Promyelocytic leukemia zinc finger protein (*PLZF*) enhances glucocorticoid-induced apoptosis in leukemic cell line NALM6

Muhamamd Wasim[1*], Muhammad Mansha[2], Anita Kofler[3], Ali Raza Awan[1], Masroor Ellahi Babar[1] and Reinhard Kofler[3]

[1]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[2]Department of Zoology, Div. of Science & Technology, University of Education, Lahore, Pakistan
[3]Tyrolean Cancer Research Institute, Innrain 66, Innsbruck, Austria

Abstract: Glucocorticoids (GC) actuate apoptosis as well as cell cycle arrest in lymphocytes, and included as core element in the lymphoid malignancy treatment. Despite clinical significance of GC and considerable efforts to understand it, the molecular basis of GC regulated cell death and the resistance phenomenon remains, however, poorly understood. Using Affymetrix-based whole genome expression profiling our group has previously identified a number of prominent glucocorticoid-response genes (Blood 107: 2061, 2006). Promyelocytic leukemia zinc finger (*PLZF*) was one of the best candidate genes. This study was proposed to investigate the possible role of *PLZF* in GC regulated cell death in leukemic model cell line NALM6. To this end, we generated NALM6 cell line (bulk) transduced with a retroviral expression vectors, pHR-SFFV-PLZF-IRES-Puro (U426) and pHR-SFFV-Venus-IRES-Puro (U417), as control, for constitutive gene-expression. HEK293T cells were transfected transiently to generate viral particles. These cell lines were characterized by Western blotting and used to assay the effect of constitutive *PLZF* expression. In conclusion, we report that *bona fide* transcription repressor *PLZF*, which turned out as prominent GC-regulated gene both *in vivo* and *in vitro* situations was found to enhance the GC-induced cell death (basal) in leukemic model cell line NALM6 after 48 and 72h time points.

Keywords: *PLZF*, glucocorticoid-induced apoptosis, leukemia cell line, NALM6, functional analysis of gene.

INTRODUCTION

Apoptosis, induced by glucocorticoids (GCs), is a phenomenon of significant biological as well as has clinical consequences. Biologically apoptosis has been associated with immune repertoire generation as well as the regulation of immune repression (Ashwell *et al*., 2000; Jondal *et al.,* 2001) while clinically, this cell death pathway has been utilized to treat lymphoid malignancies (Pui and Evans, 2006; Schmidt *et al*., 2004). A long list of GC-regulated genes have been recognized in lymphoid lineages in leukemic cell lines (Schmidt *et al.,* 2004) along with related clinical samples (Schmidt *et al*., 2006b; Tissing *et al*., 2007). The most promising GC-regulated gene identified, among them, was promyelocytic leukemia zinc finger (*PLZF*) (Costoya, 2007; McConnell and Licht, 2007). This gene was found to be GC-regulated in most of the child acute lymphoblastic leukemia (ALL) patients, in one adult ALL patient and in two healthy donors treated with GC (Schmidt *et al*., 2006b).

The transcriptional repressor *PLZF* is representative of POK protein family having a BTB domain and nine C_2H_2 *Zn* fingers at the NH_2-terminal and COOH-terminal end of the protein respectively. The BTB domain is involved in both homodimerization of *PLZF* and heterodimerization with other proteins whereas zinc fingers bind to specific response elements for transcriptional repression. The BTB domain has a charged pocket which renders the protein–protein interaction capability to this domain (Melnick *et al*., 2000a).

The human *PLZF* gene is located on chromosome 11q22–q23 (Baysal *et al*., 1997) with a genomic region of 120 kb and seven exons (van Schothorst *et al*., 1999). National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) has also described these two transcript variants (RefSeq) of *PLZF,* NM_006006.4 (2417 bp) and NM_001018011.1 (2262 bp) with same coding sequence and encoded protein (fig. 1).

