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Clinical and biological relevance of *CREB3L1* in Philadelphia chromosome-negative myeloproliferative neoplasms

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ABSTRACT

Cyclic AMP-response element-binding protein 3-like 1 *(CREB3L1)* is a gene involved in the unfolded protein response (UPR). Recently, we demonstrated that *CREB3L1* is specifically overexpressed in the platelets of patients with Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs). In this study, we aimed to show the clinical and biological relevance of *CREB3L1* in these hematological diseases. Overexpression of *CREB3L1* was specific to platelets in MPNs and associated with a higher risk of thrombosis and fibrotic transformation in essential thrombocythemia (ET) and polycythemia vera (PV) cases, respectively. Furthermore, we found that UPR genes were downregulated in platelets of patients with ET and PV, which were more pronounced in patients harboring the *JAK2* V617F mutation. However, *CREB3L1* overexpression does not alter UPR gene expression or cell proliferation in UT-7/TPO/CALRm cells exogenously expressing mutated calreticulin and HEL cells harboring endogenous JAK2 V617F. Furthermore, *CREB3L1* overexpression did not modulate sensitivity to endoplasmic reticulum stress in these cell lines. Taken together, our data show 1) a potential role of *CREB3L1* expression in platelets as a new marker of high-risk MPNs and 2) an association between *CREB3L1* overexpression and UPR gene downregulation in these patients' platelets, with *CREB3L1* not altering UPR in our *in vitro* models and possibly further *in vivo* mechanisms being involved.

1. Introduction

Cyclic AMP-response element-binding protein 3-like 1 (CREB3L1), a member of the *CREB/ATF* transcription factor family [1–3], plays a role in the unfolded protein response (UPR), a cellular system active in response to endoplasmic reticulum (ER) stress generated by the accumulation of misfolded protein [4,5]. It acts initially as a pro-survival mechanism and ultimately leads to apoptosis in case misfolded protein accumulation is not resolved; UPR is implicated in the oncogenesis of several cancers, including glioblastoma multiforme, prostate, and breast cancer [6], acting in response to different extrinsic (hypoxia, nutrient deprivation, acidosis) and intrinsic factors (oncogene activation) that cause ER stress in neoplastic proliferation.

CREB3L1 is a transmembrane protein that resides in the ER membrane; ER stress induces CREB3L1 intramembrane cleavage followed by N-terminal transportation to the nucleus, where it acts as a transcription factor contributing to the UPR and additional mechanisms including bone and collagen formation, cellular differentiation, and viral infection control [7–10]. *CREB3L1* is expressed in the majority of solid tumors [11,12] and associated with metastasis in breast carcinoma [13,14]. The role of CREB3L1 in hematological diseases is still unknown; however, our group recently showed by RNA-sequencing analysis that *CREB3L1*

Abbreviations: CREB3L1, Cyclic AMP-response element-binding protein 3-like 1; JAK2, Janus kinase 2; CALR, Calreticulin; MPNs, Philadelphia chromosome-negative myeloproliferative neoplasms; UPR, unfolded protein response.

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expression is significantly higher in platelets of patients with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), subgroups of Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) [15] and that it is useful to discriminate reactive cases from neoplastic cases. Despite this, the biological significance of this overexpression and the clinical relevance of *CREB3L1* in MPNs remains elusive.

In this study, we analyzed *CREB3L1* expression in MPN cases in association with clinical parameters to define *CREB3L1* relevance in characterizing MPNs and investigated the association between *CREB3L1* expression and expression of other UPR genes in MPN cases. Furthermore, to investigate the association between *CREB3L1* overexpression and UPR deregulation or neoplastic cellular growth in MPNs, we studied UPR gene expression, ER stress-inducing drug sensitivity, and cell proliferation in MPN-restricted driver gene mutation-positive cell lines.

2. Materials and methods

2.1. Patients' clinical data analysis

Patient data were collected in accordance with the Declaration of Helsinki [16] and written informed consent was obtained from all participants before study inclusion and sample collection. All patients with MPNs who revisited our institution between Oct 2000 and Jun 2021 were retrospectively diagnosed according to the 2016 World Health Organization (WHO) criteria [17–19]; 77 patients with ET, 29 with PV and 16 with PMF were included in the clinical data analysis. Randomly chosen patients (10 with *JAK2* V617F and 10 with *CALR* mutations (*CALRm*)) were prospectively analyzed for peripheral blood (PB) cell fractions *CREB3L1* expression. As healthy controls, we analyzed PB from volunteers without history of hematological disorders but with physiological blood parameters at a semestral medical check-ups. *JAK2* V617F and *CALRm* were analyzed using a previously published method [20,21]. This study was approved by the ethics committee of Juntendo University (IRB#M12–0895) before sample collection and data collection.

2.2. Sample collection

Platelet samples were collected during patient's follow-up [15] and stored frozen at − 80 °C until use. For cell fraction *CREB3L1* expression, platelets, red blood cells (RBCs), lymphocytes (Lym), and granulocytes (Gran) were isolated from 10 mL PB as follows: platelets were first isolated [15] with limited number of PB cells as determined by FACS (FACS Celesta, BD Biosciences) (Supplemental Fig. 1); then the remaining platelet-depleted sample was mixed, and 3 mL of this sample was treated for 15 min with 5 volumes of RBC lysis buffer (QIAGEN), washed twice with phosphate-buffered saline solution (PBS) to eliminate RBCs from the sample. Lym and Gran were sorted by FACS (FACSAria II, BD Biosciences) based on doublet exclusion and SSC-A/FSC-A cell complexity and dimension discrimination gating (minimum 10^5 cells per sample). The sorted Lym and Gran were washed once with PBS, resuspended in TRIzol reagent (Thermo Fisher Scientific), and stored at − 80 °C. The remaining 7 mL of platelet-depleted PB was carefully layered on 3 mL each of two different lymphocyte separation density solutions (1119 and 1077 g/L, respectively, Nacalai Tesque) in a 15 mL tube and centrifuged for 30 min at 2000 rpm (716 g) without braking during deceleration. The RBC fraction deposited at the bottom of the tube was then collected with a disposable dropper, resuspended in TRIzol LS reagent, and stored at − 80 °C. RNA was then extracted from platelets, Lym, Gran, and RBC fractions using the Purelink RNA Mini Kit (Thermo Fisher).

