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The F-box Protein FBX4 Targets PIN2/TRF1 for Ubiquitin-mediated Degradation and Regulates Telomere Maintenance*

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Pin2/TRF1 was identified previously as both a protein (TRF1) that binds to telomeric DNA repeats and as a protein (Pin2) that associates with the kinase NIMA and suppresses its mitosis inducing activity. Pin2/TRF1 negatively regulates telomere length and also plays a critical role in cell cycle checkpoint control. Pin2/TRF1 is down-regulated in many human cancers and may be degraded by the ubiquitin-proteasome pathway, but components of the pathway involved in Pin2/TRF1 turnover have not been elucidated. By using the two-hybrid system, we recently identified Pin2/TRF1-interacting proteins, PinX1–4, and we demonstrated that PinX1 is a conserved telomerase inhibitor and a putative tumor suppressor. Here we report the characterization of PinX3. PinX3 was later found to be identical to FBX4, a member of the F-box family of proteins, which function as substrate-specific adaptors of Cul1-based ubiquitin ligases. FBX4 interacts with both Pin2 and TRF1 isoforms and promotes their ubiquitination in vitro and in vivo. Moreover, overexpression of FBX4 reduces endogenous Pin2/TRF1 protein levels and causes progressive telomere elongation in human cells. In contrast, inhibition of FBX4 by RNA interference stabilizes Pin2/TRF1 and promotes telomere shortening, thereby impairing cell growth. These results demonstrate that FBX4 is a central regulator of Pin2/TRF1 protein abundance and that alterations in the stability of Pin2/TRF1 can have a dramatic impact on telomere length. Thus, FBX4 may play a critical role in telomere maintenance.

Telomeres are DNA-protein structures that cap chromosome ends, and their deregulation has been implicated in cancer and aging. Telomeres are maintained by a network of telomeric factors, including telomerase, TRF1/Pin2, TRF2, tankyrases, Tin2, Rap1, Pin1, Pot1, and Ptop/Pip1/Tint1 (1–9). TRF1 and its interacting proteins, such as tankyrases and Tin2, have been shown to control telomere length (10–12). For example, overexpression of TRF1 results in a gradual telomere shortening, whereas overexpression of dominant-negative mutants leads to telomere elongation in human cells (10, 13–15). These results indicate that TRF1 negatively regulates telomere elongation and also suggests that Pin2/TRF1 function is tightly regulated. Consistent with this idea, the ability of TRF1 to bind telomeres has been shown to be modulated by tankyrases (11, 16). Furthermore, TRF1 has been shown to be degraded via the ubiquitin-proteasome pathway (17). However, the mechanisms controlling TRF1 degradation and the significance of this pathway for telomere maintenance are unknown.

Previously, we isolated a telomere-binding protein Pin2 as a protein that interacts with the mitotic protein kinase NIMA (18, 19). Pin2 is identical to TRF1, with the exception of a 20-amino acid internal deletion, but is expressed at 5–10-fold higher levels than TRF1 in various cells examined (20). Pin2 and TRF1 appear to be generated by alternative splicing, as they are likely generated from the same gene (PIN2/TRF1) (21). For clarity, we shall refer to the long isoform as TRF1 and the short isoform as Pin2 (18, 22), but we will refer to endogenous proteins as Pin2/TRF1 because of the difficulty in separating them physically or functionally, as described previously (23–25).

In addition to the central role of Pin2/TRF1 in telomere maintenance, we have also demonstrated a role for Pin2/TRF1 in mitotic regulation (18). Pin2/TRF1 protein levels are tightly regulated during the cell cycle, and their overexpression induces mitotic entry and apoptosis (20, 24). Furthermore, this apoptosis promoting activity is enhanced by disruption of the mitotic spindle checkpoint but is suppressed by ATM through phosphorylation of Pin2/TRF1 (24). In fact, inhibition of endogenous Pin2/TRF1 function in ATM-negative cells is able to restore telomere shortening and mitotic checkpoint defects in response to DNA damage (26). Thus, ATM-mediated phosphorylation of Pin2/TRF1 appears to be critical for the ability of ATM to control telomere maintenance in response to DNA damage. Moreover, Pin2/TRF1 is found on the mitotic spindle during mitosis and contributes to the maintenance of the mitotic spindle checkpoint (25, 27). The finding that Pin2/TRF1-deficient mice die during early embryonic development without an obvious effect on telomere length (28) suggests that this lethality may primarily reflect its cell cycle functions.

