Mitotic Arrest by Tumor Suppressor RASSF1A Is Regulated via CHK1 Phosphorylation

Lingyan Jiang, Rong Rong, M. Saeed Sheikh, and Ying Huang

Abstract

The tumor suppressor RAS-association domain family 1 isoform A (RASSF1A), by gene deletion or promoter hypermethylation has been observed in more than 40 types of human malignancies (1). Loss or altered expression of RASSF1A either by gene deletion or promoter hypermethylation has been observed in more than 40 types of human malignancies (1–4). Reintroduction of RASSF1A in RASSF1A-negative tumor cells suppresses their growth in vitro and in nude mice (reviewed in ref. 5).

RASSF1A plays an important role in cell-cycle regulation. Studies have shown that RASSF1A regulates both G1 and mitotic cell-cycle progression (6–12). RASSF1A-mediated cell-cycle regulation in G1 has been attributed to its ability to regulate different aspects of RASSF1A function (16). We and others have shown that RASSF1A-mediated mitotic cell-cycle regulation is closely associated with its ability to interact with and stabilize mitotic progression and overexpression of RASSF1A causes mitotic arrest (6, 7). We and others have shown that RASSF1A-mediated mitotic cell-cycle regulation is closely associated with its ability to interact with and stabilize microtubules (6, 7). Deletion of microtubule association region affects the ability of RASSF1A to regulate mitosis (6). In addition, RASSF1A is also shown to regulate mitosis via modulation of CDC20/anaphase-promoting complex to control anaphase phase cell-cycle progression (12).

Following more than a decade of its discovery, little is known about RASSF1A regulation at the protein level. We have recently reported the identification of Aurora-A as the first RASSF1A kinase (16). We showed that Aurora-A phosphorylated RASSF1A and modulated its ability to associate with microtubules (16). We further noted that RASSF1A phosphorylation by Aurora-A altered RASSF1A-induced mitotic arrest (16). Recently, several other kinases including cyclin-dependent kinase-4 (CDK4; ref. 17), protein kinase C (PKC; ref. 18), macrophage stimulating-1 (MST1; ref. 19), and Aurora-B (20) have also been identified to phosphorylate RASSF1A. Each of these kinases seems to regulate different aspects of RASSF1A function, suggesting that regulation of RASSF1A is a multiplex event involving multiple kinases that modulate RASSF1A function needed for various cellular processes.


Introduction

RAS-association domain family 1 isoform A (RASSF1A), the major transcript of RASSF1 gene is an important tumor suppressor (1). Loss or altered expression of RASSF1A either by gene deletion or promoter hypermethylation has been observed in more than 40 types of human malignancies (1–4). Reintroduction of RASSF1A in RASSF1A-negative tumor cells suppresses their growth in vitro and in nude mice (reviewed in ref. 5).

RASSF1A plays an important role in cell-cycle regulation. Studies have shown that RASSF1A regulates both G1 and mitotic cell-cycle progression (6–12). RASSF1A-mediated cell-cycle regulation in G1 has been attributed to its ability to regulate different aspects of RASSF1A function, suggesting that regulation of RASSF1A is a multiplex event involving multiple kinases that modulate RASSF1A function needed for various cellular processes.

Checkpoint kinase 1 (CHK1), a threonine/serine protein kinase, regulates cell-cycle checkpoint at G1–S, intra-S-phase, and G2–M transitions (21). Members of the Cdc25 family of dual-specificity phosphatases (Cdc25A, Cdc25B, Cdc25C) are believed to be crucial substrates of CHK1 for...
the regulation of DNA damage-induced cell-cycle checkpoints (22, 23). CHK1 is not only important in cell-cycle checkpoint activation in cells under stress, but also required for normal cell-cycle progression in uniastrressed cells (22–24). Studies have shown that CHK1 kinase activity is elevated during late-G1-phase as well as upon mitotic entry (24), and CHK1 depletion causes cell-cycle arrest at metaphase and chromosome misalignment (25).

A number of CHK1 substrates have been identified including the aforementioned Cdc25 family of dual-specificity phosphatases (Cdc25A, Cdc25B, Cdc25C; ref. 26), p53 (27), and Histone H3 (28). The common CHK1 phosphorylation motif has been identified as “R/K-X-X-S/T” with Lys or Arg located at -3 position of the threonine or serine resides (29). However, the key substrate of CHK1 during normal cell-cycle progression remains elusive.