2.3. RT-qPCR analysis

Complementary DNA (cDNA) was synthesized using the ReverTra Ace qPCR kit (Toyobo) according to the manufacturer's protocol. The starting amount of RNA for cDNA synthesis was 120 ng for each of the four PB fractions described above and 1 μg for patients' platelet for the cohort study and cell lines. RT-qPCR was performed by using Thunderbird SYBR qPCR Mix (Toyobo) with the following sets of primers: *CREB3L1* forward primer: GGA GAA TGC CAA CAG GAC, *CREB3L1* reverse primer: ACC AGA ACA AAG CAC AAG G[15]; *PERK* forward primer: ACG ATG AGA CAG AGT TGC GAC, *PERK* reverse primer: ATC CAA GGC AGC AAT TCT CCC; *ATF6* forward primer: TCC TCG GTC AGT GGACTC TTA, *ATF6* reverse primer: CTT GGG CTG AAT TGA AGG TTT TG; *IRE1* forward primer: CAC AGT GAC GCT TCC TGA AAC, *IRE1* reverse primer: GCC ATC ATT AGG ATC TGG GAG A, and *GAPDH* forward primer: AGC CAC ATC GCT CAG ACA C, *GAPDH* reverse primer: GCC CAA TAC GAC CAA ATC C. RT-qPCR thermal conditions were as follows: initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 30 s for 45 cycles, followed by melting curve analysis at the reaction end. The relative expression levels of the gene of interest were calculated by the ΔΔCt method for each sample by comparison with the mean expression level of healthy controls in the case of patient experiments or with empty vector (EV) cell lines in the case of *in vitro* cell line analysis. *GAPDH* was used as the internal control gene for all samples [22].

2.4. Cell lines generation and proliferation assay

CREB3L1 cDNA was subcloned into the pMSCV-IRES-green fluorescence protein (GFP) vector (Addgene #20672). Retroviral particles were produced as previously described [23]. HEL cells and SET-2 cells harboring endogenous JAK2 V617F [24], and UT-7/TPO/CALRm cells exogenously expressing either CALR Del52 or Ins5 mutations, previously generated by retroviral transfection of pMSCV-IRES-mOrange vector (Addgene #54568) containing *CALR*m sequences [23,25], were then virally infected at an efficiency of ~20%, as previously described [26]. GFP-positive cells were then sorted 72 h after transfection using the FACSAria II (BD Biosciences) to obtain CREB3L1 expressing cells and EV controls for each cell line [26]. Cell proliferation assay was performed with Cell Count Reagent SF (Nacalai Tesque), as described previously [23].

2.5. Immunoblot analysis

Immunoblot analysis was performed as described previously [27]. The following primary antibodies were used for immunoblotting: anti-CREB3L1 (R&D Systems, AF4080), anti-CALRm (Oncodianova, DIA-CAL), anti-JAK2 (Cell Signaling, #3230) and anti-β-Actin (Cell Signaling, #4967). The following horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) were used: goat anti-mouse immunoglobulin G (IgG; #sc-2005) and anti-rabbit IgG (#sc-2004).

2.6. Compounds cell sensitivity

UT-7/TPO, SET-2, and HEL cells were cultured for 48 h in the presence of different concentrations of tunicamycin (Sigma-Aldrich) [28], MG-132 (Nacalai Tesque) [29] or brefeldin A (Fujifilm-WAKO) [30] (1, 3, 10, 30, 100, 300, and 1000 nM in dimethyl sulfoxide) or vehicle. Cell proliferation was quantified as described previously [23] relative to dimethyl sulfoxide-treated controls.

2.7. Statistical analysis

Boxplots were generated using BoxPlotR [31] and statistical analyses were performed using R software [32]. Significance between samples was calculated using non-parametric tests, such as Kruskal–Wallis and Mann–Whitney *U* test, and parametric tests such as Student's *t*-test. Correlation coefficients were calculated using Pearson's method. Survival analysis was carried out using Kaplan–Meier curves, and groups were compared by log-rank test. P levels of significance are indicated in

