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ORIGINAL ARTICLE

Parafibromin tumor suppressor enhances cell growth in the cells expressing SV40 large T antigen

T Iwata[1], N Mizusawa[1], Y Taketani[2], M Itakura[3] and K Yoshimoto[1]

[1]*Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan;* [2]*Department of Clinical Nutrition, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan and* [3]*Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Tokushima, Japan*

Parafibromin (PF) is a 531-amino acid protein encoded by *HRPT2*, a putative tumor suppressor gene recently implicated in the autosomal-dominant hyperparathyroidism-jaw tumor familial cancer syndrome and sporadic parathyroid carcinoma. To investigate effects of PF's overexpression on cell proliferation, we performed assays in four different cell lines. The transient overexpression of PF inhibited cell growth in HEK293 and NIH3T3 cells, but enhanced cell growth in the SV40 large T antigen-expressing cell lines such as 293FT and COS7 cells. In 293FT cells, PF was found to interact with SV40 large T antigen and its overexpression promoted entry into the S phase, implying that the interaction enhanced progression through the cell cycle. The tumor suppressor protein PF acts as a positive regulator of cell growth similar to an oncoprotein in the presence of SV40 large T antigen.
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Introduction

Primary hyperparathyroidism is characterized by the calcium-insensitive hypersecretion of parathyroid hormone and the formation of parathyroid tumors (Grimelius and Johansson, 1997). The disease usually results from a single parathyroid adenoma, but in a minority of cases is part of hereditary syndromes, namely, multiple endocrine neoplasia types 1 and 2A, familial isolated hyperparathyroidism and hyperparathyroidism-jaw tumor (HPT-JT) syndrome (Kassem *et al*., 1994; Marx *et al*., 1998; Schuffenecker *et al*., 1998; Wassif *et al*., 1999). HPT-JT syndrome is characterized by parathyroid tumors, fibro-osseous lesions of the mandible and maxilla, and renal cysts and tumors (Jackson *et al*., 1990; Szabo *et al*., 1995; Carpten *et al*., 2002). Interestingly, it is associated with a high incidence of parathyroid carcinoma in contrast to sporadic and other familial forms of primary hyperparathyroidism. The gene whose inactivation is directly associated with the pathogenesis of HPT-JT syndrome has been identified as the tumor suppressor gene *HRPT2* (Carpten *et al*., 2002). In addition, somatic mutations of *HRPT2* have been frequently found in patients with sporadic parathyroid carcinoma (Howell *et al*., 2003; Shattuck *et al*., 2003).

The *HRPT2* gene is ubiquitously expressed and encodes a protein consisting of 531 amino acids, termed PF. PF is thought to be a tumor-suppressor protein since disease-associated *HRPT2* mutations result uniformly in a loss of function of PF (Carpten *et al*., 2002; Howell *et al*., 2003; Shattuck *et al*., 2003; Simonds *et al*., 2004; Villablanca *et al*., 2004; Mizusawa *et al*., 2006). PF has two putative nuclear localization sequences (NLSs) and a partial homology with the yeast Cdc73p, a component of the RNA polymerase II-associated Paf1 complex in budding yeast. In fact, PF physically interacts with human orthologs of components of the yeast Paf1 complex including Paf1, Leo1, Ctr9 and Rtf1 that are involved in transcription, elongation, and 3′ end-processing (Rozenblatt-Rosen *et al*., 2005; Yart *et al*., 2005; Zhu *et al*., 2005). However, it remains unknown whether the human Paf1 complex is directly involved in tumor suppression.

SV40, a small DNA virus belonging to the polyomavirus family, has served as a powerful model system for dissecting fundamental biological processes including DNA replication, transcription, and neoplastic transformation (Simmons, 2000). After infection, SV40 large T antigen (LT) alters the gene expression and growth of host cells by binding to cellular transcription factors, components of the replication machinery and of the cell cycle regulatory apparatus including p53 and retinoblastoma family proteins such as pRb, p107 and p130 (Ali and DeCaprio, 2001). Most of the interactions of LT with these cellular proteins are crucial for tumorigenesis (Fanning and Knippers, 1992). Thus, LT is useful for studying oncogenic transformation; however, LT has not been regarded as a common cause of human tumors, because no clear evidence that

