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Priority Report

Aurora B–Mediated Phosphorylation of RASSF1A Maintains Proper Cytokinesis by Recruiting Syntaxin16 to the Midzone and Midbody

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Abstract

Aurora B is critically involved in ensuring proper cytokinesis and maintaining genomic stability. The tumor suppressor RASSF1A regulates cell cycle progression by regulating mitotic progression, G_1-S transition, and microtubule stability. We previously reported that both Aurora A and Aurora B phosphorylate RASSF1A, and showed that phosphorylation of RASSF1A by Aurora A blocks the inhibitory function of RASSF1A toward anaphase-promoting complex-Cdc20. However, the role of Aurora B–mediated RASSF1A phosphorylation remains unknown. Here, we show that phosphorylation of RASSF1A on Ser203 by Aurora B during late mitosis has a critical role in regulating cytokinesis. Notably, RASSF1A interacts with Syntaxin16, a member of the t-SNARE family, at the midzone and midbody during late mitosis. Aurora B is required for this interaction and for the subsequent recruitment of Syntaxin16 to the midzone and midbody, a prerequisite for the successful completion of cytokinesis. Furthermore, Aurora B depletion results in a failure of Syntaxin16 to properly localize to the midzone and midbody, a mislocalization that was prevented by overexpression of the phosphomimetic RASSF1A (S203D) mutant. Finally, either depletion of Syntaxin16 or expression of the nonphosphorylatable RASSF1A (S203A) mutant results in cytokinesis defects. Our findings implicate Aurora B–mediated phosphorylation of RASSF1A in the regulation of cytokinesis. [Cancer Res 2009;69(22):8540–4]

Introduction

Cytokinesis is the physical process of cell division after mitotic segregation of the genetic material (1), a process that includes furrow ingression and abscission in mammalian cells (2). Protein signaling networks regulated by Rho GTPase and SNARE-dependent membrane fusion proteins have been implicated in the regulatory mechanisms of cytokinesis (1, 3). Failure of cytokinesis can lead to tetraploidy and thereby contribute to tumorigenesis (4).

Aurora B interacts with INCENP, survivin, and borealin to form a chromosome passenger complex to allow the spindle midzone/midbody localization of MKLP1/MgcRacGAP by Aurora B–mediated phosphorylation (5–7).

RASSF1A has important roles in regulating both anaphase-promoting complex (APC)-Cdc20 activity and microtubule stability (8, 9). RASSF1A is phosphorylated by both Aurora A and Aurora B (10, 11), and phosphorylation of RASSF1A by Aurora A prevents RASSF1A from exerting its inhibitory function on APC-Cdc20 (11). The role of phosphorylation of RASSF1A by Aurora B, which has remained unknown, is addressed here.

Materials and Methods

Yeast two-hybrid screen. Yeast two-hybrid screening was done as previously described (12) using pGBKT7-RASSF1A as bait. Positive interactions were confirmed by growing double transformants of PJ69-4A cells in selective media plates [–Lue, –His, –Trp, in the presence of 5 mmol/L 3-aminotriazole (3AT)] and control media plates (–Lue, –Trp).

Antibodies. Antibodies to RASSF1A and Ser203-phosphorylated RASSF1A have been previously described (11). Guinea pig polyclonal antibodies to Syntaxin16 were prepared against glutathione *S*-transferase fusion proteins of full-length Syntaxin16. Specific antibodies were affinity purified using the appropriate antigen. Other antibodies used include anti-HA (Roche), anti-Flag, anti–β-actin (Sigma), anti–Aurora A (Santa Cruz Biotechnology), anti–Aurora B (Transduction Laboratories), anti-Syntaxin16 (Synaptic System), and 58K Golgi protein marker (Abcam) antibodies.

Immunoprecipitation, immunofluorescence, and time-lapse microscopy. Immunoprecipitation and immunofluorescence experiments were done as previously described (8). For time-lapse phase-contrast microscopy, images were collected from cells grown in a microincubation chamber (maintained at 37°C and supplied with CO_2) placed on the stage of an Olympus IX-71 microscope equipped with a Hamamatsu Orca charge-coupled device camera. Data were processed using Metamorph software.

