc20 Activity

Su Jung Song, Min Sup Song, Soon Jung Kim, Seo Yeon Kim, Seung Hae Kwon, Jhin Gook Kim, Diego F. Calvisi, Dongmin Kang, and Dae-Sik Lim

Abstract

The Aurora (Ipl) kinase family plays important roles in the regulation of mitosis and tumorigenesis. The tumor suppressor RASSF1A controls mitotic progression by regulating anaphase-promoting complex (APC)-Cdc20 activity and microtubule stability, but the mechanism by which this action is regulated has not been previously established. Here, we show that Aurora A and B associate with and phosphorylate RASSF1A on serine 203 in vivo at different times and in different subcellular compartments during mitosis. Notably, both depletion of Aurora A by RNA interference and expression of a nonphosphorylatable RASSF1A (S203A) mutant gene led to a marked delay in prometaphase progression. This is likely because of the failure of RASSF1A to dissociate from Cdc20, constitutive inhibition of APC-Cdc20, and accumulation of mitotic cyclins. In contrast, the delay in prometaphase progression caused by Aurora A depletion was largely normalized by phosphomimetic RASSF1A (S203D).

Introduction

Mitosis is the fundamental process by which duplicated chromosomes are divided equally into two daughter cells (1). Aurora kinases regulate cell cycle progression from G2 phase to cytokinesis in a coordinated manner (1–3). Aurora A contributes to centrosome maturation and the establishment of spindle bipolarity by phosphorylating several substrates, including Eg5, TACC, CENP-A, and Ajuba (4–7). Aurora A localizes to centrosomes during interphase and to spindle poles and fibers from prometaphase to anaphase (3). Inactivation of Aurora A delays, but does not block, mitotic entry (2, 6, 8); however, little is known of the role of Aurora A in mitotic progression beyond this point (6, 9).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(20); human Aurora A, 5'-AUGGCCUGCUAUGCUGA-3' (30); and human Aurora B, 5'-GGUGAGGGAGUACGAGU-3' (31).

**Immunoprecipitation.** Cells were lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L MgCl2, 0.5% Triton X-100, protease inhibitors, and phosphatase inhibitors. Cell debris was removed by centrifugation and the lysates were incubated for 1 h at 4°C with protein A-agarose–coupled antibodies (Sigma). The immunoprecipitates were washed four times with lysis buffer and then analyzed by immunoblotting.

**In vitro kinase assays.** Immunoprecipitates prepared from cell lysates or purified Aurora A (Crystall Genomics, Inc.) or Aurora B (Upstate Biotechnology) were incubated for 30 min at 30°C with purified GST fusion proteins of wild-type RASSF1A or various RASSF1A mutants and [γ-32P]ATP. Reaction mixtures were resolved by SDS-PAGE and phosphorylated proteins were detected by autoradiography.

**Immunofluorescence and time-lapse microscopy.** Immunofluorescence experiments were performed as previously described (20). For time-lapse phase-contrast microscopy, images were collected from cells grown in a microincubation chamber that was maintained at 37°C, supplied with CO2, and placed on the stage of a Carl Zeiss LSM510 META microscope (C-Apochromat 40×/1.2 W corr) coupled with a PDM detector connected at the side port. The GFP and RFP channels used 488- and 543-nm argon laser lines for excitation, and differential interference contrast microscopy images were also recorded. Data were processed with LSM 510 META System software. The total cell fluorescence minus background was quantified for each cell in successive images of a time series and plotted over time. Initial pixel values are indicated in the figure legends.

**Cell cycle analysis.** Cells transfected with pcDNA-CD4 (surface marker) and vectors for siRNAs specific for Aurora kinases or wild-type or serine 203 mutant forms (S203A, S203D) of RASSF1A at a mass ratio of 1:10 were collected at various times after release from thymidine block and incubated for 1 h at 4°C with FITC-conjugated anti-CD4 (Boston Biochem). The cells were then washed thrice with PBS, fixed with 70% ethanol, stained with propidium iodide (25 μg/mL, Sigma), and incubated for 30 min at 37°C with RNase A (20 μg/mL, Roche). The DNA content of cells was then evaluated by flow cytometry using a FACScan instrument. Linear red fluorescence (FL2) in green (CD4-expressing) cells was analyzed.

