**Tumor and Stem Cell Biology**

**TRF1 Mediates Mitotic Abnormalities Induced by Aurora-A Overexpression**

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**Abstract**

Aurora-A, a conserved serine-threonine kinase, plays essential roles in mitosis. Aberrant upregulation of Aurora-A perturbs proper mitotic progression and results in a generation of multinucleated cells with centrosome amplification. The molecular mechanisms for these mitotic defects remain elusive. Here, we show that the overexpressed Aurora-A-induced mitotic defects depend on the telomeric protein TRF1. Live and fixed cell analyses revealed that Aurora-A overexpression in HeLa cells compromises chromosome biorientation, which leads to cytokinetic failure and tetraploidization with increased centrosome numbers. TRF1 depletion by small interfering RNAs or by tankyrase-1 overexpression suppresses Aurora-A-induced occurrence of unaligned chromosomes in metaphase, thus preventing the subsequent abnormalities. We found that Aurora-A binds and phosphorylates TRF1. When TRF1 knockdown cells are complemented with wild-type TRF1, Aurora-A–induced mitotic defects recur. By contrast, a TRF1 mutant that is not phosphorylatable by Aurora-A does not restore such Aurora-A–induced phenotype. We propose that TRF1 phosphorylation by excessive Aurora-A may provoke abnormal mitosis and chromosomal instability. Cancer Res 70(5); 2041–52. ©2010 AACR.

**Introduction**

In mitosis, proper chromosome segregation is ensured by bipolar spindle attachment to all sister kinetochores, which is followed by anaphase and cytokinesis. Aurora-A is a conserved serine-threonine kinase that plays multiple roles in mitosis, including centrosome maturation and separation, bipolar spindle assembly, chromosome alignment, and cytokinesis (1). Under physiologic conditions, the protein amount and enzyme activity of Aurora-A are elevated from G2 phase to metaphase. The subcellular localization of Aurora-A seems to be important for its function: Aurora-A is first concentrated at the centrosomes, where it mediates centrosome maturation in late G2/prophase, and following the nuclear envelope breakdown (NEBD), Aurora-A localizes to the mitotic spindles and controls their function (1–4).

Human Aurora-A maps to chromosomal region 20q13.2, which is amplified in various cancers (5–7). Aurora-A is also overexpressed at the mRNA and protein levels in cancers. Ectopic overexpression of Aurora-A transforms rodent cell lines (6, 7), establishing it as a proto-oncogene. Aurora-A overexpression induces checkpoint disruption and cytokinetic failure, which causes tetraploidy and centrosome overduplication (8–10). These abnormalities perturb proper chromosome segregation and thereby lead to chromosomal instability, which is closely associated with carcinogenesis. Whereas Aurora-A phosphorylates various substrates, including p53, TPX2, TACC, CENP-A, and Ajuba, the critical substrate that mediates mitotic abnormalities remains elusive (1).

TRF1 is a double-stranded telomere DNA-binding protein (11). It forms a homodimer and nucleates the protein complexes called shelterin at the telomeric TTAGGG repeats (12). Because DNA polymerase–dependent replication machinery cannot replicate the very ends of linear DNA, telomeres gradually shorten after every replication cycle. Critically shortened telomeres cannot protect the chromosome ends and thus trigger a DNA damage response, resulting in replicative senescence (13). Most immortal cells escape from replicative senescence by activating telomerase (14–16). Longer telomeres contain more shelterin and render the chromosome ends to more closed configuration, with less accessibility to telomerase (17, 18). Accordingly, the balance between telomerase-mediated extension and its blockade by TRF1 maintains the stable, average telomere length in telomerase-positive cells.

Telomere binding and protein stability of TRF1 are negatively regulated by tankyrase-1 (19–21). Tankyrase-1 poly(ADP-ribosyl)ates (PARsylate) TRF1, and this posttranslational modification dissociates TRF1 from telomeres (19, 22). The released TRF1 undergoes proteasomal degradation.
(20). Tankyrase-1–mediated dissociation of TRF1 allows easier telomerase access to telomeres. Consistently, forced expression of tankyrase-1 in nuclei of human cells elongates telomeres in a telomerase-dependent manner (20, 22, 23).

