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Analysis of the Interaction between hPFTAIRE1 and PLZF in a Yeast Two-hybrid System

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Abstract hPFTAIRE1 is a Cdc2-related kinase family member. To search its substrates and regulatory proteins, hPFTAIRE1 was fused to LexA and used as a bait to screen a human brain LexA two-hybrid library. In this screening, seven hPFTAIRE1 interacting proteins, including promyelocytic leukemia zinc finger (PLZF), were obtained. The interaction between PLZF and hPFTAIRE1 was confirmed by β-galactosidase assay and Leu growth activity. PLZF encodes a transcription factor belonging to the POZ/BTB domain and Krüppel zinc finger (POK) family. The highly conserved POZ/BTB domain plays a critical role in protein-protein interaction. We deleted the POZ/BTB and Krüppel zinc finger domains, respectively, and observed the interaction between hPFTAIRE1 and truncated PLZFs by liquid β-galactosidase activity assay. A weak interaction was detected between hPFTAIRE1 and PLZF. We also observed the interaction between PLZF and another Cdc2-related kinase, PCTAIRE1. A similar result was observed. The interaction between PLZF and hPFTAIRE1 or PCTAIRE1 was confirmed by co-immunoprecipitation assay in a yeast system. PLZF is a phosphoprotein and plays multiple roles during cell growth. Our results suggest that hPFTAIRE1 and PCTAIRE1 may play important roles in the functional regulation of PLZF.

Key words hPFTAIRE1; PLZF; PCTIARE1; yeast two-hybrid; interaction

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that have been shown to be key players in the control of cell cycle progression [1]. These various CDKs share high similarity in their amino acid sequence, and contain a PSTAIRE motif for binding of their cyclin partners. Over the last few years, a number of kinases have been identified in mammalians based on structural similarity with $p34^{cdc2}$ [2], but their cyclin partners remain unidentified. These Cdc2-related protein kinases derived their names from the presence of an amino acid for serine substitution in the cyclin binding sequence PSTAIRE. Some of these Cdc2-related kinases, such as PFTAIRE, PCTAIRE, PITSIRE and Cdk5, have been shown to play important roles during cell division, gametogenesis and differentiation [3–6]. Two mouse *PFTAIRE* (*PFTAIRE* and *mPFTAIRE1*) genes have been reported [7,8]. mPFTAIRE1 has a 46 amino acid extension compared to PFTAIRE at its N-terminus. *PFTAIRE* is expressed primarily in the postnatal and adult nervous system by *in situ* hybridization and indirect immunofluorescence. In neurons, the PFTAIRE protein was localized in the nucleus and cytoplasm of cell bodies. *mPFTAIRE1* is ubiquitously expressed in murine tissues and highly expressed in brain, testis and embryo. mPFTAIRE1 is implicated in the process of meiosis as well as neuron differentiation. We have isolated a Cdc2-related protein kinase, PFTAIRE1, from humans (hPFTAIRE1) [9]. *hPFTAIRE1* was highly expressed in brain, pancreas, kidney, heart, testis and ovary. To search for its substrate, we screened an adult human brain two-hybrid cDNA library using hPFTAIRE1 as bait and identified several hPFTAIRE1 interacting proteins, including septin family members KIAA0202 [10] and PLZF.

The *PLZF* gene was initially identified by virtue of its fusion with RARα as a result of a variant (11;17) chro-

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mosomal translocation that occurs in a small subset of acute promyelocytic leukemia (APL) patients [11,12]. The PLZF protein, highly conserved among humans, mice and chickens [13], is a nuclear protein containing nine Krüppel-type zinc finger domains at the carboxyl terminus, which bind directly to DNA [14]. The amino terminus of PLZF contains a BTB/POZ domain that mediates self-dimerization and transcriptional repression through binding to nuclear corepressors [15]. The target genes of PLZF include cyclin A and the interleukin (IL)-3 receptor alpha chain [16]. PLZF overexpression in hematopoietic cell lines leads to growth suppression, apoptosis, G1/S phase cell cycle arrest, and differentiation blockade [17]. Suppression of cyclin A expression by PLZF may contribute to the growth suppressive properties of PLZF [16]. Analysis of PLZF knockout mice demonstrate that PLZF is largely implicated in development, especially in limb and axial skeleton patterning, and Hox gene regulation *in vivo* [18]. The PLZF protein was postulated to play a specific role in early hematopoiesis [17]. PLZF is a negative regulator of cell cycle progression, ultimately leading to growth suppression.