Members of POK protein family have been proposed to interact through their POZ domain as for relevant example *in vitro* interaction between *PLZF* and *BCL6*. *BCL6* has been shown to be involved in the pathogenesis of a large subgroup of non-Hodgking lymphoma (NHLs) where a chromosomal translocation which juxta-poses its coding region to heterologous promoters is responsible for its over-expression (Dhordain *et al*., 2000). Although, *PLZF* was originally thought to play a role in non-solid tumors, some studies reported putative role played by *PLZF* in solid tumors. Thus, *PLZF* appears to have implication in melanoma tumorigenesis, where its expression levels are linked with better prognosis

**Corresponding author:* e-mail: muhammad.wasim@uvas.edu.pk

(Shiraishi *et al*., 2007; Felicetti *et al*., 2004). The mechanism of *PLZF* action in this cellular context is, however, still uncertain. *PLZF* has been proposed to represses *HOXB7,* but both proteins appear to act functionally independently (Felicetti *et al*., 2004).

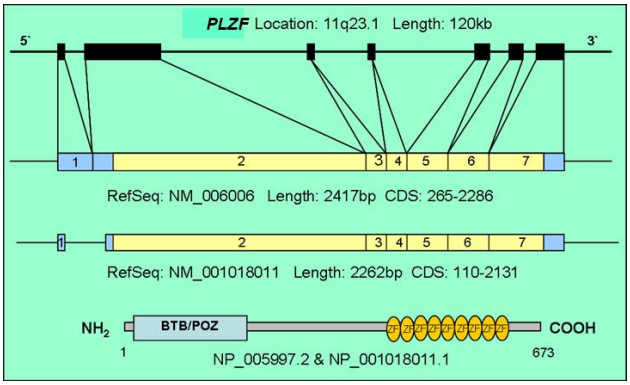

Fig. 1: *PLZF*-Genomic locus, transcript variants and protein. *PLZF* gene spans over a 120 kb stretch of DNA consisting of 7 exons. It has two transcript variants NM_006006.4 (2417 bp) and NM_001018011.1 (2262 bp) with same coding sequence (2021 bp) and encoded protein of 673 amino acids. The difference between two is in the 5` untranslated region (UTR) which is short in NM_001018011.1.

Cervical cancer is another tumor type where *PLZF* has been implicated as possible tumor suppressor. In this malignancy a novel gene, *CCS-3*, was found to be down-regulated compared to normal tissues, and has been shown to interact with *PLZF*. It has been suggested that the possible tumor suppressor role of *CCS-3* may be due to its interaction with *PLZF* (Rho *et al*., 2006).

Pathogenesis of different types of leukemia has been reported to be enhanced by impaired *PLZF* function, e.g., myeloid leukemia resulted due to a fusion protein product of *AML-1/ETO*. Reports have proven that *in vitro* transrepression activity of *PLZF* is inhibited by *AML-1/ETO* fusion protein product with *ETO* zinc finger domain. This is potentially done by exclusion of PLZF from nucleus to prohibit its binding with response elements on regulatory areas of target genes (Melnick *et al*., 2000b).

GCs are steroid hormones extensively used in the therapy of children with ALL by virtue of their property to mediate apoptosis and cell cycle inhibition. *PLZF* is a promising GC-induced candidate gene and is tunrned out during our Affimetryx based microarray analysis performed on children with ALL under GC therapy (Schmidt *et al*., 2006b). The herein described study is carried out to ascertain the effect of transgenic *PLZF* on GC-mediated cell death in NALM 6, a commonly used leukemic model cell line.

MATERIALS AND METHODS

Tissue culture and cell lines

NALM6 (ACC 128, DSMZ) and HEK293T (ATCC), cell lines used in this study, were tested for mycoplasma infection and their authenticity was verified by DNA fingerprinting (Parson *et al*., 2005). NALM6 (suspension cells) were maintained in RPMI 1640 medium, and HEK293T (adherent) cells in DMEM. FCS 10%, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin were added to these culture media. Cells were cultured at 37°C, 5% CO_2, saturated humidity and diluted 1:2 daily or 1:3 every other day to maintain mid-log-phase cultures (2.5-6x10^5cells/ml). Cell numbers were determined in a CASY1 TT cell counter (Schärfe System Germany), using 1:200 dilution in isotonic CASYton.