Here we found that Aurora-A does not induce centrosome amplification and tetraploidization if tankyrase-1 is overexpressed in nuclei of human cells elongates easier telomerase access to telomeres. Consistently, forced expression of tankyrase-1 in nuclei of human cells elongates easier telomerase access to telomeres. Consistently, forced expression of tankyrase-1 in nuclei of human cells elongates easier telomerase access to telomeres. Consistently, forced expression of tankyrase-1 in nuclei of human cells elongates easier telomerase access to telomeres. Consistently, forced expression of tankyrase-1 in nuclei of human cells elongates easier telomerase access to telomeres. Consistently, forced expression of tankyrase-1 in nuclei of human cells elongates easier telomerase access to telomeres.

Materials and Methods

Immunofluorescence microscopy. For Aurora-A and tubulin staining, cells were fixed with methanol at −20°C for 5 min. For TRF1 and tankyrase-1 staining, cells were fixed with 2% paraformaldehyde/PBS for 10 min and permeabilized with 0.5% NP40/PBS. Indirect immunofluorescence staining was performed as described (24) with the antibodies listed in Supplementary Materials and Methods.

Western blot analysis. Western blot analysis was performed as described previously (25) with peroxidase-conjugated anti-HA antibody (3F10; Roche; 0.05 μg/mL) or the primary antibodies listed in Supplementary Materials and Methods.

Small interfering RNA transfection. The small interfering RNAs (siRNA) were purchased from Qiagen: TRF1, 5′-AACGUAUUCUGUAAAGCTT-3′ (#6) or 5′-ACAGTAGTGTCCCTTGAT-3′ (#7); TIN2, 5′-AACGCUCUUGAUAGCCCUU-3′ (26); Aurora-A, 5′-UGCCCUUGUACUGUCA-3′ (2). Myc–TRF1 constructs used here lack the 3′ untranslated region, wherein the siRNA #7 target site was located. Nonsilencing control siRNA (D-001210-02) was purchased from Dharmacon. Cells were transfected with the siRNAs using Lipofectamine RNAmax (Invitrogen).

Time-lapse microscopy. Time-lapse microscopy was performed with a confocal laser scanning microscope (Fluoview FV-1000, Olympus) equipped with an incubation chamber. Cells were transfected with a total of 1 μg of plasmids for histone H2B–green fluorescent protein (GFP) and Aurora-A (molar ratio, 1:5) or histone H2B–GFP, Aurora-A, and TRF1 (molar ratio, 1:5:5). These molar ratios were determined to be optimal to make a cell express histone H2B–GFP simultaneously with Aurora-A or with Aurora-A and TRF1.

Roscovitine treatment assay. Cells were transfected with histone H2B–GFP, with or without Aurora-A. After a 24-h culture, the cells were treated with 500 ng/mL of nocodazole for 24 h. Then to induce premature mitotic exit, cells were exposed to 20 μmol/L roscovitine for 8 h (27) in the absence of nocodazole. After fixing with 2% paraformaldehyde/PBS, cells were analyzed with a confocal microscope (IX-71, Olympus).

In vitro kinase assay. In vitro kinase assay was performed by using the recombinant Aurora-A (Upstate) according to the manufacturer’s instructions. In brief, 30 μL of the reaction containing 1.5 μg of glutathione S-transferase (GST) fusion proteins, 20 ng recombinant Aurora-A, and [γ-32P]ATP were incubated at 30°C for 30 min. The reactions were terminated by adding SDS sample buffer and subjected to SDS-PAGE and autoradiography.

Detection of TRF1 phosphorylation in intact cells. Cells were transfected with siRNAs. After a 24-h culture, the cells were fixed with Míc–TRF1 constructs for 18 h and incubated in a phosphate-free medium supplemented with 32P-labeled phosphoric acid for 6 h. The cells were directly lysed by adding immunoprecipitation buffer comprising 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 20 mmol/L β-glycerophosphate, 5 mmol/L MgCl2, 5% glycerol, 0.5% NP40, 1 mmol/L DTT, 1/50 volume of the protease inhibitor cocktail (Nacalai tesque), and 1/100 volume of phosphatase inhibitor cocktails 1 and 2 (Sigma). Cell lysate was immunoprecipitated with anti-Myc. The immunocomplex was subjected to SDS-PAGE, followed by autoradiography.