In this study, we screened hPFTAIRE1 interacting proteins in a two-hybrid system and identified PLZF. The interaction between hPFTAIRE1 and PLZF was analyzed. We also analyzed the interaction between PLZF and another CDK family member, PCTAIRE1.

Materials and Methods

Strains and growth conditions

The *Escherichia coli* strain used in this study was DH5α (*supE44ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) [24]. The *Saccharomyces cerevisiae* strain used in this study was CJY151 (*MATα, his3, trp1*, $LexA_{op(\times 6)}$-*leu2*, $LexA_{op(\times 8)}$-*lacZ*), which was generated by integrating the LacZ reporter and its upstream modulation sequence to the strain EGY48 (*MATα, trp1, ura3, his3, leu2*, $LexA_{op(\times 6)}$-*leu2*). *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) at 37 ºC. *S. cerevisiae* strains were cultured in YPD medium (2% tryptone, 1% yeast extract, 2% glucose) at 30 ºC or SC/Glu or SC/Gal at 30 ºC.

Plasmid construction

The full open reading frame of the *hPFTAIRE1* gene was inserted into the *Eco*RI and *Bam*HI sites of the pGilda vector to generate pGilda-PFT, which was used as a bait plasmid for two-hybrid screening. The full open reading frame of the *PCTAIRE1* coding region was inserted into the *Eco*RI and *Bam*HI sites of the pGilda vector to generate pGilda-PCT. Full-length *PLZF* was cloned into the *Eco*RI and *Xho*I sites of pB42AD to construct pB42AD-PLZF. All the constructed plasmids were confirmed by DNA sequencing.

Yeast two-hybrid screening

A human adult brain LexA two-hybrid cDNA library (Clontech, Mountain View, USA) was used for two-hybrid screening, in which cDNA fragments were inserted into pB42AD. pGilda-PFT (LexA-PFTAIRE1 fusion) was used as a bait to screen the library. All procedures followed the manufacturer's protocol.

The pGilda-PFT and library plasmids were sequentially transformed into yeast host strain CJY151 (*MATα, his3, trp1*, $LexA_{op(\times 6)}$-*leu2*, $LexA_{op(\times 8)}$-*lacZ*) [19]. A total of 1×10^8 transformants were plated onto the yeast galactose drop-out minimal medium lacking histidine, tryptophan and leucine, and incubated for 3 d at 30 ºC. The Leu^+ colonies were collected and assayed for β-galactosidase activity. The library plasmids from the positive colonies (Leu^+ and β-Gal^+) were confirmed by polymerase chain reaction (PCR) based restriction map and DNA sequence analysis.

Mutagenesis and β-galactosidase activity assay

Three PLZF mutants, PLZFΔBTB (PLZF with BTB domain deleted), PLZFΔzinc finger (PLZF with zinc finger domain deleted) and PLZFΔN (PLZF with only zinc finger domain available), were constructed into the *Eco*RI and *Xho*I sites of pB42AD by PCR amplification using the human full-length *PLZF* as a template. All plasmids were sequenced for correct construction. Various pairs of the pGilda and pB42AD plasmids were co-transformed into yeast reporter strain CJY151 (*MATα, his3, trp1*, $LexA_{op(\times 6)}$-*leu2*, $LexA_{op(\times 8)}$-*lacZ*) [19] to test for β-galactosidase activity. β-galactosidase assays with ONPG and X-gal as substrates, respectively, were performed, as described previously (MATCHMAKER two-hybrid system, Clontech).

Co-immunoprecipitation assay

Approximately 2 mg of total protein extracts was used for each co-immunoprecipitation assay. Each sample was incubated with 3 μg antibody (Santa Cruz, Santa Cruz, USA) for 2 h at 0 ºC, then pulled down with protein G (Sigma, St. Louis, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% (*W*/*V*) polyacrylamide gels. The relative molecular mass

of proteins was estimated using pre-stained protein markers (molecular weight 27–180 kDa; Sigma-Aldrich, St. Louis, USA). Pulled-down proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond-C; Amersham, Piscataway, USA). The transfer condition was 100 V, 1 h, 4 ºC. Appropriate antibodies were used to detect the corresponding proteins according to Sambrook. Secondary antibody was visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA). The anti-HA and anti-LexA, antibodies were purchased from Santa Cruz.

