The PU.1 transcription factor induces cyclin D2 expression in U937 cells

Leukemia (2006) **20,** 2208–2210. doi:10.1038/sj.leu.2404447; published online 19 October 2006

The PU.1 transcription factor is expressed in a wide variety of haematopoietic precursors including long-and short-term reconstituting stem cells, the common myeloid and lymphoid progenitors (CMP, CLP), granulocyte–monocyte progenitors (GMP), monocytes, neutrophils and B-lymphocytes. Compelling evidence gathered from both mouse models and human studies has demonstrated that PU.1 is a pivotal component of the intricate network regulating normal and neoplastic haematopoiesis.[1]

Physiologically, PU.1 expression contributes to the commitment of the CMPs to granulocytes and monocytes and to B-cell differentiation. These observations derive from studies on *PU.1* knockout mice showing that PU.1-null animals present normal erythroid and megakaryocytic cells, but display embryonic/newborn lethality owing to the lack of monocytes, neutrophils and B-lymphocytes.[2] More recently, the role of PU.1 in normal haematopoiesis has been further defined by conditional knockout mice demonstrating that PU.1 expression is critical to perpetuate the haematopoietic stem cell pool and to allow the generation of CMPs and CLPs.[3] PU.1 disruption in CMPs or GMPs blocks their maturation, whereas PU.1 deficiency in CLPs does not affect their ability to differentiate in mature B cells. These results are not surprising considering that PU.1 induces the expression of multiple proteins critically involved in the commitment of the myeloid and lymphoid lineages, including the granulocyte/macrophage colony-stimulating factor receptor (CSF), the macrophage CSF receptor, the granulocyte CSF receptor and the interleukin-7 receptor.[1]

PU.1 is also directly involved in the pathogenesis of human leukaemias. Overexpression of PU.1 in mice exposed to the Friend virus results in the development of erythroleukaemia.[4] Likewise, mice harbouring deletions of the upstream regulatory element of the PU.1 promoter that lead to reduced PU.1 expression develop acute myeloid leukaemia (AML) or T-cell lymphoma.[5,6] Moreover, mutations impairing PU.1 transcriptional activity have been reported in 7% of 126 patients diagnosed with AML.[7] Recent findings have also shown that both AML1/ETO and FLT3-ITD oncoproteins inhibit PU.1 activity, therefore reinforcing the notion that reduced PU.1 function (possibly coupled with mutation-induced haploinsufficiency) may contribute to the development of AML.

Although it is well established that PU.1 modulates the proliferation and differentiation of several haematopoietic precursors, the mechanisms responsible for these activities are still partially unclear. As D-type cyclins are critical modulators of cell proliferation favouring cell cycle transition from the G1 to the S phase, we investigated if PU.1 could regulate the levels of D-cyclins. We report that PU.1 induces the expression of cyclin D2 in the U937 promonocytic cell line.

A preliminary sequence analysis of the promoter for human cyclin D2 (accession number U47284) revealed two *GAGGAA* consensus sites for PU.1, one of which is conserved in mice (accession number AFO15788) (Figure 1a, in bold). To determine if PU.1 is able to transactivate this sequence, we transiently expressed the full-length human cyclin D2 promoter (cloned in the PGL2 luciferase vector, gift of Professor M Eilers, Marburg, Germany) in human embryonic kidney (HEK) 293 cells either alone or with PU.1. Luciferase assays performed normalizing transfection efficiency with an expression vector for Renilla demonstrated a reproducible 2.7-fold increase in the activity of the cyclin D2 promoter in the presence of PU.1 (Figure 1b). However, as PU.1 is expressed exclusively in haematopoietic lineages, we wanted to ascertain if these results could be reproduced in a haematopoietic cell line.

Previous evidence has shown that treatment of U937 cells with tetradecanoyl-phorbol-13 acetate (TPA) leads to the phosphorylation of PU.1 and induction of its transcriptional activity. Indeed, as had been reported previously, when we carried out an electrophoretic mobility shift assay on U937 cells incubated with 20 ng/ml TPA, we observed an increase in PU.1 transcriptional activity after 9–12 h (not shown). Interestingly, PU.1 phosphorylation by serine–threonine kinases can be monitored by immunoblot because hyperphosphorylated (active) PU.1 exhibits a supershift compared to the unphosphorylated (inactive) protein. We therefore performed an anti-PU.1 Western blot on U937 cell lysates derived from cells incubated with TPA and noticed a reproducible increase of the hyperphosphorylated PU.1 protein after 9 h. This increase reached a plateau after a 21-h stimulation (Figure 1c, left panel). When we repeated the immunoblot employing an anti-cyclin D2 antibody, we found a marked increase in cyclin D2 levels starting from 12 h of TPA incubation, suggesting that in U937 cells activation of PU.1 leads to increased expression of cyclin D2 (Figure 1c, right panel).