Results

Aurora-A overexpression. Transient overexpression of Aurora-A in HeLa I.2.11 cells (28) produced multinucleated cells coupled with increased centrosome number as reported (refs. 7, 8; Fig. 1A). Among Aurora-A–overexpressing cells, frequencies of multinucleation and centrosome amplification were ∼20% (Fig. 1C and D), similar to the previous reports (7, 8).

Our preliminary speculation was that Aurora-A–induced mitotic abnormalities might be affected by tankyrase-1, because, in mitosis, tankyrase-1 is phosphorylated (29, 30) and colocalizes with Aurora-A on the centrosomes (31, 32). To examine this possibility, we established a series of HeLa I.2.11–derived cell lines that overexpressed tankyrase-1 (Fig. 1B). Tankyrase-1 is present in the cytoplasm and the nucleus, wherein each pool presumably has a distinct function (25, 33, 34). Thus, we overexpressed tankyrase-1 either in the cytoplasm (F–tankyrase-1; FLAG-tagged at the NH2 terminus) or in the nucleus (FN–tankyrase-1; tagged with a FLAG and an NLS at the NH2 terminus; Supplementary Fig. S1). Remarkably, tankyrase-1 overexpression in the nucleus repressed both Aurora-A–induced multinucleation and centrosome amplification. This effect depended on the poly(ADP-ribose) polymerase (PARP) activity of tankyrase-1, because the PARP dead mutant (H1184A/E1291AHE/A; ref. 35) did not suppress the abnormalities (Fig. 1A, C, and D). By contrast, cytoplasmic tankyrase-1 did not affect the Aurora-A–induced abnormalities. Tankyrase-1 overexpression did not affect the levels of exogenous Aurora-A expression (Fig. 1B). These results were reproducible with fibrosarcoma HTC75 cells (Supplementary Fig. S2A–C). It seems unlikely that Aurora-A is directly inhibited by tankyrase-1–mediated PARylation because tankyrase-1 did not PARylate Aurora-A (Supplementary Fig. S3).

TRF1 is required for Aurora-A–induced multinucleation and centrosome amplification. Tankyrase-1 in the nuclei PARylates and releases TRF1 from telomeres, a process that allows telomerase to elongate telomeres (22, 35). Consistently, nuclear TRF1 foci disappeared in FN–tankyrase-1–overexpressing cells (Supplementary Fig. S1). Therefore we...
wanted to know if the telomere length control is causally related to the suppression of the Aurora-A-induced phenotype. To address this possibility, we compared the suppression efficiency in cells with different telomere lengths. First, FN-tankyrase-1 inhibited the Aurora-A-induced phenotype in cells with both long (HeLa I.2.11; 15–25 kb; ref. 28) and short telomeres (HTC75; ∼5 kb; ref. 17; Fig. 1 and Supplementary Fig. S2A–C). Second, a suppressive effect of FN-tankyrase-1 on Aurora-A-induced multinucleation was observed in HTC75 cells at ∼30 population doublings (PD) and at >100 PD, which retained shorter and longer telomeres, respectively (ref. 24; Supplementary Fig. S2D and E). Thus, suppression of Aurora-A–induced mitotic defects does not depend on telomere length.

Because telomere-unbound TRF1 is subjected to proteasomal degradation (20), FN-tankyrase-1 decreased the TRF1 protein level (Fig. 1B and Supplementary Fig. S2A). This observation prompted us to examine the possibility that TRF1 is required for Aurora-A–induced phenotypes. To deplete TRF1, we transfected HeLa I.2.11 cells with siRNA 24 h before

