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Mitotic perturbations induced by Nek2 overexpression require interaction with TRF1 in breast cancer cells

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Abbreviations: APC/C, anaphase-promoting complex/cyclosome; Cdc20, cell-division cycle protein 20; CIN, chromosome instability; Co-IP, co-immunoprecipitation; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; Hec1, highly expressed in cancer protein 1; λPPase, lambda protein phosphatase; Mad1, mitotic arrest deficient 1; Mad2, mitotic arrest deficient 2; Nek2, NIMA-related kinase 2; NIMA, never in mitosis A; SAC, spindle assembly checkpoint; siRNA, small interfering RNA; TRF1, telomeric repeat binding factor 1

NIMA-related kinase 2 (Nek2), a serine–threonine protein kinase, plays a major role in mitotic progression, including timing of mitotic entry, chromatin condensation, spindle organization, and cytokinesis. Nek2 overexpression results in premature centrosome separation, while kinase death Nek2 mutant expression or Nek2-depleted cells lead to centrosome separation failure. In addition, it has been revealed that telomeric repeat binding factor 1 (TRF1) interacts directly with Nek2. TRF1 not only regulates telomere length, but is also associated with cell cycle regulation. However, the interactions and correlations between Nek2 and TRF1 are far from clear. Here, we show that mitotic aberrations through Nek2 overexpression are likely to require TRF1. Our results demonstrate that Nek2 directly binds and phosphorylates TRF1 through multiple sites on TRF1. Nek2 overexpression in breast cancer cells, MDA-MB-231 and MCF7, results in increased numbers of centrosomes and multinucleated cells, which leads to cytokinetic failure and aneuploidization. Additionally, TRF1 depletion by siRNA prevents the phenomenon of unaligned chromosomes by Nek2 overexpression during metaphase. Concurrent Nek2 overexpression and TRF1-depleted cells demonstrated ≤ 2 centrosomes per cell, similar to mock plasmid and negative control siRNA-transfected cells. Interestingly, when exogenous TRF1 was added back in Nek2-overexpressed cells with endogenous TRF1 depletion, cells had re-induced cytokinetic failure. Therefore, we propose that TRF1 is required for overexpressed Nek2 to trigger abnormal mitosis and chromosomal instability.

Introduction

Chromosome instability (CIN) is a hallmark of tumor initiation and progression.[1] CIN is a result of alterations in mitotic timing, mitotic checkpoint control, disruption of microtubule or centrosome dynamics.[2] The mitotic checkpoint, performed by the spindle assembly checkpoint (SAC), monitors proper microtubule binding from opposite spindle poles to all sister kinetochores during metaphase. Perturbations in the mitotic checkpoint pathway result in mitotic CIN. It has been suggested that disruption of the SAC results in loss of chromosome integrity or premature exit from mitosis leading to aneuploidy.[3]

NIMA-related kinase 2 (Nek2) is a conserved mammalian serine–threonine kinase that plays an important role in cell cycle regulation. The protein expression level of Nek2 is quickly degraded at the prometaphase to metaphase transition of mitosis in an APC/C-dependent manner. Increased activity of Nek2 during the G_2 phase of cell cycle leads to centrosome separation via phosphorylation of C-Nap1 and rootletin through intercentriolar linker proteins.[4-6] Depending upon the phase of cell cycle, Nek2 is distributed within the nucleus and/or cytoplasm and also demonstrates a variety of other cell cycle-related functions. Nek2 is present in chromosomes from prophase to metaphase. However, Nek2 begins to separate from chromosomes upon entering into anaphase. This ubiquitous expression of Nek2 is involved in multicellular functions throughout cell cycle progression.[7]

Recently, studies confirmed that Nek2 interacts with Mad1 to regulate signaling of the SAC.[8] Mad1 may play a pivotal role in the formation of the SAC, because depletion of Mad1 resulted in defective checkpoint signaling and increased chromosome missegregation.[9,10] Mad1 appears to facilitate Mad2 localization to the kinetochore and promotes a structural change to the activated form. Activated Mad2 then complexes with Cdc20, Bub3

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and BubR1.[10,11] Furthermore, it has been reported that mouse telomere repeat binding factor 1 (Trf1) is also an interaction partner for Mad1.[12]

Telomeric repeat binding factor 1 (TRF1) is a double-stranded telomere DNA-binding protein.[13] TRF1 plays dual roles in telomere maintenance and cell cycle control. The function of TRF1 in telomere maintenance has been well-established. Recently, however, many studies suggest that TRF1 is not only involved in cell cycle regulation, but also plays a specific role in cell cycle progression, especially mitotic progression.[14,15] Cell cycle-specific expression and localization of TRF1 suggests that TRF1 may influence cell cycle at specific points. Studies also reported that overexpression of TRF1 affected cell cycle progression.[14,16] Indeed, while cells with TRF1 overexpression do not alter the onset of mitosis, the cells cannot progress through normal mitosis.[16] Additionally, it has been shown that the concentration of TRF1 free from telomeres may be important for cell cycle progression. TRF1 may induce mitotic entry or apoptosis, depending on the average telomere length in the cell.[16] TRF1 induces apoptosis in cell lines with short telomeres, while it does not induce apoptosis in cell lines with long telomeres. A high level of telomere-unbound TRF1 in cells with short telomere implies that telomeres may be too short for cells to divide. Loss of endogenous TRF1 inhibited NIMA induction of premature mitotic entry and apoptosis.[17,18]

In this study, we demonstrated that TRF1 was responsible for the cytokinetic failure induced by Nek2 overexpression. Nek2 overexpression displayed abnormal centrosome amplification and multi-nucleation in breast cancer cells, which induced chromosomal instability. Moreover, we observed lagging chromosomes in Nek2-overexpressing cells resulting from prolonged mitosis. However, the results of TRF1 knockdown suggested that chromosome instability induced by Nek2 overexpression was mediated by TRF1. When TRF1 was knocked down in Nek2 overexpressing cells, cells exhibiting centrosome amplification and cytokinetic failure were decreased. Furthermore, TRF1 addback in Nek2-overexpressed cells showed an increase in cytokinetic failure. Taken together, our studies revealed that TRF1 is required for cytokinetic failure induced by Nek2 overexpression.

Results

Cell cycle regulates Nek2 and TRF1 binding

In order to clarify the binding relationship of Nek2 and TRF1, co-immunoprecipitation (Co-IP) was performed with cell cycle-synchronized cell lysates. MCF7 cells were synchronized in S, G_2/M, or G_1 phase. Equivalent amounts of nuclear lysate from G_1, S, or M phase cells were incubated with protein A/G agarose beads coupled to the anti-TRF1 antibody. These results confirmed that Nek2 and TRF1 protein levels were regulated during cell cycle (**Fig. 1A**). In the nuclear extracted fraction, TRF1 levels remained relatively low during G_1 and S, but were increased when cells transitioned to G_2/M phase. Also, Nek2 protein expression levels were dramatically increased as cells moved from G_1/S phase to G_2/M phase in both cytoplasmic and nuclear lysates. Immunoblotting analysis of immunoprecipitated samples with antibodies against TRF1 and Nek2 showed results similar to those from the cell lysates. Comparable amounts of Nek2 and TRF1 were brought down from G_1, S, and G_2/M phase immunoprecipitation. Co-IP data indicated that Nek2 co-precipitated with TRF1, and that their association was highest when cells progressed through G_2/M phase. This was followed by a decrease in association as cells moved to the next G_1.

Next, double thymidine or a thymidine–nocodazole block was used to arrest MCF7 cells at the G_1/S or G_2/M phase boundaries. Cell cycle was monitored by flow cytometry (**Fig. 1B**). The expression levels of Nek2 and TRF1 changed with cell cycle progression. The results showed that Nek2 and TRF1 protein levels were higher in cell populations at S and M phase. Additionally, Nek2 and TRF1 complexes were observed to precipitate most during M phase. These results indicated that Nek2 and TRF1 binding was enhanced during mitosis. This is consistent with the idea that the nucleolus, dissembled during mitosis, releases more Nek2 into the nucleoplasm, making it more available for TRF1 binding. Furthermore, Nek2 and TRF1 expression levels are highest in G_2/M phase, further increasing the possibility of binding.

The next step was to determine whether Nek2 could form a complex with exogenous TRF1 in breast cancer cells. To that end, Co-IP following transfection of GFP-tagged TRF1 into MCF7 cells using an anti-GFP antibody was performed. Results showed that the Nek2 protein co-precipitated with GFP-TRF1 (**Fig. 1C**). These Co-IP results suggested that Nek2 and TRF1 indeed coexist in a protein complex in breast cancer cells.

After determining that Nek2 and TRF1 coexisted, validation of the physiological relevance of the interaction between Nek2 and TRF1 was performed. Immunofluorescence analysis was performed to compare the subcellular localization of Nek2 and TRF1 at different stages of cell cycle. M phase cells were arrested by thymidine–nocodazole treatment. Nek2 and TRF1 proteins were stained with antibodies against Nek2 and TRF1, respectively. The analysis of Nek2 and TRF1 subcellular localization during cell cycle progression revealed that Nek2 and TRF1 mainly co-localized throughout interphase and prophase (**Fig. 1D**). Immunofluorescence microscopy was then used to determine in which cell cycle stages Nek2 and TRF1 colocalized. As demonstrated, Nek2 and TRF1 displayed the same colocalization patterns at interphase and prophase. In addition, Nek2 and TRF1 were observed to localize to the condensed chromosomes in the prophase cells as the chromatin begins to compress (**Fig. 1D**).

In contrast, Nek2 and TRF1 did not colocalize after metaphase. Indeed, previous studies on the regulation of Nek2 expression reported that Nek2 is degraded by means of the APC/C in prometaphase.[19] Furthermore, the levels of TRF1 are tightly regulated during cell cycle, remaining relatively low during G_1 and S, but increasing through G_2 and early M phase.[14] This suggested that expression of Nek2 and TRF1 was regulated by cell cycle progression and colocalized throughout interphase and prophase. Taken together, these results indicate that Nek2 and TRF1 colocalize within the nucleus of cells during interphase and prometaphase.

