

A specific interaction between the telomeric protein Pin2/TRF1 and the mitotic spindle

Masafumi Nakamura*, Xiao Zhen Zhou*, Shuji Kishi*, Isao Kosugi†, Yoshihiro Tsutsui† and Kun Ping Lu*

Pin2/TRF1 was independently identified as a telomeric DNA binding protein (TRF1) [1] and as a protein (Pin2) that can bind the mitotic kinase NIMA and suppress its ability to induce mitotic catastrophe [2, 3]. Pin2/TRF1 has been shown to bind telomeric DNA as a dimer [3–7] and to negatively regulate telomere length [8–11]. Interestingly, Pin2/TRF1 levels are regulated during the cell cycle, being increased in late G2 and mitosis and degraded as cells exit from mitosis [3]. Furthermore, overexpression of Pin2/TRF1 induces mitotic entry and then apoptosis [12]. This Pin2/TRF1 activity can be significantly potentiated by the microtubule-disrupting agent nocodazole [12] but is suppressed by phosphorylation of Pin2/TRF1 by ATM; this negative regulation is important for preventing apoptosis upon DNA damage [13]. These results suggest a role for Pin2/TRF1 in mitosis. However, nothing is known about how Pin2/TRF1 is involved in mitotic progression. Here, we describe a surprising physical interaction between Pin2/TRF1 and microtubules in a cell cycle-specific manner. Both expressed and endogenous Pin2/TRF1 proteins were localized to the mitotic spindle during mitosis. Furthermore, Pin2/TRF1 directly bound microtubules via its C-terminal domain. Moreover, Pin2/TRF1 also promoted microtubule polymerization in vitro. These results demonstrate for the first time a specific interaction between Pin2/TRF1 and microtubules in a mitosis-specific manner, and they suggest a new role for Pin2/TRF1 in modulating the function of microtubules during mitosis.

Addresses: *Cancer Biology Program, Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA. †Second Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan.

Correspondence: Kun Ping Lu
E-mail: klu@caregroup.harvard.edu

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Results and discussion

Pin2/TRF1 localizes to the mitotic spindle in cells

To investigate how Pin2/TRF1 affects the cell cycle, we examined whether Pin2/TRF1 is localized on some cell cycle-related structures besides telomeres. Pin2 is identical to TRF1 with the exception of a 20 amino acid internal deletion, and it is expressed at a rate 5- to 10-fold higher than TRF1 in various cells examined [3]; TRF1 and Pin2 are likely to be generated from the same gene *PIN2/TRF1* [14]. For clarity, we will here use Pin2 for the 20 amino acid-deleted isoform and TRF1 for the 20 amino acid-containing isoform, but we will refer to endogenous proteins as Pin2/TRF1. To examine the localization of Pin2/TRF1 during the cell cycle, we first transfected HeLa cells with either a GFP-Pin2 fusion construct or the control GFP vector and then visualized the localization of the GFP signal at 18–20 hr posttransfection before induction of apoptosis [12]. Although GFP was diffusely distributed to whole cells both in interphase and mitotic cells, GFP-Pin2 localized at telomeric nuclear speckles during interphase (Figure 1a,b; data not shown), as shown previously [1, 3, 13]. Surprisingly, in mitotic cells, GFP-Pin2 localized to the chromosome and also colocalized with the mitotic spindle, as revealed by immunostaining with anti-tubulin monoclonal antibody (mAb) (Figure 1b). Since neither GFP nor a GFP-Pin2^{1–316} mutant was localized at the same structure (Figure 1a,b,d), these results indicate that exogenously expressed Pin2 is specifically localized to the mitotic spindle in mitotic cells.