**In vitro ubiquitination assays.** The APC was immunopurified from lysates of mitotic HeLa cells using anti-Cdc27 beads (Santa Cruz Biotechnology). In vitro assays of NH2-terminal (amino acids 1–102) ubiquitination of Xenopus cyclin B1 were performed as previously described (20).

**Human tissue sample analysis.** Patient samples were evaluated by immunohistochemistry using anti–Aurora A and anti–p-RASSF1A (pS203) antibodies. The study cohort comprised consecutive lung tumors ascertained at the Samsung Biomedical Center. All biopsies were evaluated at the Samsung Biomedical Center, and histologic diagnoses were based on established criteria. Human hepatocellular carcinoma (HCC) and matched surrounding nontumor liver tissues were analyzed by immunoblotting with established criteria. Human hepatocellular carcinoma (HCC) and matched surrounding nontumor liver tissues were analyzed by immunoblotting.

**Results**

**RASSF1A specifically interacts with Aurora A and Aurora B at different times and in different subcellular compartments during mitosis.** The localization of both RASSF1A and Aurora kinases at mitotic spindles and poles during mitosis led us to investigate possible functional interactions between these proteins. In vitro and in vivo binding assays revealed that Aurora A and B specifically interact with RASSF1A (Fig. 1A; Supplementary Fig. S1B). RASSF1A also colocalized with Aurora A and Aurora B to spindle poles and midbody, respectively (Supplementary Fig. S1A). Notably, a communoprecipitation analysis of HeLa cell lysates obtained at various stages of mitosis revealed that endogenous RASSF1A interacts with endogenous Aurora A during early mitosis and with Aurora B during late mitosis (Fig. 1A).

Intriguingly, the interaction of RASSF1A with Cdc20 occurred at earlier time during mitosis than did the interaction of RASSF1A with Aurora A. Finally, we found that the COOH terminus of Aurora A is essential for binding to RASSF1A (Supplementary Fig. S2A), which interacts via its COOH-terminal C2 domain (amino acids 170–229) with Aurora A (Supplementary Fig. S2B).

**RASSF1A is primarily phosphorylated on serine 203 by Aurora A and Aurora B in vivo.** We next investigated the ability of Aurora kinases to phosphorylate RASSF1A. An in vitro kinase assay revealed that wild-type, but not kinase-dead, Aurora A and Aurora B phosphorylated purified GST-RASSF1A (Fig. 1B), predominantly in the C2 domain (amino acids 170–229; Supplementary Fig. S3A). To identify the primary site phosphorylated by Aurora kinases, we created mutants in which all eight serine/threonine/tyrosine sites within the C2 domain were individually changed to alanine. Phosphorylation of the S203A mutant was almost undetectable, whereas other C2-domain mutants were clearly phosphorylated (Supplementary Fig. S3B). Similarly, the full-length GST-RASSF1A (S203A) mutant was not phosphorylated by purified protein Aurora A or Aurora B (Fig. 1C, left). Thus, serine 203 of RASSF1A is the principal phosphorylation site for Aurora A and Aurora B. It was recently shown that Aurora A phosphorylates RASSF1A at both threonine 202 and serine 203 (28). However, we found that the GST-RASSF1A (T202A) mutant was still phosphorylated and phosphorylation of full-length or C2-domain constructs of GST-RASSF1A (S203A) was undetectable in an in vitro Aurora kinase assay (Fig. 1D; Supplementary Fig. S3B). These results suggest that serine-203 is the primary site of RASSF1A phosphorylation by Aurora kinases.