CHK1 is shown to be activated via different mechanisms in various cellular conditions. For example, it is phosphorylated and activated by (i) ATM- and Rad3-related (ATR; ref. 23) and (ii) caspase-dependent cleavage when cells undergo apoptosis (30). In the case of caspase-dependent activation, caspase-mediated cleavage removes the C-terminal autoinhibitory domain of CHK1 and generates a truncated version composed of amino acids 1 to 299 (31). The truncated CHK1 possesses much higher kinase activity, that is about 8- to 20-fold higher than its full-length counterpart (30). Ectopic expression of this super-active (SA)-CHK1 has been shown to induce apoptosis (30), Thus, these studies suggest that CHK1 is crucial for both cell-cycle and cell-death regulation. However, the question as to how CHK1 exerts its function to regulate these cellular processes remains to be further elucidated.

In this study, we have identified CHK1 as a novel kinase for RASSF1A. We show that CHK1 phosphorylates RASSF1A at Serine 184 and ectopic expression of SA-CHK1 further enhances RASSF1A phosphorylation at Serine 184. We also found that CHK1 modulates the ability of RASSF1A to associate with microtubules and induce mitotic arrest. Our studies thus provide valuable new information about regulation of RASSF1A and demonstrate that phosphorylation of RASSF1A is one of the molecular mechanisms via which CHK1 regulates cell-cycle progression.

Materials and Methods

Plasmid constructs and proteins

S-tag RASSF1A protein were expressed and purified from a pET32b bacterial vector expression system following the protocol provided by the manufacturer (Novagen), pSRt-HA-S-F1A expression vector, containing both HA- and S-tags on the same vector, was generated, by fusing the RASSF1A cDNA downstream of the HA- and S-tags. GFP- and Myc-tagged RASSF1A expression vectors were generated in our previous studies (6). The RASSF1A point mutation and deletion variants were generated with the QuikChange XL mutagenesis kit (Stratagene). Flag-tagged CHK1 expression vectors were kindly provided by Dr. J. Lukas (Danish Cancer Society, Denmark; ref. 32) and Myc-tagged CHK1 expression vectors were kindly provided by Dr. T. Matsunaga (Kanazawa University, Japan; ref. 30). Active CHK1 proteins for the in vitro kinase assay were purchased from Millipore.

Antibodies and reagents

The Ser-184 phosphor-specific RASSF1A antibodies were generated commercially (Genscript) by immunizing rabbits with a synthetic peptide containing phospho-Ser-184 (CPSSKKPP[S]LQDARR). Total RASSF1A protein was analyzed using 2 different RASSF1A antibodies; one as reported in our previous study (6) and the other from eBioscience Inc., which was used for immunoprecipitation. The following antibodies were also used: CHK1 (G-4; Santa Cruz Biotechnology), α-tubulin (Sigma-Aldrich, Inc.), β-actin (AC-15; Sigma-Aldrich, Inc.), Flag (M2; Sigma-Aldrich, Inc.), GFP (clone 7.1 and 8.3; Roche Applied Science), HA (3F10; Roche Applied Science), Myc (9E10; Santa Cruz Biotechnology). Reagents, including nocodazole, taxol, thymidine, polybrene, and puromycin were purchased from Sigma-Aldrich, Inc.

Cell-culture conditions

Human cell lines MCF-7 (breast cancer), HeLa (cervical cancer), and 293T (human embryonic kidney) were regularly maintained in Dulbecco’s Modified Eagle Medium with supplementation of 10% FBS. All 3 cell lines used in this study were from the National Institutes of Health and are also available from the American Type Culture Collection. These cell lines were not further authenticated in our laboratories. All cell transfections were performed using Lipofectamine 2000 reagent (Invitrogen) per the manufacturer’s protocol.

Cell synchronization

To synchronize cells in mitotic phase, we used nocodazole- or taxol-mediated M-phase-block approach as previously described (16). Briefly, MCF-7 cells grown at 50% confluence were treated with nocodazole (0.5 μg/mL) or taxol (50 nmol/L) for 18 or 24 hours respectively, and the M-phase cells were harvested for subsequent experiments.

Immunoprecipitation and S-tag protein pull down

Immunoprecipitation and S-tag protein pull down assays were performed as we have previously described (33).

Immunostaining

Immunostaining was performed as we have previously described (16). Briefly, cells grown on glass slides were fixed and labeled with the indicated primary antibodies followed by rhodamine-anti-mouse or anti-rabbit secondary antibodies (Pierce Biotechnology, Inc.). DNA was counterstained with 4’,6-diamidino-2-phenylindole (DAPI) as previously described (16).