As part of our efforts to elucidate further the function of Pin2/TRF1, we previously employed a yeast two-hybrid screen to identify new Pin2/TRF1-interacting proteins (23). From 10^8 clones screened, we identified six known genes, including the microtubule-binding protein EB1 involved in mitotic regulation, and four previously uncharacterized genes (PinX1–4) (5, 23, 29). PinX1 is a highly conserved protein that directly interacts with and potently inhibits telomerase activity in vitro and in vivo and also forces cancer cells into crisis. In contrast, depletion of the endogenous PinX1 protein significantly increases telomerase activity and tumorigenicity of human cancer cells (23). These results indicate that PinX1 is a potent telomerase inhibitor (23). PinX1 can also

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F-box Protein Essential for Pin2/TRF1 Degradation

bind to the telomerase RNA component, and its telomerase inhibitory activity is conserved in budding yeast (30).

In this study, we report the characterization of PinX3 was later found to be identical to Fbx4, a member of the F-box family of proteins (31). F-box proteins function as substrate-specific receptors for Cullin-based SCF ubiquitin ligases (31–36). Culli assembles with the RING finger protein Rbx1 via its C terminus and with a specificity module composed of Skp1 and an F-box protein through its N terminus. Rbx1 recruits E2 ubiquitin-conjugating enzymes, whereas F-box proteins recruit substrates via C-terminal protein interaction domains. Fbx4 interacts with both Pin2 and TRF1 isoforms in vitro and in vivo. Furthermore, Fbx4 promotes ubiquitination of both Pin2 and TRF1 isoforms in vitro and in vivo. Moreover, overexpression of Fbx4 promotes degradation of endogenous Pin2/TRF1 and results in progressive telomere elongation in human cells, whereas inhibition of Fbx4 by RNA interference (RNAi) stabilizes endogenous Pin2/TRF1 levels and leads to telomere shortening and impaired cell growth. These findings indicate that Fbx4 targets Pin2/TRF1 for ubiquitin-mediated degradation, and this post-translational regulation is critical for proper control of telomere length.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins and Production of Fbx4 Antibodies—GST-Pin2 proteins were produced and purified as described previously (20, 23). To generate GST-Fbx4 fusion proteins, cDNAs encoding full-length Fbx4 and its mutants were subcloned into a pGEX vector, and the resulting fusion proteins were expressed in Escherichia coli and purified by glutathione beads, as described previously (23, 37, 38). To raise antibodies against Fbx4, a recombinant GST-Fbx4 C-terminal 150-residue fragment was used to immunize rabbits as described (23). Anti-Fbx4 antibodies were purified by using immobilized GST-Fbx4 as described (23).

GST Pulldown, Immunoprecipitation, and Immunoblotting Analyses—These assays were performed as described previously (23, 37). Briefly, relevant proteins were expressed in HeLa or HT1080 cells by transient transfection or were translated in vitro. Analyses—These assays were performed as described previously (23, 37). Anti-Fbx4 antibodies were purified by using immobilized GST-Fbx4 as described (23).

GST Pulldown, Immunoprecipitation, and Immunoblotting Analyses—These assays were performed as described previously (23, 37). Briefly, relevant proteins were expressed in HeLa or HT1080 cells by transient transfection or were translated in vitro by using the coupled transcription/translation system (Promega) in the presence of [35S]Met, followed by lysis or dilution in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The cellular supernatants were incubated with 1 μM GST or GST fusion proteins for 1 h at 4 °C, and 15 μl of glutathione-agarose beads were then added, followed by further incubation for 2 h at 4 °C. The precipitated proteins were washed four times in the same buffer and subjected to immunoblotting analysis. In Figs. 1 and 2, the samples labeled input were typically 25–40% of the samples employed in binding experiments.

Ubiquitination Assay and Protein Degradation Assay—Ubiquitination and protein degradation assays were carried out as described previously (39, 40). Briefly, Pin2 or TRF1 was translated in vitro using the coupled transcription/translation system (Promega) in the presence of [35S]Met. [35S]-Pin2 protein (2.5 μl) was incubated with ubiquitin-activating enzyme (E1) (50 ng), Ubch5a (200 ng), ubiquitin (1 mg/ml), 1 μM ubiquitin aldehyde, 2.3 μl of in vitro translated Fbx4 or Fbx4AE, and 4 mM ATP in a total volume of 10 μl (60 min, 30 °C). To investigate the polyubiquitination of Pin2 in vivo, we co-transfected HA-Pin2 and His-tagged ubiquitin into HeLa cells, followed by MG-132 treatment to inhibit proteasome function. Cell lysates were collected and subjected to the pulldown analysis with nickel-agarose beads to isolate His-tagged ubiquitin conjugates using a urea-containing buffer to remove any Pin2-associating proteins, followed by immunoblotting analysis with anti-Pin2 antibodies. For protein degradation assays, cells were transfected stably or transiently with expression constructs, as indicated. Cycloheximide (100 μg/ml) was added to the media to block new protein synthesis. Cells were harvested at each time point, and total lysates were analyzed by immunoblotting analysis with anti-Pin2/TRF1 or -HA antibodies. The immunoblots were scanned and semi-quantitated by using the software NIH image 1.6.2, as described previously (39). The results of three independent experiments were plotted such that protein levels at the 0-h time point were defined as 100%.