Correspondence: Dr K Yoshimoto, Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15, Kuramoto-cho, Tokushima City, 770-8504 Japan.
E-mail: yoshimot@dent.tokushima-u.ac.jp
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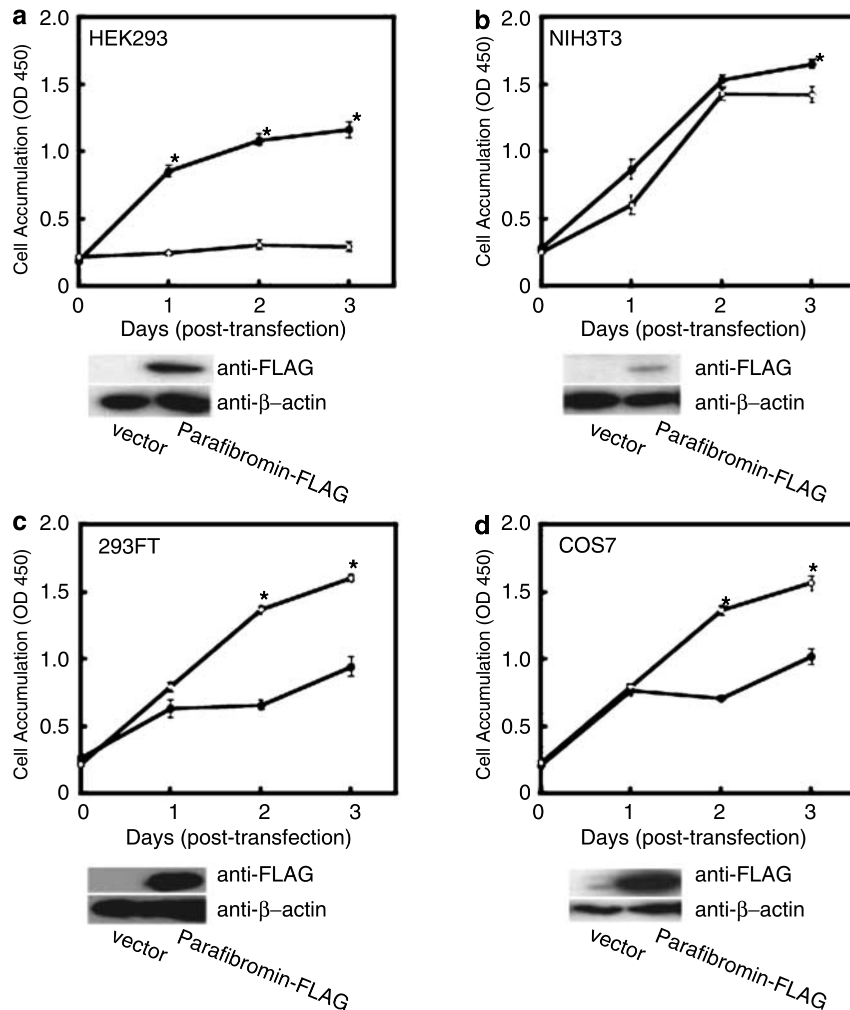

Figure 1 Upper panels: Effects of the overexpression of PF on proliferation in fibroblastic cell lines: HEK293 (**a**), NIH3T3 (**b**), 293FT (**c**) and COS7 (**d**). The proliferation of cultured cells transiently transfected with a vector (filled circle) or cDNA (open circle) encoding PF as indicated was assayed. Each value represents the mean (bars, s.d.) of three individual experiments. $*P<0.05$, determined with Student's *t*-test. Lower panels: Western blot analysis of each cell line transfected with the vector and PF cDNA.

infection with SV40 contributes to tumorigenesis in humans has been obtained.

In this study, we analysed effects of the overexpression of PF on cell growth in four cell lines. We found that PF increased the growth of cells expressing LT, although it suppressed that of cells not expressing LT. Consequently, we hypothesized that the different effects of PF on cell growth between the cell lines with or without LT expression arises from interaction between PF and LT. To test this hypothesis, we investigated the interaction between PF as a component of the human Paf1 complex and LT.

Results

PF enhances growth in cell lines expressing LT

PF is thought to be a tumor suppressor protein since disease-associated *HRPT2* mutations uniformly predict loss of PF function. To investigate whether PF has a direct effect on cell proliferation, we performed assays in different cell lines overexpressing PF. The efficiency with which the enhanced green fluorescent protein-expression vector was introduced into HEK293, NIH3T3, 293FT and COS7 cells was approximately 80, 20, 80 and 80%, respectively (data not shown). In HEK293 cells, the transient overexpression of PF inhibited proliferation strongly (Figure 1a). Although the inhibitory effect of PF in NIH3T3 cells was weaker than that in HEK293 cells because of the low transfection efficiency, a significant inhibitory effect on cell growth was observed (Figure 1b). The results regarding the tumor suppressor activity of PF were consistent with the findings made by Woodard *et al.* (2004). Moreover, PF unexpectedly enhanced cell growth in 293FT and COS7 cells (Figure 1c and d). Because 293FT and COS7 cells are derived from SV40-transformed embryonic kidney fibroblasts, we speculated that interaction between PF and LT caused an acceleration of cell growth.