In vitro kinase assay

Two sources of CHK1 protein were used for in vitro kinase assays: (i) immunoprecipitated exogenously expressed CHK1
and (ii) recombinant active CHK1 protein purified from insect cells (Millipore). To immunoprecipitate exogenously expressed CHK1, cells were first lysed in a buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP-40, 1 mmol/L dithiothreitol (DTT), 1 mmol/L NaF, 1 mmol/L Na3VO4, 10 μg/mL leupeptin, 10 μg/mL Aprotinin, 400 μmol/L phenylmethylsulfonylfluoride, and 0.1 μg/mL okadaic acid. The exogenous Flag-tagged CHK1 kinase was immunoprecipitated from 1.5 mg of total cell lysates by 5 μg of anti-Flag antibodies. The immunocomplex or alternatively 100 ng of active CHK1 was then incubated together with 1 μg of purified RASSF1A in 50 μL of CHK1 kinase cocktail [20 mmol/L MOPS (pH 7.2), 10 mmol/L MgCl2, 5 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na3VO4, 25 mmol/L β-glycerophosphate, 10 μmol/L ATP, and 5 μCi γ[32P]ATP] at 30°C for 30 minutes. The reaction products were separated on a SDS-PAGE gel and subjected to autoradiography.

**In-gel kinase assay**

Detailed procedure describing in-gel kinase assay is mentioned in our previous study (16).

**Cell-cycle analysis**

Cell-cycle analyses were performed as we have previously described (16). Briefly, 293T or MCF-7 cells seeded at ~30% to 40% confluence were transiently transfected with either GFP-alone vector or constructs carrying the GFP-tagged wild type or mutant RASSF1A. Cells were fixed and subjected to flow cytometry analysis. The DNA content of the GFP-positive cells was evaluated.

**Results**

**CHK1 directly phosphorylates RASSF1A in vitro and in the cells**

We have previously used an *in vitro* in-gel kinase assay as a screening approach to search for novel RASSF1A kinase(s). Those studies led to the identification of Aurora-A as an RASSF1A kinase (16). We used similar approach to search for additional novel RASSF1A kinases. Two different SDS-PAGE gels, generated by embedding the purified RASSF1A recombinant protein or bovine serum albumin (BSA; as negative control) into the polyacrylamide gels, were used to separate the MCF-7 cell lysates. After electrophoresis, kinase assays were performed directly on the electrophoresed gels (see Materials and Methods). As shown in Fig. 1A, a 32P-labeled band of ~ 54 kDa was seen only in the RASSF1A-embedded gel (lanes 1–3) but not in the BSA-embedded gel (lanes 4–6); and the band intensity was stronger with cell lysates of taxol- or nocodazole- M-phase-arrested cells than that of the untreated cells (compare lanes 2 and 3 with lane 1). These findings suggested that the purified RASSF1A protein was phosphorylated by a kinase of approximately 54 kDa that was activated during mitosis. We then performed literature and database searches to gain some insight into the identity of this possible RASSF1A kinase. Among all kinases that are activated during mitosis, CHK1 has a molecular mass of 54.4 kDa (34, 35). Previous studies have shown that CHK1 is activated during mitosis and required for mitotic progression; its depletion causes metaphase block and apoptosis (25). To further determine whether CHK1 was indeed an RASSF1A kinase, we performed *in vitro* CHK1 kinase assay using the immunoprecipitated Flag-tagged wild-type (WT)-CHK1 and the purified recombinant RASSF1A (as substrate). The kinase-dead (KD)-CHK1 (CH1-KD130A) and myelin basic protein (MBP, a generic substrate) were also utilized as a proper negative and positive controls. Figure 1B shows that WT-CHK1 strongly phosphorylated MBP (top, lane 1), demonstrating the kinase activity of the immunoprecipitated-CHK1; and similarly, CHK1 also strongly phosphorylated the recombinant RASSF1A (lane 3). By contrast, RASSF1A phosphorylation was significantly reduced when KD-CHK1 was used instead of WT-CHK1 (lane 4). The efficiency of immunoprecipitation of exogenous WT- and KD-CHK1 was also shown by Western blot analysis of the immunoprecipitation products (Fig. 1B, bottom). These results demonstrate that RASSF1A is phosphorylated by the WT-CHK1 in *vitro* whereas the kinase-dead mutation in CHK1 significantly diminishes such ability.