Establishment of Stable HT1080 Cell Lines and Telomere Length Analysis—Stable HT1080 cell lines were established, and telomere length was determined as described previously (23). Briefly, an HA-Fbx4 cDNA was subcloned into an expression vector and then transfected into fibrosarcoma cell line HT1080, along with the empty vector as a control. Two different Fbx4 RNAi constructs (CCGATTGATGTAAGCTATAT and CAACATAAATTCAACATTCTA) and control viral vector pSM2C were purchased from Open Biosystem (41). For virus production, pSM2C plasmids were transfected into Phoenix packaging cells, and viral supernatants were harvested 48 h after transfection. After selection with antibiotics and limiting dilution, multiple independent single clones were isolated and checked for protein expression by immunoblotting analysis with anti-HA or anti-Fbx4 antibodies. We maintained the stable cell lines continuously in culture, splitting on every 4th day and seeding at the concentration of 6 × 105 cells per 10-cm culture dish. Multiple independent stable clones were established from each transfection, and they expressed the expected proteins and exhibited similar properties.

RESULTS

Fbx4 Interacts with Pin2 and TRF1 Isoforms in Vitro and in Vivo—In our original yeast two-hybrid screen for Pin2-interacting proteins (23), three positive clones contained PINX3 (Pin2-interacting protein X3 gene). The longest clone encodes a 387-amino acid polypeptide that was found to be identical to Fbx4. cDNAs encoding this F-box protein were independently identified by virtue of the presence of an F-box motif located near the N terminus of the protein (42, 43). Fbx4 has been shown recently to bind to αβ-crystallin, a nuclear speckle protein, and Fbx4 overexpression appears to increase ubiquitination of as yet unidentified cellular proteins (44–46), but to date, specific Fbx4 targets have not been identified.

To confirm the apparent interaction between Fbx4 and Pin2, we performed co-immunoprecipitation experiments. HeLa cells were co-transfected with HA-Pin2 and Xpress-Fbx4 expression constructs, and cell extracts were then subjected to immunoprecipitation with anti-HA 12CA5 antibody or nonspecific anti-GFP monoclonal antibody, followed by immunoblotting analysis with an anti-Xpress monoclonal antibody. Fbx4 was detected in anti-Pin2 immunoprecipitates but not in control immunoprecipitates (Fig. 1A). Co-immunoprecipitation of Fbx4 and Pin2 was not because of cross-reactivity of Fbx4 with the 12CA5 antibody, because Fbx4 was not present in immunoprecipitation reactions performed with this antibody on extracts that lacked HA-Pin2 or in immunoprecipitation reactions using anti-GFP control antibodies (Fig. 1A). To confirm these results, HeLa cells were transfected with HA-Fbx4 and then subjected to immunoprecipitation with anti-HA or anti-GFP control antibodies, followed by immunoblotting analysis with anti-Pin2/TRF1 antibodies that recognized both Pin2 and TRF1 isoforms at approximately a 10:1 ratio, as shown previously (20). More importantly, both endogenous Pin2 and TRF1 isoforms were
immunoprecipitated by anti-HA antibodies (Fig. 1B). These results indicate that expressed Fbx4 forms stable complexes with endogenous Pin2/TRF1 in cells.

To confirm further the interactions between endogenous Pin2/TRF1 and Fbx4 in cells, we raised polyclonal antibodies against recombinant glutathione S-transferase (GST)-Fbx4-C-terminal fragment fusion protein. Affinity-purified immune sera, but not pre-immune control antibodies, recognized an endogenous Fbx4 as well as ectopically expressed HA protein with the expected molecular weight in HeLa and various other cells (Fig. 1C, data not shown), indicating that these antibodies are specific. By using these anti-Fbx4 antibodies, we found that endogenous Fbx4 was immunoprecipitated with anti-Pin2/TRF1 antibodies but not the preimmune controls (Fig. 1C). These results indicate that endogenous Fbx4 and Pin2/TRF1 interact in mammalian cells.

To examine the in vitro interaction between Fbx4 and Pin2 or TRF1, we used the GST pulldown approach, as described previously (23). 35S-Labeled Fbx4, -Pin2, or -TRF1 was synthesized by using an in vitro transcription and translation system (TNT) and tested for their ability to bind to GST-Pin2 or GST-Fbx4 that was immobilized on glutathione-agarose beads. GST-Fbx4, but not GST, bound to in vitro synthesized 35S-Pin2 or -TRF1 (Fig. 1D). Conversely, Fbx4 was retained by GST-Pin2 but not by GST (Fig. 1E). These results indicate that Fbx4 interacts with both Pin2 and TRF1 isoforms in vitro.