To confirm this phosphorylation of RASSF1A by Aurora kinases in vivo, we generated phosphoryserine-203–RASSF1A (p-RASSF1A)–specific antibodies. These antibodies reacted only with purified RASSF1A that had been phosphorylated on serine 203 by Aurora kinases; this reactivity could be blocked by preincubation of the antibodies with the phosphopeptide antigen but not with the nonphosphopeptide (Fig. 1C, right). We further confirmed the specificity of these antibodies by using RASSF1A−/− mouse embryonic fibroblasts (MEF) and RASSF1A siRNA-transfected HeLa cells (Supplementary Fig. S4A). Of interest, p-RASSF1A was low in asynchronous cells, whereas its levels were higher in mitotic cells; this p-RASSF1A disappeared after treatment with X-protein phosphatase (Supplementary Fig. S4A).

An immunofluorescence analysis revealed that p-RASSF1A was localized to metaphase spindle poles, a staining pattern that was abolished in the presence of the phosphopeptide antigen but not the nonphosphopeptide (Supplementary Fig. S4B). Thus, Aurora A and Aurora B indeed specifically phosphorylate RASSF1A on serine 203 in vivo.

**Aurora A and Aurora B differentially phosphorylate RASSF1A on serine 203 during mitosis.** Because both Aurora A and Aurora B phosphorylate RASSF1A at the same site, we hypothesized that they can differentially phosphorylate RASSF1A during mitosis. To show this, we depleted either endogenous Aurora A or Aurora B using RNA interference (RNAi; Supplementary Fig. S4C) and performed an immunofluorescence analysis with p-RASSF1A antibodies. In control cells, p-RASSF1A colocalized with Aurora A to spindle poles during early mitosis and with Aurora B to the spindle midzone and midbody during late mitosis (Fig. 2A). Interestingly, in cells depleted of Aurora A, p-RASSF1A was undetectable at spindle poles during early mitosis, whereas...
p-RASSF1A localized to the spindle midzone and midbody during late mitosis (Fig. 2B, left). Likewise, Aurora B depletion eliminated p-RASSF1A localization to the spindle midzone and midbody during late mitosis without affecting p-RASSF1A localization to spindle poles during early mitosis (Fig. 2B, right). Taken together, these data suggest that, in vivo, Aurora A and Aurora B specifically phosphorylate RASSF1A on serine 203 during mitosis at different times and in different cellular compartments and may play important roles in at least two distinct mitotic regulatory mechanisms.

Phosphorylation of RASSF1A on serine 203 is required for mitotic progression. To gain insight into the functional relevance of mitotic phosphorylation of RASSF1A on serine 203, we first examined the effects of RASSF1A phosphorylation on mitotic progression. We found that reintroducing RASSF1A (S203A) mutant into RASSF1A−/− MEFs led to a profound elongation of mitosis accompanied by accumulation of cyclins A and B compared with wild-type cells (Fig. 3A). Overexpression of RASSF1A (S203A) in HeLa cells also resulted in a marked delay in mitotic progression that was accompanied by an extended accumulation of cyclins A and B (Supplementary Figs. S5B and S6A). Of interest, exogenous RASSF1A (S203A) retained the ability to localize to spindle poles, whereas endogenous p-RASSF1A was hardly detectable, suggesting that the exogenous S203A mutant inhibited phosphorylation of endogenous RASSF1A in a dominant-negative manner (Supplementary Fig. S5A).

We next examined the effects of Aurora kinases on RASSF1A-mediated regulation of mitotic progression. During the preparation
of this article, Rong and colleagues (28) reported that a phosphomimetic mutant of RASSF1A (T202E/S203E) did not associate with microtubules during interphase and proposed that Aurora A regulates RASSF1A-microtubule interaction and mitotic progression. Our data contradict this finding. Using two different phosphomimetic mutants of RASSF1A (T202E/S203E and T202D/S203D) and the nonphosphorylatable RASSF1A (S203A) mutant, we found that the phosphorylation status of serine 203 had no effect on the localization of RASSF1A to either cytoplasmic microtubules in interphase or to the mitotic apparatus in mitosis (Fig. 3B; Supplementary Fig. S6B; see also Discussion). Consistently, a coimmunoprecipitation analysis revealed that any of the RASSF1A phosphorylation residues could be substituted without affecting RASSF1A association with tubulin (Fig. 3C). Taken together, these data suggest that phosphorylation of RASSF1A on serine 203 by Aurora kinases is required for normal mitotic progression independent of the ability of RASSF1A to associate with microtubules.