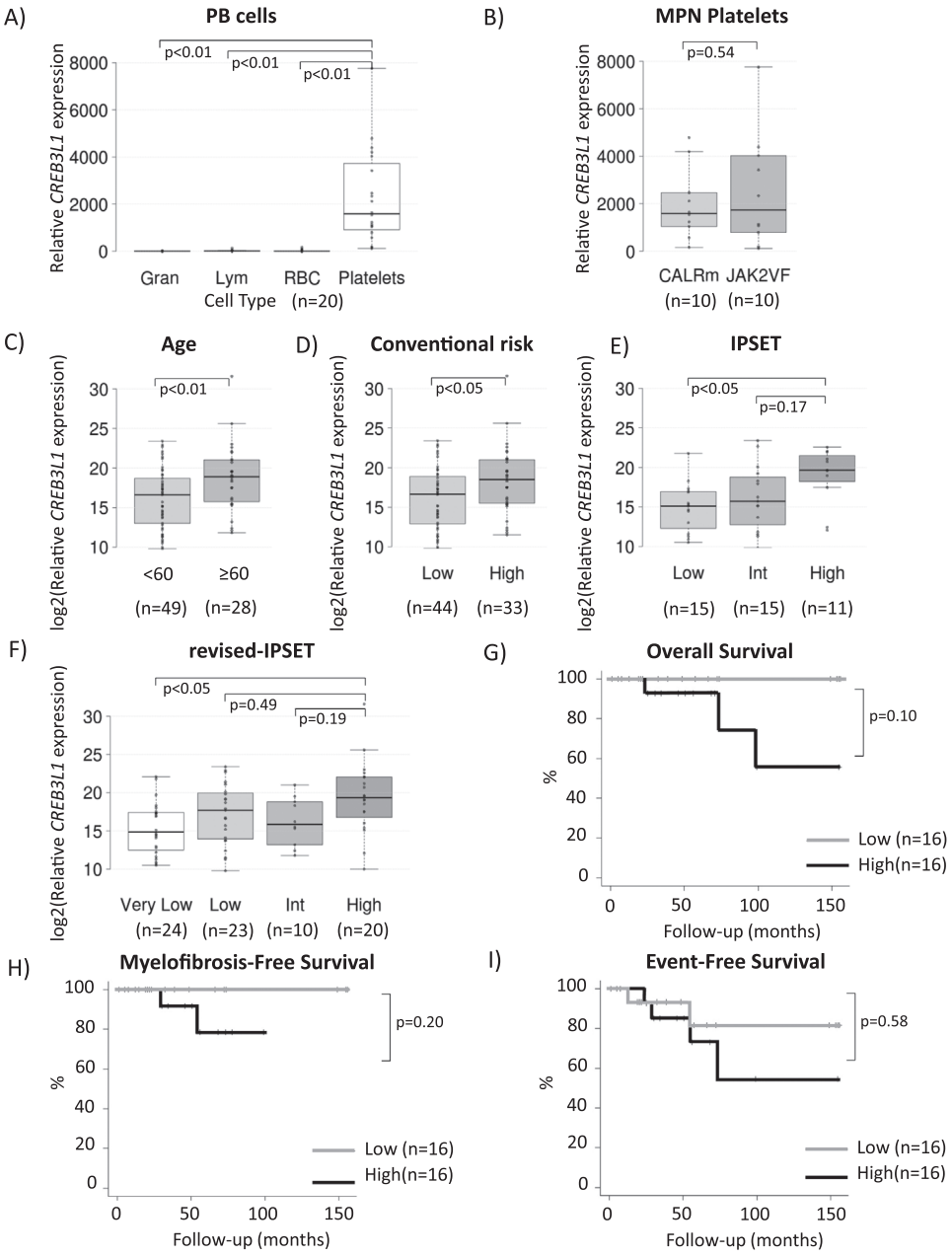

Fig. 1. Clinical relevance of *CREB3L1* expression in platelets in myeloproliferative neoplasms (MPNs). Box plot comparing *CREB3L1* mRNA expression levels determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) between granulocytes (Gran), lymphocytes (Lym), red blood cells (RBC) and platelets (A), *CREB3L1* expression levels in platelet RNA between MPN cases harboring *CALR* mutation (CALRm) and *JAK2* V617F (JAK2VF) (B), *CREB3L1* expression levels in platelet RNA among patients with essential thrombocytopenia (ET) grouped by age (< 60 or ≥ 60 years at the time of sample collection) (C), conventional risk score (D), IPSET (E), and r-IPSET risk score (F). Kaplan–Meier probability curves for overall survival (G), myelofibrosis-free survival (H), and event-free survival (I) in patients with ET expressing lower (first quartile) or higher (fourth quartile) *CREB3L1* mRNA levels in platelets, determined by RT-qPCR.

each figure, and a p-value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1. *CREB3L1 is specifically overexpressed in platelets of patients with Ph-negative MPN*

To determine in which specific cell type *CREB3L1* is upregulated, we analyzed its expression in PB cell fractions obtained from patients with MPN; 10 patients harboring *JAK2* V617F (6 PV, 4 ET) and 10 patients harboring *CALR*m (5 ET and 1 PMF for Del52, and 3 ET and 1 PMF for Ins5) were analyzed. *MPL* mutated cases were excluded due to lack of access of any of these rare cases during prospective sample collections. Only the platelet fraction showed highly significant expression of *CREB3L1*, while the gene was not overexpressed in other PB fractions (platelets *vs.* Lym/Gran/RBC, $p < 0.01$, Fig. 1A). Furthermore, to determine whether there was a difference in *CREB3L1* expression depending on the status of MPN driver gene mutations, we analyzed *CREB3L1* expression in platelets from patients harboring *JAK2* V617F or *CALR*m and found no statistically significant difference between the two groups (Fig. 1B). There was no significant difference on *CREB3L1* expression between disease types (data not shown), the same with our larger cohort study [15]. Since *CREB3L1* was not detectable in other PB fractions, the comparison between different diseases for granulocyte, lymphocyte, and RBC was not performed. Nevertheless, these findings indicate that *CREB3L1* is highly expressed specifically in MPN platelets, without a clear association with a specific MPN driver gene mutation, and is absent in other distinct PB cellular fractions.

3.2. *CREB3L1 expression is associated with higher clinical risk in ET and PV cases*

Since patients with ET are characterized by increased levels of platelets where *CREB3L1* is specifically overexpressed, we analyzed correlations between *CREB3L1* expression and clinical parameters from