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Figure 1. Aurora-A–induced centrosomal amplification and multinucleation are blocked by tankyrase-1. A, centrosomal amplification and binucleation by Aurora-A overexpression. HeLa I.2.11 cells were infected with tankyrase-1 retroviruses. The resulting cell lines were transfected with HA-Aurora-A. After 48 h, HA-Aurora-A and γ-tubulin were detected by indirect immunofluorescence staining. Blue, 4′,6-Diamidino-2-phenylindole (DAPI) staining of DNA. Insets, magnified images of γ-tubulin dots. F, FLAG; N, nuclear localization signal; (HE/A), catalytically inactive. B, Western blots of the cell lysates in A. WCE, whole cell extract; NE, nuclear extract. C and D, quantitation of cells that exhibit centrosome amplification (defined by the presence of more than two centrosomes; C) or multinucleated cells (D) in A. The graphs show the averages of at least three experiments. Asterisk indicates statistical significance (χ² test). Error bar, SD. Total numbers of examined cells are indicated in parentheses.
Aurora-A transduction. Two different siRNAs against TRF1 downregulated the protein level (Fig. 2A). They did not decrease the level of Aurora-A overexpression compared with control siRNA. Similar to FN-tankyrase-1 overexpression, TRF1 knockdown suppressed the occurrence of multinucleated cells and centrosome amplification led by Aurora-A (Fig. 2B-D). By contrast, nonsilencing control siRNA did not affect Aurora-A–induced phenotypes. It is unlikely that these results are due to the consequence of cell cycle defects, because these siRNAs had no detectable effects on cell cycle distribution (Supplementary Fig. S4). TRF1-G18A, a non-PARsylatable mutant, in which the tankyrase-1–binding motif [RXX(P/A)DG] was disrupted, fully restored the Aurora-A–induced phenotypes even in FN-tankyrase-1–overexpressing cells (data not shown). These observations suggest that FN-tankyrase-1 suppresses the mitotic abnormalities by means of TRF1 downregulation.

**TRF1 knockdown suppresses cytokinetic failure induced by Aurora-A overexpression.** Aurora-A overexpression does not deregulate centrosome duplication in S phase but causes cytokinetic failure, which results in tetraploidy and doubled centrosome number (8, 9). Consistently, most cells that exhibited centrosome amplification had four centrosomes instead of three or five (Figs. 1A and 2B; Supplementary Fig. S2B). To further examine the effect of TRF1 knockdown, we performed live cell imaging of cells expressing histone H2B–GFP. In a control mitosis, separation of sister chromatids at the anaphase onset was followed by ingression of the cleavage furrow at midzone and completion of cytokinesis (Fig. 3A, top; Supplementary Movie S1).
In Aurora-A–overexpressing cells (Fig. 3B), the cleavage furrow was initially formed but eventually regressed in ~20% of dividing cells. These cells produced binucleated cells that retained unsegregated, two daughter nuclei, which would also retain duplicated centrosomes (Fig. 3A, top middle and D; Supplementary Movie S2). These observations favor the idea that excess Aurora-A increases the centrosome number through cytokinetic failure rather than deregulated centrosome duplication. Among the cells that failed to divide, we also observed multinucleated cells, which resulted from irregular nuclear division and cytokinetic failure (Fig. 3A, bottom middle; Supplementary Movie S3). Frequencies of these abnormalities (Fig. 3D) in live cell analyses were comparable with those in the fixed preparations (Figs. 1 and 2).

We then asked if this cytokinetic failure could be alleviated by TRF1 depletion. In TRF1 knockdown cells (Fig. 3B), Aurora-A overexpression did not induce cytokinetic failure.

**Figure 3.** TRF1 is required for Aurora-A–induced cytokinetic failure. A, time-lapse images of HeLa I.2.11 cells. Cells were transfected with the indicated siRNAs. Twenty-four hours after transfection, the cells were further transfected with histone H2B–GFP (green), with control or FLAG–Aurora-A expression vector. The elapsed time in minutes is shown at the bottom left in each panel. Scale bar, 10 μm. B, introduction of an siRNA-resistant TRF1 into HeLa I.2.11 cells. Cells were transfected with TRF1 siRNA. After 24 h, the cells were transfected with Myc-TRF1 and HA–Aurora-A. After an additional incubation for 24 h, Western blot analysis was performed. C, TIN2 knockdown downregulates TRF1. HeLa I.2.11 cells were sequentially transfected with TIN2 siRNA and HA–Aurora-A as in B. After 24 h, Western blot analysis was performed. D, HeLa I.2.11 cells were transfected as in B and C. Mitotic defects were analyzed as in A. The graph shows the averages of at least three experiments. Asterisk indicates statistical significance (χ² test). Error bar, SD. Parentheses indicate total cell numbers examined.
cytokinetic failure reappeared when an siRNA-resistant TRF1 (Myc-TRF1) was introduced into the cells (Fig. 3B and D). Myc-TRF1 per se did not induce mitotic defects (Fig. 3D).