Next, we used CHK1-specific inhibitor UCN-01 to investigate possible CHK1-mediated RASSF1A phosphorylation in cells. For this purpose, 293T cells transfected with HA-tagged RASSF1A were treated with CHK1-specific inhibitor UCN-01, and RASSF1A phosphorylation status was determined by presence of the upshifted RASSF1A-specific band. As shown in Fig. 1C, in cells that were not treated with CHK1 inhibitor (lane 1), RASSF1A was detected both in phosphorylated (upper band) and unphosphorylated (lower band) states. However, treatment with UCN-01 reduced phosphorylated RASSF1A levels in a dose-dependent manner (lanes 2 and 3). These results suggest that CHK1 phosphorylates RASSF1A inside the cells. We further sought to determine whether CHK1 can directly phosphorylate RASSF1A. To that end, recombinant versions of purified CHK1 and RASSF1A were used in *in vitro* kinase assay. As expected, the purified CHK1 autophosphorylated itself (Fig. 1D, lanes 1, 2, and 4, top arrow) as well as the purified pan-substrate MBP (lane 4). Importantly, CHK1 also strongly phosphorylated the purified S-tagged RASSF1A (lane 2), whereas the S-tag-alone peptide, used as negative control, displayed background level signal (lane 1). RASSF1A phosphorylation was also not noted when purified BSA was used instead of CHK1 (Fig. 1D, lane 3). Together these results indicate that CHK1 directly phosphorylates RASSF1A.

**CHK1 interacts with RASSF1A**

We also sought to determine the mutual interactions between RASSF1A and CHK1. Co-immunoprecipitation assay was performed using the exogenously expressed Myc-tagged RASSF1A and Flag-tagged CHK1. As shown in Fig. 2A, Flag-tagged CHK1 (lane 2), but not Flag-tag-only (lane 1), pulled down the Myc-tagged RASSF1A. Similarly, when co-immunoprecipitation assay was performed using anti-Flag antibody and control immunoglobulin G (IgG), Myc-tagged RASSF1A protein was only detected from the anti-Flag immunoprecipitant (for Flag-tagged CHK1; Fig. 2B,
lane 2) but not from the anti-IgG immunoprecipitant (Fig. 2B, lane 1). Thus, our results demonstrate that CHK1 interacts with RASSF1A.

CHK1 phosphorylates RASSF1A at Ser-184

Next, we explored the identity of the amino acid(s) within RASSF1A that is/are phosphorylated by CHK1. We used a set of recombinant full-length or deletion variants of RASSF1A (16) to first map the region on RASSF1A that exhibited CHK1-mediated phosphorylation. For each of these RASSF1A variants, a region of ~30 to 60 amino acids harboring a known functional domain of the protein was deleted (Fig. 3A; ref. 16) and the expression of the purified variants was confirmed (Fig. 3B). In *vitro* kinase assay was then performed using purified recombinant CHK1 and RASSF1A proteins. As shown in Fig. 3C, deletion of amino acids 165-200 (Δ165-200) significantly diminished CHK1-dependent phosphorylation of RASSF1A (lane 6) whereas other RASSF1A variants were not similarly affected. These results hinted that the region containing amino acids 165 to 200 could harbor the potential site of CHK1-mediated RASSF1A phosphorylation. Next, we used the mass spectrometry analyses coupled with *in vitro* kinase assay to further define the CHK1-dependent phosphorylation site(s) within RASSF1A.
RASSF1A. First, in vitro kinase assay was performed using the recombinant CHK1 and full-length RASSF1A to obtain the phosphorylated-RASSF1A; the phosphorylated-RASSF1A was then subjected to tandem MS-MS mass spectrometry analysis to ascertain the identity of the phosphorylated residue(s). Through these analyses, 3 RASSF1A residues including Thr-38, Thr-43, and Ser-184 were reproducibly identified, Ser-184 seemed to be the likely candidate residue for CHK1-mediated phosphorylation. This conclusion is based on our findings that removal of the Ser-184-containing fragment corresponding to residues 165 to 200 but not the Thr-38 and Thr-43-containing fragment altered RASSF1A phosphorylation in vitro (Fig. 3C). Interestingly, amino acid sequence adjacent to Ser184 (KPPS184; Fig. 4A) also seems to closely resemble the CHK1 phosphorylation consensus motif (RXXT/S) with a characteristic that a basic residue at “-3” position of the phosphorylation site (Thr or Ser; ref. 29). The amino acid sequences adjacent to the 2 other potential phosphorylation sites (Thr-38 and Thr-43) do not possess such characteristics (Fig. 4A). Together, these results suggest that residue Ser-184 within RASSF1A seems to be a potential site of CHK1-mediated phosphorylation.