To map the domains in Pin2 and Fbx4 that are important for their interaction, a series of Fbx4 (Fig. 2A) and Pin2 (Fig. 2C) mutants were generated and used in the in vitro binding assay. GST-Pin2 bound to Fbx4 fragments, including amino acid residues 172–306, but did not associate with fragments containing amino acid residues 1–117, 118–171, or 307–387 (Fig. 2B). Furthermore, a deletion of the F-box from Fbx4 (Fbx4ΔF) did not affect its binding to Pin2 (Fig. 2B). These results indicate that Pin2 binds C-terminal to the F-box motif. All targets of F-box proteins identified to date interact with sequences located C-terminal to the F-box motif. Upon examination of the Fbx4-binding domain in Pin2, we found that GST-Pin2 fragments encompassing residues 1–419, 48–155, or 1–155 bound to Fbx4 (Fig. 2D). In contrast, GST-Pin2 fragments encompassing residues 1–47, 156–316, 317–419, or 269–316 all failed to bind to Fbx4 (Fig. 2D). Most interestingly, based on the TRF1 crystal structure 1H60 (47), the amino acid residues from 48 to 155 correspond to the first three α-helices in the dimerization domain and are present in both Pin2...
and TRF1. These results indicate that the first three α-helices in the dimerization domain of Pin2 and TRF1 are required for association with Fbx4.

**Fbx4 and UbcH5a Promote Ubiquitination of Both Pin2 and TRF1 in Vitro and in Vivo**—The results presented thus far indicated that Fbx4 interacts with Pin2 and TRF1 in vitro and in vivo. Given that many F-box proteins can target certain regulatory proteins for degradation by the ubiquitin-proteasome pathway (31–36) and that TRF1 is regulated by proteolysis (17), we hypothesized that Fbx4 might target Pin2 and TRF1 for SCF-mediated ubiquitination and thereby promote its turnover by the proteasome. To examine this possibility, we first examined whether Fbx4 promotes ubiquitination of Pin2 and TRF1 in vitro. To this end, we employed a reticulocyte extract system wherein in vitro translated F-box proteins assemble into SCF complexes composed of neddylated Cul1, Rbx1, and Skp1, which pre-exist in reticulocyte extracts (40, 48). In vitro translated and 35S-labeled Pin2 or TRF1 was incubated with ATP, ubiquitin, E1 in the absence or presence of Fbx4 (produced in reticulocyte extracts), and/or the ubiquitin-conjugating enzyme (E2) UbcH5a. 35S-Labeled Pin2 and TRF1 were polyubiquitinated only when Fbx4 and UbcH5a were present (Fig. 3A). In contrast, an F-box deletion mutant Fbx4ΔF failed to promote ubiquitination of Pin2 or TRF1 in this assay (Fig. 3A). To examine whether other E2 enzymes could catalyze the ubiquitination of Pin2 and TRF1 in vitro, we incubated 35S-labeled Pin2 or TRF1 with the in vitro ubiquitination system supplemented with various E2 proteins, as described (49). Out of eight E2 enzymes examined, only UbcH5a effectively catalyzed polyubiquitination of Pin2 (Fig. 3C) or TRF1 (Fig. 3D). These results indicate that Fbx4 and UbcH5a promote ubiquitination of both Pin2 and TRF1 isoforms in vitro.

To examine whether Fbx4 promotes ubiquitination of Pin2 in cells, we first examined the effects of Fbx4 on ubiquitination of Pin2 by cotransfecting Pin2, His-tagged ubiquitin, and Fbx4 or its F-box deletion mutant into HeLa cells, followed by addition of MG-132 to block proteasome activity. Cells were collected and lysed in urea-containing buffer, and ubiquitin conjugates were purified using nickel-agarose beads. Ubiquitinated proteins were separated by SDS-PAGE and then blotted for Pin2 (39). In the presence of MG-132, His ubiquitin, and Fbx4, Pin2 was substantially polyubiquitinated (Fig. 3B). However, no ubiquitinated Pin2 was detected in the presence of Fbx4 in the absence of His ubiquitin or Fbx4 (Fig. 3B), indicating the specificity of the in vivo ubiquitination assay. Thus, Fbx4 promotes ubiquitination of Pin2 in cells, and this function requires the F-box motif.