Results

hPFTAIRE1 used as bait to screen interacting proteins

The protein kinase *hPFTAIRE1* was highly expressed in human brain [9]. Therefore an adult human brain LexA two-hybrid library was chosen to search for its putative substrates. A full-length *hPFTAIRE1* was fused with LexA and inserted into the pGilda vector to generate pGilda-PFT for expression of LexA-hPFTAIRE1 protein (**Fig. 1**).

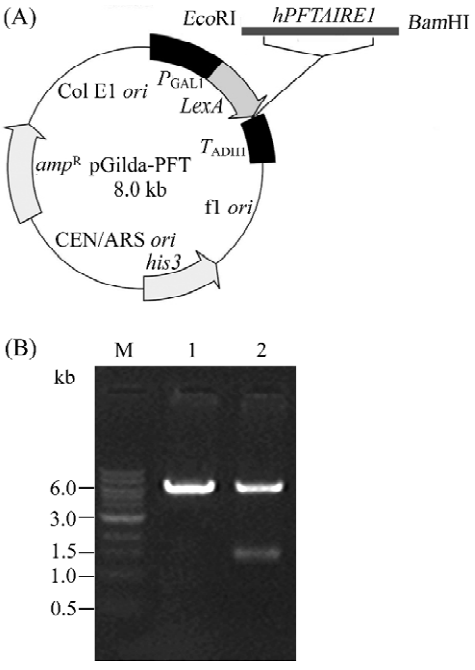

Fig. 1 Vector pGilda-PFT and electrophoresis analysis
(A) Diagram of vector pGilda-PFT. Enzyme sites EcoRI and BamHI were used for hPFTAIRE1 cloning. (B) Agarose gel electrophoresis analysis of recombinant pGilda-PFT digested with EcoRI and BamHI. M, DNA maker; 1, negative control; 2, pGilda-PFT digested with EcoRI and BamHI.

pGilda-PFT, co-transformed with control vector, had no detectable β-galactosidase activity [**Fig. 2(A)**]. Results indicated that the hPFTAIRE1 protein could be used as bait for two-hybrid screening.

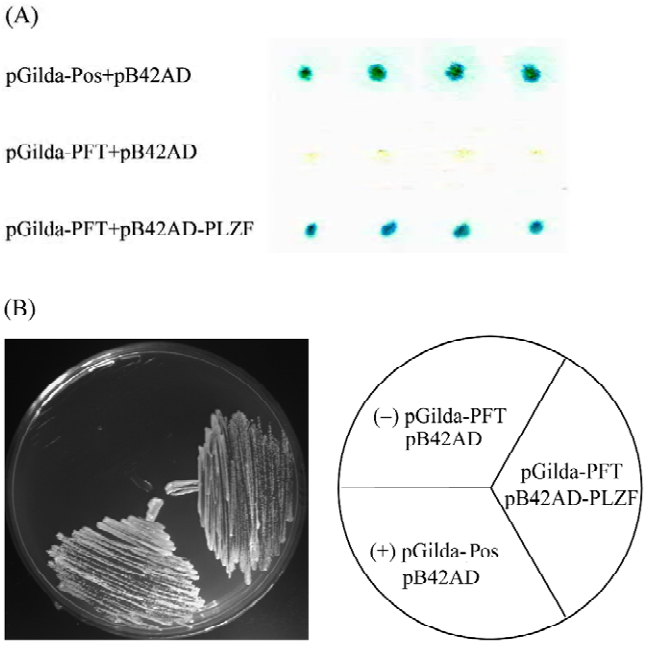

Fig. 2 β-galactosidase activity assay and growth ability
(A) β-galactosidase activity assay of pGilda-PFT. LexA-hPFTAIRE1 and B42AD were co-expressed in CJY151. pGilda-positive was used as a control. (B) Growth ability of transformants on SD/Gal/$His^-Trp^-Leu^-$ plate.