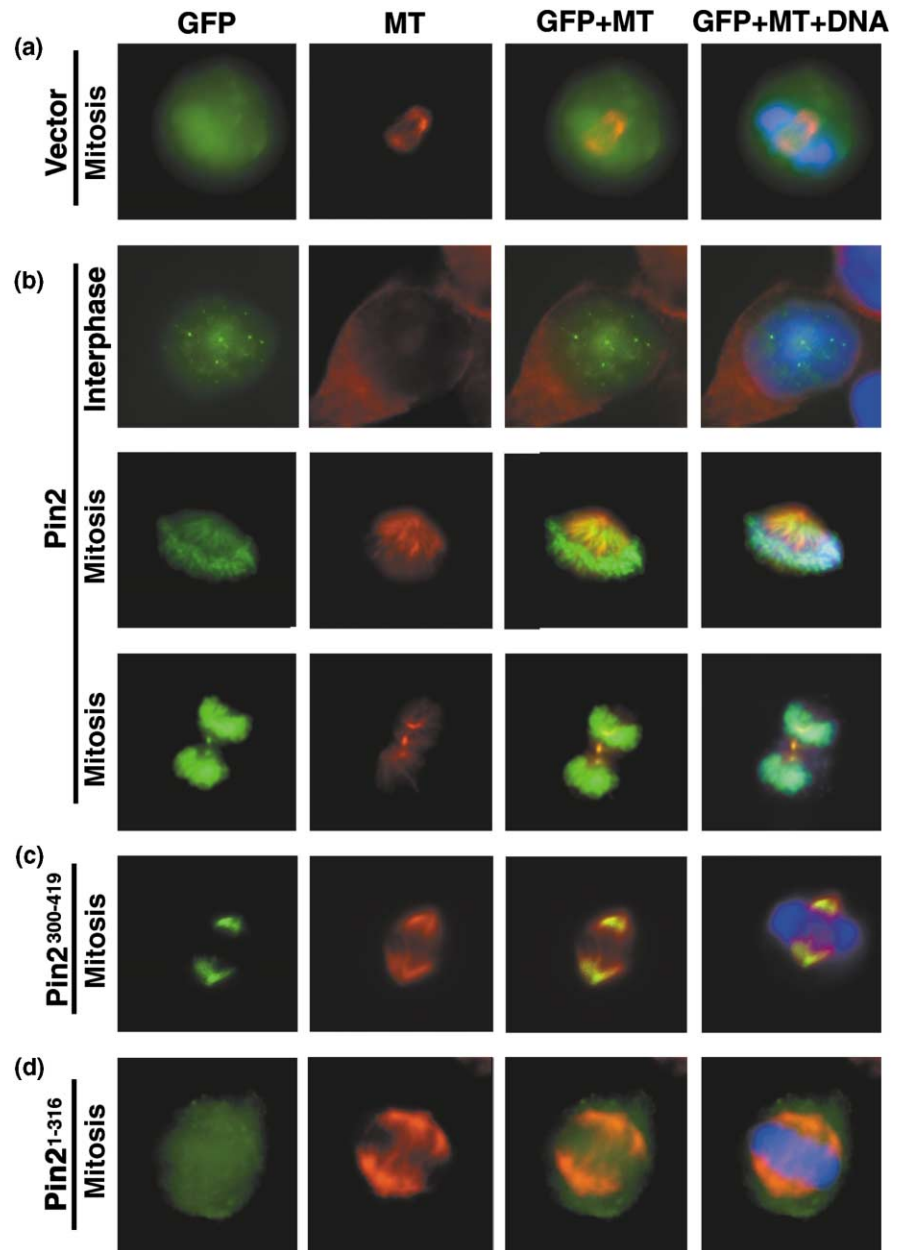
We next asked whether endogenous Pin2/TRF1 is localized to microtubules. Exponentially growing HeLa cells were fixed with methanol and then stained with anti-tubulin mAb and affinity-purified anti-Pin2/TRF1 antibodies [3, 15]. During interphase, at which time microtubules localized to the cytoplasm, Pin2/TRF1 was localized at nuclear telomeric speckles (Figure 2a), as shown previously [1, 3, 13]. However, in mitotic cells, Pin2/TRF1 again colocalized with microtubules to the mitotic spindle (Figure 2b). These data indicate that both exogenous and endogenous Pin2/TRF1 specifically localizes to the mitotic spindle during mitosis.

Pin2/TRF1 directly binds microtubules via its C-terminal domain

To determine whether cellular Pin2/TRF1 can bind microtubules, HeLa cell lysates were incubated with Taxol-stabilized microtubules, and the binding of cellular Pin2/TRF1 to microtubules was assayed by cosedimentation, as described [15]. Pin2/TRF1 was cosedimented in a mi-

Figure 1

GFP-Pin2 localizes to the mitotic spindle. (a) GFP, (b) GFP-Pin2, (c) GFP-Pin2³⁰⁰⁻⁴¹⁹, and (d) GFP-Pin2¹⁻³¹⁶ were separately transfected into HeLa cells for 18–20 hr (before inducing apoptosis), followed by staining microtubules and DNA with anti- α -tubulin mAb and DAPI, respectively. The colors refer to the following: green, GFP or GFP-Pin2; red, microtubules (MT); yellow, colocalization of the GFP and microtubule signals; and blue, DNA.