To examine whether Fbx4 and UbcH5a promote turnover of Pin2 and TRF1 in vivo, we co-transfected Pin2 or TRF1 with Fbx4 and UbcH5a or its dominant-negative mutant (UbcH5aDN) (50) and then monitored Pin2 or TRF1 protein stability after the cycloheximide treatment to stop new protein synthesis, as described previously (39, 40, 51). Co-transfection of Fbx4 and UbcH5a significantly reduced the half-lives of Pin2 and TRF1, which were stabilized by the proteasome inhibitor MG-132 (Fig. 3, E and F). Furthermore, UbcH5aDN stabilized Pin2 protein (Fig. 3, E and F). In contrast, the stability of an unrelated protein was not affected under either condition (data not shown). These results indicate that Fbx4 and UbcH5a not only promote ubiquitination of Pin2 and TRF1, but also reduce their half-lives in cells.
FIGURE 3. Fbx4 and UbcH5a promote ubiquitination of Pin2 and TRF1 isoforms in vitro and in vivo. A, Fbx4 promotes ubiquitination of Pin2 and TRF1 in vitro. 35S-Labeled Pin2 or TRF1 was incubated with E1, ATP, and ubiquitin in the presence or absence of Fbx4 (or its F-box deletion mutant) produced in rabbit reticulocyte extracts and/or bacterial UbcH5a. Reaction mixtures were separated by SDS-PAGE, followed by autoradiography. B, Fbx4 promotes ubiquitination of Pin2 in vivo. HeLa cells were transfected with HA-Pin2, Fbx4, its F-box deletion mutant, and/or His-tagged ubiquitin or vector control, followed by lysing cells in a buffer containing 6 M urea. Ubiquitin-conjugated proteins were captured with nickel-agarose beads and subjected to immunoblotting analysis with anti-HA antibody. C and D, UbcH5a promotes ubiquitination of Pin2 and TRF1 in vitro. 35S-Pin2 or -TRF1 was synthesized by TNT and subjected to ubiquitination assay in vitro using different E2 enzymes as indicated. E and F, Fbx4 and UbcH5a modulate Pin2 or TRF1 protein stability in cells. HeLa cells were co-transfected with HA-Pin2 or HA-TRF1, HA-Fbx4, and UbcH5a or its dominant-negative mutant and then treated with cycloheximide (100 μg/ml) for the indicated times, followed by immunoblot analysis with anti-HA or tubulin antibody (E). Pin2 or TRF1 levels were semi-quantitated using tubulin as a loading control and the relative levels at time 0 defined as 100% (F).
Stable Overexpression of Fbx4 Reduces Endogenous Pin2/TRF1 Protein Levels and Leads to Progressive Telomere Elongation in Human Cells—Given that overexpression of Fbx4 reduces the half-lives of Pin2 and TRF1, an important question is whether Fbx4 affects their function in cells. One of the most well accepted functions of Pin2/TRF1 proteins is their ability to regulate telomere length in the telomerase-positive fibrosarcoma cell line HT1080 (10, 13–15). To examine whether Fbx4 affects this function of endogenous Pin2/TRF1, we transfected Fbx4 or control vector into HT1080 cells and established three independent stably expressing clones in order to rule out the effects of clonal variation (Fig. 4A and data not shown). Although endogenous Pin2/TRF1 proteins were readily detectable in total lysates prepared from vector control cells with the expected 10:1 ratio, as shown previously (20), they were expressed at much lower levels, approaching the limits of detection, in all Fbx4-expressing clones examined (Fig. 4A and data not shown). These results further support the finding that overexpression of Fbx4 leads to reduction of endogenous Pin2/TRF1 expression in tissue culture cells.

Reduction of Pin2/TRF1 would be expected to affect telomere length. To examine whether overexpression of Fbx4 affects telomere maintenance, we measured TRF length in stable HT1080 cells lines at various population doublings (PD). As shown previously (10, 12, 23), HT1080 cells stably transfected with the control vector maintained an average TRF length of ~4 kb. More importantly, this TRF length was similar to the TRF length (4.3 kb) in HT1080 cells before transfection and did not change significantly over 72 PD (Fig. 4, B and C), as described previously (23). More importantly, a similar TRF length was also detected in at least three HT1080 cell lines stably expressing Fbx4 at early PDs (= 8 PD, actually 24–30 PD due to clonal expansion), but TRF length progressively increased at late PDs (≥24 PD), approaching 9 kb after 40 PDs (Fig. 4, B and C, and data not shown). Elongation of telomeric sequence was also evident from the increase in the TTAGGG hybridization signal (Fig. 4B). Given that multiple independent Fbx4-expressing clones started with similar TRF lengths at 0 PD and then underwent progressive telomere elongation, it is unlikely that the effects of Fbx4 overexpression reflect clonal variation, as shown under some conditions (52). Most interestingly, these longer telomeres were not lengthened further, but appeared to be stabilized with a maximal length of 9 kb, suggesting that feedback mechanisms limiting overall telomere length might be operating, as shown previously in HT1080 cells (10, 23) and in budding yeast (53–55). These results indicate that overexpression of Fbx4 reduces endogenous Pin2/TRF1 levels and causes progressive elongation of telomeres. These results are consistent with previous experiments showing that inhibition of Pin2/TRF1 function by overexpression of a dominant-negative mutant results in progressive telomere elongation (10).