Depletion of Aurora A inhibits prometaphase progression after the G2-M transition, a delay that is largely recovered by the phosphomimetic RASSF1A (S203D) mutant. To understand the effects of Aurora kinases on RASSF1A-mediated regulation of mitotic progression, we analyzed mitotic progression in Aurora kinase–depleted cells. In agreement with previous findings (6), measuring the mitotic index showed that depletion of Aurora A, but not Aurora B, delayed the G2-M transition (Fig. 4A). Interestingly, Aurora A depletion also inhibited mitotic progression beyond entry into mitosis (Fig. 4A) and was accompanied by the accumulation of cyclins A and B (Supplementary Fig. S7A). Notably, progression after mitotic entry, but not G2-M transition, was restored in Aurora A–depleted cells by the expression of the phosphomimetic RASSF1A (S203D) mutant. To exclude the possibility that the delay of mitotic progression might simply reflect the elongation of the G2 phase by Aurora A depletion (6), we next examined the mitotic progression of individual cells immediately after mitotic entry in real time by monitoring the degradation of a GFP–cyclin A fusion protein (33–35). Indeed, the depletion of Aurora A, but not Aurora B, significantly delayed prometaphase progression (average = 107 min; n = 8). This Aurora A depletion–mediated delay was largely normalized by the expression of phosphomimetic RASSF1A (S203D) (average = 56 min; n = 8), but not by wild-type RASSF1A (average = 100 min;
n = 8; Fig. 4B and C; Supplementary Fig. S7B). Thus, exogenous RASSF1A (S203D) is likely sufficient to inhibit the function of endogenous nonphosphorylated RASSF1A on serine 203 in cells depleted of Aurora A. Taken together, these data suggest that mitotic phosphorylation of RASSF1A on serine 203 by Aurora A specifically contributes to normal prometaphase progression.

Aurora A prevents RASSF1A from inhibiting APC-Cdc20 by phosphorylating RASSF1A on serine 203. RASSF1A exhibits spatial and temporal control over APC and Cdc20, binding to Cdc20 and inhibiting APC-Cdc20 activity during early prometaphase (20, 36, 37). Thus, we tested whether phosphorylation of RASSF1A by Aurora A affects its ability to interact with Cdc20. Notably, the phosphomimetic RASSF1A (S203D) mutant did not seem to interact with Cdc20 (Fig. 5A, left). We also performed an in vitro binding assay using a mixture of Cdc20 and purified wild-type GST-RASSF1A that has been previously phosphorylated by Aurora A. The interaction of Aurora A–treated GST-RASSF1A with Cdc20 was weak compared with that of GST-RASSF1A that had not been treated with Aurora A (Fig. 5A, right). Indeed, endogenous p-RASSF1A was barely detectable in immunoprecipitates of endogenous Cdc20 (Fig. 5B). Notably, the amount of endogenous RASSF1A bound to endogenous Cdc20 was increased in Aurora A–depleted HeLa cells compared with control or Aurora B–depleted cells (Supplementary Fig. S6C). Collectively, these data suggest that Aurora A regulates the interaction between RASSF1A and Cdc20.