To determine further if TRF1 is necessary for abnormal mitosis, we downregulated TRF1 by means of TIN2 knockdown. TIN2 is a TRF1-binding partner (36) and inhibits tankyrase-1–mediated PARylation of TRF1 (37). Accordingly, TIN2 knockdown derepresses tankyrase-1, which in turn strips out TRF1 from telomeres and enhances its degradation (26, 37). As expected, TIN2 knockdown was accompanied by TRF1 downregulation (Fig. 3C) and alleviated Aurora-A–induced cytokinetic failure (Fig. 3D). This effect was not statistically significant, presumably because the level of TRF1 downregulation was moderate compared with direct knockdown of TRF1. Together, these observations indicate that TRF1 is required for Aurora-A–induced cytokinetic failure.

**Aurora-A overexpression induces chromosome misalignment and prolonged mitosis.** We next monitored the mitotic duration in Aurora-A–overexpressing cells. In untreated or control siRNA-treated cells, cytokinesis was completed in 80 to 100 minutes after the onset of NEBD (Fig. 4A, top; Supplementary Movie S5). In those cells, average time from NEBD to anaphase was 60 minutes (Fig. 4B). By contrast, Aurora-A–overexpressing cells showed varied time...
from NEBD to anaphase onset: 40% of the cells took 80 minutes or longer (Fig. 4A, middle and B; Supplementary Movie S6). We noticed that cytokinetic failure in Fig. 3 was frequently observed in cells that exhibited normal mitotic progression time (<80 minutes). By contrast, cells with prolonged mitosis (>80 minutes) exhibited a higher tendency to complete cytokinesis successfully in spite of Aurora-A overexpression (Fig. 4A, middle; Supplementary Movie S6). It has been proposed that excess Aurora-A inhibits the kinetochore attachment to microtubules, producing unaligned chromosomes (9). Consistently, we often detected unaligned chromosomes in Aurora-A–overexpressing cells (Supplementary Movie S6). These results suggest that prolonged mitosis by Aurora-A may be due to the presence of unaligned chromosomes, and subsequent cytokinesis failure is suppressed by prolonged mitosis.

**TRF1 mediates Aurora-A–induced chromosome misalignment and kinetochore-microtubule attachment failure.** Prolonged mitosis is associated with the failure of chromosomes to align at the metaphase plate, which keeps the spindle assembly checkpoint (SAC) active and blocks anaphase onset (38). Importantly, TRF1 knockdown suppressed the prolonged mitosis in spite of Aurora-A overexpression (Fig. 4A, bottom and B; Supplementary Movie S7). This observation led us to hypothesize that TRF1 mediates Aurora-A–induced perturbation of proper kinetochore-microtubule attachments, leading to sustained SAC activation and prolonged mitosis. As shown in Fig. 5A, Aurora-A

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**Figure 5.** TRF1 downregulation suppresses Aurora-A–induced unaligned chromosomes. HeLa I.2.11 cells were treated with the indicated siRNAs for 24 h and then transfected with FLAG–Aurora-A (and histone H2B–GFP in B). The graphs show the averages of at least three experiments. Asterisk indicates statistical significance (χ² test). Error bar, SD. Parentheses indicate total cell numbers examined. A, Aurora-A (FLAG) and α-tubulin were detected by immunofluorescence staining. Arrowheads indicate unaligned chromosomes at metaphase. B, after 24 h, the cells were treated with 500 ng/mL nocodazole for 12 h and then exposed to the nocodazole-free medium that contained 20 μmol/L roscovitine for 8 h.
Figure 6. Aurora-A–mediated phosphorylation of TRF1 is crucial for the mitotic defects. A, in vitro binding assay. The bead-bound GST or GST–Aurora-A was incubated with the in vitro translated Myc-TRF1. The bead-bound proteins were subjected to Western blot analysis with anti-Myc. B, TRF1 has two consensus motifs for Aurora-A phosphorylation. Bold red letters, conserved amino acid residues; numbers, positions of amino acids. C, TRF1 phosphorylation by Aurora-A. Left, in vitro kinase assay. Recombinant Aurora-A was incubated with either GST or GST-TRF1 in the presence of [γ-32P]ATP. The reaction mixtures were fractionated on SDS-PAGE and visualized by autoradiography or Coomassie Blue staining. Asterisk indicates bovine serum albumin in the reaction mixture. Right, TRF1 phosphorylation in HeLa I.2.11 cells was detected as described in Materials and Methods. D, left, HeLa I.2.11 cells were transfected with TRF1 siRNA. After 24 h, the cells were transfected with siRNA-resistant Myc-TRF1 constructs. After an additional incubation for 24 h, nuclear extracts were subjected to Western blot analysis. Right, Aurora-A–induced mitotic abnormalities were analyzed as in Fig. 3A. The graph indicates the average of three experiments. Asterisk indicates statistical significance ($\chi^2$ test). Error bar, SD. Parentheses indicate total cell numbers examined.
overexpression caused unaligned chromosomes in metaphase, a hallmark of kinetochore-microtubule attachment failure. As expected, TRF1 knockdown suppressed the occurrence of these unaligned chromosomes. Furthermore, Aurora-A overexpression caused multiple spindle pole formation, which was suppressed by TRF1 knockdown (Fig. 5A).