Results and Discussion

RASSF1A interacts with the SNARE protein Syntaxin16 *in vitro* and *in vivo*. We previously reported that RASSF1A is phosphorylated by Aurora B at the midzone and midbody during late mitosis and by Aurora A at the spindle pole during early mitosis (11). Because RASSF1A is not involved in either the spindle checkpoint or the localization to the kinetochore (8, 11), we reasoned that phosphorylation of RASSF1A on Ser203 by Aurora B might be required for cytokinesis. Among the positive clones isolated from a yeast two-hybrid screen (12), one was identified as human Syntaxin16, a member of the t-SNARE membrane fusion protein family. The interaction of RASSF1A with Syntaxin16 was reconfirmed in a yeast two-hybrid screen (Fig. 1*A*). A coimmunoprecipitation experiment using *in vitro*–translated RASFF1A and Syntaxin16 revealed that RASSF1A interacts directly with Syntaxin16 (Fig. 1*B*). Importantly, RASSF1A seemed to interact with Syntaxin16 during late mitosis in HeLa cells (Fig. 1*C*). We also identified that the COOH-terminal region (residues 200–324) of Syntaxin16 binds to the COOH-terminal region (residues 110–344) of RASSF1A (Supplementary Fig. S2). Importantly, Syntaxin16 specifically localized not only to Golgi apparatus in interphase but also to the midzone and midbody during late mitosis, as determined by coimmunostaining with proteins including Aurora B, MKLP1, and INCENP, which are localized to midzone and midbody

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

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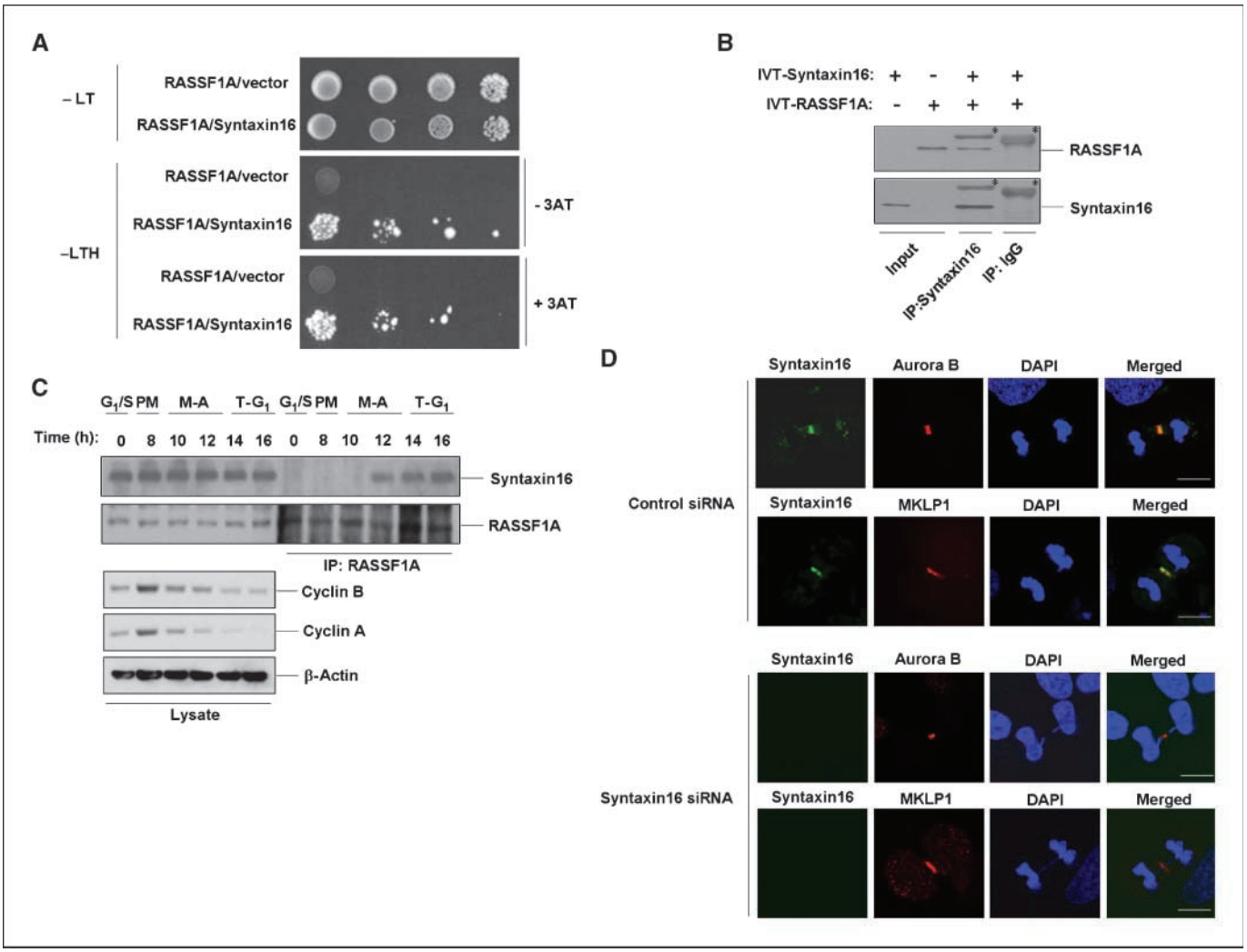