In contrast to our current and previous (5) results, which showed that RASSF1A interacts with Cdc20 at prometaphase, Liu and colleagues failed to detect an interaction between RASSF1A and Cdc20 using a yeast two-hybrid assay (27). Because we had found that only a small portion of RASSF1A interact weakly with Cdc20 (20), it is possible that the apparent absence of an interaction in the study of Liu and colleagues could be explained by the assay conditions used. Using a yeast two-hybrid assay, we found that RASSF1A and Cdc20 indeed interacted under the low-stringency conditions (<10 mmol/L 3AT) generally used to detect low-affinity interactions. By comparison, the interaction of RASSF1A with Mst1, with which it forms a heterodimer through strong interactions with...
the SARAH domain (38), was clearly evident even in a high-stringency selective medium (>10 mmol/L 3AT; Fig. 5C and data not shown). Liu and colleagues also reported that RASSF1A did not bind Cdc20 in nocodazole-treated mitotic cells. Because RASSF1A interacts with Cdc20 at early prometaphase before spindle checkpoint activation (20, 36), RASSF1A should be largely dissociated from Cdc20 by the time the spindle checkpoint is activated by nocodazole treatment. Consistently, we also found

![Figure 4. Phosphorylation of RASSF1A on serine 203 by Aurora A regulates prometaphase progression. A, HeLa cells cotransfected with siRNAs specific for Aurora A or Aurora B and HA-tagged wild-type or the phosphomimetic mutant (S203D) of RASSF1A were immunostained with anti-phospho-histone H3 (p-Ser10) antibody and DAPI at the indicated times after release from thymidine block. The numbers of p-Ser10-positive cells were measured to establish the mitotic indices. B, HeLa cells cotransfected with Aurora A siRNA, GFP-cyclin A (33, 34), and HA-WT or the mutant (S203D) of RASSF1A were analyzed by time-lapse fluorescence microscopy. Stacks of images were taken at 4-min intervals. Selected individual frames and time points are shown. Scale bars, 10 μm (top). Time is shown in minute relative to nuclear envelope breakdown (NEBD). The total cell fluorescence minus background was quantified for each cell in successive images of a time series and plotted over time. Initial pixel values of GFP-cyclin A: 425.2 for scrambled (negative control) siRNA, 426.7 for Aurora A siRNA, 431.8 for Aurora A siRNA + RASSF1A-WT, and 427.4 for Aurora A siRNA + RASSF1A-S203D (bottom). C, the duration of mitotic progression from nuclear envelope breakdown to anaphase was quantified from all independent results for each experiment in B.](https://www.aacrjournals.org/canres/article-lookup/doi/10.1158/0008-5472.CAN-08-3984)
that RASSF1A did not interact with Cdc20 in nocodazole-treated mitotic cells (Fig. 5D). Thus, differences in sample preparation and time points selected could account for discrepancies between our results and the results of Liu and colleagues.

Finally, we investigated whether Aurora A–mediated RASSF1A phosphorylation affects the ubiquitin ligase activity of APC-Cdc20. An in vivo ubiquitination assay revealed that the ubiquitination of cyclin B, a target of APC-Cdc20, was markedly reduced by Aurora A depletion (Fig. 5E). To exclude the possibility that these in vivo data result from the elongation of G2 phase by Aurora A depletion, we performed an in vitro ubiquitination assay and found that the APC-Cdc20 activity was markedly reduced by nonphosphorylatable RASSF1A (S203A) compared with wild-type RASSF1A or the phosphomimetic RASSF1A (S203D) mutant (Fig. 5F). Taken together, these data suggest that Aurora A regulates APC-Cdc20 activity by inhibiting the interaction between RASSF1A and Cdc20.

Phosphorylation of RASSF1A on serine 203 is up-regulated by Aurora A overexpression in human tumors. Because Aurora A is overexpressed in many human cancers (10–12) and phosphorylates RASSF1A on serine 203, we examined whether phosphorylation of RASSF1A on serine 203 is up-regulated in human tumors that overexpress Aurora A. An immunohistochemical