To exclude that PU.1-independent biological events may be responsible for the observed TPA-mediated induction of cyclin D2 expression, we retrovirally transduced U937 cells with a dominant-negative form of PU.1 (ΔNPU.1 provided by Professor R Kawara, University of Nebraska Medical Center, USA) that retains the DNA-binding domain of the wild-type protein, but is devoid of both the transactivation and the PEST domains. Control Western blots showed that ΔNPU.1-infected cells (lanes 5–8) but not mock-infected cells (lanes 1–4) expressed the dominant-negative form of the transcription factor (Figure 1d, left panel). Moreover, this experiment demonstrated that expression of ΔNPU.1 did not affect TPA-induced phosphorylation of the endogenous PU.1 as incubation with the drug caused PU.1 hyperphosphorylation both in the absence and in the presence of ΔNPU.1. However, when we analysed cyclin D2 expression levels, we found a marked difference in the two U937 cell populations. In mock-infected U937 exposure to TPA increased cyclin D2 expression as had been observed previously (Figure 1d, right panel, lanes 1–4). On the contrary, U937 cells expressing ΔNPU.1 did not present increased levels of cyclin D2 upon PU.1 phosphorylation (Figure 1d, right panel, lanes 5–8), indicating that the dominant-negative form of the transcription factor successfully antagonized the effect of the endogenous PU.1 on the cyclin D2 promoter.

To further determine the biological effect of PU.1 regulation of cyclin D2 expression in U937 cells, we transduced this cell line with viral particles encoding for ΔNPU.1 and cyclin D2, alone or in combination. Immunoblotting experiments confirmed proper expression of the expected proteins in each experimental condition (Figure 1e, left panels). Fluorescent-activated cell sorting (FACS) analysis of these different cell populations after an 18-h treatment with TPA revealed that ΔNPU.1 did not alter the cell cycle distribution of these cells, as