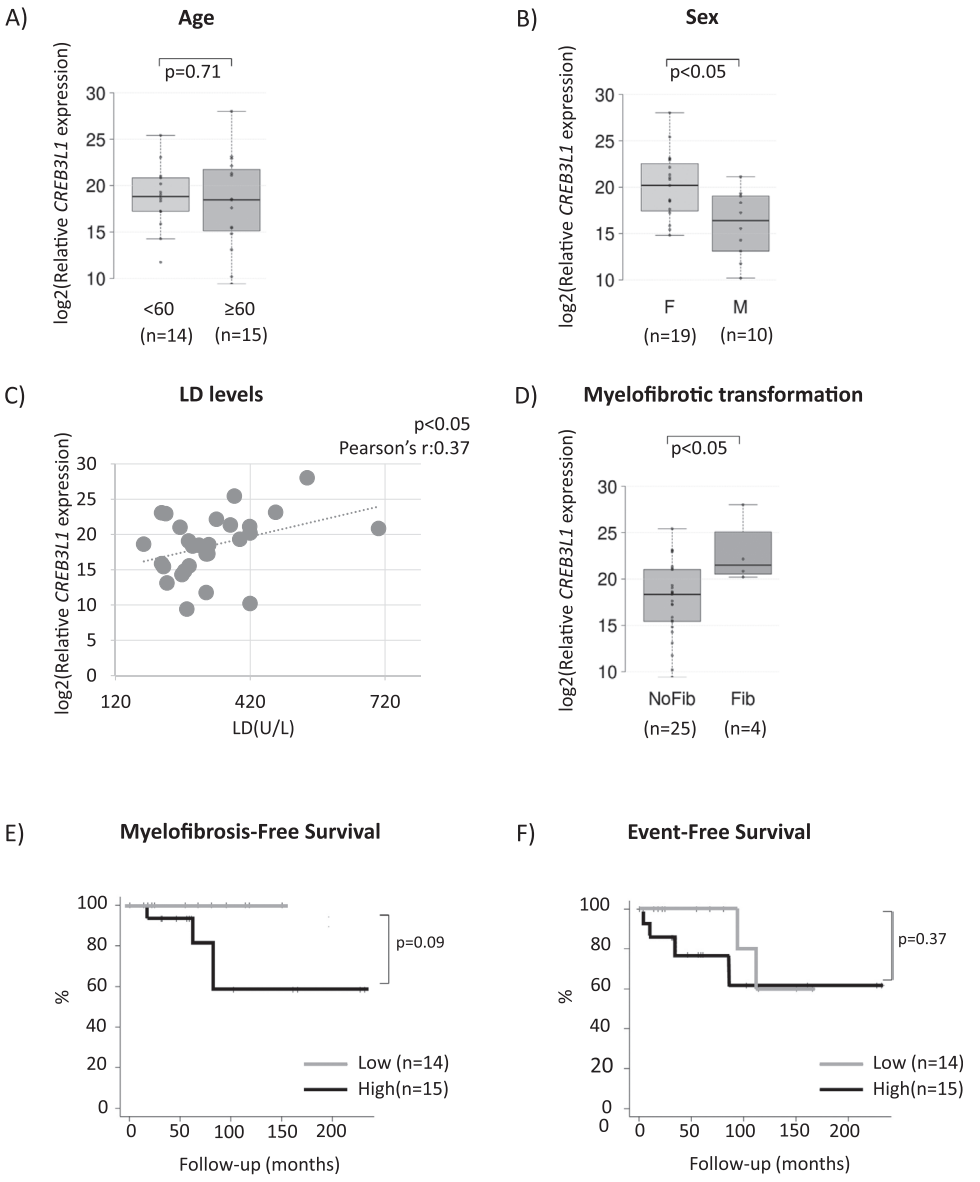

Fig. 2. Clinical relevance of *CREB3L1* expression in platelets of patients with polycythemia vera (PV). Box plot comparing *CREB3L1* mRNA expression levels by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) in platelets of patients with PV grouped according to age < 60 or ≥ 60 years at the time of sample collection (A) and sex (B). Correlation between *CREB3L1* mRNA expression levels in platelets determined by RT-qPCR and lactate dehydrogenase (LD) levels (C). Box plot comparing *CREB3L1* mRNA expression levels determined by RT-qPCR in platelets grouped according to myelofibrotic transformation (D). Kaplan–Meier probability curves for myelofibrosis-free survival (E) and event-free survival (F) in patients with PV expressing lower (below median) and higher (above median) *CREB3L1* mRNA levels in platelets determined by RT-qPCR.

a cohort of 77 ET cases, defined by the WHO 2016 diagnostic criteria. There was no correlation between *CREB3L1* levels and sex, white blood cell count (WBC), platelet count, hemoglobin (Hb) level, hematocrit (Hct) level, and lactate dehydrogenase (LD) level (Supplemental Fig. 2A, B; Supplemental Table 1). However, when we divided the patients according to age at the time of sample collection, we found that patients older than 60 years in ET cases expressed significantly higher levels of *CREB3L1* compared with younger patients ($p < 0.05$, Fig. 1C).

Older age is a factor associated with increased risk in ET cases [33] therefore, we investigated the association of *CREB3L1* expression and ET cases stratified by thrombosis risk classification. Grouping the patients according to three major models, including ET conventional risk score (low/high) [34], international prognostic score for thrombosis in ET (IPSET) (low/intermediate/high) [35] and revised-IPSET (very low/-low/intermediate/high) [36], we found that *CREB3L1* was significantly more expressed in the highest risk group for all three risk scores (Fig. 1D-F). To further examine the association between *CREB3L1* and clinical outcome, we calculated the overall survival (OS) probability by grouping patients with ET according to low and high *CREB3L1* expression (first and fourth quartiles, respectively). A decrease in survival probability in patients with higher *CREB3L1* levels was depicted by the Kaplan–Meier curve and log-rank test (Fig. 1G). The same group of patients also showed a tendency to develop fibrosis over time, one of the characteristics of disease progression, and an indicator of poorer outcome in ET (Fig. 1H) [37]. This tendency does not remain if we calculate the event-free survival (EFS) probability, where events are defined as death, transformation to leukemia and myelofibrosis, or thrombosis (Fig. 1I).