Interaction between Nek2 and TRF1 is mediated by phosphorylation

In order to determine the interaction between Nek2 and TRF1 in vitro, a GST pull-down assay was performed. Total MCF7 cell lysates were added to the GST-TRF1 or GST immobilized beads. Immunoblotting results using anti-TRF1 and anti-Nek2 antibodies are shown in **Figure 2**. From these results, it was observed that TRF1 was detected in total cell lysates of MCF7, GST-TRF1 with MCF7 lysates, and GST-TRF1 without MCF7 lysates. Furthermore, Nek2 protein signal was observed only in the GST-TRF1 with MCF7 lysates, which indicated that GST-TRF1 brought down Nek2 from total cell lysates (**Fig. 2A**). Thus, these results showed that TRF1 interacts with Nek2 in vitro.

The results from the colocalization, immunoprecipitation, and pull-down assay suggested a functional connection between Nek2 and TRF1. Since it is known that Nek2 phosphorylates its substrates, we hypothesized that inhibition of kinase activity would lead to the disruption of Nek2 binding to TRF1. To test this hypothesis, anti-TRF1 antibody-coupled beads were immunoprecipitated with MCF7 cell lysates. The co-precipitated beads were subjected to incubation with or without λ protein phosphatase (λ PPase). Following incubation, samples were analyzed by immunoblotting with anti-Nek2 and anti-TRF1 antibodies to verify binding status. This analysis showed that treatment of the immunoprecipitates with λ PPase resulted in loss of binding between Nek2 and TRF1, confirming that the interaction between Nek2 and TRF1 was due to protein phosphorylation (**Fig. 2B**).

While the interaction between Nek2 and TRF1 was investigated by various methods, the question remained as to whether TRF1 was a direct binding partner and substrate of Nek2 kinase. To address this, an in vitro kinase assay using both purified Nek2 and TRF1 was performed. Toward this end, purified full-length TRF1 (1–420 amino acids) was incubated with purified Nek2 or kinase dead mutant Nek2 (T179A Nek2KD) in a kinase buffer containing 1 mM ATP for 1 h at 30 °C. Results were analyzed by western blot using anti-phosphoserine, anti-phosphothreonine, or anti-phosphotyrosine antibodies. Since Nek2 is a serine/threonine kinase, phosphorylation would be detected using an anti-phosphoserine or threonine antibody. Anti-phosphotyrosine was used as a negative control for this experiment as Nek2 cannot phosphorylate tyrosine. Results were positive for both the anti-phosphoserine and anti-phosphothreonine antibodies, indicating that Nek2 phosphorylated TRF1. In addition, no signal was detected for the anti-phosphotyrosine antibody. However, these phosphorylation results were unexpected, because TRF1 was not

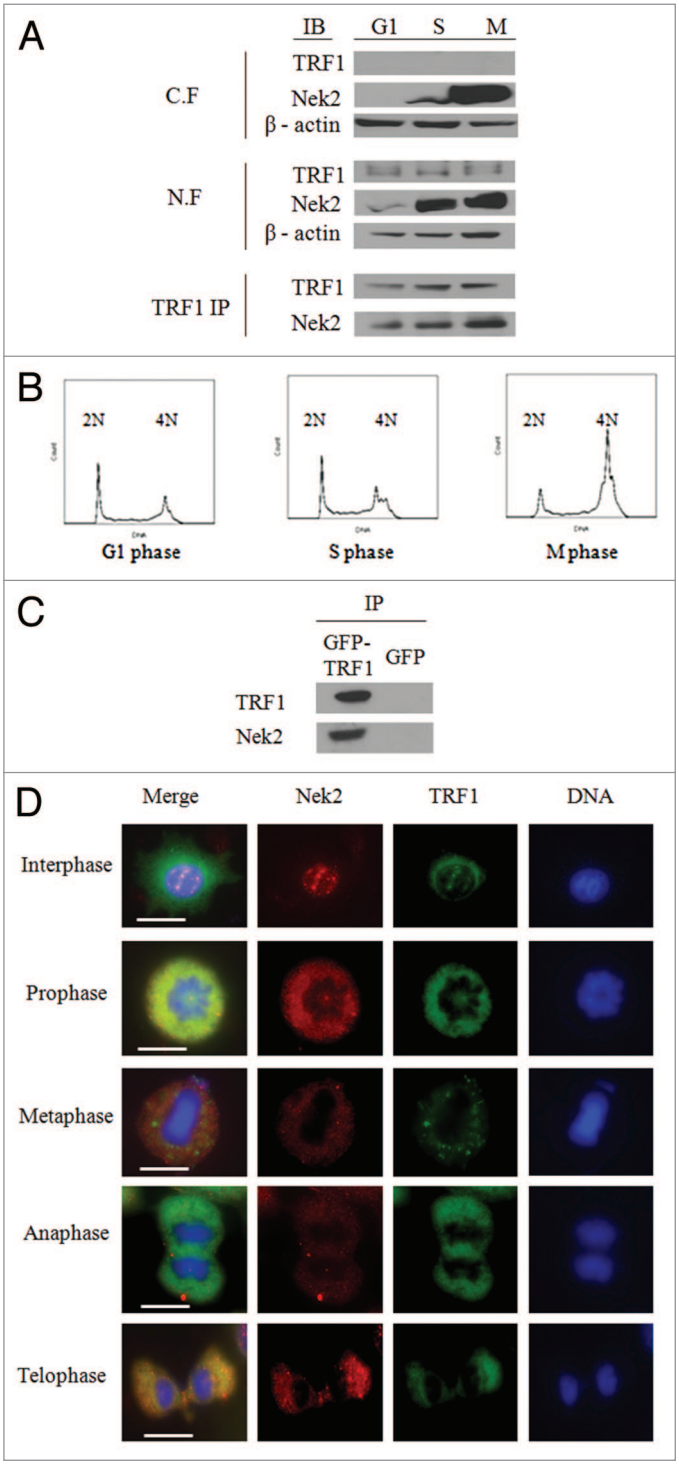

Figure 1. The interaction between Nek2 and TRF1. (**A**) Cell cycle-dependent interaction of Nek2 and TRF1. Cells were synchronized using the double thymidine block (S phase), and thymidine/nocodazole block (M phase). G_1 cells were acquired after 5 h of extra culture upon thymidine/nocodazole release. Cytoplasmic fractions (C.F.) and nuclear fractions (N.F.) were extracted. The N.F. was used for Co-IP. TRF1 was only detected in the N.F. with increased expression at M phase, while expression levels of Nek2 in C.F. and N.F. varied with cell cycle progression. Also, interactions between Nek2 and TRF1 increased from G_1 to M phase. (**B**) Cell cycle profiles of synchronized cells. Cell cycle-synchronized cells were stained with propidium iodide and analyzed by flow cytometry. In M phase cells, 4n DNA content increased. (**C**) Co-IP with overexpressed TRF1 in MCF7 cells transiently transfected with GFP-TRF1 or GFP alone. N.F. were immunoprecipitated with anti-GFP antibody. Precipitated proteins were detected by western with anti-TRF1 or anti-Nek2 antibody. TRF1 and Nek2 were detected only in the GFP-TRF1 transfected cells. (**D**) Subcellular localization of Nek2 and TRF1 at interphase and M phase of MCF7 cells. Cells were co-immunostained with antibodies against Nek2 (red) and TRF1 (green). The nucleus was stained with DAPI (blue). Representative cells at each stage were shown: interphase, prophase, metaphase, anaphase, and telophase. Nek2 protein and TRF1 proteins colocalized at interphase and prophase (scale bar = 25 μm).

phosphorylated by Nek2 at one specific serine or threonine site. These results suggested that TRF1 might be phosphorylated by Nek2 at multiple serine/threonine sites (**Fig. 2C**). Moreover, TRF1 was phosphorylated with Nek2KD as well. One possible explanation is that since the T179A point mutated Nek2 still phosphorylated TRF1, other residues on Nek2 are involved in phosphorylation of TRF1. Indeed, several point mutations of Nek2 have been developed to silence its kinase activity.[20,21] Thus, these data demonstrated that Nek2 likely phosphorylates TRF1 at multiple residues.

Even though TRF1 is suspected of possessing potential multiple Nek2 phosphorylated sites, to map the interactive portion of TRF1, the N-terminal (1–214 amino acids) and C-terminal (215–420 amino acids) of TRF1 were subjected to a kinase reaction, followed by western analysis using anti-phosphoserine or anti-phosphothreonine antibody. Compared with the phosphorylation level observed on the N-terminal of TRF1, the phosphorylation levels of both the serine and threonine residues located in the C-terminal were almost abolished (**Fig. 2D**). This is mostly likely due to the presence of multiple Nek2 phosphorylation sites in TRF1. Also, these results indicated that phosphorylated residues of TRF1 are mainly located in the N terminus, which has a D/E-rich region and dimerization domain. In summary, these binding data demonstrated that TRF1 is a binding partner and substrate for Nek2 both in vitro and in vivo.