Figure 1. RASSF1A interacts with Syntaxin16 *in vitro* and *in vivo*. *A,* yeast strain PJ69-4A cells cotransformed with pGBKT7-Syntaxin16 and pACTII-RASSF1A were grown in selective media plates (–Lue, –His, –Trp; *LTH*) with or without 3AT and in control media plates (–Lue, –Trp; *LT*). *B, in vitro*–translated (*IVT*) RASSF1A was incubated with *in vitro*–translated Syntaxin16 for 1 h at 4°C, after which the binding mixture was immunoprecipitated (*IP*) with anti-Syntaxin16 antibody or control IgG. *C,* lysates of HeLa cells released from double-thymidine block for the indicated times were immunoprecipitated with an anti-RASSF1A antibody, and the resulting immunoprecipitates were analyzed by immunoblotting with anti-Syntaxin16 and anti-RASSF1A antibodies. Lysates were also subjected to immunoblotting with antibodies for cell cycle marker proteins. *D,* HeLa cells were transfected with control or Syntaxin16 siRNA, fixed, and then stained with anti-Syntaxin16 (*green*), anti–Aurora B (*red*) or anti-MKLP1 (*red*), and 4′,6-diamidino-2-phenylindole (DAPI; *blue*). *Bar,* 10 μm.

(Fig. 1*D*; Supplementary Fig. S3). Surprisingly, the localization of Syntaxin16 to the midzone and midbody was abrogated in either *Rassf1a*$^{-/-}$ MEFs or HeLa cells transfected with small interfering RNA (siRNA) targeting RASSF1A (Supplementary Fig. S1 and data not shown). Taken together, these results indicate that Syntaxin16 binds to RASSF1A and localizes to the midzone and midbody during late mitosis in a RASSF1A-dependent manner.

Phosphorylation of RASSF1A on Ser203 by Aurora B is required for the recruitment of Syntaxin16 to the midzone and midbody. We next examined the effect of Aurora B on the association of RASSF1A with Syntaxin16. The phosphomimetic RASSF1A (S203D) mutant interacted with Syntaxin16 to a greater extent than did wild-type RASSF1A or the nonphosphorylatable RASSF1A (S203A) mutant (Fig. 2*A*). We also found that Syntaxin16 specifically colocalized with endogenous Ser203-phosphorylated RASSF1A to the midzone and midbody during late mitosis in HeLa cells and *Rassf1a*$^{+/+}$ MEFs, but not in *Rassf1a*$^{-/-}$ MEFs (Fig. 2*B*; Supplementary Fig. S4). Thus, these data suggest that the interaction between RASSF1A and Syntaxin16 is regulated by Aurora B–mediated RASSF1A phosphorylation. Indeed, Syntaxin16 colocalized with the RASSF1A (S203D) mutant to the midzone and midbody, but did not colocalize with RASSF1A (S203A) (Supplementary Fig. S5). Although we still exactly do not know why endogenous RASSF1A did not permit Syntaxin16 association with the midzone and midbody in cells overexpressing RASSF1A (S203A), it is possible that RASSF1A (S203A) mutant functions as a dominant-negative mutant by forming oligomers with endogenous RASSF1A. In fact, this possibility can be supported by the previous observation that overexpression of RASSF1A (S203A) mutant blocks endogenous RASSF1A phosphorylation by Aurora A kinase (11).