Subsequently, we applied the same analysis to 29 WHO 2016 defined patients with PV, a group of cases characterized by a lower extent of thrombocytosis than ET cases, an increase in Hb levels, and the presence of *JAK2* mutation in virtually all cases [38]. The level of expression of *CREB3L1* between ET and PV was not statistically different (data not shown). In contrast to ET cases, in PV, there was no association between *CREB3L1* expression in platelets and older age (Fig. 2A), and female patients were associated with higher *CREB3L1* levels when compared with male patients ($p < 0.05$, Fig. 2B). There was no correlation between *CREB3L1* and other PB parameters (Supplemental Fig. 3; Supplemental Table 1), except for LD levels, which showed a significant positive correlation (Pearson's coefficient $= 0.37$, $p < 0.05$, Fig. 2C). *CREB3L1* expression was also increased in patients with PV who developed myelofibrosis during the course of the disease ($p < 0.05$,

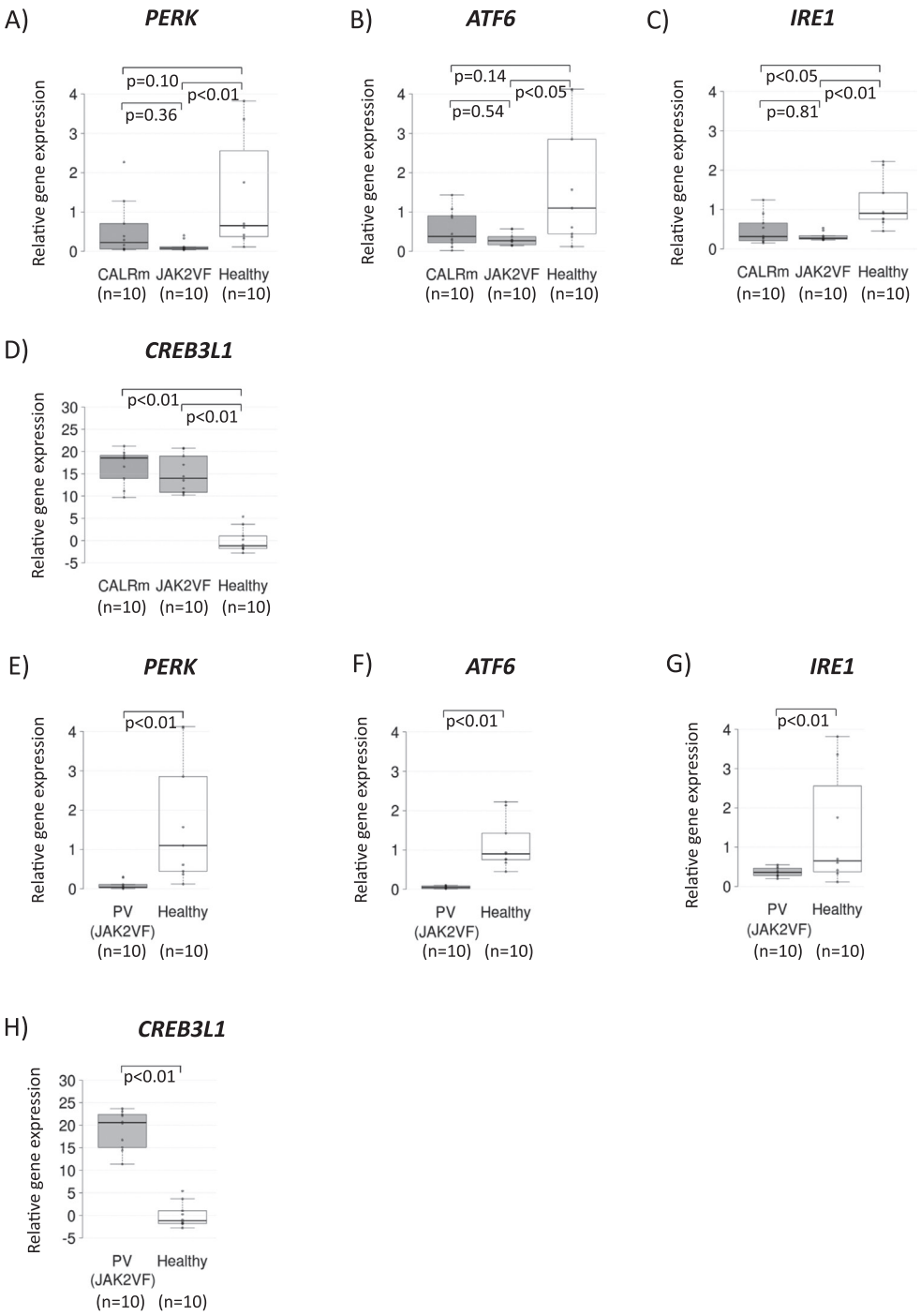

Fig. 3. Downregulation of UPR genes expression in platelets of patients with essential thrombocytopenia (ET) and patients with polycythemia vera (PV). Box plot comparing *PERK* (A), *ATF6* (B), *IRE1* (C) and *CREB3L1* (D) mRNA expression levels determined by reverse-transcription quantitative polymerase chain reaction in platelet RNA of patients with ET grouped by driver mutations (*CALR* mutation; CALRm, and *JAK2* V617F; JAK2VF) and those of patients with PV (*PERK* (E), *ATF6* (F), *IRE1* (G), and *CREB3L1* (H)) relative to healthy controls.

Fig. 2D). This association was also shown in myelofibrosis-free survival probability, similar to ET (Fig. 1H), where the patients that expressed higher levels of *CREB3L1* showed an increased chance for the development of fibrosis during the course of their disease (Fig. 2E). No strong correlation between elevated *CREB3L1* expression and EFS was observed (Fig. 2F). OS was not calculated in this cohort, because no death events were reported.

A similar analysis was performed in an available PMF cohort of 16 cases in which *CREB3L1* expression levels were analyzed. Due to the limited number of patients, we could not detect any meaningful association between *CREB3L1* expression and other clinical parameters (Supplemental Table 1).