Inhibition of Fbx4 through RNA Interference Increases Endogenous Pin2/TRF1 Protein Stability in Human Cells—The results presented thus far demonstrate that overexpression of Fbx4 promotes ubiquitin-mediated proteolysis of both endogenously and exogenously expressed Pin2/TRF1, leading to telomere elongation. The central question is whether endogenous Fbx4 is important for regulating Pin2/TRF1 protein stability and telomere maintenance. To address this issue, we inhibited endogenous Fbx4 using two different RNA interference (RNAi)

![Image of Figure 4](http://www.jbc.org/)

**FIGURE 4.** Overexpression of Fbx4 reduces endogenous Pin2/TRF1 levels and leads to gradual and progressive telomere elongation in human cells. A, overexpression of Fbx4 reduces endogenous Pin2/TRF1 levels. HT1080 cell lines were transfected with the control vector or HA-Fbx4 expression construct, and multiple independent stable cell lines were selected, followed by immunoblot analysis with anti-HA or anti-tubulin antibody, or with anit-Pin2/TRF1 antibodies that can recognize both Pin2 and TRF1 isoforms. B and C, overexpression of Fbx4 leads to telomere elongation. The stable cell lines were maintained continuously in culture, splitting every 4th day and seeding at the same number of cells at each subculture. Cells were harvested at population doublings (PDs) as indicated, and genomic DNA was isolated and digested with HinfI and RsaI, followed by Southern blot analysis using a TTAGGG oligonucleotide as a probe. Prior to hybridization, the gels were stained with ethidium bromide to ensure equal loading of genomic DNA, with a segment of the gels being shown in the lower panels (B). Average TRF length versus PD number from at least two independent measurements from the same clones was quantified using ImageQuant and presented in C.
F-box Protein Essential for Pin2/TRF1 Degradation

FIGURE 5. Depletion of Fbx4 increases the stability of endogenous Pin2/TRF1 proteins even in the presence of tankyrase overexpression. A, inhibition of Fbx4 increases Pin2/TRF1 levels. HT1080 cell lines were infected with viruses expressing two different Fbx4 RNAi constructs (Fbx4-RNAi-1 and -2) or the control vector and multiple independent stable lines selected, followed by reverse transcription-PCR for measuring Fbx4 mRNA, and immunoblotting analysis with anti-Fbx4, anti-Pin2/TRF1, or tubulin antibodies. B and C, depletion of Fbx4 reduces Pin2/TRF1 turnover. Stable clones were treated with cycloheximide for the indicated times, followed by immunoblotting analysis with anti-Pin2/TRF1 or anti-tubulin antibody (B) and semi-quantification (C). D and E, requirement of Fbx4 for tankyrase 1 to promote Pin2/TRF1 turnover. Stable clones were transfected with tankyrase 1 and then treated with cycloheximide, followed by immunoblotting analysis with anti-Pin2/TRF1 or anti-tubulin antibody (D) and semi-quantification (E).

constructs in a retroviral vector pSM2c, as described under "Experimental Procedures." HT1080 cells were infected with two different Fbx4 RNAi viral constructs or with a control vector, and multiple independent stable clones were selected for each construct. When compared with cells harboring the control vector, two different Fbx4 RNAi constructs reduced Fbx4 expression in multiple independent clones, as measured by reverse transcription-PCR (Fig. 5A, and data not shown). Furthermore, as indicated by immunoblotting analysis with anti-Fbx4 antibodies, Fbx4 protein levels were also significantly reduced in multiple HT1080 cell lines stably expressing Fbx4 RNAi down to 4–25% of endogenous levels, although the extent of the Fbx4 reduction was variable among individual clones (Fig. 5A). More importantly, the steady-state levels of endogenous Pin2/TRF1 were significantly elevated in multiple cell lines where Fbx4 was inhibited (Fig. 5A, and data not shown). These results suggest that inhibition of Fbx4 enhances the stability of Pin2/TRF1 in HT1080 cells.

To examine directly whether depletion of Fbx4 stabilizes Pin2/TRF1, we monitored Pin2/TRF1 protein abundance in these cells after cyclo-
heximide treatment, as described above. As shown in Fig. 5, B and C, the half-life of Pin2/TRF1 proteins in Fbx4-depleted cells was significantly extended, when compared with vector control cells. More importantly, turnover of Pin2/TRF1 in vector control cells was blocked by the proteasome inhibitor MG132 (Fig. 5, B and C), confirming that Pin2/TRF1 degradation is mediated by the proteasome. We also note that the half-life of endogenous Pin2/TRF1 was similar to that of overexpressed protein when Fbx4 was co-expressed (Fig. 5, B and C, versus Fig. 3, E and F), further supporting the notion that Fbx4 is a critical factor in determining the stability of Pin2/TRF1. These results indicate that inhibition of endogenous Fbx4 enhances the stability of endogenous Pin2/TRF1 in HT1080 cells.