To further determine that the residue Ser-184 within RASSF1A is indeed phosphorylated by CHK1 inside the cells, we sought to generate RASSF1A Ser184 phospho-specific antibodies. For this purpose, animals were immunized with an RASSF1A peptide (CPSSKKPF[Ser]LQDARR) that harbored phosphorylated Ser184. The 293T cells, transfected with either the wild-type or the non-phosphorylatable mutant (Ser184A) RASSF1A constructs, were used for detection of phosphorylated RASSF1A by the Ser184 phosphor-specific antibody. As shown in Fig. 4B, the Ser184 phosphor-specific antibody clearly recognized phospho-specific signals in cells expressing GFP-RASSF1A (top, lane 1); and that signal was significantly reduced or diminished when cells were treated with CHK1 inhibitor UCN-01 (lane 2) or phosphatase (lane 3). In addition, no phosphor-specific signal was detected in cells expressing the nonphosphorylatable mutant-RASSF1A (F1A-S184A; top, lane 4). These results demonstrate the specificity of the phosphor-specific antibodies and also indicate that RASSF1A is phosphorylated at Ser-184 inside the cells. Furthermore, the finding that CHK1-specific inhibitor UCN-01 markedly reduced RASSF1A phosphorylation at Ser-184 (Fig. 4B, lane 2) also suggests that CHK1 phosphorylates this residue. To further examine the effect of CHK1 on Ser184 phosphorylation inside the cells, we introduced 293T cells with expression vectors carrying WT-CHK1 or its SA or KD (D130A) variants together with the GFP-RASSF1A expression construct. It is of note that deletion of the C-terminal autoinhibitory domain of CHK1 generates a SA variant of CHK1 with more than 20-fold increase in kinase activity (30, 31). As shown in Fig. 4C, cells coexpressing GFP-RASSF1A with empty vector showed background levels of phosphorylation on the residue Ser-184 (lane 1). Expression of exogenous WT-CHK1 clearly increased RASSF1A phosphorylation at Ser-184 (top, lane 2), however, the signal was further strongly enhanced (8-fold higher) in cells expressing the SA-CHK1 (top, lane 3). By contrast, KD-CHK1 did not further increase RASSF1A phosphorylation on Ser-184 beyond the basal levels (top, lane 4). Together, these data indicate that CHK1 phosphorylates RASSF1A at Ser-184 in the cells.

**CHK1-mediated phosphorylation alters RASSF1A cellular distribution**

It has been shown that phosphorylation regulates protein distribution and function. Next, we sought to determine whether RASSF1A localization was influenced by CHK1-mediated phosphorylation. We analyzed RASSF1A cellular localization in cells expressing the WT-CHK1 or SA-CHK1 or KD-CHK1. As shown in Fig. 5, when cells expressing GFP-RASSF1A without exogenous CHK1, RASSF1A distribution in majority (>95%) of the cells exhibited a network- and fiber-like pattern (Fig. 5, top, #1). This is consistent with our previous findings that RASSF1A colocalizes with microtubules and displayed fiber-like distribution (6, 16). When WT-CHK1 (Myc-WT-CHK1) was coexpressed, ~10% of cells exhibited GFP-RASSF1A distribution as a punctate pattern (Fig. 5, top, #2, arrow). Interestingly, with SA-CHK1 (Myc-SA-CHK1) coexpression, the proportion of cells with punctate pattern of RASSF1A distribution dramatically increased to ~90%; RASSF1A distribution seemed to be varied, showing ring-like (denoted by double arrows) or droplet-like patterns (denoted by single arrows; Fig. 5, top,
By contrast, punctate distribution pattern for RASSF1A was not noted in cells expressing the KD-CHK1 (Myc-KD-CHK1); RASSF1A still exhibited fiber-like distribution pattern in these cells. Thus, these results indicate that CHK1-mediated RASSF1A phosphorylation seems to alter RASSF1A distribution in the cells.

Next, we investigated whether CHK1-mediated phosphorylation affects RASSF1A association with microtubules. For this purpose, we generated 2 GFP-tagged RASSF1A mutants. One is a phosphorylation-mimic-mutant (GFP-F1A S184D) that has an alteration at amino acid 184 (Ser → Asp substitution), which mimics the phosphorylated state of RASSF1A by CHK1. The other one is phosphorylation-null-mutant (GFP-F1A S184A; Ser → Ala substitution; Fig. 6A).