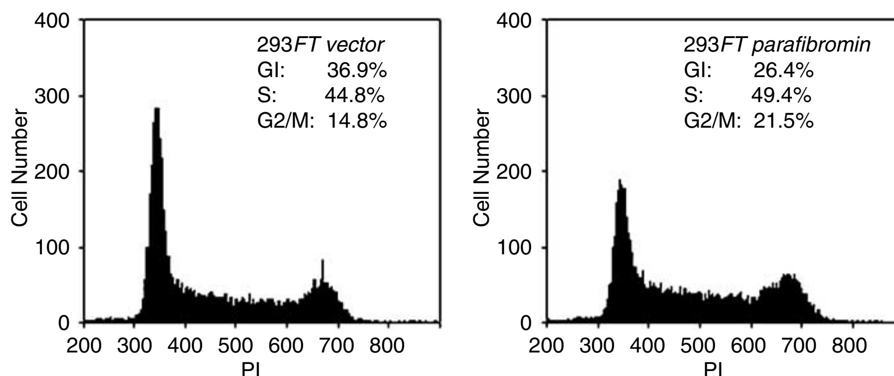

Figure 2 Effects of PF's overexpression on the cell cycle in 293FT cells. 293FT cells were transfected with a vector or cDNA-encoding PF. Twenty-four hours post-transfection, cells were trypsinized, fixed and stained with PI for flow cytometric analysis. The flow cytometric profiles are representative of at least three independent experiments.

PF enhances progression to the S phase in cells expressing LT

To identify the nature of the cell cycle behavior, cells transiently transfected with PF were examined for DNA content by flow cytometry. PF-transfected cells were compared with vector-transfected cells. Analysis of propidium iodide (PI) incorporation showed that PF caused an increase in the fraction of cells in phase S or M2 with a concomitant reduction in number of cells in G1 in the 293FT cell line (Figure 2), suggesting that PF promoted entering into S in LT-expressing cells.

PF interacts with LT

To demonstrate interaction between PF and LT, we performed reciprocal immunoprecipitation experiments. Immunoprecipitation assays were performed using extracts from HEK293 and 293FT cells that had been transfected with FLAG-tagged PF. FLAG-tagged PF and LT were co-immunoprecipitated in 293FT cells, but not in HEK293 cells lacking expression of LT (Figure 3a). In addition, to verify that endogenous PF interacts with LT, immunoprecipitation of 293FT cell lysates using anti-LT antibody was performed. Western blotting using anti-PF antibody revealed that endogenous PF also interacted with LT in 293FT cells (Figure 3b).

PF directly binds with LT in human Paf1 complex-LT interaction

PF composes the human Paf1 complex together with hPaf1, hCtr9, hLeo1 and hRtf1. To confirm if PF interacts with LT by itself or through other components of this complex, immunoprecipitation and Western blot analyses using lysates of 293FT cells transfected with hemagglutinin (HA)-tagged forms of each component were performed. They revealed that all the components interacted with LT in 293FT cells (Figure 4a), indicating that LT interacts with the human Paf1 complex *in vivo*. To determine which protein directly interacts with LT, the HA-tagged components including PF and LT were synthesized *in vitro* and subjected to immunoprecipitation with anti-HA antibody. Subsequent Western