Identification of PLZF from screening

pGilda-PFT was co-transformed with the two-hybrid library in yeast strain CJY151. Among 1×10^8 colonies screened, 243 colonies could grow on the minimal medium (SC/Gal/Raff *ura*$^-$ *his*$^-$ *trp*$^-$ *leu*$^-$), and 81 of these showed a positive response in the β-galactosidase assay. Restriction map and DNA sequence analysis demonstrated one of the inserts encoding for PLZF. The interaction between hPFTAIRE1 and PLZF was confirmed by β-galactosidase assay and Leu growth activity [**Fig. 2(B)**]. From the screening, we totally isolated 16 inserts, representing five kinds of fragments of PLZF (**Fig. 3**).

Test of the autonomous activating activity of PLZF and its mutants

To verify the interaction between hPFTAIRE1 and PLZF

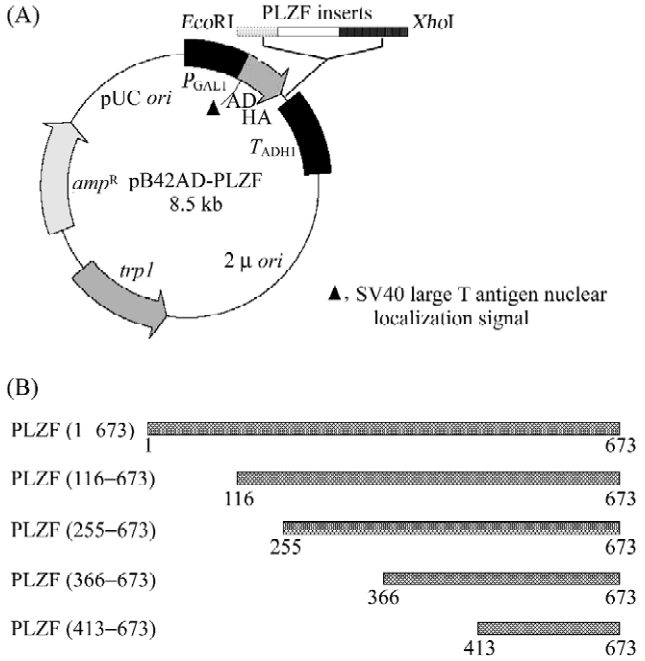

Fig. 3 Schematic diagram of PLZF inserts

(A) Diagram of vector pB42AD-PLZF. (B) Schematic diagram of the PLZF inserts from the yeast two-hybrid screen.

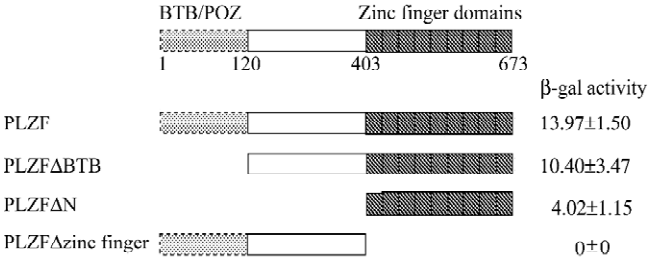

Fig. 4 Schematic diagram of truncated PLZF

The BTB/POZ domain is shown in dotted box and the zinc finger domains are shaded. The autonomous activation of PLZF and PLZF mutants were also shown in β-galactosidase activity assay by using X-gal and ONPG as substrate.

in yeast, we transformed pB42AD-PLZF with control vector. We found that PLZF had an autonomous activating activity.

From primary structure analysis, PLZF contains two conserved domains. At its N-terminus, PLZF contains a BTB/POZ domain and a nine Krüppel-type zinc finger domain located at its C-terminus (amino acids 403–673). To determine which domain is crucial for autonomous activation, three PLZF deletion fragments (PLZFΔBTB, PLZFΔzinc finger and PLZFΔN) were inserted into pB42AD to express HA fusion proteins (**Fig. 4**). The three mutants were each co-transformed with pGilda into yeast strain CJY151, and wild-type PLZF was used as a control. Liquid β-galactosidase activity assays were performed. As shown in **Fig. 4**, the C-terminal zinc finger domain is crucial for autonomous activation. The central region of PLZF plays a minor role and deletion of the BTB/POZ domain contributes little to autonomous activation.