3.3. UPR gene expression is downregulated in mRNA of Ph-MPN platelets

Our results show that *CREB3L1* is overexpressed in platelets of patients with MPNs, but the cause of this increase in expression has not yet been described. Since *CREB3L1* is a known part of the UPR stress response [39], we hypothesized a link between its increase and deregulation of the UPR pathway. Therefore, we investigated the expression of the three main genes of the UPR signaling (*PERK*, *ATF6*, and *IRE1*) [40] and *CREB3L1* expression in mRNAs of platelets of patients with ET and PV by RT-qPCR, compared with healthy controls. All three genes were significantly downregulated in ET harboring *JAK2* V617F ($p < 0.01$), while there was a trend of downregulation in *CALR*m ET cases (Fig. 3A-C), *CREB3L1* was overexpressed in both cases (Fig. 3D).

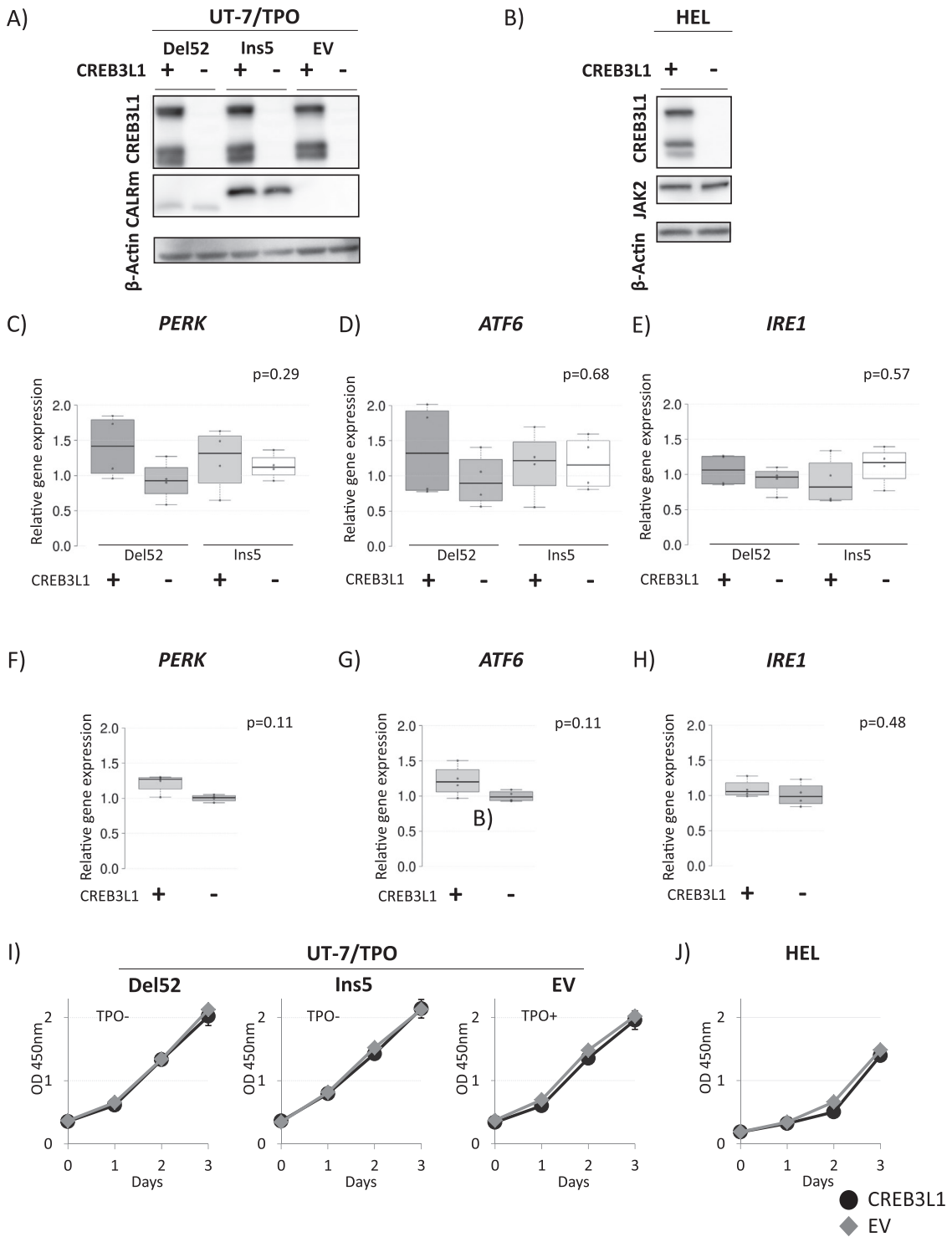

Fig. 4. No impact on UPR gene expression and cell proliferation by *CREB3L1* expression in UT-7/TPO cells expressing mutant CALR and HEL cells. Immunoblot analysis of extracts prepared from UT-7/TPO cells infected with virus expressing mutant CALR (Del52 or Ins5) infected with *CREB3L1* (A) and from HEL cells (JAK2 V617F positive cell line) infected with *CREB3L1* (B). β-Actin was shown as the loading control. Box plot comparing *PERK, ATF6*, and *IRE1* mRNA expression levels determined by reverse-transcription quantitative polymerase chain reaction in UT-7/TPO CALR Del52 and Ins5 with or without CREB3L1, and those in HEL cells (C-E and F-H, respectively). Cell proliferation assay of UT-7/TPO cells expressing CALR Del52, Ins5, or EV with (black circle) or without (gray diamond) CREB3L1 expression (I) and cell proliferation assay of HEL cells (J) (Data point from four individual experiments). The absorbance at 450 nm for counting viable cells by formazan dye measurement and the mean ± standard deviation of three replicates is shown. EV; empty vector.

In PV cases, with all the patients harboring *JAK2* V617F, all three UPR genes were significantly downregulated with *CREB3L1* over-expression when compared with healthy controls ($p < 0.01$, Fig. 3 E- H).