**Figure 5.** Phosphorylation of RASSF1A on serine 203 by Aurora A induces dissociation from Cdc20 and prevents RASSF1A from inhibiting APC-Cdc20 activity. A, lysates from HeLa cells transfected with HA-WT or the mutants (S203D, S203A) of RASSF1A were immunoprecipitated with anti-Cdc20 antibody (left). Relative amounts of RASSF1A immunoprecipitated with anti-Cdc20 were quantified. Purified Aurora A immunoprecipitated with anti-Cdc20 were quantified. Purified Aurora A was incubated with GST-RASSF1A and ATP. The kinase reactions were incubated with in vitro translated (IVT) HA-Cdc20 and then subjected to GST pull-down assay (right). B, lysates from HeLa cells at the indicated times after release from thymidine block were immunoprecipitated with anti-Cdc20 antibody. C, for yeast two-hybrid assays, PJ69-4A strain cotransformed with pGBK7-Mst1 or Cdc20 and pACTII-RASSF1A were grown in selective medium (-Lue, -His, -Trp) or control medium (-Lue, -Trp). D, lysates from HeLa cells asynchronous (Asn) or treated with nocodazole (200 ng/mL, 16 h), were immunoprecipitated with an anti-Cdc20 antibody. E, lysates from HeLa cells cotransfected with siRNAs specific for Aurora A or Aurora B and HA-ubiquitin were analyzed for cyclin B ubiquitination in vivo. F, the APC purified from mitotic HeLa cells was assayed for ubiquitin ligase activity using an 35S-labeled fragment of Xenopus cyclin B1 in the presence of in vitro translated Cdc20 and wild-type or the mutants (S203A, S203D) of RASSF1A (top). The amounts of Cdc20 and RASSF1A in the reaction mixtures were also analyzed (bottom).
analysis revealed that Aurora A was overexpressed and the level of RASSF1A phosphorylated on serine 203 was elevated in human lung cancer biopsies in which RASSF1A had not been inactivated by promoter hypermethylation (Fig. 6A). We also confirmed the up-regulation of Aurora A–mediated RASSF1A phosphorylation in human lung biopsies by immunoblot analysis (Fig. 6B). In addition, we analyzed the Aurora A–RASSF1A regulatory pathway in human HCC and found that RASSF1A was expressed in all 15 HCC samples (of 60) in which RASSF1A was not inactivated by promoter hypermethylation (Supplementary Fig. S8). Notably, 11 of these 15 HCC samples showed a significant correlation between Aurora A expression and level of serine 203–phosphorylated RASSF1A (Fig. 6C). These results suggest that Aurora A–mediated phosphorylation of RASSF1A on serine 203 might be related to tumorigenesis.

**Discussion**

Abundant evidence suggests roles for Aurora A in centrosome maturation (39), spindle formation (1, 3), and G2–M transition (2, 6). Here, we show that Aurora A also interacts with and phosphorylates RASSF1A during early mitosis, playing a role in mitotic progression. Importantly, a marked delay in prometaphase progression induced by Aurora A depletion was largely normalized by the phosphomimetic RASSF1A (S203D) mutant (Fig. 4; Supplementary Fig. S7). Accordingly, we propose that Aurora A regulates prometaphase progression by inhibiting the ability of RASSF1A to suppress APC-Cdc20 activity (Fig. 6D).

Emi1, Mad2, and RASSF1A have been recently proposed to act as APC-Cdc20 inhibitors (37, 40). These inhibitors function at specific times and at particular cellular sites by binding to Cdc20 during mitosis (36). The inactivation of Emi1 at prometaphase is dependent on Plk activity and is required for degradation of cyclin A (41, 42). However, inhibition of Plk1 activity may not delay the timing of cyclin A degradation (34, 43, 44), suggesting that Emi1 degradation alone is not sufficient for cyclin A destruction during prometaphase. In addition, an excess of RASSF1A has been shown to induce the accumulation of cyclin A in Emi1-depleted cells (20). Considering these results, it is conceivable that the regulation of RASSF1A by Aurora A during prometaphase could initiate APC-Cdc20–mediated cyclin A degradation at the appropriate time. The activated spindle checkpoint prevents the degradation of cyclin B but not destruction of cyclin A (45, 46). Consistent with this observation, we found that nonphosphorylatable (S203A), but not phosphomimetic (S203D), RASSF1A inhibited the degradation of both cyclins A and B after nocodazole treatment (data not shown). Also, these mutants did not activate the spindle checkpoint (data not shown), suggesting that phosphorylation of RASSF1A by Aurora A does not regulate spindle checkpoint activation.