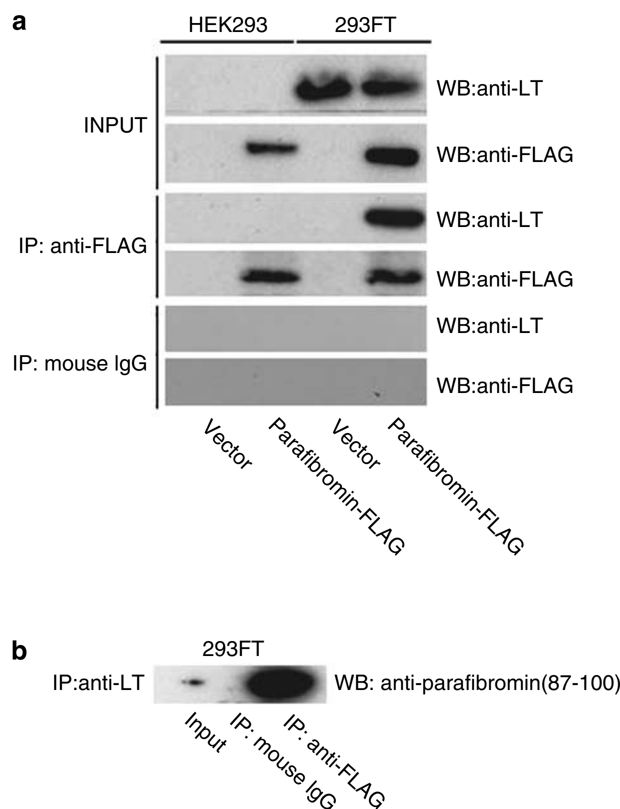

Figure 3 Interaction between LT and PF. (**a**) Immunoprecipitation with anti-FLAG antibody or normal mouse IgG from lysates of HEK293 (lacking LT) and 293FT (expressing LT) cells transfected with the vector or FLAG-tagged PF. Western blot analyses (WB) of input samples and immunoprecipitants (IP) with anti-LT and anti-FLAG antibodies were performed. (**b**) Analysing the interaction of endogenous PF and LT in 293FT cells. Immunoprecipitation of 293FT cell lysates with anti-LT antibody or normal mouse IgG and Western blotting with anti-PF antibody were performed.

blotting of the immunoprecipitates with anti-LT antibody revealed that LT was efficiently co-precipitated with PF or hRtf1, whereas no direct interaction with hPaf1, hCtr9, or hLeo1 was observed (Figure 4b).

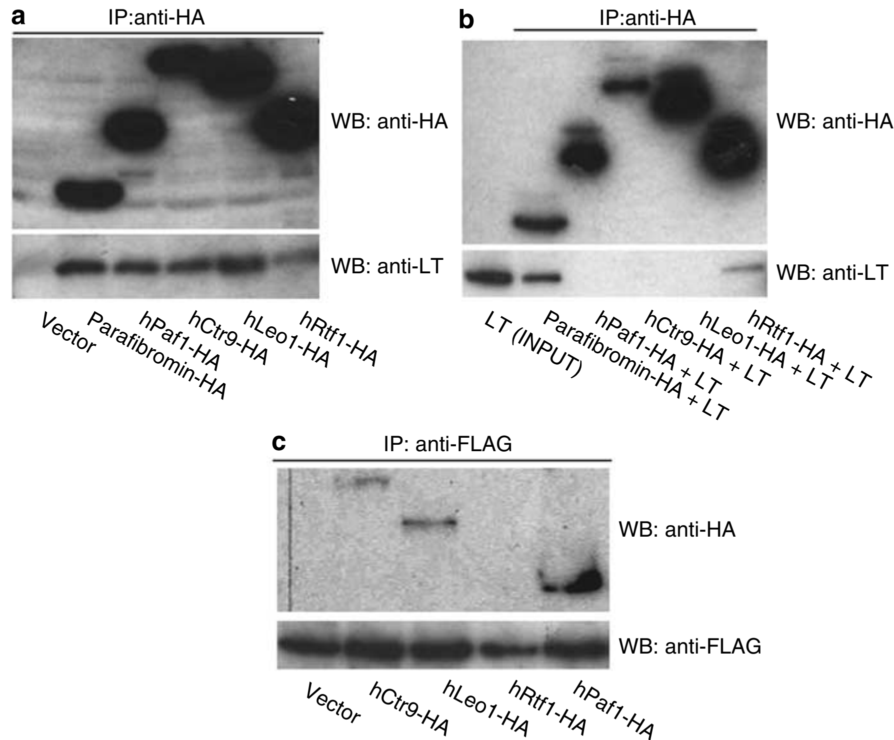

Figure 4 Interaction between LT and components of the human Paf1 complex. (**a**) Immunoprecipitation with anti-HA antibody from lysate of 293FT cells transfected with each HA-tagged component of the Paf1 complex. The immunoprecipitated proteins were immunoblotted with anti-HA antibody (top) and with anti-LT antibody (bottom). (**b**) Interaction of each component of the human Paf1 complex with LT. Each HA-tagged component of the complex and LT were independently translated *in vitro*. The mixtures of reticulocyte lysates were immunoprecipitated with anti-HA antibody and subsequently subjected to Western blotting with anti-HA (top) and anti-LT (bottom) antibodies. (**c**) Interaction of each component of the human Paf1 complex with PF. Immunoprecipitation with anti-FLAG antibody from 293FT lysates co-transfected with each HA-tagged component of the Paf1 complex and with FLAG-tagged PF. The immunoprecipitated proteins were immunoblotted with anti-HA antibody (top) and with anti-FLAG antibody (bottom).

This suggested that LT directly interacted with PF. Interestingly, hRtf1 also directly interacted with LT, while the interaction of hRtf1 with other human Paf1 components was especially weak (Zhu *et al*., 2005). In fact, the immunoprecipitation and Western blotting could not identify interaction between PF and hRtf1 in 293FT cells as in the studies of Rozenblatt-Rosen *et al*. (2005) and Yart *et al*. (2005) (Figure 4c). Therefore, the interaction of hRtf1 with LT may be independent of the human Paf1 complex.