Next, we tested whether the localization of Syntaxin16 and its interaction with the phosphorylated form of RASSF1A are dependent on Aurora B. We found that depletion of Aurora B abolished

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both the interaction between RASSF1A and Syntaxin16 and the localization of Syntaxin16 to the midzone and midbody, whereas depletion of Aurora A had no such effect (Fig. 2*C*; Supplementary Fig. S6). Intriguingly, expression of the RASSF1A (S203D) mutant significantly recovered not only the aberrant localization of Syntaxin16 but also the multinucleation that resulted from Aurora B depletion (Fig. 2*D*; Supplementary Fig. S7). However, we did not observe significant mislocalization of other Aurora B–regulated proteins including INCENP, MKLP1, and RhoA (data not shown). Taken together, these data suggest that the phosphorylation of RASSF1A by Aurora B enhances the interaction between RASSF1A and Syntaxin16 and is also required for the proper localization of Syntaxin16 to the midzone and midbody to allow a normal cytokinesis.

Phosphorylation of RASSF1A on Ser203 by Aurora B is required for completion of cytokinesis. Membrane fusion is a crucial step during cytokinesis before midbody abscission, and Syntaxin-mediated fusion of vesicles to the plasma membrane is also required for cytokinesis in *Drosophila, C. elegans*, and mammalian cells (13, 14). Thus, we investigated cell division in live cells in real time. Interestingly, Syntaxin16-depleted cells failed to complete cytokinesis due to a defect in furrowing and to furrow regression without abscission, whereas control cells successfully completed cytokinesis (Fig. 3*A, left*; Supplementary Fig. S8). A quantification of cells with abnormal cytokinesis (Fig. 3*A, right*) suggests that Syntaxin16 is required for completion of cytokinesis. Because Aurora B is essential for cytokinesis (5), we tested whether the phosphorylation of RASSF1A on Ser203 by Aurora B regulates cytokinesis. A time-lapse microscopy analysis revealed that a large population of cells transfected with the nonphosphorylatable RASSF1A (S203A) arrested at prometaphase, as previously reported (11). However, we found that a significant number of these cells slowly but eventually entered into metaphase and progressed to telophase; many of these cells (52%, $n = 40$) displayed defects in cytokinesis (Fig. 3*B*). These RASSF1A (S203A)–expressing cells ultimately exhibited multinucleation similar to cells depleted of Syntaxin16 (Fig. 3*C* and data not shown). Importantly, we also found that an NH_2-terminal mutant of Syntaxin16 that is defective in RASSF1A binding had significantly increased multinucleated cells (Supplementary Figs. S1 and S9). Finally, imaging of live cells showed that cytokinesis was abnormal in a majority (50%, $n = 35$) of *Rassf1a*$^{-/-}$ MEFs, but not in *Rassf1a*$^{+/+}$ MEFs (Fig. 3*D* and data not shown), consistent with a previous report (15). Intriguingly, the reintroduction of either wild-type or RASSF1A (S203D) mutant in *Rassf1a*$^{-/-}$ MEFs recovered a normal cytokinesis, whereas