3.4. No impact on UPR gene expression and cell proliferation by CREB3L1 expression in MPN model cell lines

Since we found that UPR genes were downregulated in MPN

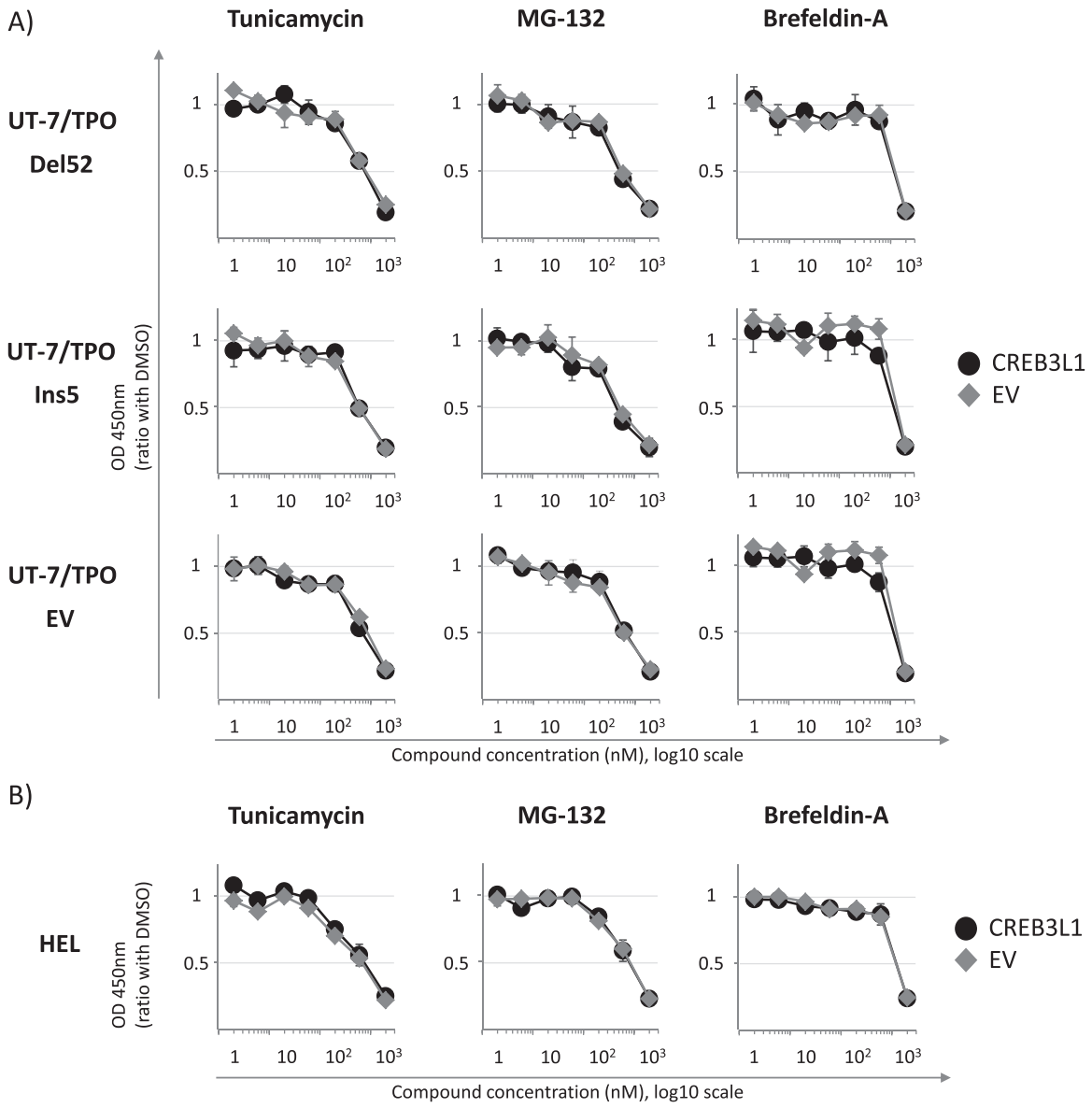

Fig. 5. No impact on sensitivity of UPR modifying drugs by CREB3L1 expression in UT-7/TPO cells expressing mutant CALR and HEL cells. Tunicamycin, MG-132, brefeldin A effects on the viability of UT-7/TPO CALR Del52, Ins5, and EV (A) with (black circle) or without (gray diamond) CREB3L1 at different concentrations and that of HEL cells (B). The absorbance at 450 nm was used to count viable cells by formazan dye measurement and compared with the relative dimethyl sulfoxide-only cell response. Mean ± standard deviation of three replicates is shown. EV; empty vector.

platelets, we hypothesized that *CREB3L1* overexpression induces UPR gene suppression. To examine this possibility in MPN model cell lines, we employed UT-7/TPO cells, a derivative acute-megakaryocytic leukemia TPO-dependent cell line that does not express *CREB3L1* [25], previously transduced with *Del52* or *Ins5 CALR* as ET models [23]. To overexpress *CREB3L1*, a second vector containing *CREB3L1* was transduced in these cells, concomitantly with another EV as a control. Specific CALRm and CREB3L1 protein expression were determined by immunoblotting (Fig. 4A) after cell line establishment. CREB3L1 blot shows three different bands, with the heaviest band being the full-length form and the two lighter being the CREB3L1 cut activated portion [41]. The same approach was used for HEL cells, a cell line that endogenously harbors a *JAK2* V617F homozygous mutation, which could function as a PV model and that does not express *CREB3L1* [24]. We transduced HEL cells with *CREB3L1*, and protein expression was determined by immunoblotting (Fig. 4B). CREB3L1 did not affect JAK2 expression, which maintained the same intensity level in both HEL cell lines, with or without CREB3L1. Since HEL is an erythroid cell line, a lineage in which *CREB3L1* was not overexpressed, we also analyzed SET-2 cells, a megakaryocytic cell line harboring a *JAK2* V617F heterozygous mutation[42], generating SET-2 expressing CREB3L1 or EV (Supplemental Fig. 4A).