MCF-7 cells introduced with these GFP-RASSF1A variants were analyzed for RASSF1A protein distribution by fluorescent microscopy. As shown in Fig. 6B, the control GFP protein was evenly distributed in the cytosol and did not codistribute with α-tubulin staining (panel 1). Wild-type RASSF1A (GFP-RASSF1A-WT, panel 2), however, largely overlapped with anti-α-tubulin immunostaining, a pattern similar to that described previously (6, 16). Similar protein distribution patterns were seen with phosphorylation-null RASSF1A (GFP-F1A-S184A; panel 4). By contrast, the...
Figure 4. CHK1 phosphorylates RASSF1A at Ser-184. A, identification of CHK1 phosphorylation sites on RASSF1A by mass spectrometry. Recombinant RASSF1A protein was first phosphorylated by purified recombinant CHK1 kinase in vitro and subsequently subjected to Tandem MS/MS mass spectrometry analysis. Residues Thr8, Thr43, and Ser-184 were identified to be phosphorylated in 2 independent experiments. B, RASSF1A Ser184 phosphorylation in the cells. 293T cells transfected with GFP-tagged WT RASSF1A or phosphorylation-null mutant (GFP-F1A-S184A) were mock treated (top, lane 1) or treated with CHK1 inhibitor UCN-01 (top, lane 2) or Calf Intestinal Alkaline Phosphatase (a pan-phosphatase; PPT, top, lane 3) as indicated. Cell lysates were analyzed by Western blotting using RASSF1A Ser184 phospho-specific antibody (top). Expression of GFP-tagged RASSF1A for all samples was also examined by anti-GFP antibody (bottom). C, CHK1-meditated RASSF1A phosphorylation at Ser184 in the cells. 293T cells expressing GFP-RASSF1A along with CHK1 variants [WT or SA or KD] were analyzed for RASSF1A phosphorylation at Ser184 by Western blotting using Ser184 phospho-specific antibody (top). The expression of total GFP-RASSF1A protein and CHK1 variants was also analyzed using the proper anti-tag antibodies (middle and bottom). It is of note that the SA-CHK1 is smaller in size because of the removal of the C-terminal sequence (after residues Asp-299) that enhances its activity (31).

Discussion

In this study, we have identified CHK1 as a novel RASSF1A kinase that modulates the ability of RASSF1A to regulate mitosis. We show that WT-CHK1 but not the KD-CHK1 phosphorylates RASSF1A in vitro and in cells. We also show that recombinant purified CHK1 phosphorylates the recombinant purified RASSF1A in vitro demonstrating that RASSF1A is a direct substrate of CHK1 kinase. We further mapped Ser-184 to be the CHK1 phosphorylation site on RASSF1A; using phosphor-specific antibodies we confirmed that residue Ser-184 is phosphorylated inside the cells and the SA-CHK1 but not the KD-CHK1 mutant further enhances RASSF1A Ser-184 phosphorylation inside the cells. RASSF1A phosphorylation by CHK1 at Ser-184 has functional importance as our results show that increased CHK1 activity alters RASSF1A association with microtubules and affects the ability of RASSF1A in inducing mitotic arrest. Our results thus suggest that phosphorylation by CHK1 is important for RASSF1A-mediated regulation during mitosis. Interestingly, a previous study has demonstrated that a somatic mutation of RASSF1A gene affecting Serine 184 occurred in about 13% of human primary nasopharyngeal carcinoma examined (36). Based on our cellular distribution of the phosphorylation-mimicking RASSF1A mutant (GFP-F1A-S184D) did not overlap with tubulin immunostaining; rather exhibited the punctate pattern (Fig. 6B, panel 3), similar to that of RASSF1A distribution in the cells expressing the WT- or SA-CHK1 (Fig. 5, panels 2 and 3). Thus, these results indicate that CHK1-mediated RASSF1A phosphorylation at Ser-184 seems to alter RASSF1A distribution and causes RASSF1A to disassociate from microtubules.