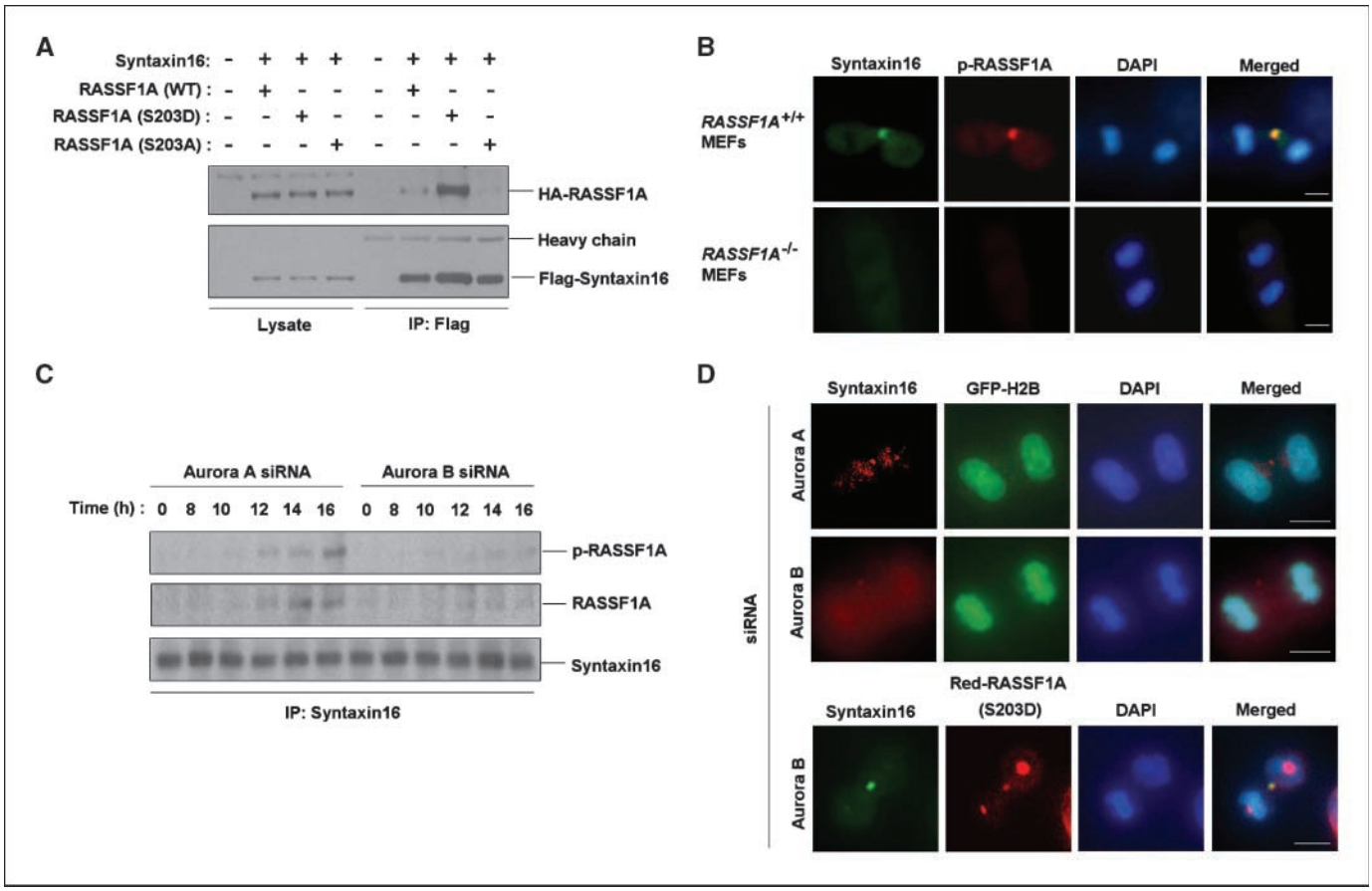

Figure 2. Aurora B regulates the interaction between RASSF1A and Syntaxin16 at the midzone and midbody during late mitosis. *A,* lysates of 293T cells expressing Flag-tagged Syntaxin16 and HA-tagged wild-type, S203D, or S203A mutants of RASSF1A were immunoprecipitated with an anti-Flag antibody, and the resulting immunoprecipitates (*IP*) were analyzed by immunoblotting with anti-HA and anti-Flag. *B, Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ MEFs were stained with anti–p-RASSF1A (*red*), anti-Syntaxin16 (*green*), and DAPI (*blue*). *Bar,* 10 μm. *C,* HeLa cells transfected