Identification of binding regions of PF and LT

To establish the structural requirements for interaction between PF and LT, a series of deletion mutants (Figure 5a) were subjected to immunoprecipitation and Western blotting. The 1–315 FLAG-tagged PF mutant readily interacted with LT, whereas the 1–232 mutant was not co-immunoprecipitated with LT (Figure 5b). Moreover, the synthesized 218–263 and 218–531 FLAG-tagged PF mutants were found to interact with LT synthesized *in vitro* (Figure 5c). Thus, residues located between amino acids 218 and 263 appear to contribute to the PF/LT complex's formation.

The detection of the PF-binding site on LT was also attempted. Immunoprecipitation and Western blot analyses of lysates from HEK293 cells co-transfected with a series of FLAG-tagged mutant LT with wild-type HA-tagged PF revealed that the 1–360 LT mutants could not interact with PF (Figure 5d). The *in vitro* translated 361–481 and 361–708 FLAG-tagged LT mutants interacted efficiently with *in vitro* translated HA-tagged PF (Figure 5e). These results suggested that a domain conferring the ability to interact with PF was located between amino acids 361 and 481 of LT.

Discussion

PF is thought to be a tumor suppressor protein. The action of many tumor suppressors and oncogenes in neoplasia involves the disruption of one or more cell cycle checkpoint proteins. Recently, it was documented that wild-type PF has antiproliferative activity and inhibits cyclin D1 expression (Woodard *et al*., 2004). The inhibitory effect of PF on cyclin D1 expression would be lost upon biallelic *HRPT2* inactivation, and disinhibition of cyclin D1 expression could initiate neoplastic transformation in certain susceptible tissues such as parathyroid glands (Arnold *et al*., 2002). This suggests an obvious model for neoplasia resulting from

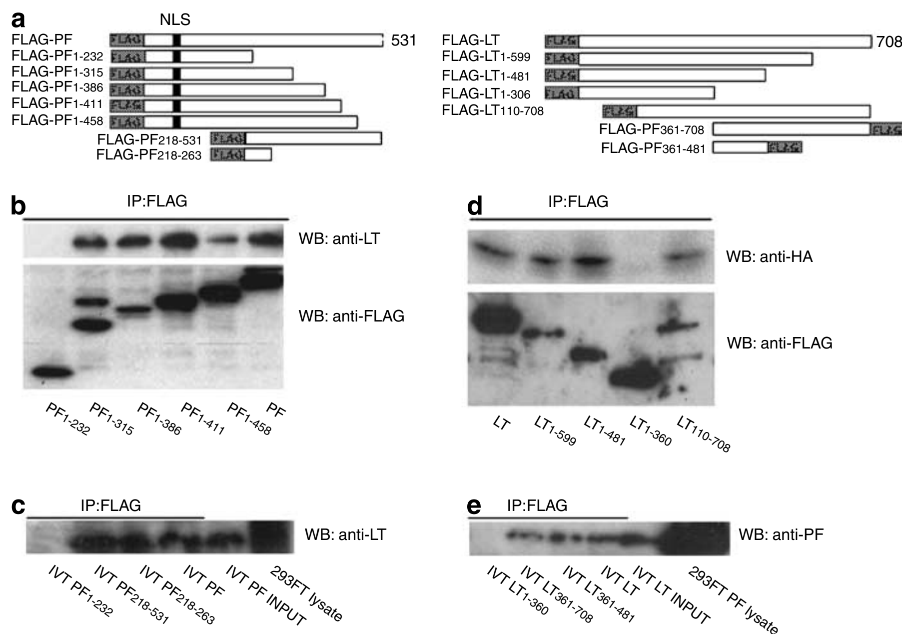

Figure 5 Mutational analysis of PF and LT. (**a**) Schematic diagrams of FLAG-tagged PF (left) and LT (right) deletion mutant constructs. NLS, nuclear localization signal. (**b**) Detection of the LT-binding site on PF. Immunoprecipitation with anti-FLAG antibody from lysates of 293FT cells transfected with FLAG-tagged PF or the indicated FLAG-tagged PF deletion mutant constructs. Immunoprecipitated proteins were immunoblotted with anti-LT antibody (top) or with anti-FLAG antibody (bottom). (**c**) Each FLAG-tagged PF mutant and LT were independently translated *in vitro* (IVT). The mixtures of reticulocyte lysates were immunoprecipitated with anti-FLAG antibody and subsequently subjected to Western blotting with anti-LT. The lysate of 293FT cells was used as a positive control. (**d**) Detection of the PF-binding site on LT. Immunoprecipitation with anti-FLAG antibody from lysates of HEK293 cells co-transfected with FLAG-tagged LT or the indicated FLAG-tagged LT deletion mutant constructs together with HA-tagged PF. Immunoprecipitated proteins were immunoblotted with anti-HA antibody (top) or with anti-FLAG antibody (bottom). (**e**) Each FLAG-tagged LT mutant and PF were independently translated IVT. The mixtures of reticulocyte lysates were immunoprecipitated with anti-FLAG antibody and subsequently subjected to Western blotting with anti-PF antibody. The lysate of 293FT cells transfected with PF (293FT PF lysate) was used as a positive control.