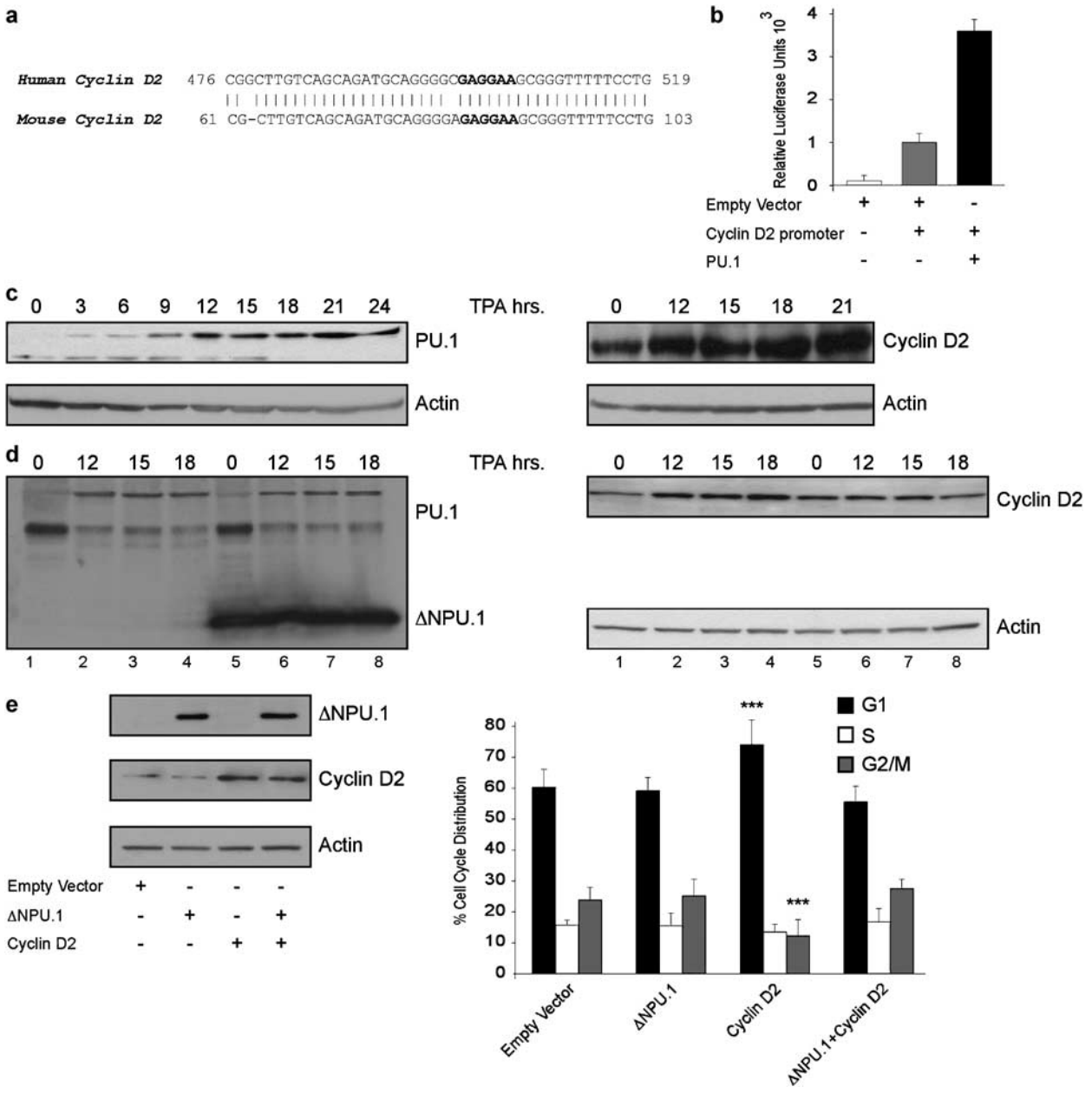

Figure 1 (**a**) Alignment of the cyclin D2 promoter sequence from the specified organisms identifies a conserved consensus (in bold) for the PU.1 transcription factor. (**b**) HEK 293 cells were transiently transfected with the indicated constructs and cell extracts employed to perform a luciferase assay. Results shown are mean ± s.d. of three independent experiments performed in triplicates. (**c**) Immunoblot showing progressive PU.1 hyperphosphorylation (higher band) in response to incubation with 20 ng/ml TPA (left panel). The same lysates from selected time points were probed with an anti-cyclin D2 antibody (right panel). (**d**) Western blot for PU.1 on lysates derived from U937 cells retrovirally transduced with empty vector (lanes 1–4) or with dominant-negative PU.1 (ΔNPU.1, lanes 5–8) and then incubated with TPA for the indicated time (left panel). The right panel shows the same lysates blotted for cyclin D2. (**e**) Immunoblot showing protein expression of U937 cells infected with virus coding for the indicated cDNAs (left panel). The same U937 populations were analysed by FACS to determine their cell cycle distribution (right panel). Statistical significance of the observed variations was calculated using a single-tail *t*-test comparing each condition to mock-infected cells. Shown are mean ± s.d. of three independent experiments. ***$P<0.001$.

compared to mock-infected U937. However, cyclin D2 overexpression caused a statistically significant increase in the G1 population as reported previously (Figure 1e, right panel).[8] Strikingly, this effect was completely abolished by the coexpression of ΔNPU.1, again suggesting that a dominant-negative form of the transcription factor antagonizes the effect of the endogenous PU.1 on cyclin D2 expression.

Taken together, our results indicate that in U937 cells, the PU.1 transcription factor contributes to the regulation of cyclin D2 expression, therefore participating in the control of cell cycle progression. These findings identify a novel mechanism by which PU.1 may influence cell proliferation, favouring or antagonizing the commitment of multiple haematopoietic progenitors toward renewal or differentiation.

L Vicari[1,2], A Eramo[3], L Manzella[2], L Malaguarnera[2], G Iannolo[1], M Gulisano[4], R De Maria[1,3], A Messina[2] and P Vigneri[2]

[1]Dipartimento di Oncologia Sperimentale, Istituto Oncologico del Mediterraneo, Viagrande, Italy;
[2]Dipartimento di Scienze Biomediche, Università di Catania,Catania, Italy;
[3]Dipartimento di Ematologia, Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Roma, Italy and
[4]IOM Ricerca srl, Viagrande, Catania, Italy
E-mail: pvigneri@libero.it

References

1 Oikawa T, Yamada T, Kihara-Negishi F, Yamamoto H, Kondoh N, Hitomi Y *et al.* The role of Ets family transcription factor PU.1 in hematopoietic cell differentiation, proliferation and apoptosis. *Cell Death Differ* 1999; **6**: 599–608.
2 Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 1994; **265**: 1573–1577.
3 Iwasaki H, Somoza C, Shigematsu H, Duprez EA, Iwasaki-Arai J, Mizuno S *et al.* Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 2005; **106**: 1590–1600.
4 Ben-David Y, Bernstein A. Friend virus-induced erythroleukemia and the multistage nature of cancer. *Cell* 1991; **66**: 831–834.
5 Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y *et al.* Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat Genet* 2004; **36**: 624–630.
6 Rosenbauer F, Owens BM, Yu L, Tumang JR, Steidl U, Kutok JL *et al.* Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1. *Nat Genet* 2006; **38**: 27–37.
7 Mueller BU, Pabst T, Osato M, Asou N, Johansen LM, Minden MD *et al.* Heterozygous PU.1 mutations are associated with acute myeloid leukemia. *Blood* 2002; **100**: 998–1007.
8 Meyyappan M, Wong H, Hull C, Riabowol KT. Increased expression of cyclin D2 during multiple states of growth arrest in primary and established cells. *Mol Cell Biol* 1998; **18**: 3163–3172.