with Aurora A or Aurora B siRNA were released from double-thymidine block for the indicated times and immunoprecipitated with an anti-Syntaxin16 antibody. The resulting immunoprecipitates were analyzed by immunoblotting with anti–p-RASSF1A, anti-RASSF1A, and anti-Syntaxin16. *D,* HeLa cells cotransfected with Aurora A or Aurora B siRNA and GFP-H2B (*green*; *top* and *middle*) or DsRed2-RASSF1A (S203D; *bottom*), as indicated, were stained with an anti-Syntaxin16 antibody (*red* or *green*) and DAPI (*blue*). *Bar,* 10 μm.

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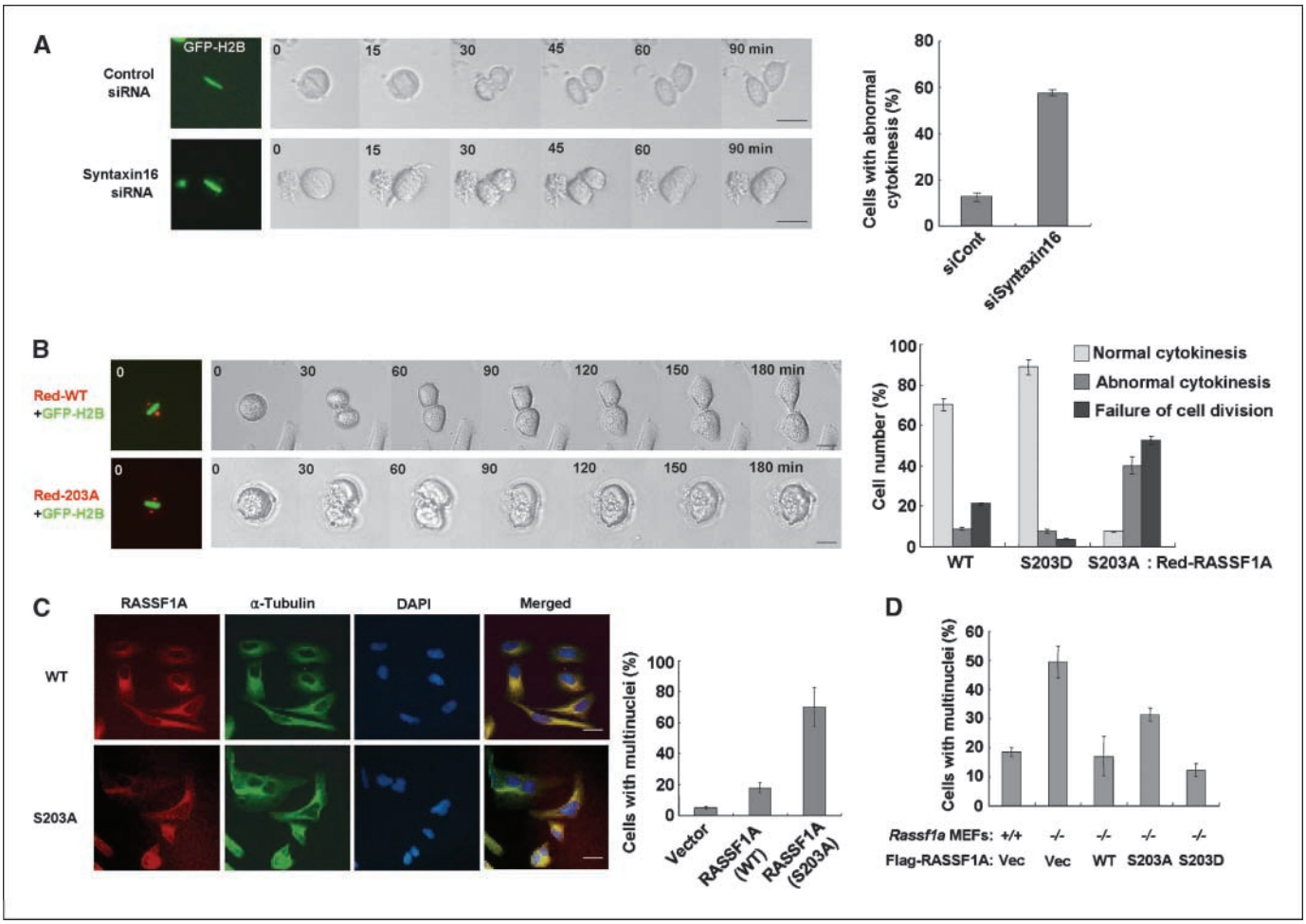