a loss of PF's function. In this report, we reconfirmed that the transient overexpression of PF inhibited the growth of HEK293 or NIH3T3 cells. However, it actually enhanced the proliferation of LT-expressing cells such as 293FT and COS7.

Interestingly, PF overexpression in LT-expressing cells enhanced rather than inhibited cell proliferation. This result was ascertained by a flow cytometric analysis showing that PF enhanced the entering into phase S of the cell cycle. PF may therefore lead to tumorigenesis in LT-expressing cells. The complexity of alterations in cancer is further increased by recently emerging evidence that some genes seem to have dual functions: the same gene can have tumor suppressor-like activity and functions as an oncogene. For example, a member of the Krüppel-like factor (KLF) family, the gene that encodes the transcription factor KLF4, can function as a tumor suppressor gene and context-dependent oncogene (Rowland and Peeper, 2006). Moreover, the menin tumor suppressor protein was reported to be an essential oncogenic cofactor for proto-oncoprotein of mixed-lineage leukemia that is targeted by chromosomal translocations in a diverse subset of leukemia (Yokoyama *et al*., 2005). Thus, PF may also act as an oncogenic cofactor.

Human PF has 32% sequence identity with yeast Cdc73 in the C-terminal region. Cdc73 is a component of the yeast Paf1 protein complex that interacts with RNA polymerase II. This complex is composed of five subunits: Paf1, Cdc73, Leo1, Ctr9 and Rtf1, indicating that PF is part of the human Paf1 complex. In fact, PF interacts with human counterparts to the yeast Paf1 complex including homologs of Leo1, Paf1, Ctr9 and Rtf1. The human Paf1 complex is involved in histone H3 methylation and transcription with events downstream of RNA synthesis (Rozenblatt-Rosen *et al*., 2005, Yart *et al*., 2005). The yeast Paf1 complex is also known to participate in cell cycle control (Porter *et al*., 2002). Interestingly, it was reported that hPaf1 overexpression resulted in an enhancement of cell proliferation in opposition to the tumor suppressor activity of PF (Moniaux *et al*., 2006). Thus, overexpression of PF or hPaf1 affects the regulation of cell growth. One possibility is that PF or hPaf1 as a component of the human Paf1 complex participates in cell growth regulation. The human Paf1 complex may be crucial in maintaining cellular homeostasis and its function may be altered by a change in stoichiometry. The tumor suppressor activity of PF disappeared in LT-expressing cells; therefore, the tumorigenic activity of hPaf1 may overcome the tumor suppressor activity of PF in the human Paf1 complex. Another possibility is that PF or Paf1 itself has an effect on cell growth. However, in the present study, it could not be uncovered whether PF or