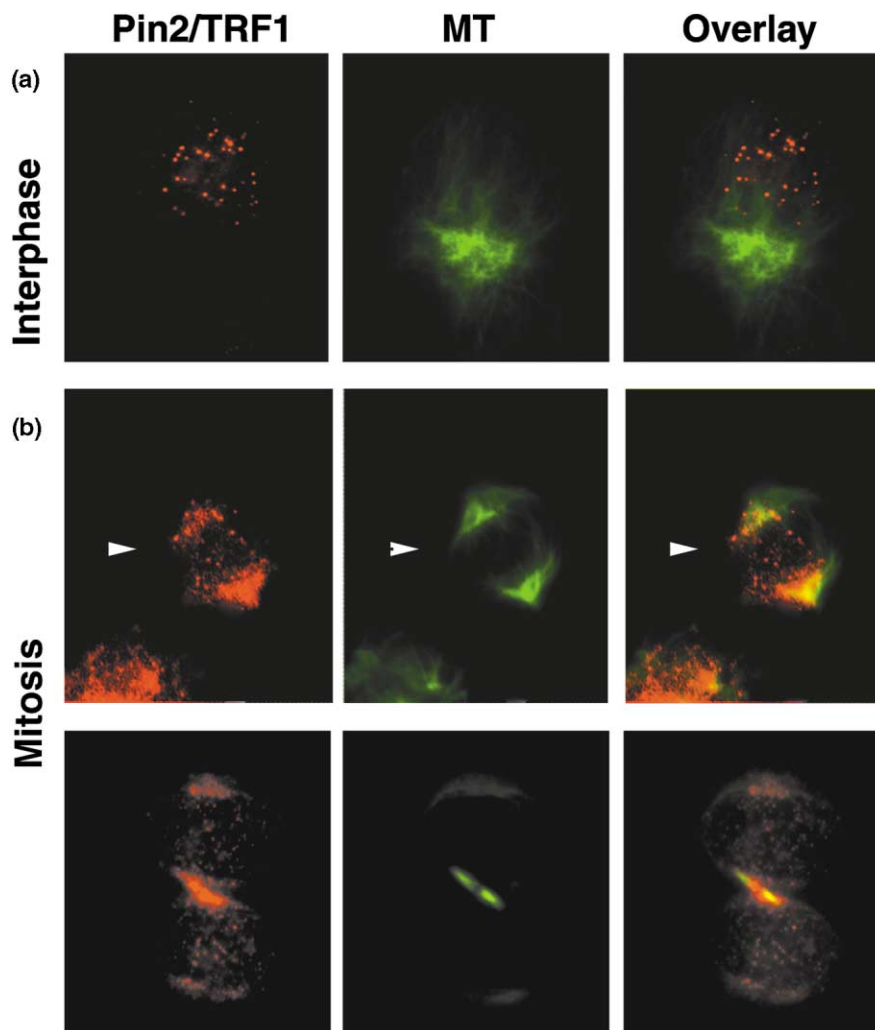
cro-tubule-dependent manner (Figure 3a), suggesting an interaction between cellular Pin2/TRF1 and microtubules. To further confirm this interaction, GST-Pin2 was incubated with Taxol-stabilized microtubules and subjected to cosedimentation. GST-Pin2, but neither GST nor GST-Pin2¹⁻³⁰⁰, cosedimented, and this was observed only in the presence of microtubules (Figure 3b). To examine whether TRF1 also binds to microtubules, we performed the microtubule cosedimentation experiments using extracts of HeLa cells transiently expressing HA-TRF1, HA-Pin2, and HA-Pin2¹⁻³¹⁶. When pellets were subjected to immunoblotting with anti-HA mAb, both HA-TRF1 and HA-Pin2 but not HA-Pin2¹⁻³¹⁶ were cosedi-

mented with microtubules (Figure 3c). In addition, we found that GFP-TRF1 was also localized at the mitotic spindle when expressed in HeLa cells (data not shown). These results indicate that both Pin2 and TRF1 proteins can directly bind to microtubules.

