CBL mutations in myeloproliferative neoplasms are also found in the gene's *proline-rich* domain and in patients with the V617F*JAK2*

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ABSTRACT

Background
Despite the discovery of the p.V617F in *JAK2*, the molecular pathogenesis of some chronic myeloproliferative neoplasms remains unclear. Although very rare, different studies have identified *CBL* (Cas-Br-Murine ecotropic retroviral transforming sequence) mutations in V617F*JAK2*-negative patients, mainly located in the *RING finger* domain. In order to determine the frequency of *CBL* mutations in these diseases, we studied different regions of all CBL family genes (*CBL*, *CBLB* and *CBLC*) in a selected group of patients with myeloproliferative neoplasms. We also included V617F*JAK2*-positive patients to check whether mutations in *CBL* and *JAK2* are mutually exclusive events.

Design and Methods
Using denaturing high performance liquid chromatography, we screened for mutations in *CBL*, *CBLB* and *CBLC* in a group of 172 V617F*JAK2*-negative and 232 V617F*JAK2*-positive patients with myeloproliferative neoplasms not selected for loss of heterozygosity. The effect on cell proliferation of the mutations detected was analyzed on a 32D(FLT3) cell model.

Results
An initial screening of all coding exons of *CBL*, *CBLB* and *CBLC* in 44 V617F*JAK2*-negative samples revealed two new *CBL* mutations (p.C416W in the *RING finger* domain and p.A678V in the *proline-rich* domain). Analyses performed on 128 additional V617F*JAK2*-negative and 232 V617F*JAK2*-positive samples detected three *CBL* changes (p.T402HfsX29, p.P417R and p.S675C in two cases) in four V617F*JAK2*-positive patients. None of these mutations was found in 200 control samples. Cell proliferation assays showed that all of the mutations promoted hypersensitivity to interleukin-3 in 32D(FLT3) cells.

Conclusions
Although mutations described to date have been found in the *RING finger* domain and in the *linker* region of CBL, we found a similar frequency of mutations in the *proline-rich* domain. In addition, we found *CBL* mutations in both V617F*JAK2*-positive (4/232; 1.7%) and negative (2/172; 1.2%) patients and all of them promoted hypersensitivity to interleukin-3.

Key words: CBL, MPN, mutation analysis.

Citation: Aranaz P, Hurtado C, Erquiaga I, Miguéliz I, Ormazábal C, Cristobal I, García-Delgado M, Novo FJ, and Vizmanos JL. CBL *mutations in myeloproliferative neoplasms are also found in the gene's proline-rich domain and in patients with the V617F*JAK2. *Haematologica 2012;97(8):1234-1241. doi:10.3324/haematol.2011.052605*

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Funding: this work was funded with the help of the Spanish Ministry of Science and Innovation (SAF 2007-62473), the PIUNA Program of the University of Navarra, the Caja Navarra Foundation through the Program "You choose, you decide" (Project 10.830) and ISCIII-RTICC (RD06/0020/0078). PA received a predoctoral grant from the Government of Navarra.

Manuscript received on July 26, 2011. Revised version arrived on January 23, 2012. Manuscript accepted January 26, 2012.

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The online version of this article has a Supplementary Appendix.

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Introduction

BCR-ABL1-negative chronic myeloproliferative neoplasms (MPN) are a heterogeneous group of clonal hematologic malignancies characterized by abnormal proliferation and survival of one or more myeloid lineage cells. In some cases these diseases evolve to acute myeloid leukemia (AML). These hematologic neoplasms include both *classic* MPN [essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF)] and *atypical* MPN (such as chronic eosinophilic leukemia, chronic neutrophilic leukemia, hypereosinophilic syndrome, mast cell disease and myeloid neoplasms with eosinophilia, among others).[1]

In the late 1990s some genetic aberrations were described as molecular disease-causing events in these neoplasms, most of them via fusion genes resulting from reciprocal chromosomal translocations. Such fusions activate tyrosine kinases, playing a role similar to ABL1 in chronic myeloid leukemia.[2,3] However these fusions are very rare and most of them have been reported in one or two cases worldwide.[4]

This situation changed in 2005 with the description of the p.V617F mutation (valine to phenylalanine in amino acid 617) in *JAK2*, found not only in classic MPN but also in a small number of atypical MPN and other myeloid neoplasms.[5] Furthermore, it was found that most of the V617F*JAK2*-negative cases of PV had other transforming mutations in exon 12 of *JAK2*. Other gain-of-function mutations have also been described in genes coding for JAK-STAT receptors, such as *MPL* or *EPOR* in familial and sporadic cases of MPN.[6-10] However, to date it is not known whether these mutations cause the full phenotype or whether they cooperate with other still uncharacterized mutations. Thus, there is still a significant proportion of patients in whom the molecular disease-causing event remains to be discovered.