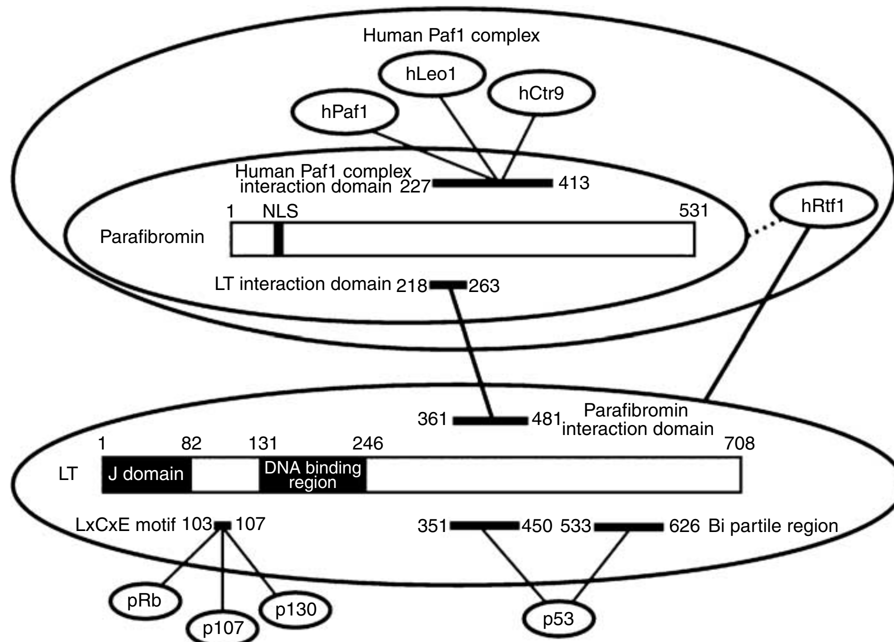

Figure 6 Functional domain of PF as a component of the human Paf1 complex and LT. PF interacts with other components of the Paf1 complex, hPaf1, hCtr9 and hLeo1 at residues 227–413 (Rozenblatt-Rosen *et al*., 2005). Although hRtf1, another component of the complex, slightly interacts with PF and directly interacts with LT, the interaction domains are unknown. PF–LT interaction domains are located between amino acids 218–263 and 361–481 in PF and LT, respectively. The LxCxE motif and the bipartite region of LT are involved in binding with the pRb family and p53, respectively.

hPaf1 is involved in regulating cell growth either as a component of the human Paf1 complex or on its own.

Recently, a positive role for PF in the Wnt pathway was reported (Mosimann *et al*., 2006). The constitutive Wnt pathway is causally involved in many different tumor types. Therefore, PF overexpression may have the potential to enhance Wnt signaling in LT-expressing cells. Cyclin D1 is the downstream target in the Wnt signaling pathway and its expression is known to be inhibited by PF overexpression in normal cell lines. We investigated whether mRNA levels of cyclin D1 were increased by PF overexpression in 293FT cells. We did not detect an increase of cyclin D1 mRNA levels in 293FT cells overexpressing PF by real-time quantitative polymerase chain reaction (PCR) (data not shown), suggesting that the cell cycle progression caused by PF overexpression in LT-expressing cells was regulated by mechanisms other than cyclin D1's induction. Further experiments are needed to clarify whether downstream targets other than cyclin D1 in the Wnt signaling pathway are involved in the difference of cell cycle progression between the cells with and without LT expression.

We showed that PF interacted with LT in the LT-expressing cells. LT interacts with a number of host cell proteins and disrupts their functions. LT has thus proven to be a useful tool for the identification of important cancer-associated proteins including pRb family members and p53. Here, we show the functional domain of PF as a component of the human Paf1 complex and LT in Figure 6. The LxCxE motif of LT, residues 103 to 107, mediates binding of LT to the pRb family proteins (Chen and Paucha, 1990). We showed that LT interacted with PF at residues 361–481, suggesting that interaction with the pRb family is not required for LT–PF interaction. On the other hand, two regions in the C-terminus of LT have been shown to mediate binding to p53: amino acids 351–450 and 533–626 (Kierstead and Tevethia, 1993). The PF-binding region in LT included one of the p53-binding regions. However, experiments *in vitro* revealed that PF directly interacted with LT, suggesting that PF–LT interaction was also independent of p53–LT interaction.

PF interacted with LT in LT-expressing cells, suggesting that the oncogenic activity of PF may be acquired by interaction with LT. Indeed, overexpression of PF with a deletion in the LT-binding region in 293FT cells did not have an effect on cell growth (data not shown). However, the mutant without the LT-binding region might not have the inherent ability of PF that is mediated by interaction with LT. Therefore, we could not rule out the possibility that the cell cycle progression by PF overexpression in LT-expressing cells depends on a pathway other than interaction between PF and LT.

We showed that amino acids 218–263 of PF were the LT-binding domain. Interestingly, the position is consistent with the binding site for β-catenin (Mosimann *et al*., 2006), suggesting that PF uses a common recruitment site to bind transcriptional activators. It is possible that PF assists in the transcriptional induction of LT- or β-catenin-targeting genes by interacting with them. To test this possibility, the presumptive PF targets in LT-expressing cells will have to be identified.

Our finding indicates that PF plays two different roles in tumorigenesis. One is a loss of the growth-suppressor

property of PF through biallelic inactivation of the *HRPT2* gene. The other is an enhanced progression of the cell cycle in the cells expressing LT. Further studies need to focus on the molecular mechanism by which PF impacts cell cycle progression.