Because Aurora-A-overexpressing cells with prolonged mitosis successfully completed cytokinesis, we speculated that unaligned chromosomes lead to the production of multinucleated cells. To examine this possibility, we used the cyclin-dependent kinase inhibitor roscovitine, by which the cells were forced to exit metaphase even in the presence of unaligned chromosomes after release from nocodazole. Under these conditions, Aurora-A overexpression produced binucleated or multinucleated cells (Fig. 5B). These results are consistent with the finding that Aurora-A–induced multinucleation occurred predominantly in cells that did not stay long in metaphase (Figs. 3A and 4A). Again, TRF1 knockdown alleviated these abnormalities (Fig. 5B).

**Catalytically inactive Aurora-A can induce tetraploidization without TRF1.** Although excess Aurora-A induces tetraploidization even in a kinase activity–independent manner (ref. 8; Supplementary Fig. S5A), whether the kinase activity is required for Aurora-A–induced kinetochore-microtubule attachment failure remains unknown. TRF1 depletion did not suppress the phosphorylation-independent tetraploidization. As compared with the wild type, Aurora-A(KD) less efficiently caused lagging chromosomes and multiple spindle poles, whether in the presence or in the absence of TRF1 (Supplementary Fig. SSB). These observations suggest that Aurora-A (KD) induces tetraploidization in a way that is different from how the wild-type Aurora-A does and that TRF1 mediates the phosphorylation-dependent pathway of Aurora-A–induced mitotic defects.

**TRF1 phosphorylation by Aurora-A is essential for mitotic defects.** The above observation that wild-type, but not KD mutant, Aurora-A–induced mitotic defects require TRF1 suggests that phosphorylation is a key event for TRF1 function. Therefore, we next studied the interaction between TRF1 and Aurora-A. In vitro binding assays indicated that TRF1 bound directly to Aurora-A, but not Aurora-B (Fig. 6A; Supplementary Fig. S6A and B). Aurora-A bound to the COOH terminal region of TRF1 between amino acid residues 265 and 439 (Supplementary Fig. S6C). The efficiency of GST-TRF1 to pull down Aurora-A was comparable with that of GST-p53, a known binding partner of Aurora-A (ref. 39; Supplementary Fig. S6D). Aurora-A coimmunoprecipitated with TRF1 in HeLa I2.11 cell lysate (Supplementary Fig. S6E). Furthermore, Aurora-A phosphorylated GST-TRF1 in vitro (Fig. 6C, left). As a negative control, GST–tankyrase-1 was not phosphorylated by Aurora-A (Supplementary Fig. S7). To map the phosphorylation sites, we focused on the consensus sequence RX(S/T)(L/V) for Aurora kinase in TRF1 (Fig. 6B). We substituted each serine residue (Ser296 and Ser417) in those motifs with alanine. Aurora-A–induced phosphorylation of the resulting mutant TRF1-S296A was significantly reduced, whereas TRF1-S417A phosphorylation was comparable with that of the wild-type TRF1 (Fig. 6C, left). Thus Aurora-A can bind TRF1 and phosphorylates it on Ser296. Furthermore, TRF1 was phosphorylated in intact cells (Fig. 6C, right). Either S296A mutation or Aurora-A knockdown (Supplementary Fig. S8A) decreased the level of TRF1 phosphorylation. Aurora-A knockdown had no effect on cell cycle distribution under these conditions (Supplementary Fig. S8B).