Multinucleation and centrosome amplification induced by Nek2 and TRF1 associations

Nek2 associates with the centrosome and kinetochores during prometaphase and dissociates at the onset of anaphase.[22,23] In addition, Nek2 is reported to play bipolar spindle formation.[24,25] Furthermore, it is known that TRF1 is involved in mitotic cell cycle progression.[15] Therefore, we predicted that centrosomal amplification induced by Nek2 overexpression may require TRF1. In order to show the involvement of Nek2 and TRF1 in centrosomal amplification and multinucleation in breast cancer cells, MDA-MB-231 and MCF7 cells were analyzed after overexpression of Nek2 and TRF1 siRNA treatment (**Fig. 3A–I**). Both cell lines were co-transfected with full-length Nek2 gene cloned into a pCMV-Myc vector and TRF1 siRNA. For the negative control, mock pCMV-Myc and a nonsilencing control siRNA were co-transfected. Immunofluorescence was performed using the anti-Myc tag and anti-γ-tubulin antibodies

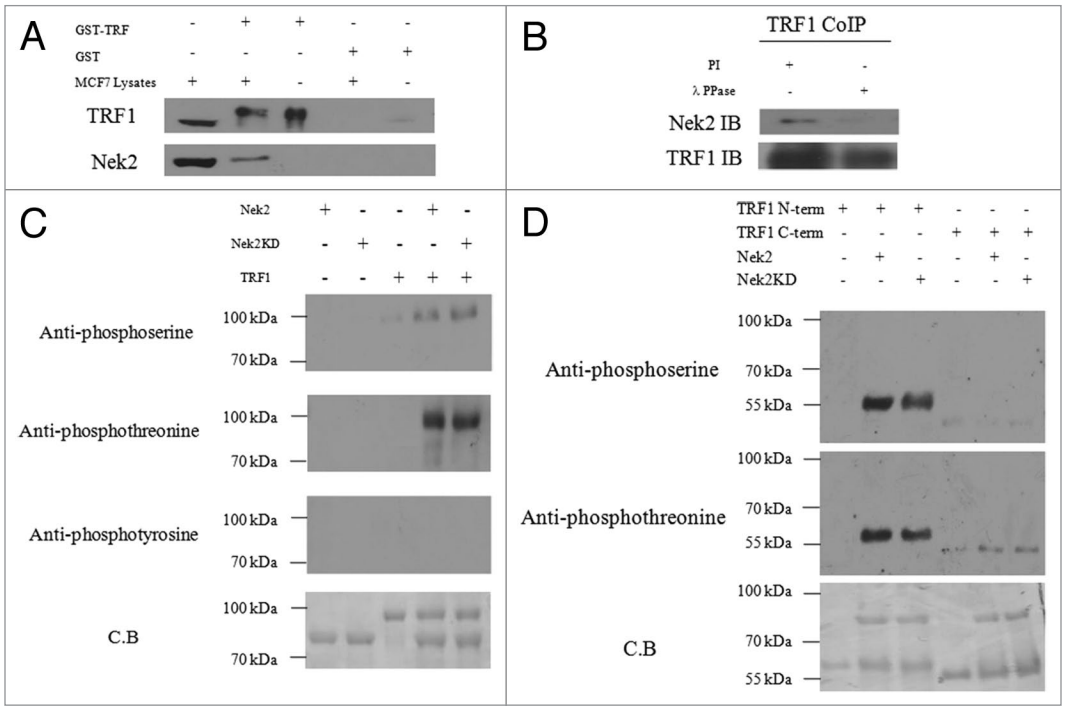

Figure 2. Nek2 directly binds to and phosphorylates TRF1 in vitro and in vivo**.** (**A**) GST-pull-down assay. Agarose bead-bound GST fusion of TRF1 or GST was incubated with the same amount of MCF7 total cell lysate. Pull-down samples were analyzed by immunoblot using anti-TRF1 or anti-Nek2 antibody, respectively. TRF1 was detected in the MCF7 lysates, GST-TRF1 with lysates, and GST-TRF1 alone samples. Nek2 was detected in the MCF7 lysates and GST-TRF1 with lysates. (**B**) Nek2 and TRF1 binding is mediated by phosphorylation. Immunoprecipitated proteins from N.F. using anti-TRF1 antibody were incubated with phosphatase inhibitor (PI) or λ protein phosphatase (λ PPase) for 30 min at 37 °C. Nek2 proteins were not detected in the presence of λ PPase, while TRF1 proteins were detected in both PI and λ PPase, suggesting that Nek2 binds to and phosphorylates TRF1. (**C**) Nek2 phosphorylates TRF1 in vitro. Purified GST-TRF1 was incubated with purified Nek2 or Nek2KD in the kinase buffer containing ATP. Kinase reactions were analyzed by immunoblotting using anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine. Both anti-phosphoserine and anti-phosphothreonine antibodies showed phosphorylation of TRF1 in Nek2 and Nek2KD, while anti-phosphotyrosine was negative for signal. Coomassie Blue staining was used to verify loading. The data indicates that Nek2 may phosphorylate TRF1 in multiple sites. (**D**) Nek2 phosphorylates N-terminus of TRF1. In vitro kinase reaction of truncated N-terminal or C-terminal of TRF1 was performed using an identical scheme. Strong phosphorylation was observed in the N terminus of TRF1 with both anti-phosphoserine and anti-phosphothreonine antibodies, whereas very weak phosphorylated signals were detected in the C terminus of TRF1.

as a centrosome marker in both breast cancer cell lines (**Fig. 3A and D**) to demonstrate whether TRF1 in conjunction with Nek2 overexpression was needed to induce chromosomal instability and aneuploidy. Cells with co-transfected vector and siRNA were fixed and immunostained with anti-γ-tubulin antibody and anti-Myc tag antibodies. Control siRNA or TRF1 siRNA-treated MDA-MB-231 and MCF7 cells with mock vector transfection demonstrated 1–2 centrosomes. In contrast, Nek2 overexpressing cells with control siRNA showed over 3 centrosomes (**Fig. 3A and D**). Interestingly, cells depleted of endogenous TRF1 by siRNA concurrent with Nek2 overexpression did not demonstrate abnormal centrosome numbers. To confirm these observations, percentages of centrosome amplification and multinucleation events in Nek2-overexpressing cells were determined. In mock vector transfections, both cell lines with either TRF1 or control siRNA demonstrated around 23% ~35% of cells with >2 centrosomes, respectively (**Fig. 3B and E**). However, MDA-MB-231 cells overexpressing Nek2 co-transfected with control siRNA showed 52% with >2 centrosomes. This was also observed in 44% of the MCF7 cells. These rates were reduced to 37% (MDA-MB-231) and 26% (MCF7) in TRF1 siRNA-treated and Nek2-overexpressing cells. Percentages of centrosomal abnormalities were comparable to multinucleated cells for both cell lines. The effect of TRF1 knockdown in Nek2-overexpressing cells was clearly distinguishable from control siRNA (**Fig. 3C and F**). Increased numbers of multinucleated cells associated with Nek2 overexpression were reduced in the cells with depleted TRF1. Results showed that most of the multinucleated cells contained supernumerary centrosomes. Finally, western analysis was performed 48 h post-transfection using anti-Nek2 and anti-TRF1 antibodies. Depleting endogenous TRF1 by siRNA did not affect the level of endogenous or exogenous Nek2 protein expression compared with control siRNA for either cell line (**Fig. 3G**).

Previous studies reported that overexpression of Nek2 induced a delay in mitosis.[26,27] It was possible that these results might be influenced by altered cell cycle distribution due to depletion of TRF1. To address how TRF1 affects cell cycle progression, MCF7 cells were transfected with either control siRNA or TRF1 siRNA. Cell cycle was synchronized at S phase with a double thymidine block. The FACS analysis revealed no obvious cell cycle arrest in TRF1 depleted or control cells (**Fig. 3H and I**). Therefore, it is likely that these results were a consequence of cell cycle defects induced by Nek2 overexpression, not TRF1 depletion.

Taken together, these results suggest that ectopic expression of Nek2 induced the accumulation of abnormal centrosome numbers through cytokinetic failure rather than downregulation of centrosomal duplication, a mechanism in which TRF1 may play an essential role.

Nek2 overexpression requires TRF1 to induce chromosome misalignment and kinetochore–microtubule attachment failure

Overexpression of Nek2 induces a delay in mitosis by increasing the activity of Mad2, one of the SAC components.[26] Moreover, Nek2 overexpression resulted in prometaphase arrest.[19] Prolonged mitosis has been suggested to promote chromosome misalignment at the metaphase plate, causing the SAC to be activated continuously, blocking the onset of anaphase.[28] Multinucleation study results shown in **Figure 3** demonstrated that TRF1 depletion suppressed the rate of multinucleated cells induced by Nek2 overexpression in breast cancer cells (**Fig. 3C and F**). Thus, we hypothesized that TRF1, in conjunction with Nek2 overexpression, plays a pivotal role in the failure of proper chromosomal alignment and kinetochore–microtubule attachment, inducing prolonged mitosis by stabilizing SAC activation. To explore this possibility, both breast cancer cell lines were co-transfected with the Myc/Myc-Nek2 vector and control/TRF1 siRNA. Twenty-four hours post-transfection, cells were treated with 100 ng/ml nocodazole for 16 h, followed by 2 h additional incubation with fresh DMEM to synchronize cells in metaphase. Treated cells were fixed with cold methanol and stained with FITC-conjugated anti-α-tubulin and anti-Myc tag antibody. Results showed that Nek2 overexpressing cells with control siRNA demonstrated misaligned chromosomes in metaphase, due to failure in kinetochore-microtubule attachment (**Fig. 4A and C**, top). Epifluorescence microscopy results showed that Nek2 overexpressing cells had a variety of abnormal chromosome alignment events at metaphase.

First, cells with overexpressed Nek2 developed lagging chromosomes during metaphase and anaphase transition, resulting in micronuclei (**Fig. 4A and 4C**, white arrowhead). Micronuclei induce DNA breakage, one of the factors in cancer development.[29] Second, unequal centrosome splitting was observed in Nek2-overexpressing cells (**Fig. 4A and 4C**, middle upper). This phenotype displayed an overdeveloped centrosome on one side, while the other side displayed a very weak or lost centrosome. This resulted in disrupted microtubule-organizing activity, possibly due to the lack of pericentriolar material.