Tankyrase 1 has been shown to promote ubiquitin-mediated turnover of Pin2/TRF1 (17). If Fbx4 is required for ubiquitin-mediated Pin2/TRF1 turnover, depletion of Fbx4 would be expected to suppress the ability of tankyrase 1 to increase Pin2/TRF1 turnover. To test this possibility, we examined the effects of tankyrase 1 overexpression on the degradation of endogenous Pin2/TRF1 proteins in Fbx4-depleted cells. As shown previously (17), overexpression of tankyrase 1 significantly increased turnover of endogenous Pin2/TRF1 in control cells (Fig. 5, D and E). However, this effect was almost completely blocked in Fbx4-depleted cells (Fig. 5, D and E). These results indicate that Fbx4 is essential for tankyrase 1 to enhance Pin2/TRF1 turnover and places Fbx4 downstream of tankyrase in the Pin2/TRF1 degradation pathway.

Inhibition of Fbx4 Causes Progressive Telomere Shortening and Impaired Cell Growth in Human Cells—Given that inhibition of Fbx4 results in stabilization of endogenous Pin2/TRF1 protein in cells, we next asked whether inhibition of Fbx4 affects telomere maintenance by measuring TRF length in HT1080 cells lines stably expressing Fbx4 shRNA at various PD. As shown previously (10, 12, 23), TRF length was relatively stable in cells expressing the control vector (Fig. 6, A and B). However, TRF length in Fbx4-inhibited cells was already shortened by ~1 kb even at 8 PD (actually 20–25 PD), the earliest passage when individual clones were expanded enough for telomere length analysis. More importantly, the TRF length continued to be progressively shortened in multiple independent stable lines examined (Fig. 6, A and B, and data not shown). The loss of telomeric sequences was evident not only from the shortening of TRF length but also from a reduction in the TTAGGG hybridization signal (Fig. 6, A and B, and data not shown). These results indicate that inhibition of Fbx4 not only increases Pin2/TRF1 protein stability but also leads to progressive telomere shortening. Consistent with this, Fbx4-inhibited stable cell lines initially grew at the same rates as those of control vector cells, but after 20–30 PD, their growth rates were significantly reduced (Fig. 6C).
lated with an increase in the percentage of cells in the G1 phase of the cell cycle (Fig. 6D). The slower cell growth was also expected based on our previous analyses of cells in which telomere elongation was inhibited by expression of PinX1 (23). Of note, stable overexpression of TRF1 has not been shown to have obvious effects on cell growth (10), which might be due to the findings that overexpressed TRF1 is still subject to Fbx4-mediated degradation (Fig. 3) and that telomeres in TRF1-overexpressing cells (10) apparently do not shorten as much as observed here in Fbx4-inhibited cells (Fig. 6). Taken together, our data indicate that the levels of Fbx4 can play a major role in determining the abundance of Pin2/TRF1. High levels of Pin2/TRF1 promote telomere shortening and a decrease in cell growth, whereas low levels of Pin2/TRF1 leads to progressively longer telomeres.

**DISCUSSION**

Pin2/TRF1 proteins have been shown to play important roles in telomere maintenance, cell division, and the response to DNA damage (1–6). Consistent with these functions, Pin2/TRF1 undergoes post-translational modifications in response to particular signaling pathways. Pin2/TRF1 is ribosylated by tankyrases (11, 16) and is phosphorylated by protein kinases such as ATM (26, 56). Furthermore, Pin2/TRF1 function may be also regulated at the level of protein abundance. Pin2/TRF1 levels fluctuate during the cell cycle, increasing at the G2/M transition followed by degradation as cells exit mitosis (20). In addition, overexpression of Pin2/TRF1 induces telomere shortening (10) and promotes mitotic entry and apoptosis in cells containing short telomeres (24). Pin2/TRF1 abundance is reduced in human cancer tissues, a situation that would be expected to promote proliferation due to ensuing telomere lengthening (24). Recently, it was reported that Pin2/TRF1 is degraded through the ubiquitin-proteasome pathway, a process that appears to be accelerated by tankyrase 1 (17). However, the mechanism by which Pin2/TRF1 is targeted for degradation and the importance of this pathway for telomere maintenance is unknown.