We examined whether TRF1 phosphorylation on Ser296 is required for Aurora-A–induced mitotic abnormalities. After depleting the endogenous TRF1, HeLa I2.11 cells were complemented by TRF1-S296A (Fig. 6D, left). Under these conditions, Aurora-A did not induce cytokinetic failure (Fig. 6D, right). We performed indirect immunofluorescence staining and confirmed that Aurora-A–induced multinucleation was restored by TRF1, but not by TRF1-S296A. In this assay, we verified that ~80% of Aurora-A–overexpressing cells simultaneously express the exogenous TRF1 and monitored only the cells that expressed both (data not shown). These results suggest that Aurora-A–mediated phosphorylation of TRF1 on Ser296 is required for mitotic defects.

**Discussion**

TRF1 has been established as a negative regulator for telomere length (11, 17). Here, we showed that TRF1 downregulation, either by tankyrase-1 overexpression or depletion of TRF1 itself or TIN2, represses the aberrant mitotic phenotypes led by the oncogenic Aurora-A. Thus TRF1 mediates Aurora-A–induced mitotic abnormalities in a pathologic context.

**Function of TRF1 in kinetochore-microtubule attachment.** Our observation that TRF1 depletion suppressed Aurora-A–induced lagging chromosomes indicates that TRF1, as a downstream effector of Aurora-A, affects the kinetochore capture by mitotic spindles. Intriguingly, TRF1 has also been identified as a binding partner for the mitotic NIMA (never-in-mitosis A) kinase (40). TRF1 localizes not only to telomeres but also to mitotic spindles and promotes microtubule polymerization in vitro (41). Furthermore, TRF1 binds the microtubule plus-end tracking protein EB1 (42), which is required for proper movement and orientation of sister chromatids at the metaphase plate (43). Although the mechanisms remain elusive, EB1 seems to regulate spindle formation bidirectionally: EB1 promotes microtubule polymerization (44), but it inhibits TRF1-mediated microtubule polymerization (42). Collectively, these observations suggest that TRF1 and EB1 function in a balanced manner for proper chromosome alignment and segregation. Excess Aurora-A could unbalance EB1-mediated control of the microtubule plus-end dynamics and/or its coupling to the kinetochore-microtubule attachment by means of TRF1 binding and phosphorylation. Recently, Munoz and colleagues reported on the functional importance of TRF1 in chromosome alignment (45). Using a transgenic mouse model, they showed that TRF1 overexpression induces aberrant spindle formation and misaligned chromosomes. These results are in good agreement with our notion that TRF1 is involved in the kinetochore-microtubule attachment.
Interestingly, a GTP-binding protein, GN3L3, represses ubiquitination and subsequent degradation of TRF1 during mitosis. The resulting sustained level of TRF1 was found to promote the metaphase to anaphase transition (46). We found that Aurora-A contributes to the increased level of TRF1 in mitosis, and the protein amounts of Aurora-A show significant positive correlation with those of TRF1 in non–small cell lung cancer cell lines. These observations could provide a basis for further inquiry into the contribution of TRF1 in mitosis and on the consequences of Aurora-A overexpression.

The balance between SAC and premature anaphase onset as a determinant of Aurora-A–induced cytokinetic failure. SAC represses the metaphase-anaphase transition until the microtubules complete bipolar capture of all sister kinetochores. Therefore, the prolonged mitotic progression time observed in 40% of Aurora-A–overexpressing cells would be due to sustained SAC elicited by long-lasting lagging chromosomes. This situation would increase the probability that all sister kinetochores satisfy bipolar spindle attachment even under the conditions when excess Aurora-A disturbs efficient kinetochore-microtubule attachment. In fact, cells with prolonged mitosis executed proper cytokinesis in spite of Aurora-A overexpression. It is likely that TRF1 knockdown corrected the mitotic progression time simply by alleviating the occurrence of lagging chromosomes.