The last aspect observed in Nek2-overexpressing cells involved multipolar spindles (**Fig. 4A and 4C**, middle lower). Results demonstrated the presence of multiple spindles in the cells with lagging chromosomes as well as unequal centrosome splitting. This is most likely due to deregulation of centrosome number and function, leading to aneuploidy and asymmetric cell division. In contrast, cells treated with TRF1 siRNA exhibited normal chromosome alignment and microtubule–kinetochore attachment at metaphase. The frequencies of >3 spindle poles significantly increased in both MDA-MB-231 and MCF7 cells (29% and 26%, respectively). In addition, misaligned chromosomes also increased significantly, to 61% in both cell lines, in comparison to control siRNA. In contrast, depletion of TRF1 in cells overexpressing Nek2 was comparable to controls and reduced the number of cells with 3 spindle poles and misaligned chromosomes (**Fig. 4B and 4D**). Taken together, these results indicate that Nek2 overexpression induced abnormal spindle pole numbers and chromosomal misalignments. However, depletion of TRF1 attenuated the abnormalities caused by Nek2 overexpression.

TRF1 knockdown decreases multinucleation and cytokinetic failure in Nek2-overexpressing cells

In this study, we demonstrated that 71% of MDA-MB-231 and 64% of MCF7 cells with Nek2 overexpression completed cytokinesis with multinucleated chromosomes (**Fig. 3**). Also,

Nek2 overexpression prolongs mitosis,[19,26] resulting in misaligned chromosomes (**Fig. 4**). These observations along with previous reports led us to hypothesize that the misalignment of chromosomes would cause the production of multinucleated cells.

To address this idea, a cyclin-dependent kinase inhibitor, purvalanol A was used. Purvalanol A allows the cells to exit mitosis even in the presence of misaligned chromosomes.[30] After 24 h co-transfection with vectors and siRNAs, both MDA-MB-231 and MCF7 cells were arrested at M phase using 100 ng/ml nocodazole for 16 h, followed by 6 h additional incubation with nocodazole-free media containing 10 μM of purvalanol A. Treated cells were fixed with cold methanol and immunofluorescence analysis with

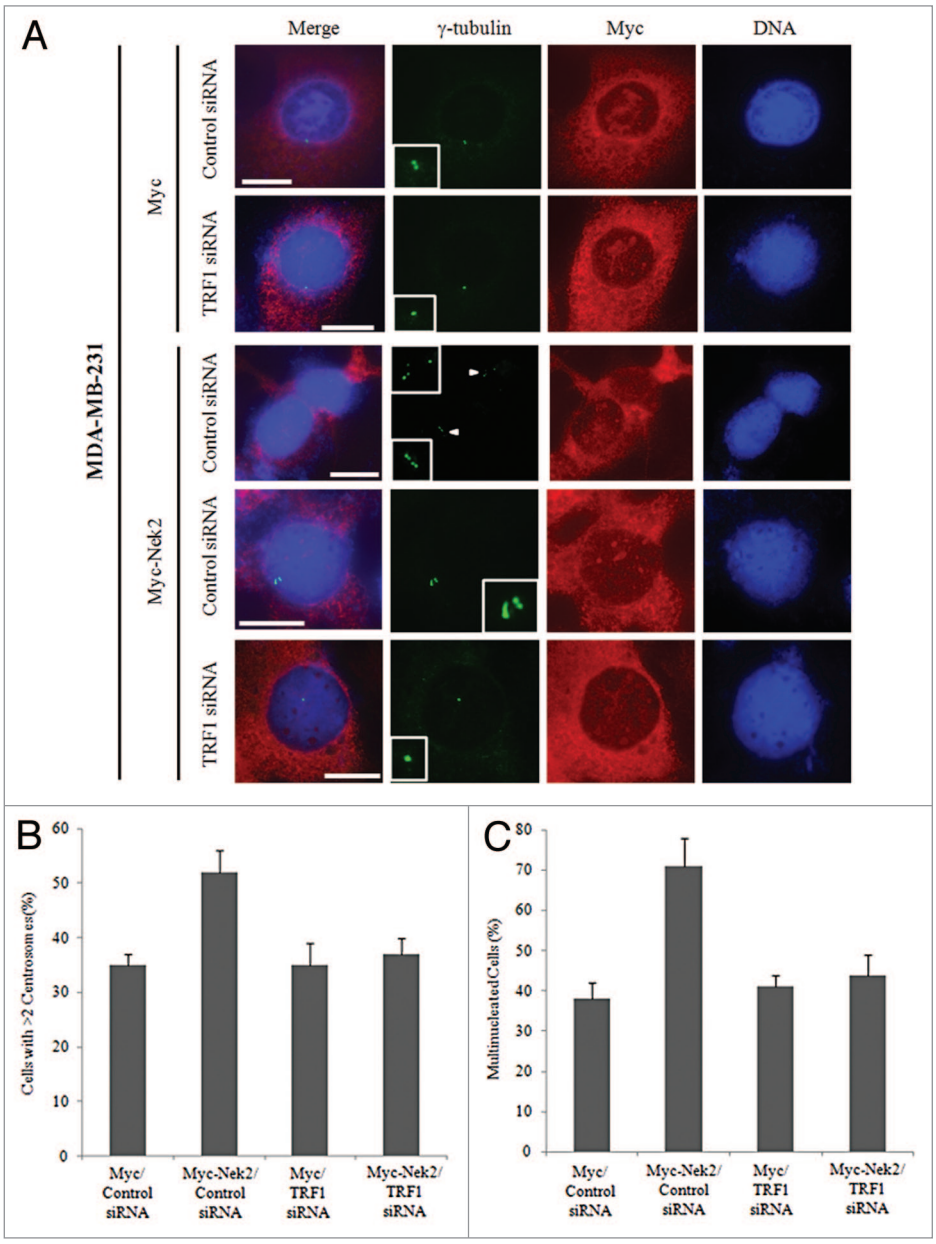

Figure 3A–C. TRF1 knockdown induces abnormal centrosome amplification and multinucleation in Nek2 overexpressing cells. (**A and D**) MDA-MB-231 (**A**) and MCF7 cells (**D**) were co-transfected with pCMV-Myc or pCMV-Myc-Nek2 vectors and control siRNA or TRF1 siRNA respectively. Forty-eight hours after co-transfection, cells were stained for γ-tubulin (green), Myc (red), and DNA (blue). Photomicrographs show typical features for each case. Nek2-overexpressing cells displayed abnormal numbers of centrosomes, while TRF1 downregulation suppressed abnormal centrosome amplification and multinucleation in the cells. Inserts show magnified images of centrosomes. Arrowheads indicate magnified insets (scale bar = 20 μm). (**B and E**) percentage of cells with > 2 centrosomes in MDA-MB-231 (**B**) and MCF7 (**E**). (**C and F**) percentage of MDA-MB-231 (**C**) and MCF7 (**F**) cells with multinucleation. The graphs show the average of 3 experiments. Nek2-induced centrosome amplification and multinucleation were decreased in TRF1-deficient cells. (**G**) MDA-MB-231 and MCF7 cells were introduced with indicated siRNA or vectors. Forty-eight hours post-transfection, whole-cell lysates were analyzed by immunoblot with anti-Nek2, anti-TRF1, or β-actin antibody. (**H**) FACS analysis of MCF7 cells. MCF7 cells were transfected with control siRNA or TRF1 siRNA. At 24 h post-transfection, cell cycle was synchronized at G_1/S phase. I, immunoblot analysis of siRNA transfected MCF7 cells. Forty-eight hours post-transfection, cells were harvested to determine efficiency of TRF1 depletion by western.

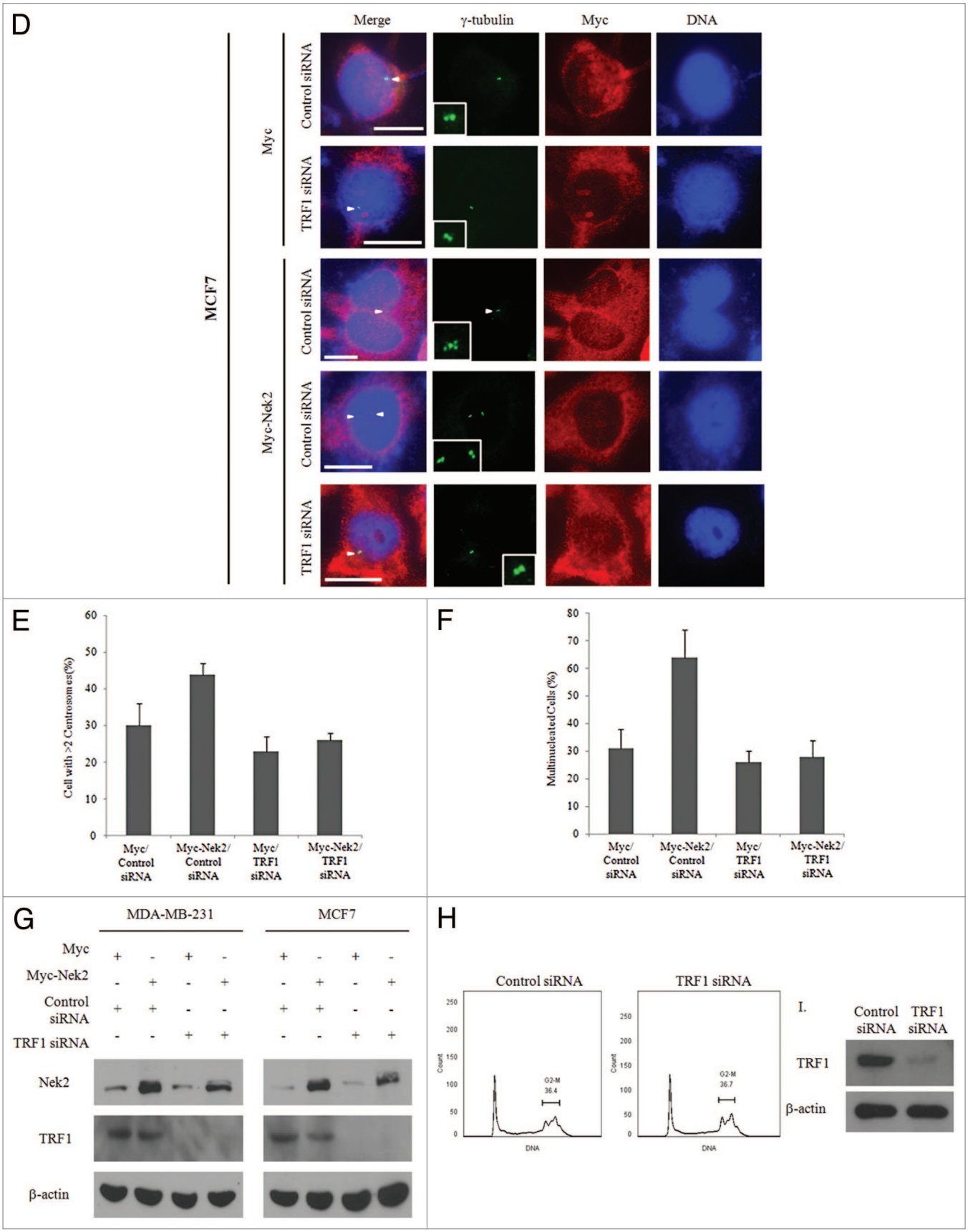

Figure 3D–H. For figure legend, see page 3604.