Immunoblotting

Cell pellet (5x10^6) was washed by phosphate buffered saline (PBS), pelleted by centrifugation at 1200rpm and lysed on ice in 100µl RIPA-buffer (1% NP-40, 0.1% SDS, 0.5% Na-Deoxycholate, 150mM NaCl, 50mM TRIS pH7.5, supplemented with a protease inhibitor mix (complete Tabs, Roche)). Lysates were incubated on ice for 60 minutes then centrifuged at 15,000g and 4°C for 20 minutes to them clarified. Bradford analysis was employed to quantify the protein by mixing 100µl supernatant with 40µl 4xSSB buffer containing 5% β-mercaptoethanol. The protein samples were denaturated at 98°C for 5 min.

Proteins (50µg) were size fractioned by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) (12.5%) in a mini gel chamber. Then the proteins were electro-blotted onto nitrocellulose membranes (0.45µm) by a Bio-Rad semi-dry transfer apparatus (constant 1.36 mA per cm^2). To assess the transfer efficiency and locate the size marker the membranes were stained with Ponceau-S-red. After 2h blocking (PBS / 1% NP-40, 5% milk powder), the membranes were incubated overnight with rabbit polyclonal antibody recognizing *PLZF* (Prestige Antibodies, SIGMA, HPA00149) diluted (1:200) in blocking buffer. Each blot was also probed using either polyclonal antisera against *α-tubulin* (DM1A, CalBiochem, Nottingham, UK) as a protein loading control. The membranes were washed 2 times for 10 minutes in PBS / 1% NP-40 and then incubated with the anti-mouse or anti-rabbit horseradish-peroxidase-conjugated secondary antibody for 45 minutes at room temperature. After another washing step (2x10min) immune complexes were visualised using an enhanced chemiluminescence reagent, ECL plus (Amersham Pharmacia Biotech) and exposure to AGFA Curix X-ray films.

Generation of transduced NALM6 cell line (Bulk)

For functional analysis, NALM6 cells were transduced with a retroviral expression vectors, pHR-SFFV-PLZF-IRES-Puro (U426) (unpublished data) and pHR-SFFV-Venus-IRES-Puro (U417) for constitutive gene-expression. HEK293T cells were transfected transiently to generate viral particles.

Retroviral transduction

PLZF and *Venus* (yellow fluorescent protein as control) expressing cell lines (bulk) were generated by retroviral transduction as previously described (Ausserlechner *et al*., 2004; Riml *et al*., 2004). Briefly, $1x10^6$ HEK293T packaging cells were transiently transfected with 2µg each of pHR-SFFV-PLZF-IRES-Puro, 1µg pMD-G and 1.25µg pCMV8.91 in one well and with 2µg each of pHR-SFFV-Venus-IRES-Puro, 1µg pMD-G and 1.25µg pCMV8.91 in other well. After 48h, supernatants, from both wells, were sterile filtered (0.45µm), supplemented with 4µg/ml polybrene and used to infect $5x10^5$ NALM6 cells. After additional 48h, infected cells were selected by addition of puromycin (1µg/ml) for 4 days.

Apoptosis determination

Propidium iodide (PI) is a DNA intercalating dye which was used to stain the cell nuclei before FACS analysis. FACS analysis was employed to assay the rate of apoptosis of permeabilized PI-treated cells (Nicoletti *et al*., 1991). Cells, $2\text{-}5x10^5$, were centrifuged at 1200rpm, resuspended in 600 µl FACS buffer (50 µg/ml propidium–iodide, 0.1% sodium citrate, 0.1% Triton X-100), kept for a minimum of 6h at 4°C protected from light. At least 20,000 events were assayed with a FACScan cytometer (Becton Dickinson) in association with CellQuest Pro software (Becton Dickinson). In this method FSC (forward scatter)/SSC (sideward scatter), FL-2 (log) and FL-3 (linear) were recorded. In FL-2 nuclei with shrunk DNA-content (subG1 peaks), in percent, were assayed. Cell debris was excluded from analysis.

RESULTS

Characterization of NALM6 cell line transduced with PLZF

We generated stably transduced (with pHR-SFFV-PLZF-IRES-Puro (U426)) derivative of the NALM6 cell line (bulk) with constitutive expression of *PLZF* for characterization and functional analysis. As revealed by western blot analysis, *PLZF* protein was expressed well in this cell line as well as in HEK 293T as recognized by *PLZF* antibody (1:200 dilution). But we could not detect *PLZF* protein in cells transfected with pHR-SFFV-Venus-IRES-Puro (U417), used as control when assayed by anti *PLZF* antibody (fig. 2). These results are in agreement as reported (Wasim *et al*., 2010) earlier where basal *PLZF* expression was very low on mRNA level, so undetectable by immunoblotting, however, its expression was regulated by GCs in leukemic model cell line NALM6.