To identify the Pin2/TRF1 domain that is responsible for microtubule binding, we expressed Pin2, N-terminal Pin2¹⁻³¹⁶, and C-terminal Pin2³⁰⁰⁻⁴¹⁹ as GFP fusion proteins in HeLa cells, followed by cosedimentation with microtubules. Both Pin2 and Pin2³⁰⁰⁻⁴¹⁹, but neither GFP nor Pin2¹⁻³¹⁶, cosedimented with microtubules (Figure 3d), indicating that the microtubule binding domain is located

Figure 2

Endogenous Pin2/TRF1 localizes to the mitotic spindle. Exponentially growing HeLa cells were fixed with methanol and doubly stained with anti-Pin2/TRF1 antibodies and anti-tubulin mAb. Images from **(a)** interphase cells and **(b)** mitotic cells. The colors refer to the following: red, the Pin2/TRF1 signal; green, the microtubules signal; and yellow, colocalization of Pin2/TRF1 and microtubule signals.



at the C-terminal 119 amino acid fragment of Pin2. To further demonstrate that this domain can indeed bind microtubules *in vivo*, we expressed GFP-Pin2¹⁻³¹⁶ and GFP-Pin2³⁰⁰⁻⁴¹⁹ in HeLa cells and determined their localization during mitosis. Neither GFP-Pin2¹⁻³¹⁶ nor GFP-Pin2³⁰⁰⁻⁴¹⁹ localized to chromosomes (Figure 1c,d). This is as expected because Pin2¹⁻³¹⁶ does not contain the telomeric DNA binding domain, whereas Pin2³⁰⁰⁻⁴¹⁹ does not contain the dimerization domain that is required for Pin2 to bind DNA, as shown previously [3, 4]. Importantly, only Pin2³⁰⁰⁻⁴¹⁹ but not Pin2¹⁻³¹⁶ localized to the mitotic spindle (Figure 1c,d), consistent with our findings that only Pin2³⁰⁰⁻⁴¹⁹ but not Pin2¹⁻³¹⁶ bound microtubules *in vitro* (Figure 3d). These results show that Pin2/TRF1 binds microtubules directly via its C-terminal 119 amino acids.

Pin2/TRF1 promotes microtubule polymerization *in vitro*

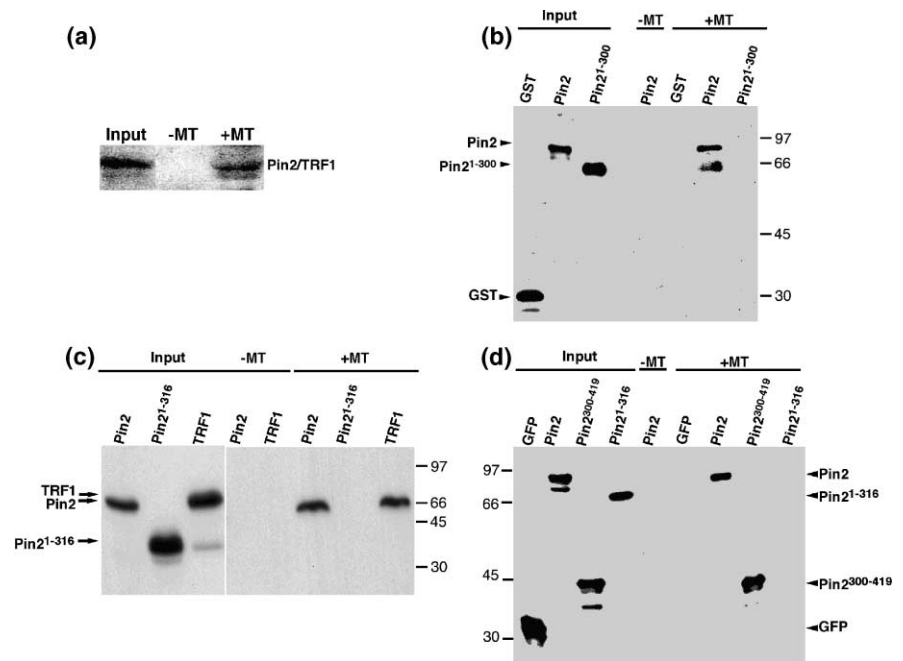
To investigate whether the interaction between Pin2/TRF1 and microtubules affects microtubule function, we