FITC conjugated anti-α-tubulin antibody and DAPI counterstain was performed (**Fig. 5A and 5C**). For both Nek2-overexpressing breast cancer cell lines, Nek2 overexpression increased the frequency of binucleated or multinucleated cells (**Fig. 5B and 5D**). In control cells with mock vector and control siRNA treatments, approximately 30% of the cells were binucleated, and 5% were multinucleated. However, Nek2-overexpressed MDA-MB-231 and MCF7 cells increased the number of binucleated cells and multinucleated cells to approximately 56% and 12%, respectively. Interestingly, the frequencies of binucleated cells and multinucleated cells with TRF1 depletion decreased to levels comparable with control cells. The formation of bi/multinucleated cells may be due to a delay in meeting the requirements of SAC activation due to attenuation induced by Nek2 overexpression. Therefore,

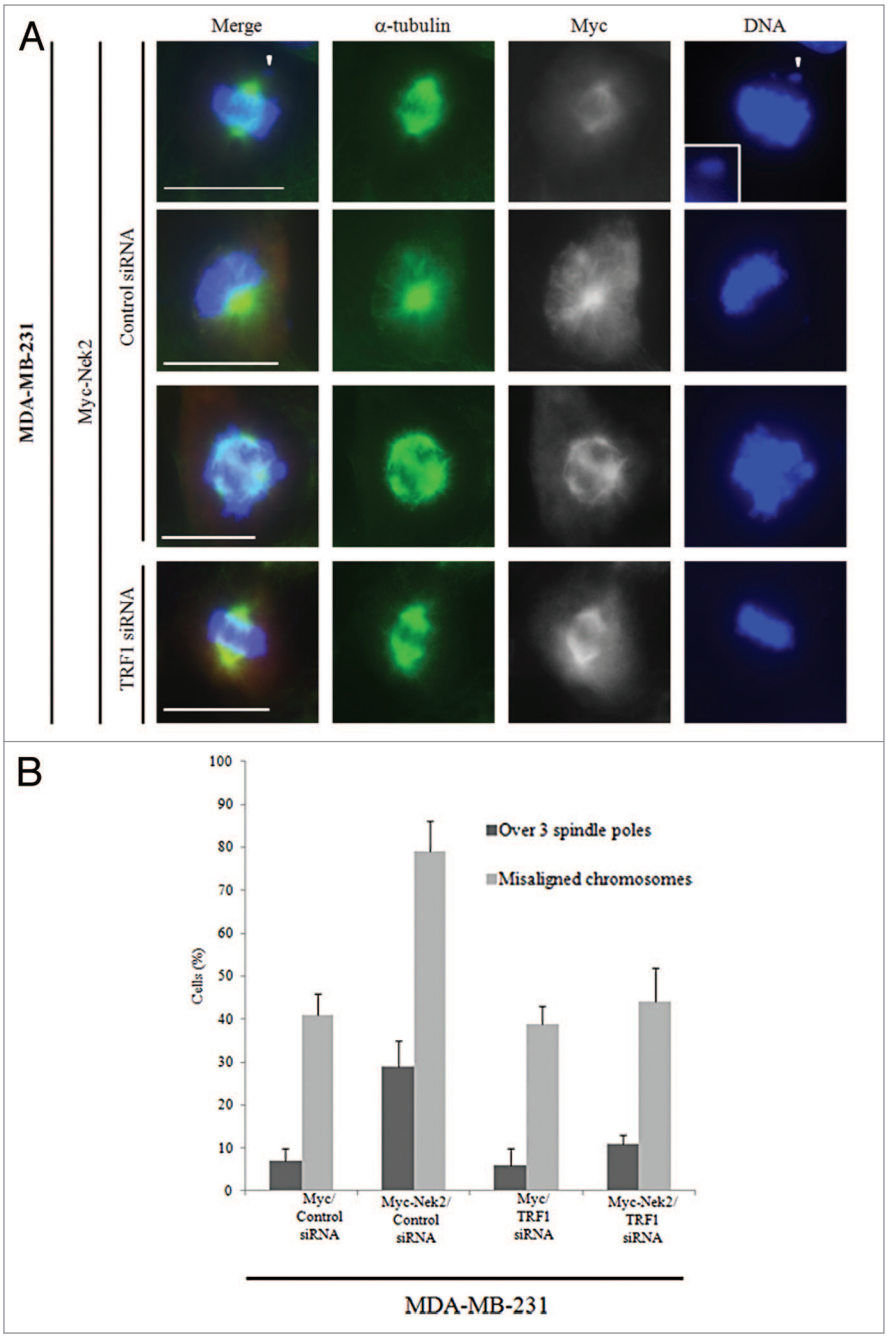

Figure 4A and B. Nek2 overexpression in conjunction with TRF1 interactions can result in chromosomal misalignment. MDA-MB-231 (**A**) and MCF7 (**C**) cells co-transfected with indicated vectors and siRNAs. Transfected cells were arrested at metaphase using nocodazole. Treated cells were fixed and immunostained with FITC conjugated anti-α-tubulin and anti-Myc antibodies. Photomicrographs represent α-tubulin (green), Myc (white/black), and DNA (blue). Nek2 overexpression induced various abnormal spindle pole formations and misaligned chromosomes. However, TRF1 siRNA suppressed formation of these abnormalities. Inserts are magnified images of arrowhead indicating a lagging chromosome (scale bar = 20 μm). (**B and D**) Percentage of the cells with <3 spindle poles (dark gray bars) and misaligned chromosomes (light gray bars) in MDA-MB-231 (**B**) and MCF7 (**D**). Results collected from 3 independent experiments.

results suggest that Nek2 overexpression caused abnormal nucleation, which was suppressed by TRF1 knockdown.

To verify that Nek2-induced cytokinetic failure was mediated by TRF1, we complemented TRF1-depleted cells with a siRNA-resistant wild-type TRF1 tagged with HA, expecting reappearance of the cytokinetic failure in Nek2-overexpressed cells. The TRF1 gene was inserted into the pCMV-HA vector. As a control group, Myc mock vector was transfected with HA mock vector/control siRNA or HA-tagged TRF1 vector/control siRNA. Also, HA mock vector/TRF siRNA or HA-tagged TRF1 vector/TRF siRNA were introduced into the Myc mock transfected cells. To compare with a control group, Nek2-overexpressing cells were generated. HA or HA-TRF1 transfected cells retaining Myc-Nek2 vectors were treated with control siRNA or TRF1 siRNA, respectively. Forty-eight hours post-transfection, cells were harvested for immunoblotting using anti-Nek2 or anti-TRF1 antibody to confirm the expression of exogenous genes. Results are shown in **Figure 6**. Immunoblotting results demonstrated expression of endogenous or exogenous Nek2 and TRF1 (**Fig. 6A and 6C**). Western results for HA-TRF1 with TRF1 siRNA transfected cells showed only expression of exogenous TRF1 under both conditions.

To assess the effect of TRF1 knockdown on cytokinetic failure, depletion, and add-back of TRF1 in the presence of Nek2