Recently, the application of single nucleotide polymorphism and comparative genomic hybridization array technologies has led to the identification of new mutations in loss of heterozygosity regions affecting genes such as *TET2*,[11] *ASXL1*,[12] *IKZF1*,[13] *RUNX1*,[14] *IDH1* and *IDH2*,[15] *EZH2*,[16] *NF1*,[17] and *CBL*.[18-23]

CBL (11q23) codes for a protein of the Cbl family of E3-ubiquitin ligases (CBL, CBLB and CBLC) that acts as a negative regulator of some cell signaling pathways, by promoting the ubiquitination of several signaling molecules including some tyrosine kinases. CBL proteins share a common structure, with a highly conserved tyrosine kinase-binding domain in the amino-terminal region that determines substrate specificity. The catalytic E3-ubiquitin ligase activity resides in the *RING finger* domain, which is separated from the tyroskine kinase binding domain by a *linker* region. CBL and CBLB have two other domains that are not well conserved in CBLC: a *proline-rich* region involved in the recognition of SH3-proteins, and the carboxy-terminal UBA domain that interacts with ubiquitin molecules allowing dimer formation.[24] CBL and CBLB play an important role in cell signaling in the majority of tissues, while CBLC activity seems to be restricted to epithelial cells.[25-27]

Over the last few years several groups have identified *CBL* mutations in different hematologic neoplasms, although most commonly in myelodysplastic syndromes (MDS)/MPN such as chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia.[18-23,28-39] These changes cause the loss of E3-ubiquitin ligase activity, resulting in deregulation of downstream targets and an increase in cell proliferation rates. To our knowledge, CBL mutations seem to be mutually exclusive with other mutations frequently found in these diseases such as *Ras* mutations, *FLT3*-ITD or V617F*JAK2*. In this study we searched for mutations in *CBL*, *CBLB* and *CBLC* in a group of 172 V617F*JAK2*-negative and 232 V617F*JAK2*-positive MPN patients not selected for loss of heterozygosity, using a denaturing high performance liquid chromatography (dHPLC) method. Although most of the mutations described previously have been found in the *RING finger* domain and in the *linker* region of CBL, we found novel mutations also in the *proline-rich* domain, both in V617F*JAK2*-positive and -negative patients.

Design and Methods

Samples

Blood samples were collected from 404 different Caucasian MPN patients without the *BCR-ABL1* fusion from several hospitals from the north of Spain. Informed consent was obtained from individual patients and the study was approved by the internal Ethics Committee. The first series of patients included 44 with V617F*JAK2*-negative MPN (4 diagnosed as PV, 15 as ET, 4 as PMF and 21 as atypical MPN). Later, a second series of 128 V617F*JAK2*-negative MPN patients (16 PV, 81 ET and 31 PMF) and 232 V617F*JAK2*-positive MPN patients (69 PV, 149 ET and 14 PMF) were included. The presence/absence of V617F*JAK2* mutation was determined in all patients by amplification refractory mutation system polymerase chain reaction (ARMS-PCR).[40] In addition, all 404 samples were negative for the presence of *MPL* p.W515 mutations by dHPLC. Human leukemia cell lines HEL, M07e, UKE-1 and SET-2 were also included in the study (Table 1).

Initial mutational screening by dHPLC included 20 healthy (no disease) samples used as controls in order to check the frequency of sequence changes observed in our population. For those fragments in which we found sequence variants in patients, we also included 180 additional control samples in order to rule out that the changes detected were population polymorphisms.

Cell lines

Cell proliferation assays were performed on 32Dcl3 (32D) murine myeloid cells (DSMZ N. ACC411) incubated at 37°C in 5% CO_2 and maintained in 90% RPMI 1640 medium with 10% fetal bovine serum supplemented with 10 ng/mL murine interleukin-3 (Recombinant Mouse IL3, Cat #PMC0035, Gibco®, Invitrogen Ltd., Paisley, UK).

Table 1. Frequency of *CBL* mutations found in our series.