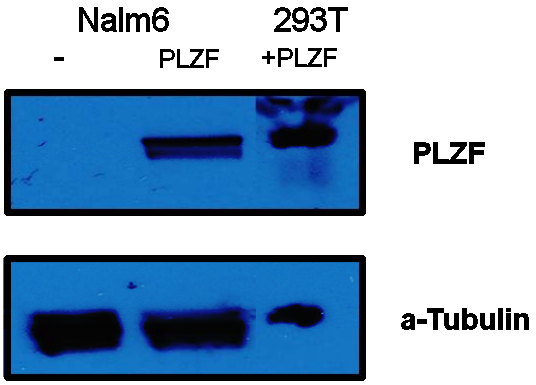

Fig. 2: Protein expression of transgenic *PLZF* in NALM6 and HEK 293T cells. A representative immunoblot of transgenic PLZF expression in NALM6 transduced with pHR-SFFV-PLZF-IRES-Puro & pHR-SFFV-Venus-IRES-Puro and HEK 293T cells in upper panel and a-tubulin as loading control in lower panel.

PLZF enhances GC-induced apoptosis in NALM6 cell line

As shown in fig. 3, transgenic expression of *PLZF* in NALM6 cell line (bulk) showed no evident effects on cell survival and cell death on its own at all time points (24h, 48h and 72h) studied. However, it significantly ($p< 0.05$) enhanced the extent of basal apoptosis induced by 10^{-7}M dexamethasone (a synthetic glucocorticoid) in this leukemic model after 48h and 72h time points compared to NALM6 cell line (bulk) with Venus transgenic expression and untransduced NALM6 cell line used as control in this assay.

DISCUSSION

To study the possible role of transgenic *PLZF*, in GC-induced apoptosis, the above NALM6 cell line (bulk) was then used to establish whether constitutive *PLZF* over-expression influences cell survival and/or modulates the apoptosis induced by GCs. GC-receptor is implicated to mediate GC effects. This transcription factor, a member of nuclear receptor super family, usually resides in cytoplasm, dimmerizes due to ligand binding and then translocates to nucleus. In the nucleus GC-receptor binds to DNA sequences specified for it or interacts with other regulatory proteins in order to mediate the target gene expression (Laudet and Gronemeyer, 2002).

Our results depict that transgenic expression of *PLZF* in NALM6 cell line (bulk) had no apparent implications on cell survival and cell death on its own after 24h, 48h and 72h (fig. 3). But it significantly ($p< 0.05$) enhanced the

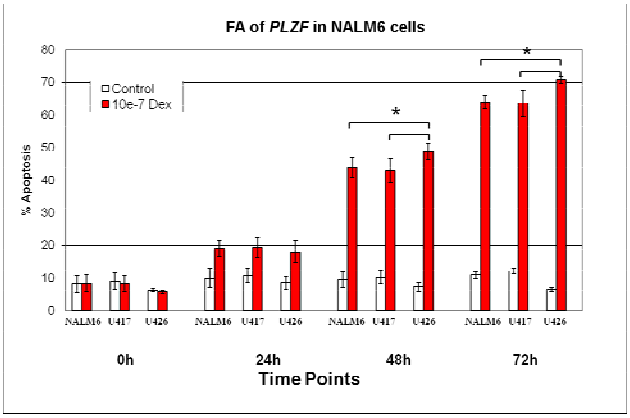

Fig. 3: *PLZF* enhances GC-induced apoptosis but on its own has no effects on cell death/survival. NALM6 cells untransduced and transduced with U426 & U417 for constitutive PLZF & Venus expression were grown in presence (red bars) & absence (white bars) of 10^{-7}M for different time points and subjected to apoptosis determination using fluorescence activated cell sorting (flow cytometry) after PI treatment. Here mean values ±SD of apoptosis are shown for cell death derived from biological triplicates. Asterisks showing the significance ($p < 0.05$) based on student t-test.