Interaction between hPFTAIRE1 and PLZF in a yeast system

Because PLZF has an autonomous activating activity in a two-hybrid system, we verified the interaction between hPFTAIRE1 and PLZF again. Full-length PLZF and three mutants were co-transformed with pGilda-PFT into yeast strain CJY151. β-galactosidase activity was assayed in liquid using ONPG as a substrate. The results are shown in **Table 1**. β-galactosidase activity in yeast strain containing pGilda-PFT and pB42AD-PLZF was 7–8-fold higher than that containing pB42AD-PLZF alone. Similar to full-length PLZF, hPFTAIRE1 showed a higher reporter activity when interacting with the PLZF mutants (**Table 1**). Results showed that the whole region of PLZF contributed to the interaction and the C-terminal zinc finger domain played an important role, both in the interaction and its autonomous activation, as deletion of this region abolished all β-galactosidase activity.

PCTAIRE1 is another Cdc2-related protein kinase and shares the highest similarity with hPFTAIRE1. More interestingly, these two kinases are highly expressed in post mitotic cells. We also analyzed the interaction between PCTAIRE1 and PLZF. PLZF and PLZF mutants were co-transformed with pGilda-PCT, respectively, and assayed in liquid for β-galactosidase activity. Results showed that PCTAIRE1 could interact with PLZF in a similar pattern (**Table 1**).

To confirm the direct interaction between hPFTAIRE1 or PCTAIRE1 and PLZF, we performed a co-immunoprecipitation experiment in yeast cells. LexA-hPFTAIRE1 or LexA-PCTAIRE1 was co-expressed with HA-PLZF proteins in CJY151. The yeast lysates were immunoprecipitated by anti-HA antibody and then detected with anti-LexA antibody using Western blot. A 77 kDa band that corresponds to hPFTAIRE1 was detected in the pGilda-PFT and pB42AD-PLZF co-transformed cells, and the control showed a negative result [**Fig. 5(A)**]. A 79 kDa band that corresponds to PCTAIRE1 was detected in the pGilda-PCT and pB42AD-PLZF co-transformed cells, and the control showed a negative result [**Fig. 5(A)**]. That the two Cdc2-related protein kinases, hPFTAIRE1 and

Table 1 Interaction of hPFTAIRE1 and PCTAIRE1 with full-length PLZF and PLZF mutants shown by β-galactosidase activity assay using X-gal and ONPG as substrates

DNA binding domain fusion	Activation domain fusion	β-galactosidase activity	
		Filter assay	Liquid assay (u)
LexA-BD	B42AD	White	0±0
LexA-BD	B42AD-PLZF	Blue	13.97±1.50
LexA-BD-PFT	B42AD-PLZF	Blue	90.62±3.40
LexA-BD-PCT	B42AD-PLZF	Blue	95.50±3.80
LexA-BD	B42AD-PLZFΔBTB	Blue	10.40±3.47
LexA-BD-PFT	B42AD-PLZFΔBTB	Blue	80.27±0.28
LexA-BD-PCT	B42AD-PLZFΔBTB	Blue	77.33±1.24
LexA-BD	B42AD-PLZFΔN	Blue	4.02±1.15
LexA-BD-PFT	B42AD-PLZFΔN	Blue	20.53±2.90
LexA-BD-PCT	B42AD-PLZFΔN	Blue	18.15±4.99
LexA-BD	B42AD-PLZFΔzinc finger	White	0±0
LexA-BD-PFT	B42AD-PLZFΔzinc finger	White	0±0
LexA-BD-PCT	B42AD-PLZFΔzinc finger	White	0±0

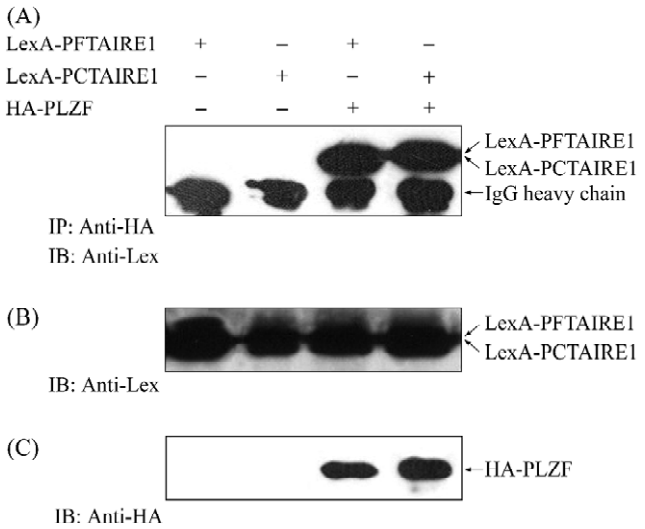

Fig. 5 Interaction of hPFTAIRE1 and PCTAIRE1 with full-length PLZF in a yeast system