		N.	% *C-CBL* mutated	Cummulative frequency
V617F*JAK2*-negative MPN (n = 172)	PV	20	0% (0/20)	2/172 (1.2%)
	ET	96	0% (0/96)	
	PMF	35	0% (0/35)	
	aMPN	21	9.5% (2/21)	
V617F*JAK2*-positive MPN (n = 232)	PV	69	1.5% (1/69)	4/232 (1.7%)
	ET	149	1.4% (2/149)	
	PMF	14	7.1% (1/14)	
Cell lines		4	0% (0/4)	0%
Healthy controls		200	0% (0/200)	0%

aMPN: atypical MPN.

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Plasmids

Plasmids with tagged human open reading frames in pCMV6-AC-GFP vectors were purchased from Origene Technologies (Cat #RG214069 for *CBL*, RG206047 for *CBLB* and RG205130 for *CBLC*). The tagged human cDNA clone for *FLT3* was also purchased from Origene Technologies as pCMV6-Entry vector (Cat #RC211459) and subcloned into pCMV6-AC-RFP vector (Cat #PS100034). These pCMV6-AC vectors carried the Neo^R gene. The pCMV–HA ubiquitin vector was a gift from Dr. Francis Grand from Wessex Regional Genetics Laboratory (Salisbury, UK).

Denaturing high performance liquid chromatography analysis

Genomic DNA was obtained from all the samples and amplified with *GenomiPhi* v2.0 (GE Healthcare, Piscataway, NJ, USA) in order to obtain enough material for mutational screening. All mutations were confirmed using the original unamplified sample and no discrepancies were observed with whole-genome amplified DNA.

We designed primers with *Primer3*[41] to amplify all coding exons of the three screened genes (*CBL*, *CBLB* and *CBLC*) in flanking introns. For each fragment we also designed a mutant primer introducing a nucleotide change in the forward or reverse primer, depending on the corresponding melting profile, to create a control mutated fragment to validate each dHPLC assay. Melting profiles for PCR fragments, solvent gradients and temperature conditions were calculated by Navigator™ Software v1.6.2 (Transgenomic Ltd., Omaha, NE, USA) and validated experimentally. All the analyses were performed on a WAVE® 4500HT System (Transgenomic Ltd., Omaha, NE, USA) with a DNASep® HT cartridge. *Online Supplementary Table S1* contains a list of primers, the sizes of the amplified fragments and dHPLC conditions.

PCR reactions were performed with AmpliTaq™ Gold (Applied Biosystems, Foster City, CA, USA) using standard protocols. After cycling, samples were subjected to several cycles of heating and cooling in order to create heteroduplex molecules to improve mutation detection by dHPLC. For each fragment, we sequenced two samples of each different elution profile. Results were analyzed with Mutation Surveyor v3.10 (SoftGenetics LLC, State College, PA, USA) and compared to genomic reference sequences (ENSG00000110395 for *CBL*, ENSG00000114423 for *CBLB* and ENSG00000142273 for *CBLC*).

All coding exons of *CBL*, *CBLB* and *CBLC* were initially analyzed in a group of 44 V617F*JAK2*-negative patients (4 PV, 4 PMF, 15 ET and 21 atypical MPN). In light of the results we analyzed the *RING finger* domain coding exons (exons 8 and 9 from *CBL*, exons 9 and 10 from *CBLB* and exons 7 and 8 from *CBLC*) in 128 V617F*JAK2*-negative (16 PV, 31 PMF, 81 ET) and in 232 V617F*JAK2*-positive MPN (69 PV, 14 PMF, 149 ET), as well as in human leukemia cell lines M07e, HEL, SET-2 and UKE-1. We also included *CBL* exon 12 in this extended analysis because we observed a p.A678V change in one sample from the initial series.

CBL *exon 8 deletions*

Some of the mutations described for *CBL* are large deletions involving exon 8 (*RING finger* domain)[18,21-23,29,30,32,37,42] and the design of our mutation screening assay was not able to detect some of them. We, therefore, designed a new PCR assay with primers located in exon 7 and intron 9 (E7Fw: 5'-TCCTGATGGACGAAATCAGA-3'; E9-Rv: 5'-CTCACAATGGATTTTGCCAGT-3') which would amplify a normal fragment of 989 bp. With this assay, any large deletion of exon 8 would be detected as a product of smaller size.