Figure 3. Syntaxin16 and phosphorylation of RASSF1A by Aurora B are required for completion of cytokinesis. *A,* HeLa cells cotransfected with control or Syntaxin16 siRNA and GFP-H2B were imaged during mitosis by time-lapse microscopy. The leftmost photos are fluorescence images and the remaining photos are phase-contrast images (*left*). *Bar,* 20 μm. Cells with abnormal cytokinesis were quantified (*right*; $n = 43$). *B,* HeLa cells expressing GFP-H2B (*green*) and DsRed2-wild-type RASSF1A or RASSF1A (S203A; *red*) were imaged during mitosis by time-lapse microscopy (*left*). *Bar,* 20 μm. Cells with abnormal cytokinesis were quantified (*right*). *C,* HeLa cells expressing DsRed2-wild-type RASSF1A or RASSF1A (S203A; *red*) were stained with an anti–α-tubulin (*green*) antibody and DAPI (*blue*). *Bar,* 10 μm (*left*). Multinucleated cells were quantified (*right*). *D, Rassf1a*$^{+/+}$ MEFs, *Rassf1a*$^{-/-}$ MEFs, and *Rassf1a*$^{-/-}$ MEFs reintroduced with wild-type or mutants of RASSF1A (S203A or S203D) were stained with anti–α-tubulin and DAPI, and then cells with multinuclei were quantified (each $n = 200$).

RASSF1A (S203A) mutant did not rescue the cytokinesis defects in *Rassf1a*$^{-/-}$ MEFs (Fig. 3*D*).

In summary, we show for the first time that phosphorylation of RASSF1A on Ser203 by Aurora B is required for the recruitment of Syntaxin16 to the midzone and midbody and the subsequent completion of cytokinesis. In addition, we found that the nonphosphorylatable RASSF1A (S203A) mutant failed to localize to the midzone and midbody, suggesting that Aurora B may also serve to recruit RASSF1A to the midzone and midbody by phosphorylating RASSF1A. Although *Rassf1a*$^{-/-}$ MEFs have been previously reported to exhibit no gross genomic instability (9), we found that *Rassf1a*$^{-/-}$ MEFs showed abnormal cytokinesis. We further showed that expression of the RASSF1A (S203A) mutant in *Rassf1a*$^{-/-}$ MEFs greatly exacerbated these cytokinesis defects, compared with *Rassf1a*$^{-/-}$ MEFs transfected with the RASSF1A (S203D) mutant or vector (Fig. 3*D* and data not shown). Therefore, it is likely that the simple protein abundance of RASSF1A is less important in the regulation of cytokinesis than the phosphorylation of RASSF1A by Aurora B.

Consistent with the finding that Syntaxin-mediated fusion of Golgi-derived vesicles to the plasma membrane is required for cytokinesis (13, 14), we found that localization of Syntaxin16 to the midzone and midbody, and thus completion of cytokinesis, depends on Aurora B–mediated phosphorylation of RASSF1A. Interestingly, Aurora B has been shown to be involved in the abscission checkpoint that protects against tetraploidization during cytokinesis (4). Thus, it would be interesting to investigate whether the interaction between RASSF1A and Syntaxin16 regulates the tetraploid checkpoint during cytokinesis in future studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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