In this study, we report that PinX3, a gene identified in a two-hybrid screen for Pin2-interacting proteins (23), is identical to Fbx4, a mammalian F-box protein of unknown function. Fbx4 interacts with both Pin2 and TRF1 isoforms and promotes their ubiquitination in vitro and in vivo. Moreover, overexpression of Fbx4 promotes turnover of endogenous Pin2/TRF1 and also causes progressive telomere lengthening in human cells. In contrast, depletion of Fbx4 by RNA interference stabilizes endogenous Pin2/TRF1 and results in gradual telomere shortening, eventually impairing cell growth. These results demonstrate that Fbx4 targets Pin2/TRF1 for ubiquitin-mediated degradation and plays an essential role for regulating telomere maintenance. In addition, tankyrase 1-dependent turnover of Pin2/TRF1 requires Fbx4, indicating that Fbx4 is downstream of tankyrase 1 in the Pin2/TRF1 degradation pathway.

F-box proteins function as substrate-specific adaptors for the SCF ubiquitin ligase (31–36), but targets of Fbx4 have remained previously unknown. F-box proteins function as rate-limiting components in degradation pathways, although in many instances the association of targets with F-box proteins relies on an upstream signaling event such as phosphorylation or glycosylation. As with other F-box proteins, association of Fbx4 with Pin2/TRF1 involves sequences located C-terminal to the F-box motif. However, unlike most characterized F-box proteins, Fbx4 lacks previously identified protein interaction domains outside of the F-box. Further studies are required to understand the structural basis for the Pin2/TRF1–Fbx4 interaction.

Several approaches were employed to elucidate the functional consequences of the interaction between Fbx4 and Pin2/TRF1. First, we demonstrated that Fbx4 together with UbcH5a promotes ubiquitination of Pin2 and TRF1 in vitro and in transfected cells. The ability of overexpressed Fbx4 to promote Pin2/TRF1 degradation was blocked by a proteasome inhibitor, indicating that Fbx4-mediated ubiquitination promotes Pin2/TRF1 degradation via the proteasome. This activity requires the F-box, indicating that Fbx4 forms a functional SCF complex. In order to demonstrate a requirement for Fbx4 in degradation of endogenous Pin2/TRF1, we created cell lines in which Fbx4 was depleted by RNAi. Consistent with a role in Pin2/TRF1 turnover, cells depleted for Fbx4 displayed stabilized Pin2/TRF1. It is interesting to note that the extent of Pin2/TRF1 stabilization was rather similar in different Fbx4-depleted stable cell lines, despite the fact that the extent of depletion was variable in the different cell lines generated. These results indicate that partial reduction of Fbx4 levels may be sufficient to stabilize a significant fraction of endogenous Pin2/TRF1. This is consistent with previous findings that Pin2/TRF1 that is bound to telomeric DNA is immune to ubiquitin-mediated degradation (17). Therefore, Fbx4 may function primarily to control the pool of Pin2/TRF1 that is not associated with telomeres. Further studies are required to understand what controls access of Fbx4 with Pin2/TRF1.

Our results also demonstrate that manipulating Fbx4 levels in HT1080 cells not only alters the abundance of Pin2/TRF1 but also has a dramatic impact on telomere maintenance, indicating that Fbx4 activity is a central component of the machinery that regulates telomere homeostasis. Reduction of endogenous Pin2/TRF1 levels by ectopic expression of Fbx4 leads to progressive telomere elongation, whereas an increase in Pin2/TRF1 levels via depletion of Fbx4 leads to progressive telomere shortening and eventually impaired cell growth. These results are consistent with the fact that Pin2/TRF1 is a negative regulator of telomere elongation (10). Previous studies have demonstrated that overexpression of TRF1 is not particularly effective in promoting telomere shortening and does not alter cell proliferation (10). This is in contrast with our results wherein depletion of Fbx4 leads to accumulation of Pin2/TRF1 and a reduction in telomere length and reduced proliferation. These findings may reflect the fact that overexpressed TRF1 is still under the control of endogenous Fbx4, and its levels may not accumulate to the point where major effects on telomere length and cell division are apparent. Most interestingly, regulation of telomere length via the ubiquitin-mediated pathway may be common to all eukaryotes. In Schizosaccharomyces pombe, mutation of the F-box protein PoF3 also leads to shortened telomeres and defects in telomeric silencing (57). However, there is no obvious sequence identity between PoF3 and Fbx4 outside of the F-box motif, so it remains to be determined whether they function through analogous mechanisms to control telomere length. Although further studies are required to determine whether Fbx4-mediated turnover of Pin2/TRF1 is regulated during the cell cycle, DNA damage response, or oncogenesis, our work has identified Fbx4 as a critical new molecule that targets Pin2/TRF1 for ubiquitin-mediated degradation and plays an essential role in telomere maintenance.

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**F-box Protein Essential for Pin2/TRF1 Degradation**
The F-box Protein FBX4 Targets PIN2/TRF1 for Ubiquitin-mediated Degradation and Regulates Telomere Maintenance

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