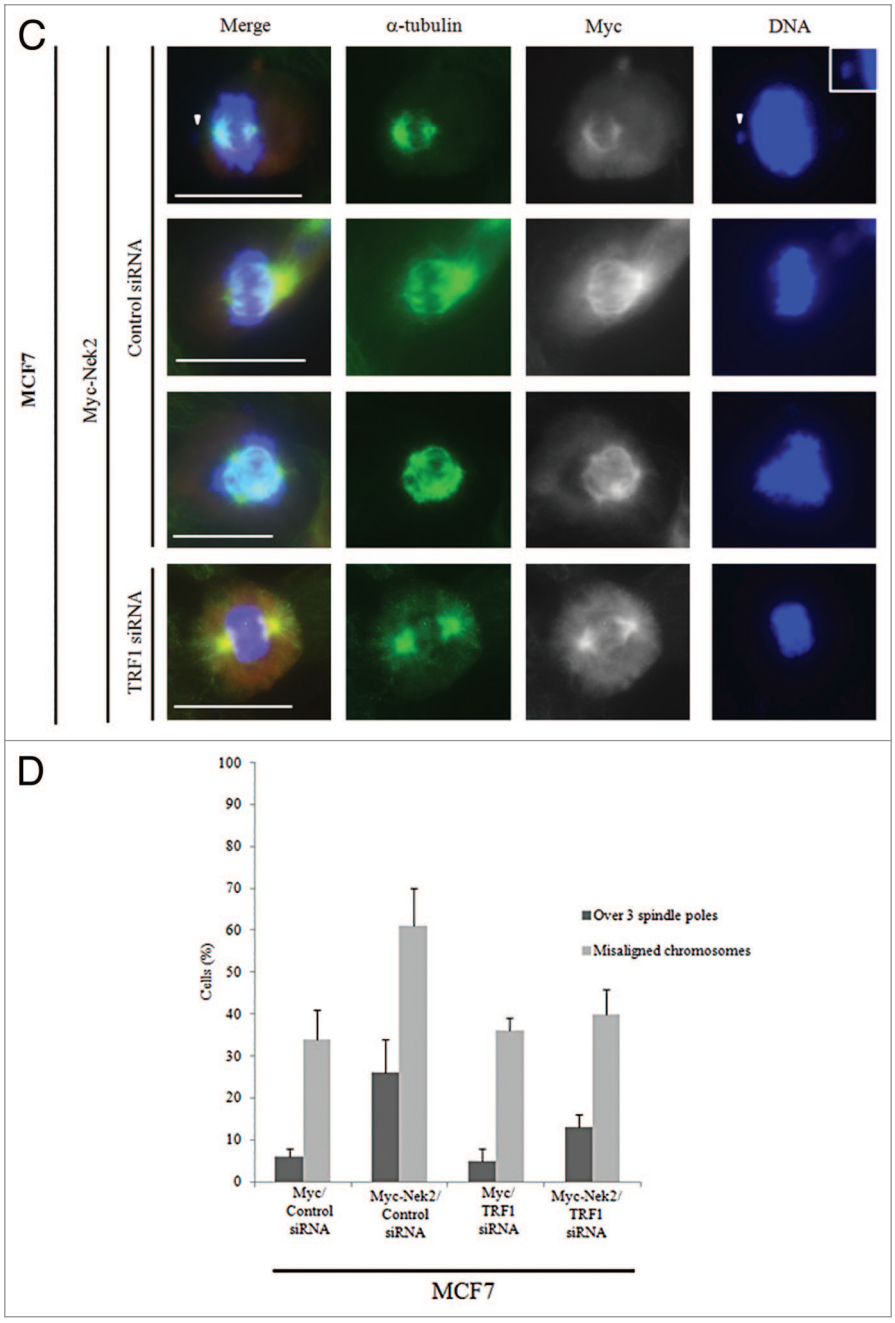

Figure 4C and D. For figure legend, see page 3606.

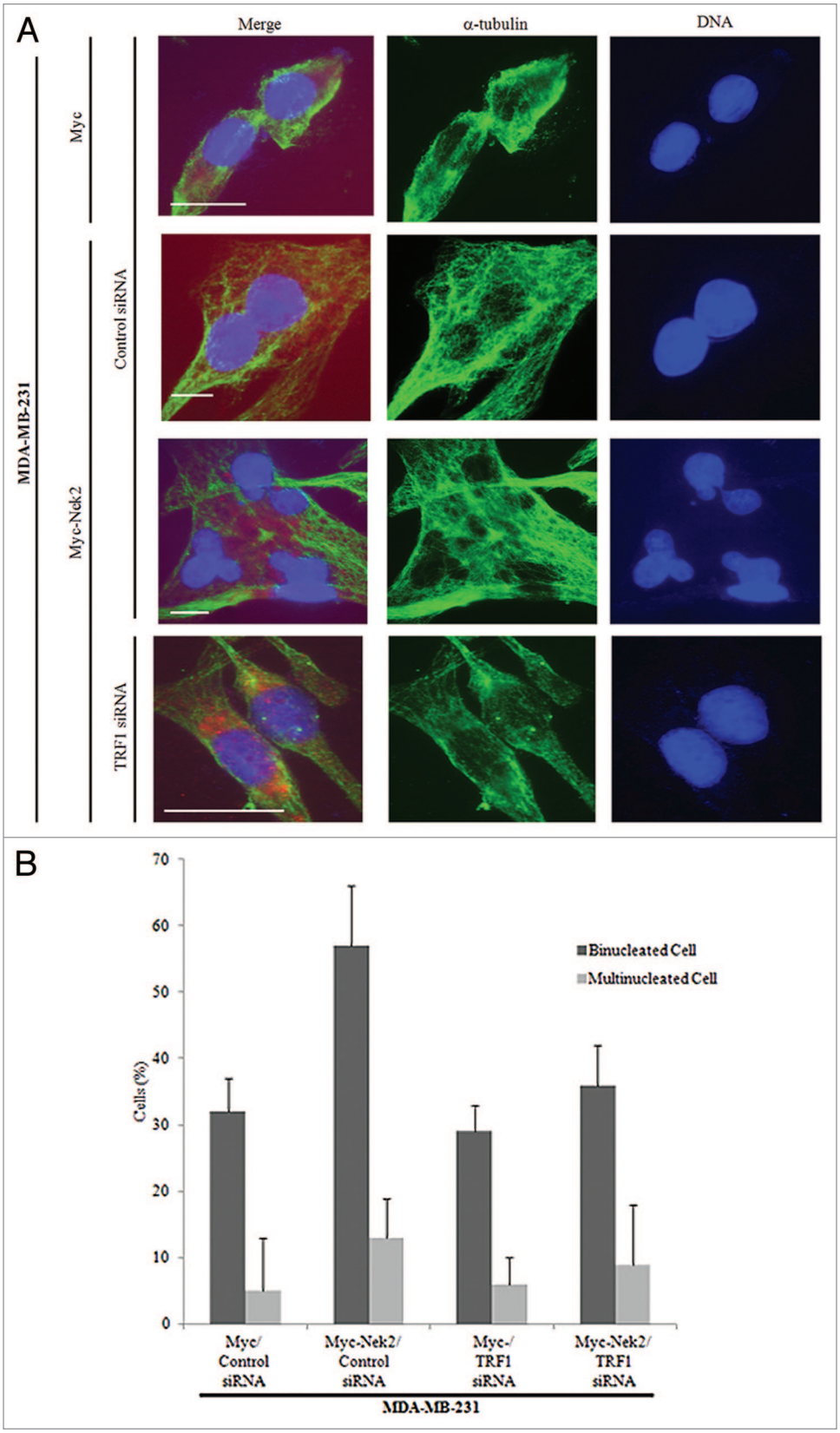

Figure 5A and B. Frequency of multinucleation in Nek2 overexpressing cells. (**A and C**) transfected MDA-MB-231 (**A**) and MCF7 (**C**) cells were arrested at prometaphase. Cells were cultured with 10 μM purvalanol A for 6 h to allow release from M phase. Treated cells were fixed and immunostaining with FITC conjugated anti-α-tubulin antibody (green) and DAPI (blue). Nek2-overexpressing cells demonstrated bi/multinucleated chromosomes (scale bar = 20 μm). (**B and C**) percentages of binucleated (dark gray bar) and multinucleated cells (light gray bar) from treated MDA-MB-231 (**B**) and MCF7 (**D**) cultures. Cells with TRF1 knockdown repressed the occurrence of bi/multinucleated cells. Results were generated from 3 independent experiments.

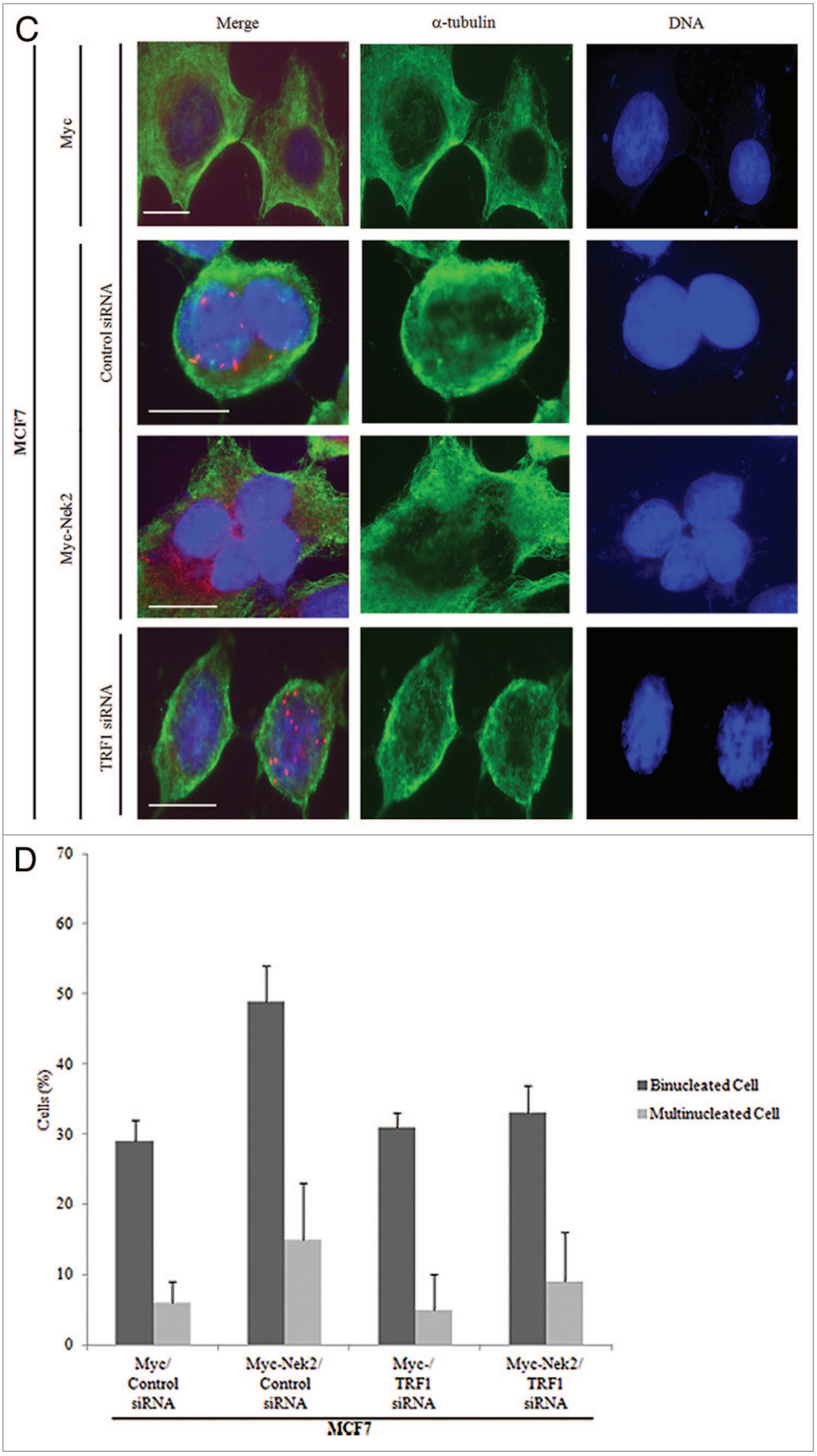

Figure 5C and D. For figure legend, see page 3608.

overexpression was performed. TRF1-silenced cells with Nek2 overexpression did not accumulate significant numbers of cytokinetic failures in either breast cancer cell line. In contrast, as anticipated, when HA-TRF1 was added back in Nek2-overexpressed cells, 63% or 49% of cells had re-induced cytokinetic failure, respectively (**Fig. 6B and 6D**). Taken together, these observations suggested that TRF1 plays a critical role in cytokinetic failure induced by Nek2 overexpression.