(A) LexA-PFTAIRE1 or LexA-PCTAIRE1 and the HA-PLZF proteins were co-expressed in CJY151. The tagged proteins were pulled down with anti-HA antibody and detected using Western blot with anti-LexA antibody as a probe. (B) LexA tagged hPFTAIRE1 or PCTAIRE1 was detected using anti-LexA antibody in Western blot analysis. (C) HA tagged PLZF was detected using anti-HA antibody in Western blot analysis.

PCTAIRE1, could interact with PLZF suggested that hPFTAIRE1 and PCTAIRE1 might play important roles in the functional regulation of PLZF.

Discussion

CDKs are a family of serine/threonine kinases, which play well-established roles in the regulation of the eukaryotic cell division cycle and have also been implicated in the control of gene transcription and other processes. hPFTAIRE1 is a member of the CDK family whose function is unknown. PCTAIRE1 also belongs to the Cdc2-related protein kinases [20], and shares high similarity with human PFTAIRE, especially in the kinase conserved subdomains. *In vitro* translated PFTAIRE or bacteria produced GST-PFTAIRE do not have kinase activity on their own protein (autophosphorylation) or other known substrates, including histone H1 and MBP. Regulatory components are believed to be required for its kinase activity. The activity and function of CDKs are governed by their regulatory subunits and phosphorylation events. We have identified several hPFTAIRE1 interacting proteins during two-hybrid screening using full-length hPFTAIRE1 as bait. In this study, we reported that PLZF was able to interact with two Cdc-2 related protein kinases, hPFTAIRE1 and PCTAIRE1, suggesting that a novel regulatory function of PLZF may be involved.

The PLZF transcriptional repressor when fused to retinoic acid receptor alpha (RARα) causes a refractory form of acute promyelocytic leukemia [21]. It was reported that PLZF could be phosphorylated on serine and threonine residues and contains two potential Cdc2 phosphorylation

consensus sequences found in the region between the POZ domain and the zinc fingers of PLZF [26]. Some researchers found that *in vitro* expressed zinc finger domains bind to a much wider variety of sequences than full-length PLZF [26]. So phosphorylation of the zinc finger domain or the upstream sequence must play an important role in PLZF DNA binding. Conversely, the substrates for hPFTAIRE1 and PCTAIRE1 kinases have not been identified. The kinase activity in the PFTAIRE immune complex, precipitated from a mouse brain cytosolic protein preparation, phosphorylated two specific sets of proteins approximately 58–60 kDa and 200–205 kDa in size. These proteins remained associated with PFTAIRE during immunoprecipitation [7]. The immunoprecipitated PFTAIRE was not able to phosphorylate histone H1, NF-H and other common substrates, suggesting that PFTAIRE may have a more restricted set of substrates than other CDKs. PLZF is a phosphoprotein, but its specific upstream phosphorylating kinases have not been reported yet. Our study suggests that hPFTAIRE1 and PCTAIRE1 could interact with PLZF, mainly through linker region and zinc finger domains. In the linker region, there is one domain termed the proline-rich domain (Pro domain) [14,27] or second repression domain (RD2) that is critical in forming a complex with ETO [28].

Recent studies identified *PLZF* as a new androgen upregulated gene in the prostate [22,23] and suggested that *PLZF* might play important roles in androgen action. It was also reported that PLZF might function in adult germline stem cell self-renewal. The PCTAIRE1 kinase has been reported to have a role in spermatogenic cells because its kinase activity was only detected in those cells [5]. It has been suggested that mouse PFTAIRE may have a role in the process of meiosis [8]. Cdc2-related protein kinase plays an important role in the regulation of gene transcription. Interaction between PLZF and two kinases suggested that PLZF might be one of the substrates of hPFTAIRE1 and/or PCTAIRE1. All these studies shed light on the role of the two Cdc2-related kinases, hPFTAIRE1 and PCTAIRE1, in the functional regulation of PLZF.

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