Detection of a new *JAK2 D620E* mutation in addition to *V617F* in a patient with polycythemia vera

Leukemia (2006) **20,** 2210–2211. doi:10.1038/sj.leu.2404419; published online 28 September 2006

Recently, a unique point mutation in the JH2 domain of the Janus kinase 2 gene (*JAK2*) was identified in several chronic myeloid disorders. This mutation leads to a *V617F* substitution of phenylalanine for valine in the protein. Affected are more than 80% of polycythemia vera (PV), 40% of essential thrombocythemia (ET) and 40% of idiopathic myelofibrosis (CIM) patients.[1]

The non-receptor Janus tyrosine kinases are involved in the signalling pathways of erythropoietin (EPO), thrombopoietin (TPO) und granulocyte colony-stimulating factor (G-CSF) which mediate anti-apoptosis, cell cycle progression and growth. The *V617F* mutation is located in the JH2 pseudokinase domain of *JAK2* which is involved in inhibition of the kinase activity.[2] The mutant *JAK2* is auto-phosphorylated in factor-dependent cell lines resulting in a cytokine independent activation of STAT5, ERK/MAP kinase and PI3K/AKT. Therefore, the *V617F* mutation is regarded as a gain-of-function mutation.[3]

Common diagnostic tests for the detection of *V617F* are sequencing of the respective gene fragment, allele-specific polymerase chain reaction (ASO-PCR) and melting curve analysis using real-time PCR devices in combination with fluorescent hybridization probes.

Here, we describe a novel point mutation located next to *V617F* in the pseudokinase domain of *JAK2*.

The genomic DNA of a 27-year-old male patient diagnosed with PV was analyzed for the *V617F* mutation by an melting curve assay using the LightCycler system (Roche, Mannheim, Germany).[4] During the interpretation of this analysis, a lower melting temperature of the mutation peak as compared to other *V617F* positive samples or the homozygously mutated HEL cell line was observed (Figure 1, Pat. 2). Subsequent, cloning and sequencing of the respective PCR product (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, 3100 genetic analyzer, ABI, Darmstadt, Germany) revealed a second C-to-A mutation at position 1860 (exon 12) of the *JAK2* gene adjacent to *V617F* at position 1849 (Figure 2a). This new mutation leads to an exchange of the amino acid aspartate for glutamate at position 620 (*D620E*) in the JH2 pseudokinase domain (Figure 2b).

To further analyze the distribution of the *V617F* and the *D620E* mutations in the peripheral blood leukocyte subpopulations of this patient, $CD15^+$ granulocytes, $CD14^+$ monocytes, $CD19^+$ B cells and $CD3^+$ T cells were purified from the mononuclear cells after Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation using MACS (magnetic associated cell sorting) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol provided by the manufacturer. Cell populations were characterized by staining with fluorescein isothiocyanate (FITC)/phycoerythrin (PE) monoclonal antibodies and flow cytometry after isolation

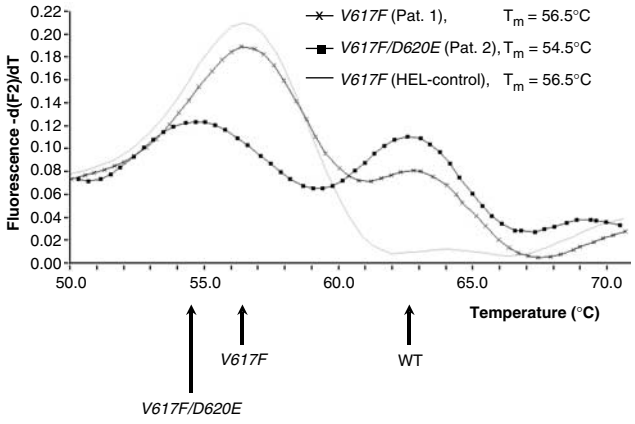

Figure 1 *JAK2* mutation detection in the DNA of two PV patients (Pat. 1, Pat. 2) by LightCycler melting curve analysis with FRET hybridization probes. As a control, HEL-DNA was included. Arrows indicate the melting temperatures (T_m) of wild-type (WT) and JAK2 mutations.