Meanwhile, increased Aurora-A activation overrides SAC (9, 10). We observed that 60% of Aurora-A–overexpressing cells showed normal mitotic progression time, in spite of the presence of uncaptured chromosomes. Chromosome nondisjunction itself induces tetraploidy through regression of the mitotic cleavage furrow (47). Consistently, we showed that roscovitine-induced mitotic exit with uncaptured chromosomes often results in cytokinetic failure. These findings suggest that uncaptured chromosomes and SAC escape are direct causes of cytokinetic failure led by Aurora-A overexpression. Although TRF1 knockdown did not affect the checkpoint protein complex formation, such as those of Mad2/Cdc20 and BubR1/Cdc20, it might affect SAC to some extent. Indeed, the time from NEBD to anaphase onset was shortened by TRF1 knockdown (Fig. 4B). Alternatively, as described above, TRF1 knockdown per se might enhance the kinetochore-microtubule attachment.

In clinical settings, higher Aurora-A expression correlates with more advanced malignancy of neuroblastoma (48). Whether there is clear threshold in the level of Aurora-A overexpression to induce mitotic abnormalities remains elusive. Our fixed cell analyses did not reveal a rigid relationship between the extent of Aurora-A overexpression and probability of mitotic abnormalities (data not shown). It is interesting to see to what extent the tumor malignancy depends on TRF1.

Dissecting mechanisms for mitotic abnormalities by Aurora-A and Aurora-A(KD). Aurora-A(KD) can induce tetraploidization in immortal cells (8; this study) but not in primary MEF (9). It is noteworthy that tetraploidization by the wild-type Aurora-A was TRF1 dependent, whereas that by Aurora-A(KD) did not require TRF1. More strikingly, whereas Aurora-A overexpression caused lagging chromosomes in a TRF1-dependent manner, Aurora-A(KD) had less ability to produce such irregular chromosomes. Thus, Aurora-A–induced dysfunction in the kinetochore-microtubule attachment depends on both its kinase activity and TRF1. Importantly, Aurora-A was able to phosphorylate TRF1. Furthermore, whereas TRF1 resumed the Aurora-A–induced mitotic abnormalities in TRF1 knockdown cells, TRF1(S296A), which cannot be phosphorylated by Aurora-A, did not. These observations strongly support the idea that TRF1 phosphorylation plays an essential role in Aurora-A–induced, kinetochore-microtubule attachment failure. Overexpression of a phosphorylation-mimic mutant, TRF1(S296D), did not induce mitotic defects, suggesting that other Aurora-A–dependent functions in addition to TRF1 phosphorylation may be involved in the mitotic abnormalities. Although Aurora-B could not directly bind TRF1, we cannot rule out the possibility that TRF1 is also phosphorylated by Aurora-B in intact cells, for instance, at unattached kinetochores upon Aurora-A overexpression. Indeed, TRF1 and Aurora-B colocalize at kinetochores in mouse cells (45).

Aurora-A controls microtubule dynamics via the phosphorylation of microtubule-destabilizing proteins MCAK and Kif2a, which regulate microtubule depolymerization and spindle dynamics (49, 50). It is also possible that TRF1 acts as a mediator of microtubule destabilization, and Aurora-A overexpression may perturb the microtubule dynamics via TRF1 phosphorylation.

Aurora-A exhibited prominent accumulation at spindles and spindle poles, whereas Aurora-A(KD) only weakly accumulated on those loci and largely distributed in the whole cell (Fig. 5A; Supplementary Fig. S5B). This difference may also explain the distinct phenotypes induced by Aurora-A and Aurora-A(KD) overexpression.

Conclusions and perspective. Here we identified TRF1 as an essential factor for Aurora-A–induced mitotic failure. TRF1 was required for unaligned chromosomes to occur in metaphase, which was an upstream event of Aurora-A–induced cytokinetic failure. Because Aurora-A is activated in many cancers, it could be a promising anticancer target (51). However, why Aurora-A inhibitors exhibit anticancer effect remains obscure. To establish an evidence-based, fruitful outcome of Aurora-A–directed therapy, it would be worthwhile to explore the involvement of TRF1 in the anticancer effects of Aurora-A inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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