Materials and methods

Plasmids
Full-length cDNAs encoding human PF and LT were amplified by performing reverse transcription-PCR with total RNA extracted from a normal human fibroblastic cell line, TIG1, and LT-expressing 293FT cells, respectively. Primers for PF (sense, 5′-CCGAATTCGGGGAAGATGGCGGACGT GC-3′, antisense, 5′-GGCGGCCGCTCAGAATCTCAAGT GCGATTTAT-3′) and LT (sense, 5′-GAGAATTCATATG GATAAAGTTTTAAACAGAGAG-3′, antisense, 5′-GTGC GGCCGCTCATGTTTCAGGTTCAGGGGGAGGTGT-3′) were used. Each sense and antisense primer was designed to contain *Eco*RI and *Not*I sites, respectively. The PCR products were cloned into the expression vector pcDNA3.1+ containing a FLAG epitope at the N-terminus or HA epitope at the C-terminus. Each deletion mutant clone of PF and LT was made by subcloning PCR products into pcDNA3.1+ containing a FLAG epitope at the N- or C-termini. Each full-length hPaf1, hCtr9, hLeo1, and hRtf1 cDNA was purchased from Invitrogen (Carlsbad, CA, USA) and subcloned into pcDNA3.1+ containing an HA epitope at the C-terminus.

Cell culture and transfection
HEK293, 293FT, NIH3T3 and COS7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotic reagent (Sigma) in an atmosphere of 5% CO_2 at 37°C. All transfections were carried out with Effectene (Qiagen, Chatsworth, CA, USA) as recommended by the manufacturer.

Antibodies
Mouse monoclonal anti-PF antibody targeting the peptide RRPDRKDLLGYLNC, corresponding to amino acid positions 87–100 (Tan *et al*., 2004), was kindly provided by Dr BT Teh (Van Andel Research Institute, Michigan, USA). Monoclonal anti-FLAG M2, anti-HA and anti-β-actin antibodies were purchased from Sigma. Monoclonoal anti-LT (Pab 101) antibody and normal mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Proliferation assay
Cell proliferation assays were carried out using a cell counting kit (Dojindo Labs, Kumamoto, Japan) according to the manufacturer's protocol. The absorbance at 450 nm of aliquots of cell supernatants was measured using an automatic plate analyzer (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was performed three times in triplicate. Results are expressed as the mean ± SD. The two-sided Student's t-test was used for statistical comparisons. A P-value <0.05 was considered statistically significant.

Flow cytometry
The DNA content of cells was determined following the fixation of cells in 70% ethanol at 4°C after trypsin-mediated detachment from the culture substrate. After the addition of 2 μg/ml of DNase-free RNase, cells were stained with 50 μg/ml of PI. DNA fluorescence was measured with an EPICS-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the percentages of cells within phases G0/G1, S and G2/M of the cell cycle were determined with FlowJo software (Tree Star, Ashland, OR, USA).

Immunoprecipitation and Western blotting
For immunoprecipitation experiments *in vitro*, cells in a 100-mm dish (endogenous or transfected) were lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl and 0.5% NP-40, pH 8.0, supplemented with complete protease-inhibitor cocktail (Roche, Basel, Switzerland)). After incubation for 30 min on ice, lysates were centrifuged at 4°C for 30 min at 15 000 g. For immunoprecipitation with anti-FLAG M2 antibody, supernatants were incubated with agarose beads coupled with FLAG M2 antibody (Sigma) at 4°C for 16 h. The beads were then washed five times with tris-buffered saline (TBS) (50 mM Tris-HCl and 150 mM NaCl, pH 8.0), and immunoprecipitated proteins were eluted by incubation with 400 μg/ml of FLAG peptide (Sigma) at 4°C for 30 min. For immunoprecipitation with the anti-LT antibody or normal mouse IgG, lysates were preincubated at 4°C for 30 min with 25 μl of protein G Sepharose (Amersham Biosciences, Uppsala, Sweden). The supernatants were incubated with protein G Sepharose coupled with anti-LT antibody or normal mouse IgG at 4°C for 16 h. The Sepharose beads were washed five times with TBS. The immunoprecipitated proteins were eluted by boiling with sodium dodecyl sulfate (SDS) sample buffer.

For immunoprecipitation experiments *in vitro*, each sample was individually synthesized *in vitro* from a pcDNA 3.1+ plasmid inserted with the indicated cDNA, using the TNT quick coupled transcription/translation system (Promega, Madison, WI, USA). For detecting interaction, the sample was mixed with TBS and then immunoprecipitation using anti-FLAG or anti-HA was performed.

Protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon transfer membranes (Millipore, Bedford, MA, USA). Membranes were blocked with blocking reagent (Blocking One: Nacalai Tesque, Kyoto, Japan) and probed with each primary antibody in 1 × ExactaCruz E dilution buffer (Santa Cruz) followed by horseradish peroxidase-conjugated secondary antibody (ExactaCruz Western Blot Reagent; Santa Cruz). Antigens were then visualized by enhanced chemiluminescence (ECL plus Western Blotting Detection; Amersham Biosciences) using Hyperfilm MP (Amersham Biosciences).

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