extent of basal apoptosis induced by 10^{-7}M dexamethasone in this leukemic model after 48h and 72h in comparison with NALM6 cell line (bulk) transduced with Venus and control NALM6 cells (untransduced). Since *PLZF* was regulated by GCs in different systems so the study of its effects this scenario would be of interest. These finding are compatible, in part, with various previous reports where ectopic *PLZF* expression in leukemic cell line Jurkat (Bernardo *et al*., 2007), histiocytic lymphoma cell line U937 (McConnell *et al*., 2003), murine myeloid cell line 32Dcl3 (Shaknovich *et al*., 1998) and meloma cells (Shiraishi *et al*., 2007) resulted in enhanced apoptosis, repression of growth and cell cycle arrest in G1/S phase.

However, the role played by transgenic *PLZF* in this leukemic model disagrees with our previous work (Wasim *et al*., 2010) carried out in another leukemic model CEM-C7H2-2C8, where tetracycline regulated conditional over-expression of *PLZF* protected the stably transduced cell lines from GC-induced cell death and knock down of *PLZF* increased the extent of GC-induced cell death. The possible explanation for this may be the difference in role of *PLZF* suggested by cell context dependent actions of it. However the previously bona fide role of *PLZF* in apoptosis, growth suppression and cell cycle arrest played on its own was not existent in this cell line too. Furthermore, *PLZF* could not transrepress the expression of its target genes e.g. HoxB7 (Shiraishi *et al*., 2007) and HoxD (Barna *et al*., 2002a), possibly due to absence of additional co-factors or post-translational modifications, for example, acetylating in CEM-C7H2-2C8 cell line and also in NALM6.

In conclusion, we report that *bonafide* transcription repressor *PLZF*, which turned out as prominent GC-regulated gene both *in vivo* and *in vitro* situations (Schmidt *et al*., 2006b; Tissing *et al*., 2007), was found to enhance the GC-induced cell death in leukemic model cell line NALM6 after 48 and 72h time points. Furthermore, this finding opens the avenues to look into the molecular mechanism of enhanced GC-induced cell death by *PLZF* in leukemic model NALM6, used in this study.

REFERENCES

Ashwell JD, Lu FW and Vacchio MS (2000). Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.,* **18**: 309-345.

Ausserlechner MJ, Obexer P, Böck G, Geley S and Kofler R (2004). Cyclin D3 and c-myc control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. *Cell Death Differ.,* **11**: 165-174.

Barna M, Merghoub T, Costoya JA, Ruggero D, Branford M, Bergia A, Samori B and Pandolfi PP (2002a). Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Dev. Cell.,* **3**: 499-510.

Baysal BE, van Schothorst EM, Farr JE, James MR, Devilee P and Richard CW (1997). A high-resolution STS, EST, and gene-based physical map of the hereditary paraganglioma region on chromosome 11q23. *Genomics.,* **44**: 214-221.

Bernardo MV, Yelo E, Gimeno L, Campillo JA and Parrado A (2007). Identification of apoptosis-related PLZF target genes. *Biochem. Biophys. Res. Commun.,* **359**: 317-322.

Costoya JA (2007). Functional analysis of the role of POK transcriptional repressors. *Brief. Funct. Genomic. Proteomic.,* **6**: 8-18.

Dhordain P, Albagli O, Honore N, Guidez F, Lantoine D, Schmid M, The MH, Zelent A and Koken MH (2000). Colocalization and heteromerization between the two human oncogene POZ/zinc finger proteins, LAZ3 (BCL6) and PLZF. *Oncogene.,* **19**: 6240-6250.

Felicetti F, Bottero L, Felli N, Mattia G, Labbaye C, Alvino E, Peschle C, Colombo MP and Care A (2004). Role of PLZF in melanoma progression. *Oncogene.,* **23**: 4567-4576.

Jondal M, Pazirandeh A and Okret S (2001). A role for glucocorticoids in the thymus? *Trends Immunol.,* **22**: 185-186.

Laudet V and Gronemeyer H (2002). The nuclear receptor facts book. Academic Press, London.

McConnell MJ and Licht JD (2007). The PLZF gene of t (11;17)-associated APL. *Curr. Top. Microbiol. Immunol.,* **313**: 31-48.