examined the effects of Pin2/TRF1 on microtubule polymerization *in vitro* by incubating Pin2 proteins with purified tubulin in a microtubule-stabilizing buffer and monitored microtubule polymerization, as described [15]. When GST-Pin2 was added at different concentrations, the rate of turbidity change was markedly increased in a concentration-dependent and time-dependent manner (Figure 4a). These results were microscopically confirmed by the formation of organized microtubule fibers (Figure 4b). In contrast, neither GST nor GST-Pin2¹⁻³⁰⁰ had any activity in promoting microtubule polymerization (Figure 4), which is consistent with their failure to bind microtubules *in vitro* or *in vivo* (Figure 1d and 3d). These results indicate that Pin2/TRF1 not only binds microtubules but also promotes microtubule assembly *in vitro*.

Our current results demonstrate the cell cycle-specific interaction between Pin2/TRF1 and the mitotic spindle, and they also provide further evidence for a role of Pin2/TRF1 in mitotic regulation. This is consistent with previ-

Figure 3

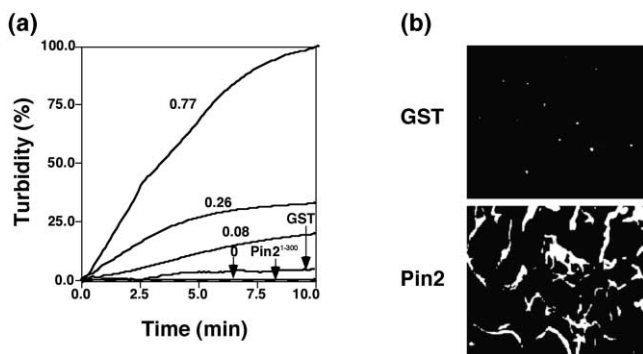
Pin2/TRF1 binds microtubules via its C-terminal domain. **(a)** Cosedimentation of cellular Pin2/TRF1 with microtubules. HeLa cell lysates were incubated with Taxol-stabilized microtubules (+MT) or control buffer (-MT), followed by centrifugation. Pellets were washed with microtubule-stabilizing buffer and boiled in SDS-sample buffer, followed by immunoblotting analysis with anti-Pin2/TRF1 antibodies. **(b)** Direct interaction of Pin2 and microtubules. Purified GST, GST-Pin2 (Pin2), and GST-Pin2¹⁻³⁰⁰ (Pin2¹⁻³⁰⁰) proteins were cosedimented with Taxol-stabilized microtubules (+MT) or control buffer (-MT), followed by immunoblotting analysis with anti-GST mAb. Note that a 66 kDa band in Pin2 lane is a degradation product. **(c)** Direct interaction of TRF1 and microtubules. Extracts of HeLa cells overexpressing HA-Pin2, HA-Pin2¹⁻³¹⁶, or HA-TRF1 were subjected to microtubule-sedimentation experiments. Sediments were analyzed by immunoblotting using anti-HA mAb. These results indicate that both Pin2 and TRF1 can bind microtubules. **(d)** The C terminus of Pin2/TRF1 binds microtubules. GFP-Pin2, -Pin2¹⁻³¹⁶, -Pin2³⁰⁰⁻⁴¹⁹, and control GFP vector were transfected into HeLa cells. Cell lysates were subjected to cosedimentation with Taxol-stabilized microtubules (+MT) or control buffer (-MT), followed by immunoblotting with anti-GFP mAb.



ous studies linking telomeres to mitotic progression in other model systems. For example, the mutation or deletion of the telomeric DNA sequence triggers mitotic entry and apoptosis in *Drosophila* [16] or causes a severe delay