CREB3L1 did not significantly change the expression of the three main UPR genes analyzed by RT-qPCR in both UT-7/TPO CALR Del52 and Ins5 cells (Fig. 4C-E), in HEL cells (Fig. 4F-H), or in SET-2 cells (Supplemental Fig. 4B-D) relative to EV control.

Even without modification of the UPR response, we hypothesized that *CREB3L1* could promote cell proliferation, acting alone or in synergy with MPN driver mutations as a possible oncogene. We analyzed cell proliferation by formazan dye absorbance during the course of 3 days from the above cell lines (Fig. 4I for UT-7/TPO Del52, Ins5, or EV; Fig. 4J for HEL cells; and Supplemental Fig. 4E for SET-2). No differences in proliferation were observed depending on the presence or absence of *CREB3L1* overexpression, which therefore did not affect cell proliferation in these models.

3.5. No impact on sensitivity of UPR modifying drugs by CREB3L1 expression

To further study the functional role of *CREB3L1* in response to ER stress in MPN model cell lines, we analyzed cell line responses to the following three drugs known to induce UPR: 1) tunicamycin, an inhibitor of the first step in the biosynthesis of N-linked glycans in protein synthesis [28]; 2) MG-132, a potent proteasome inhibitor [29]; and 3) brefeldin A, an inhibitor of protein transport from the ER to the Golgi apparatus [30]. These drugs increase the accumulation of misfolded proteins, resulting in an elevation in ER stress [43]. Consequently, we analyzed cell viability after exposure to different concentrations of each of the three compounds to test a possible link between *CREB3L1* overexpression and ER stress response. In accordance with the RT-qPCR data, *CREB3L1* overexpression did not induce significant changes in cell viability of the examined cell lines at any of the different drug concentrations (Fig. 5A-B; Supplemental Fig. 5). Therefore, CREB3L1 had little effect on ER stress in our cell line models and did not cause significant alterations in UPR gene expression or sensitivity to UPR inducers.

4. Discussion

In the current study, we showed the following: (1) *CREB3L1* overexpression is specific for MPN platelets; (2) higher *CREB3L1* expression levels are associated with higher risk of thrombosis in patients with ET and fibrotic transformation of PV; (3) expression of genes involved in UPR pathway is downregulated in MPN platelets; and (4) UPR gene expression, cell proliferation and sensitivity to ER stress are independence from *CREB3L1* overexpression in *JAK2* V617F- and *CALR*m-positive cell lines.

CREB3L1 overexpression was observed in platelets and no other PB cell fractions, implying that this gene expression is cell-specific. In the megakaryocytes maturation, endomitotic replication, cytoplasmic remodeling, and extra-membrane production occur, resulting in dynamic changes in the secretory pathway associated with UPR [44,45]. Therefore, we hypothesized that CREB3L1 is induced to make cells resistant to the extra ER stress [46] caused by ectopic activation of JAK2 signaling during megakaryocyte maturation in MPN.

Contrary to our hypothesis, the expression of other UPR genes, such as *PERK*, *ATF6*, and *IRE1,* was reduced in ET and PV patients than those in healthy controls. Correlation analysis of the pair value between *CREB3L1* and UPR genes did not show a significant correlation (Supplemental Figure 6A-C), suggesting that *CREB3L1* and other UPR genes were uncoupled in MPN megakaryocytes. It is noted, however, our platelet fraction contained some non-platelet cells (Supplemental Fig. 1), which are more than those in the previous studies[47–50]. The correlation analysis may be affected by the RNA derived from non-platelet cells. In addition to this, CREB3L1 overexpression had no impact on UPR pathway gene expression, cell proliferation, ER stress-inducing drug sensitivity, and proplatelet formation[51] in MPN model cells (Supplemental Figures 7A-C). Further study *in vivo* model is required to determine the biological meaning and underlying molecular mechanism of CREB3L1 induction in the megakaryocytes and any other, including hematopoietic stem cells, in MPN.

We found an association between *CREB3L1* overexpression, older age, and higher risk of thrombosis in ET for all risk scores, with a tendency for reduced OS and a higher risk of fibrotic transformation. Furthermore, ET is often seen in older patients in whom the course of the disease is longer, with an increase in disease burden and accumulation of several genetic alterations over time [52], which could further increase ER stress with the accumulation of unwanted proteins. In PV cases, where thrombocytosis is less prominent, *CREB3L1* expression is still associated with fibrotic transformation, an indicator of disease progression [38] and with increased LD levels, a well-known marker of cellular damage [38,53]. *CREB3L1* could indicate more advanced ET and PV diseases, in which platelet generation is accompanied by fibrotic accumulation, both leading to increased ER stress, for which *CREB3L1* could be the main counteracting response. However, fibrosis-free survival for ET and PV with high *CREB3L1* expression was statistically insignificant. In addition, the CREB3L1 level in platelet of PMF patients showed a smaller increase compared to those in PV or ET without significance (Supplemental Table 1), presumably due to the limited number of patients in this cohort.

To the best of our knowledge, this is the first study to report the *CREB3L1* relevance in MPNs, indicating specific platelet overexpression and concomitant reduction of UPR gene expression in patients' platelets. From a clinical perspective, *CREB3L1* is useful in discriminating reactive cases from neoplastic cases and in indicating high-risk diseases often associated with thrombosis and an increased probability of fibrotic transformation. Further clinical studies with a larger number of cases will help in validating the role of *CREB3L1* as a negative prognostic marker in MPNs.

Declarations of interest

Araki and Imai are employees of Meiji Seika Pharma and Komatsu has received a salary from PharmaEssentia Japan where he is a board member. All other authors have no declarations.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.leukres.2022.106883.

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