Phosphorylation-mimicking mutant RASSF1A is defective in inducing mitotic arrest

Previous studies have shown that RASSF1A association with microtubules is crucial for its ability to induce mitotic arrest (6, 16). We next investigated whether CHK1-mediated RASSF1A phosphorylation and microtubule disassociation altered the ability of RASSF1A to induce mitotic arrest. To that end, 293T cells, which have previously been shown to exhibit mitotic arrest by exogenous RASSF1A (6, 16), were transiently transfected with RASSF1A expression vectors carrying wild-type (GFP-F1A-WT) or phosphorylation-mimicking mutant (GFP-F1A-S184D) or phosphorylation-null mutant (GFP-F1A-S184A). The transfectants were then subjected to cell-cycle analyses by flow cytometry. As shown in Table 1, the fractions of G2–M phase cells were increased from 23.6% in GFP-only expressing cells to 35.7% in WT-RASSF1A expressing cells, an observation consistent with our previous findings (6, 16). The fraction of G2–M phase cells was 32.4% in phosphorylation-null RASSF1A-S184A—expressing population; similar to that for the WT-RASSF1A—expressing cells (35.7%). However, in RASSF1A-S184D (phosphomimic mutant) expressing cells, the G2–M phase cell fraction was only 24%, a value comparable to that of the control GFP-only expressing cells. Thus, these results suggest that CHK1-mediated RASSF1A phosphorylation not only modulates RASSF1A—microtubule association but also affects the ability of RASSF1A to induce mitotic arrest.
findings, we propose that the absence of CHK1-mediated phosphorylation because of Ser184 mutation may affect the functions of RASSF1A as a tumor suppressor. It will be of interest in future studies to further examine the role of RASSF1A Ser184 mutation in human cancer development.

Our studies demonstrate that RASSF1A is a novel substrate of CHK1. CHK1 is known to be very important for G2 and mitotic phase cell-cycle progression; and previous studies have shown that depletion of CHK1 caused cell-cycle arrest at G2 and M phases (25). The exact mechanism(s) as to how CHK1 mediates its effect to regulate G2–M progression was not completely clear. Our current study provides evidence suggesting that CHK1 may regulate mitosis via phosphorylation of RASSF1A. It is well established that the proper microtubule dynamics is very important for mitotic spindle formation and mitotic progression (37–39). Microtubule dynamics is known to be regulated by microtubule-associated proteins (MAP) that interact with and stabilize microtubules (reviewed in ref. 38). MAPs are shown to be phosphorylated by mitotic kinases during mitosis; and phosphorylation disassociates MAPs from microtubules and thus, promotes microtubule dynamics, mitotic spindle formation and mitotic progression (reviewed in ref. 39). RASSF1A is also a microtubule interacting and stabilizing protein and thus, its regulation during mitosis presumably is also important for increasing microtubule dynamics and M-phase cell-cycle progression. Our studies show that RASSF1A phosphorylation by CHK1 indeed can alter RASSF1A’s association with microtubules. We show that overexpression of WT-CHK1, but not the KD-CHK1 promotes RASSF1A disassociation from microtubules and such effect is much stronger in cells with the SA-CHK1 expression (Fig. 5). Similarly, RASSF1A S184D mutant, mimicking CHK1 phosphorylation on Ser-184, also causes RASSF1A disassociation from microtubules (Fig. 6). Importantly, our results also show that the phosphorylation-mimicking mutant RASSF1A loses its ability to induce M-phase cell-cycle arrest (Table 1). These results suggest that interactions with microtubules are important for RASSF1A to induce M-phase arrest; CHK1-mediated phosphorylation alters RASSF1A-microtubule association to promote mitotic progression. Interestingly, previous studies have shown that CHK1 is required for mitotic progression and

**Figure 5.** Subcellular localization of RASSF1A is affected by the activity of CHK1. The 293T cells were transfected with GFP-RASSF1A construct along with Myc-tagged expression vectors of CHK1 variants (WT or SA or KD). Cells were fixed and stained with anti-Myc antibodies for detection of Myc-tagged CHK1 variants (red) and also counterstained with DAPI nuclear-dye (blue). GFP-RASSF1A (green) is also shown (top). Arrows: GFP-RASSF1A exhibits punctate distribution pattern.
CHK1-depletion arrested cell cycle at the metaphase (25). It is possible that CHK1-mediated RASSF1A phosphorylation is a necessary event for allowing mitotic progression. Presumably, CHK1-triggered RASSF1A phosphorylation promotes RASSF1A dissociation from microtubules and prevents RASSF1A to continue stabilizing microtubules; this in

Figure 6. Subcellular localization of GFP-tagged wild type-RASSF1A, phosphorylation-mimic- and phosphorylation-null-RASSF1A variants. A, putative CHK1 phosphorylation site on WT RASSF1A and residue alteration on RASSF1A mutant variants (S184A and S184D). B, phosphorylation-mimic-mutation at Ser184 (S184D) alters cellular distribution of RASSF1A. MCF-7 cells transfected with vectors expressing either GFP-only or GFP-tagged wild-type or mutant-RASSF1A variants were fixed and stained with α-tubulin–specific antibodies (red) and with DAPI (blue). S184D, phosphorylation-mimicking mutant; S184A, phosphorylation-null mutant.