Rong and colleagues previously showed that the phosphomimetic RASSF1A (T202E/S203E) mutant did not associate with microtubules (28). In contrast, we showed that both phosphomimetic (S203D) and nonphosphorylatable (S203A) RASSF1A mutants interacted normally with microtubules and localized to microtubules in interphase and to the mitotic apparatus during mitosis (Fig. 3B and C; Supplementary Fig. S6B). Furthermore, we
showed that both endogenous p-RASSF1A and the nonphosphorylatable mutant form of RASSF1A are normally localized in spindle poles and other spindles (Fig. 2; Supplementary Fig. S5A). Thus, Aurora kinases are not required for the localization of RASSF1A to mitotic apparatus during mitosis. In fact, because Aurora A is a mitotic kinase and is inactive in interphase, it is very unlikely that phosphorylation of RASSF1A on serine 203 by Aurora A occurs during interphase, from which it follows that this pathway is not likely to regulate microtubule stability in interphase.

Our previously proposed model of RASSF1A-Cdc20 interaction (20) was recently challenged by Liu and colleagues, who suggested that RASSF1A does not interact with Cdc20 (27). However, Wang and colleagues have confirmed our results, demonstrating RASSF1A/Cdc20 interaction during early mitosis in vivo (21). In addressing these discrepancies, we also show that RASSF1A/Cdc20 interaction is relatively weak but nevertheless significant compared with Mst1/RASSF1A heterodimer interactions (Fig. 5C). Consistent with the results of Liu and colleagues, we also showed that RASSF1A/Cdc20 interaction was generally abolished by nocodazole treatment, but this was not the case following release from a double thymidine block (Fig. 5D). Consistently, RASSF1A interacts with Cdc20 at early prometaphase before spindle checkpoint activation (20, 36). It is also conceivable that this RASSF1A/Cdc20 interaction largely ceases during spindle checkpoint activation (36) and this process could be regulated by Aurora kinases.

The existence of a possible feedback mechanism was implied in a recent report suggesting that RASSF1A is an activator of Aurora A in vivo; however, this mechanism was not shown to occur in vitro (47). In fact, we also found no evidence for activation of Aurora A by RASSF1A in an in vitro kinase assay (data not shown). Further investigations will be needed to confirm the existence and regulatory significance of this proposed pathway.

Importantly, this study shows that phosphorylation of RASSF1A is mediated by both Aurora A and Aurora B at different times and in different cellular compartments in vivo during mitosis. Of interest, during interphase, p-RASSF1A is localized to nuclear bodies that are not shared with either Aurora A or Aurora B (Fig.2). Indeed, RASSF1A serine 203 is also phosphorylated by cyclin D–Cdk4 during interphase and is required for RASSF1A binding to Skp2 and subsequent ubiquitin-dependent degradation (29), suggesting that RASSF1A phosphorylation on serine 203 achieved by the actions of different kinases in different cellular compartments play distinct roles in cell cycle regulation.

Disclosed that deregulation of Aurora kinases and inactivation of RASSF1A have been implicated in tumorigenesis (12, 13, 16, 18, 19, 48), it will be important to determine whether the oncogenicity of Aurora kinases regulates the tumor-suppressor activity of RASSF1A. Indeed, we showed that phosphorylation of RASSF1A on serine 203 is increased by Aurora A overexpression in human lung cancer and hepatocarcinoma (Fig. 6A–C). Therefore, because Aurora kinase inhibitors have been proposed as new anticancer agents (49, 50), the phosphorylation status of RASSF1A in human cancers could prove to be a specific and quantifiable tumor marker.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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