McConnell MJ, Chevallier N, Berkofsky-Fessler W, Giltnane JM, Malani RB, Staudt LM and Licht JD (2003). Growth suppression by acute promyelocytic leukemia-associated protein PLZF is mediated by repression of c-myc expression. *Mol. Cell Biol.,* **23**: 9375-9388.

Melnick A, Ahmad KF, Arai S, Polinger A, Ball H, Borden KL, Carlile GW, Prive GG and Licht JD (2000a). In-depth mutational analysis of the promyelocytic leukemia zinc finger BTB/POZ domain reveals motifs and residues required for biological and transcriptional functions. *Mol. Cell Biol.,* **20**: 6550-6567.

Melnick AM, Westendorf JJ, Polinger A, Carlile GW, Arai S, Ball HJ, Lutterbach B, Hiebert SW and Licht JD (2000b). The ETO protein disrupted in t(8;21)-associated acute myeloid leukemia is a corepressor for the promyelocytic leukemia zinc finger protein. *Mol. Cell Biol.,* **20**: 2075-2086.

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods.,* **139**: 271-279.

Parson W, Kirchebner R, Muhlmann R, Renner K, Kofler A, Schmidt S and Kofler R (2005). Cancer cell line identification by short tandem repeat profiling: Power and limitations. *FASEB J.,* **19**: 434-436.

Pui CH and Evans WE (2006). Treatment of acute lymphoblastic leukemia. *N. Engl. J. Med.,* **354**: 166-178.

Rho SB, Park YG, Park K, Lee SH and Lee JH (2006). A novel cervical cancer suppressor 3 (CCS-3) interacts with the BTB domain of PLZF and inhibits the cell growth by inducing apoptosis. *FEBS Lett.,* **580**: 4073-4080.

Riml S, Schmidt S, Ausserlechner MJ, Geley S and Kofler R (2004) Glucocorticoid receptor heterozygosity combined with lack of receptor auto-induction causes glucocorticoid resistance in Jurkat acute lymphoblastic leukemia cells. *Cell Death Differ.,* **11**(Suppl 1): S65-S72.

Schmidt S, Rainer J, Ploner C, Presul E, Riml S and Kofler R (2004). Glucocorticoid-induced apoptosis and glucocorticoid resistance: Molecular mechanisms and clinical relevance. *Cell Death. Differ.,* **11**(Suppl 1): S45-S55.

Schmidt S, Rainer J, Riml S, Ploner C, Jesacher S, Achmuller C, Presul E, Skvortsov S, Crazzolara R, Fiegl M, Raivio T, Janne OA, Geley S, Meister B and Kofler R (2006b). Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia. *Blood,* **107**: 2061-2069.

Shaknovich R, Yeyati PL, Ivins S, Melnick A, Lempert C, Waxman S, Zelent A and Licht JD (1998). The promyelocytic leukemia zinc finger protein affects myeloid cell growth, differentiation, and apoptosis. *Mol. Cell Biol.,* **18**: 5533-5545.

Shiraishi K, Yamasaki K, Nanba D, Inoue H, Hanakawa Y, Shirakata Y, Hashimoto K and Higashiyama S (2007). Pre-B-cell leukemia transcription factor 1 is a major target of promyelocytic leukemia zinc-finger-mediated melanoma cell growth suppression. *Oncogene,* **26**: 339-348.

Tissing WJ, den Boer ML, Meijerink JP, Menezes RX, Swagemakers S, van der Spek PJ, Sallan SE, Armstrong SA and Pieters R (2007). Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. *Blood,* **109**: 3929-3935.

van Schothorst EM, Prins DE, Baysal BE, Beekman M, Licht JD, Waxman S, Zelent A, Cornelisse CJ, van Ommen GJ, Richard, III CW and Devilee P (1999). Genomic structure of the human PLZF gene. *Gene,* **236**: 21-24.

Wasim M, Carlet M, Mansha M, Greil R, Ploner C, Trockenbacher A, Rainer J and Kofler R (2010). PLZF/ZBTB16, a glucocorticoid response gene in acute lymphoblastic leukemia, interferes with glucocorticoid-induced apoptosis. *J. Steroid Biochem. Mol. Biol.*, **120**: 218-227.