### Table 1. Cell-cycle profile of RASSF1A wild-type and mutant variants

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<th>Cell-cycle phase</th>
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<tr>
<td>GFP-RASSF1A</td>
<td>S184D (%)</td>
<td>3.2</td>
<td>50.2</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Abbreviations: S184A, phosphorylation-null mutant in which Ser184 is replaced with Ala184; S184D, phosphorylation-mimicking mutant in which Ser184 is replaced with Asp184.

293T cells seeded on 100-mm plates were transiently transfected with 10 μg of plasmids expressing either GFP alone or various GFP-tagged wild-type or the indicated mutant forms of RASSF1A. Cells were fixed at 48 hours time point following transfection and analyzed for the DNA content of the GFP-expressing cells using flow cytometry, as we have previously described (16). The presented values represent the means of 3 independent experiments.
turn could promote microtubule dynamics and microtubule spindle formation and thus, mitotic progression. Hence, our studies have identified that modulation of RASSF1A by CHK1 seems to be a novel mechanism via which CHK1 regulates cell-cycle progression at M-phase.

In this study, we have discovered Ser184 to be the CHK1 phosphorylation site. Two other RASSF1A residues including Thr38 and Thr43 were also found to be phosphorylated by the tandem MS-MS mass spectrometry analyses using the CHK1-phosphorylated RASSF1A protein (Fig. 4A). However, RASSF1A deletion variant lacking a region corresponding to amino acids 1 to 50 that harbors Thr38 and Thr43 does not show reduction in RASSF1A phosphorylation (Fig. 3C). These results do not strongly support the notion that residues Thr38 and Thr43 could be the CHK1 phosphorylation sites. By contrast, RASSF1A deletion variant devoid of a region corresponding to amino acids 165 to 200 that contains Ser184 exhibit significantly diminished CHK1-mediated phosphorylation (Fig. 3C), suggesting Ser184 to be more likely candidate for the CHK1-mediated phosphorylation site. Indeed, we confirmed that Ser184 within RASSF1A (recognized by phosphorylation-specific antibodies) is phosphorylated in cell; and SA-CHK1 strongly enhances phosphorylation at this residue (Fig. 4B and C). These results strongly support the notion that Ser184 within RASSF1A seems to be the CHK1 phosphorylation site. Interestingly, we also note that RASSF1A phosphorylation at Ser184 was significantly reduced but not completely inhibited by CHK1 inhibitor UCN-01 although it was completely abolished in the case of nonphosphorylatable mutation (Ser184Ala) or by treatment with phosphatase (Fig. 4B). These results suggest that Ser184 residue could be phosphorylated by more than one kinases. Future studies will further investigate this possibility.

It is also worth noting that residue Ser184 residues within the "basic region" (from amino acid 164 to 258) of RASSF1A. This region harbors a high proportion of basic residues; there are 24 positively charged residues (lysine and arginine) out of a total of 94 residues in this region. Previous studies have demonstrated that this region is critical for interactions between RASSF1A and microtubules (6, 7). Remarkably, a number of known RASSF1A kinases identified thus far all phosphorylate RASSF1A residues within this region. For example, Aurora-A and MST1 phosphorylate residues Threonine 202 and Serine 203 (16, 19), Aurora-B and CDK4 phosphorylate Serine 203 (17, 20), and PKC phosphorylates residue Serine 203 and Serine 197 (18). These phosphorylation sites of RASSF1A by various kinases seem to be clustered within a relatively small region (amino acids 184–203) of the RASSF1A protein. This finding suggests that this region seems to be critical for regulation of RASSF1A function. Although RASSF1A phosphorylation by CDK4 promotes RASSF1A to undergo Skp2–dependent protein degradation during G1–S transition (17), Aurora-A– (16) and CHK1 (in the present study)-mediated RASSF1A phosphorylation causes RASSF1A to dissociate from microtubules and facilitate mitotic progression. These findings would indicate that phosphorylation-dependent modulation of RASSF1A seems to be an important event to promote cell-cycle progression during M-phase.

In summary, we have identified CHK1 as a novel RASSF1A kinase. CHK1-mediated RASSF1A phosphorylation affects RASSF1A functions particularly its interaction with microtubules and the ability to modulate mitotic arrest. Our studies thus provide valuable new insights into the regulatory mechanisms of RASSF1A and also highlight a novel function for CHK1 involving phosphorylation of RASSF1A to modulate mitotic progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: L. Jiang, R. Rong, M.S. Sheikh, Y. Huang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Jiang, Y. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Jiang, M.S. Sheikh, Y. Huang
Writing, review, and/or revision of the manuscript: (e.g., statistical analysis, biostatistics, computational analysis): L. Jiang, M.S. Sheikh, Y. Huang
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