or block in anaphase in *Tetrahymena* [17]. Further support for a role of Pin2/TRF1 in mitotic progression comes from our studies of the cell cycle-specific regulation of Pin2/TRF1 function [3, 12, 13]. Pin2/TRF1 levels are strikingly increased at the G2/M transition, followed by a decrease as cells exit from mitosis [3]. Furthermore, overexpression of Pin2/TRF1 induces mitotic entry, followed by apoptosis in cells containing short telomeres but not in cells containing long telomeres due to sequestration of Pin2/TRF1 by long telomeres [12]. Moreover, a Pin2/TRF1 point mutant that fails to bind telomeres still potently induces mitotic entry and apoptosis, indicating that the ability of Pin2/TRF1 to affect mitosis is dependent on its cellular concentration but not on telomere binding [12]. Significantly, the ability of Pin2/TRF1 to induce mitotic entry and apoptosis is potentiated by nocodazole [12] but is suppressed by ATM via phosphorylation [13]. These results together indicate that Pin2/TRF1 is specifically involved in mitotic regulation. At the present time, we can only speculate how Pin2/TRF1 affects mitotic regulation. One possibility is that upon entry into mitosis, at which time the breakdown of the nuclear envelope and the increase in Pin2/TRF1 levels normally occur, Pin2/TRF1 binds to microtubules. This interaction targets Pin2/TRF1 to the mitotic spindle, where it may affect the function of microtubules or other proteins on the

Figure 4



Pin2/TRF1 promotes microtubule polymerization in vitro. **(a)** Various concentrations of GST-Pin2 as indicated (μM) or $0.77 \mu\text{M}$ of GST-Pin2¹⁻³⁰⁰ or GST proteins were separately incubated with purified tubulin. The resulting microtubule assembly was measured by changes in the turbidity. The turbidity in the presence of $0.77 \mu\text{M}$ Pin2 at 10 min is defined as 100%. **(b)** The reaction mixtures were transferred onto coverslips and stained by anti-tubulin mAb.

mitotic spindle. This is consistent with the findings that Pin2/TRF1 promotes microtubule assembly in vitro (Figure 4) and with our preliminary results showing that expression of a dominant-negative Pin2/TRF1 mutant in ATM-negative cells restored their cell cycle checkpoint defect in response to the microtubule-affecting drug nocodazole. Further experiments are needed to determine how Pin2/TRF1 is involved in the regulation of microtubule function during mitosis.

In summary, we demonstrate that Pin2/TRF1 binds and localizes to the mitotic spindle in addition to telomeres. Furthermore, Pin2/TRF1 directly binds microtubules via its C-terminal domain. Finally, Pin2/TRF1 promotes microtubule polymerization in vitro. These results demonstrate for the first time that Pin2/TRF1 localizes to a cellular structure other than telomeres and suggest that Pin2/TRF1 may have a novel role in modulating microtubule function in mitosis.

Materials and methods

DNA construction

Various GFP-Pin2 constructs were described previously [12]. To construct GFP-Pin2⁹⁰⁰⁻⁴¹⁹, GFP-Pin2 plasmid was digested by EcoRI and XmnI, followed by self-ligation. Various GST fusion proteins were expressed in bacteria and purified, as described [18].

Immunostaining

Immunostaining was performed, as described previously [15]. In brief, HeLa cells were transfected by pEGFP or various pEGFP-Pin2 constructs and were fixed with methanol, followed by staining with the anti- α -tubulin mAb (Amersham) or affinity-purified anti-Pin2/TRF1 antibodies [3]. Although fixation with glutaraldehyde has been used to detect telomere localization of Pin2/TRF1, as shown previously [1, 3, 12], here we fixed the cells with methanol, because this fixation is an optimal and standard procedure for visualizing microtubules and detecting localization of proteins on microtubules, as shown previously [15, 19].

Microtubule binding and polymerization assays

Microtubule assembly assay was performed, as described [15, 20]. In brief, 0.77 μ M various recombinant proteins were incubated with 13 μ M of purified bovine tubulin (Cytoskeleton) in 20 μ l of microtubule-stabilizing buffer containing 1 mM GTP, 1 mM MgCl₂, and 10% glycerol at 30°C, followed by measuring changes in turbidity every 6 s. For microtubule binding assay, whole cell lysates were prepared from HeLa cells or A-T cells using the lysis buffer containing 300 mM NaCl and 0.5% Triton X-100. These whole-cell extracts or recombinant proteins were incubated with Taxol-stabilized microtubules, followed by centrifugation at 35,000 rpm for 20 min. Pellets were washed by microtubule-stabilizing buffer and boiled in SDS-sample buffer, followed by immunoblotting analysis with anti-Pin2/TRF1 or -GST antibodies (Santa Cruz), as described [15, 20].

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