Discussion

TRF1 is mainly associated with 2 different cellular functions, telomere length regulation, and mitotic regulation.[31] It was reported that human Pin2, a splice variant of TRF1, when co-expressed in mammalian cells, interacted with NIMA to block its effects on mitosis.[17] In breast cancer cells with short telomeres, overexpression of TRF1 caused entry into mitosis followed by mitotic arrest and apoptosis. This consequence of TRF1 overexpression was not observed in cells with longer telomeres.[16] Therefore, telomere length might affect the cell cycle based on the level of unbound TRF1 during mitosis.[31] Indeed, there is also evidence that TRF1 is not only tightly regulated during cell cycle, but also plays a role in mitotic cell cycle progression.[14-16,32]

Nek2 degradation is mediated by APC/C through its D-box domain.[19,33] Similarly, TRF1 contains D-box-like motif destruction recognition as well.[14] Thus, the most likely mechanisms for fluctuations in expression levels for both Nek2 and TRF1 involve an increased accumulation during G_2/M transition, followed by degradation as cells move into G_1. This observation is consistent with a previous study showing that the protein levels of endogenous TRF1 were increased in M phase, and binding with its partner was increased during mitosis.[34] Therefore, the binding between Nek2 and TRF1 mainly occurred during early mitosis, suggesting cell cycle-specific protein interactions that peaked in G_2/M phase. Even though the molecular mechanisms of cell cycle-dependent fluctuation of TRF1 remain to be elucidated, evidence from this and other studies support the concept that accumulation and degradation of TRF1, which may or may not

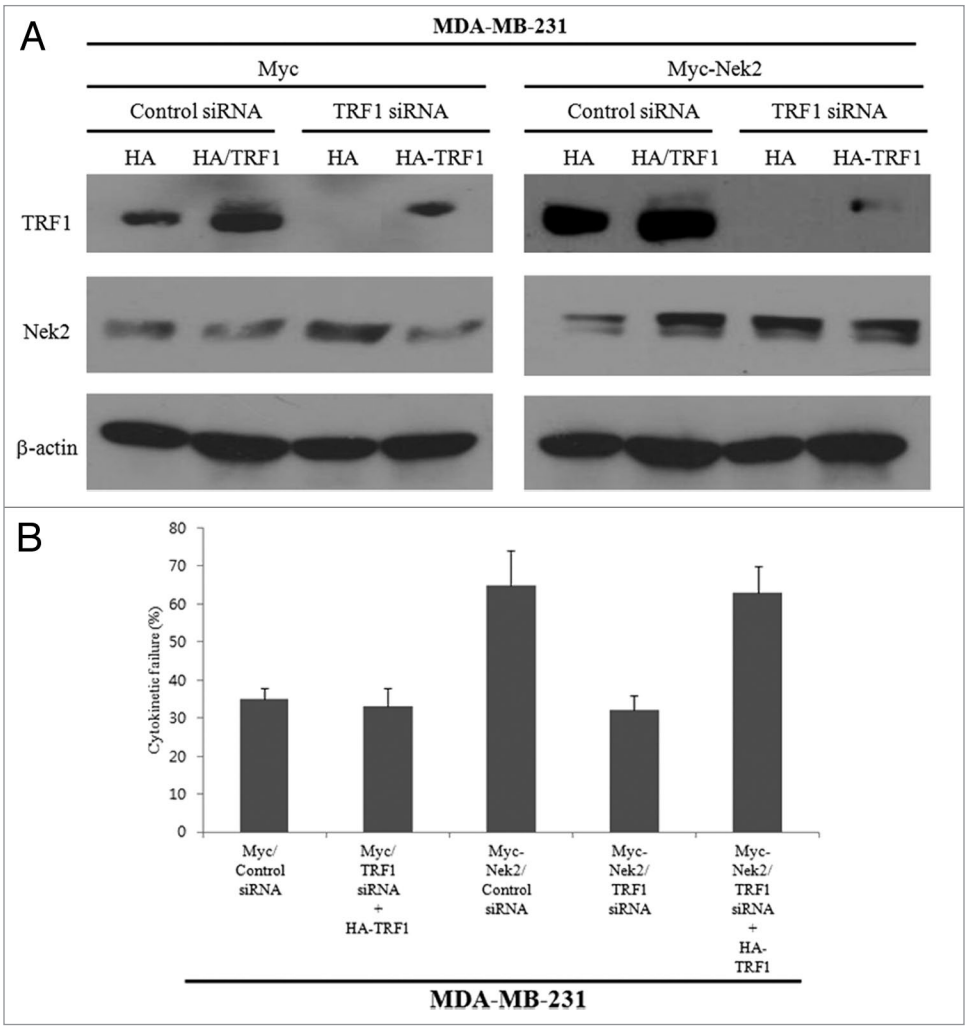

Figure 6A and B. TRF1 is responsible for the cytokinetic failure associated with Nek2 overexpression. (**A and C**) immunoblot analysis of TRF1 add-back experiments. Forty-eight hours post-co-transfection with indicated vectors and siRNAs, whole cell lysates from MDA-MB-231 (**A**) and MCF7 (**C**) cells were analyzed by immunoblotting with antibodies against TRF1, Nek2, and β-actin. (**B and D**) Percentages of MDA-MB-231 (**B**) and MCF7 (**D**) cells with cytokinetic failure (performed 3 times). Cells were transfected as in (**A and C**). The add-back of TRF1 recovered cytokinetic failure in both cell lines.

be bound to telomeres (telomere-unbound fraction), plays a role in entry and exit from M phase.

In vitro kinase assays demonstrated that full-length TRF1 was phosphorylated by Nek2. However, the phosphorylation of TRF1 was detected by both the anti-phosphoserine and anti-phosphothreonine antibodies, suggesting that TRF1 is likely to be phosphorylated by Nek2 at multiple sites. Moreover, in vitro kinase assays using truncated TRF1 showed that phosphorylated serine/threonine were mainly detected on the N terminus of TRF1, containing the D/E-rich and the dimerization domain. Identification of the phosphorylation domain is important, because these results suggest 2 possibilities. First, Nek2 may regulate the function of the D/E-rich domain and dimerization domain in the N terminus of TRF1 through phosphorylation. The D/E-rich domain in TRF1 has tankyrase 1 and tankyrase 2 binding sites.[35,36] Tankyrase 1 negatively regulates telomere binding and protein stability of TRF1,[35,37,38] allowing telomerase to easily access the telomeres via dissociation of TRF1 from the telomere. Thus, Nek2 could be a binding competitor of tankyrase 1, regulating the association of TRF1 to the telomeres and, ultimately, participating in regulation of TRF1 stability.

A second and more likely possibility is that the dimerization domain of TRF1 presents a large surface for interaction with other proteins. Indeed, several proteins, such as TIN2[39] and ATM,[16] are known to interact with the dimerization domain. This architecture of the TRF1 dimerization domain suggests that in the formation of the TRF1 dimer, 2 DNA binding domains may bind independently, in tandem, to 2 binding sites on telomeric DNA. Alternatively, dimerization of TRF1 brings 2 DNA binding domains together, increasing the affinity of TRF1 for telomeric DNA. Hence, Nek2 probably serves as a positive regulator in the stabilization of shelterin formation or mediation of TRF1 dimerization.

Nek2 is well-known for its role in centrosome duplication and separation.[40,41] Studies reported that Nek2 is an attractive target for cancer treatment[42,43] and revealed that overexpression of Nek2 led to the premature separation of centrosomes and subsequent cytokinesis failure.[4,44,45] In human cells, overexpressed Nek2 led to centrosome dispersal.[4] Consistent with these previous reports, data generated from this study demonstrated abnormal centrosome amplification and multi-nucleation in Nek2-overexpressed breast cancer cell lines.

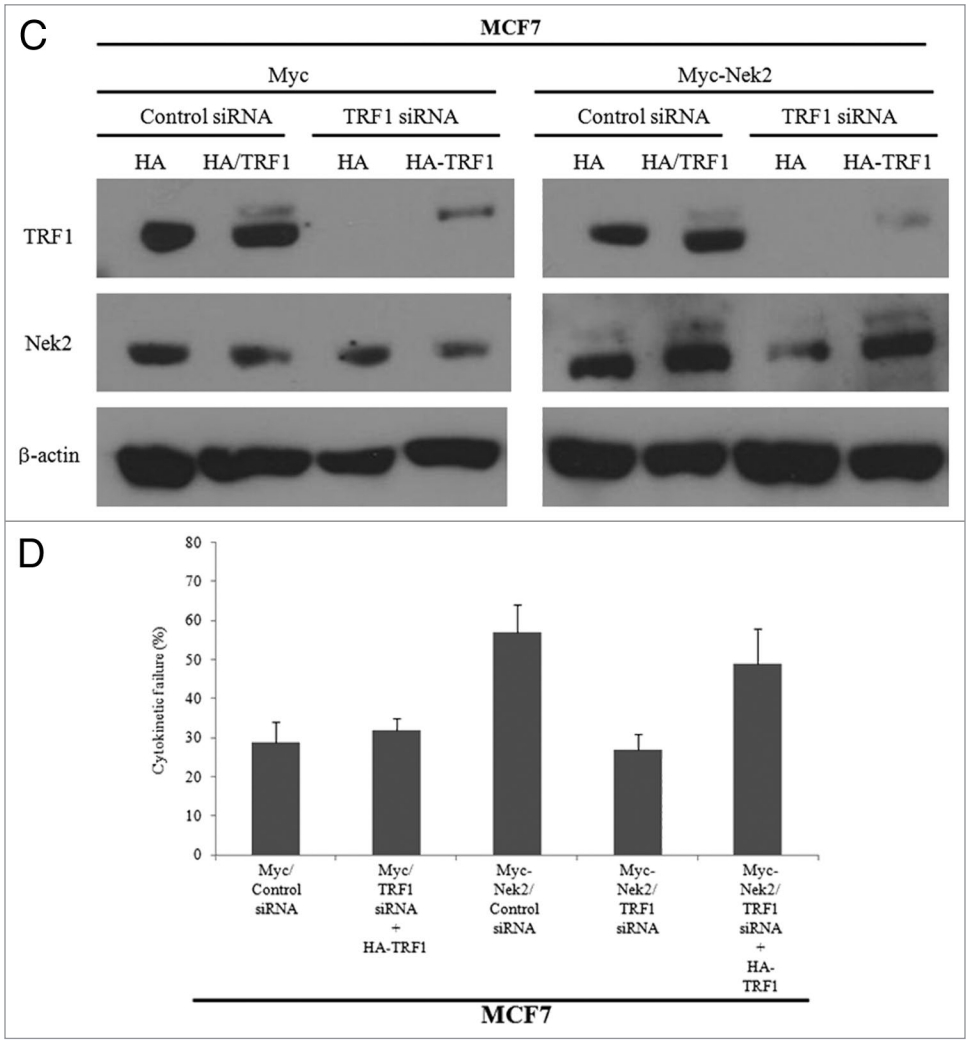

Figure 6C and D. For figure legend, see page 3610.

Since chromosome polyploidy weakens the structure of chromosomes, polyploidy, resulting from centrosome amplification, induces chromosome segregation errors.[46] Furthermore, monopolar or multipolar spindle formation in Nek2-overexpressing cells, which have numerical centrosomal abnormalities caused by mitotic aberration, was observed. During mitosis, centrosomes exert strong pulling forces due to the attachments between spindles and chromosomes. Therefore, they need to be structurally fortified before the onset of mitosis. Otherwise, centrosomes will not be able to withstand the tension from these forces. As a result, centrosome fragmentation can occur. When centrioles are not able to keep coupled, cells will undergo uncontrolled splitting, ultimately forming extra centrosomes containing one centriole and multipolar spindles.[47] Taken together, these previous studies explain how and why multinucleated polyploidy was readily observed in Nek2-overexpressing cells in this study.

The prolongation of mitosis in cancer cells is due to a delay in satisfying the requirements of the SAC. Over-activation of the SAC is likely to increase the rate of aneuploidy through accumulation of lagging chromosomes.[2] Recently, Liu et al.[26] reported that overexpression of Nek2 led to prolonged mitosis through over-activation of Mad2. Furthermore, Nek2 overexpression caused prolonged prometaphase.[19] Lagging chromosomes caused by Nek2 overexpression demonstrated that TRF1 knockdown suppressed Nek2-induced lagging chromosomes, suggesting that TRF1 may affect kinetochore capture by mitotic spindles. It has been known that lagging chromosomes at mitosis are a potential feature of aneuploidy. After cytokinesis, a lagging chromosome could be a source of chromosome instability and aneuploidy, which gives rise to the formation of micronuclei. Micronuclei play a critical role in cancer development.[48,49] The prolongation of metaphase through Nek2 overexpression may likely allow all sister kinetochores to attach to the microtubules, leading to merotelic attachment, also occurring in lagging chromosomes as well.[50] TRF1 add-back showed a recapitulation of increased cytokinetic failure, suggesting that TRF1 is required for Nek2-induced cytokinetic failure. This implies that deletion of TRF1 may improve kinetochore–microtubule attachment stability. Interestingly, Nek2 can bind with microtubules and Hec1 to facilitate kinetochore–microtubule binding,[22,23,51,52] and TRF1 directly binds with microtubules through its C terminus.[15] A previous study reported that TRF1 knockdown decreased the time from nuclear envelope breakdown to the onset of anaphase, while the checkpoint protein complex formation was not affected.[53]

In conclusion, our study indicates that Nek2 overexpression could not induce centrosome amplification and chromosome instability without expression of TRF1. Therefore, TRF1 is responsible for the cytokinetic failure due to Nek2 overexpression. This direct interaction between TRF1 and Nek2 overexpression is a newly identified, protective mechanism guarding the cell against aneuploidy and potential cancer cell progression.

Materials and Methods

Cell culture and cell cycle synchronization

MDA-MB-231 and MCF7 breast cancer cells (ATCC) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively) under 5% CO_2 in humid conditions at 37 °C. For synchronization of cells in G_1, S, or G_2/M phase, cells were treated using the double thymidine block (S phase) and thymidine/nocodazole block (G_2/M phase). Cells were transiently transfected with expression vector or siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, the cells were lysed for immunoblotting, immunoprecipitation, fixed for immunofluorescence analysis or fluorescence activated cell sorting (FACS) analysis.

Immunoprecipitation and GST-pull-down assay

For immunoprecipitation, 200 μg of nuclear extract fractions were prepared. Three μl of anti-TRF1 rabbit polyclonal antibody (Abcam) or anti-GFP rabbit polyclonal antibody was added to the sample and incubated for 1 h at 4 °C. Twenty μl of the newly washed beads slurry was added to the protein–antibody mixture and incubated at 4 °C for overnight. Immunocomplexes were subjected to western analysis. To determine whether phosphorylation mediates binding between Nek2 and TRF1, TRF1-IP was performed as described above. The protein–antibody captured beads were incubated with 400 unit of λ protein phosphatase (NEB) at 30 °C for 20 min. To stop the reaction, 50 μl of 1× Laemmli sample buffer was added and the sample was then boiled at 100 °C for 10 min. Samples were analyzed by immunoblot analysis.

For GST-pull-down assay, pGEX-4T-1 vector containing full-length, N terminus or C terminus of TRF1 was transformed in *E. coli* BL21 (DE3). IPTG induced *E. coli* cultures were grown for 5 h at 30 °C with shaking. Bacteria pellets were lysed by sonication. Forty μl of glutathione agarose beads (Pierce) were washed 3 times with cold binding buffer. The beads were incubated with GST fusion protein expressed lysates for 3 h at 4 °C. The beads were mixed with MCF7 total lysates, followed by overnight incubation on a rotating platform at 4 °C. Following washes in binding buffer, a fraction of the beads was resuspended in 100 μl of 2× Laemmli sample buffer and boiled. The beads were spun down, and supernatants were collected for further immunoblot analysis.

In vitro kinase assay

In vitro kinase assays were performed with purified Nek2 and TRF1 proteins in kinase buffer (Cell Signaling) supplemented with ATP (Teknova). Four hundred ng of Nek2 and 1 μg of TRF1 proteins were incubated for 1 h at 30 °C with kinase buffer containing 1 mM of ATP in 30 μl total volume. The kinase reactions were stopped by adding 20 mM of EDTA and 2X Laemmli sample buffer, followed by boiling at 70 °C for 5 min. Samples were resolved by SDS-PAGE and subjected to immunoblot analysis.

For immunoblotting, nitrocellulose membranes were incubated for 2 h in TBST containing 5% BSA. To detect phosphorylated amino acids, the membrane was incubated with anti-phosphoserine (Invitrogen, 1:2000 rabbit polyclonal), anti-phosphothreonine (Invitrogen, 1: 2000 rabbit polyclonal), or anti-phosphotyrosine (Invitrogen, 1:2000 mouse monoclonal) antibody at 4 °C overnight. The membranes were then incubated with secondary antibodies described above for 1 h at

room temperature, followed by signal detection and X-ray film exposure.

Immunofluorescence microscopy

Cells were grown on 8-well chamber slides (Millipore) and fixed with cold methanol for 20 min or stored at –20 °C overnight. The methanol fixed slides were washed 3 times in PBS at 5 min each to rehydrate the cells. The cells were incubated with PBS containing 0.1% of Triton X-100 for 30 min at room temperature, followed by blocking non-specific binding sites using 2% BSA in PBS for 30 min at room temperature. Slides were incubated with anti-Nek2 antibody (Abcam, 1:200 mouse monoclonal) at 4 °C overnight, followed by secondary antibody incubation using Alexa Fluor 568 goat anti-mouse antibody (Invitrogen, 1:400) for 1 h at room temperature. A second round of immunostaining was performed with anti-TRF1 antibody (Abcam, 1:200 rabbit polyclonal) and Alexa Fluor 488 goat anti-rabbit antibodies following the same protocol as the first round immunostaining. The slides were stored at 4 °C until visualization and viewed using an Olympus IX70 inverted deconvolving epifluorescence microscope under the 60× oil objective lens. SimplePCI software (Compix) was used for image capture and analysis.

Fluorescence-activated cell sorter (FACS) analysis

Cell cycle-synchronized cells were washed in cold PBS containing 1% calf serum. Cells were resuspended in 200 μl PBS, and then 800 μl of absolute ethanol was added in a slow dropwise fashion while vortexing to avoid cell clumping. Fixed cells were stored at –20°C until analysis. DNA was stained with 300 μl of PI staining solution containing 50 μg/ml of propidium iodide, 10 μg/ml of RNase A, and 1% of Triton X-100 for 30 min at 37 °C. DNA from 10 000 cells was evaluated with a FACSAria III flow cytometer (Becton Dickinson), and cell cycle phases were analyzed using Flowjo V10 software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author's Contributions

JL, the first author of this paper, conceived the study, organized it, performed experiments, analyzed and interpreted the data, and wrote the drafts of the manuscript. LG supported the research, contributed to analysis and interpretation and reviewed the manuscript. All authors read and approved the final version of manuscript.

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