Site-directed mutagenesis

All missense mutations detected for each gene were functionally tested. Mutants p.R420Q (used as the control mutant), p.C416W, p.P417R, p.T402HfsX29, p.S675C and p.A678V for *CBL*; p.R462W for *CBLB* and p.Q419PfsX81, p.P435S and p.E392K for *CBLC* were obtained using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA, USA) from the original plasmids.

Transfection

Transfections were performed with Amaxa Nucleofector® Device II technology (Lonza Cologne GmbH, Basel, Switzerland) according to standard protocols. Cells of the 32D cell line in exponential growth were first transfected with *FLT3* vector and maintained in medium until a second CBL/Ubi transfection. From the first transfection with *FLT3* vector, cells were grown with Geneticin (G-418 sulfate, Cat #11811 Gibco®, Invitrogen Ltd., Paisley, UK) to select those clones that had incorporated the vector.

Cell proliferation assays

Proliferation analysis was performed with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Ref #G3580, Promega Corp, Madison, WI, USA) according to standard protocols, comparing cells transfected with wild-type *CBL* (*CBL*, *CBLB* or *CBLC* in each case) with cells transfected with mutant *CBL* during 3 or 4 days, in triplicate. In each case we carried out four different experiments, also including 32D(FLT3) cells transfected with pCMV6-AC-GFP and mock-transfected 32D(FLT3) cells as controls. In all cases cells were supplemented with 10 ng/mL recombinant human FLT3-ligand (Cat #GF038, Millipore, Temecula, CA, USA) and with 10 ng/mL murine interleukin-3 (Recombinant Mouse IL3, Cat #PMC0035, Gibco®, Invitrogen Ltd., Paisley, UK). As the positive mutant control we used p.R420Q, a previously described *CBL* mutant with an effect on cell proliferation.[28]

Statistical analysis

Results from MTS proliferation assays were compared using the Student's t-test implemented in UNStat (a free tool available at *http://www.unav.es/departamento/genetica/unstat*).

Results

Mutational screening

In the initial screening of all coding exons of *CBL*, *CBLB* and *CBLC* in 44 patients with V617F*JAK2*-negative MPN we detected two missense changes not previously described in *CBL* (2/44; 4.5%). In this first series we also found three missense changes in *CBLC* described as single nucleotide polymorphisms (rs35457630, rs3208856, rs116023028) in the *RING finger* domain and *proline-rich* region. These changes were detected in samples from both patients and controls. No missense changes were detected in *CBLB*.

CBL changes (p.C416W or g.72251T>G and p.A678V or g.81664C>T) were found in patients diagnosed with atypical MPN, although the disease in the patient with p.A678V later evolved to CMML because of the development of dysplastic features. Whereas p.C416W affected the *RING finger* domain, like other mutations previously reported, p.A678V was located in exon 12, which codes for the *proline-rich* domain of CBL (see Figure 1). For this reason, we decided to include this exon in the analysis of *CBL* in an additional group of patients.

When we analyzed exon 12 of *CBL* and the *RING finger*

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domains of *CBL*, *CBLB* and *CBLC* in the additional series of samples (128 V617F*JAK2*-negative and 232 V617F*JAK2*-positive patients), we found three *CBL* changes in four V617F*JAK2*-positive patients (4/232, 1.7%). The first one (detected in a patient with ET) was a not previously reported g.71955_71955A deletion in exon 8. This is a frameshift change that truncates the *RING finger* domain with loss of the *proline-rich* and UBA carboxy-terminal domains (p.T402HfsX29) (Figure 1). The second change was a substitution g.72253C>G (p.P417R) in exon 9 in a patient diagnosed with PMF, also affecting the *RING finger* domain and previously identified in a patient with juvenile myelomonocytic leukemia.[37] Finally, the third *CBL* mutation was a not previously reported g.81655C>G substitution (p.S675C) in exon 12 (*proline-rich* region). Remarkably, this change was detected in two different V617F*JAK2*-positive patients, one with ET and the other with PV. None of the 200 control samples analyzed showed any of these changes. *CBL* exon 8 deletions were not observed in any case.

We also detected a not previously reported substitution (g.149486C>T, p.R462W) in the *RING finger* domain of *CBLB* in a sample from a V617F*JAK2*-positive patient with PV (1/232; 0.4%). In *CBLC*, we detected one frameshift change (g.15702_15703insC, p.Q419PfsX81), in a patient

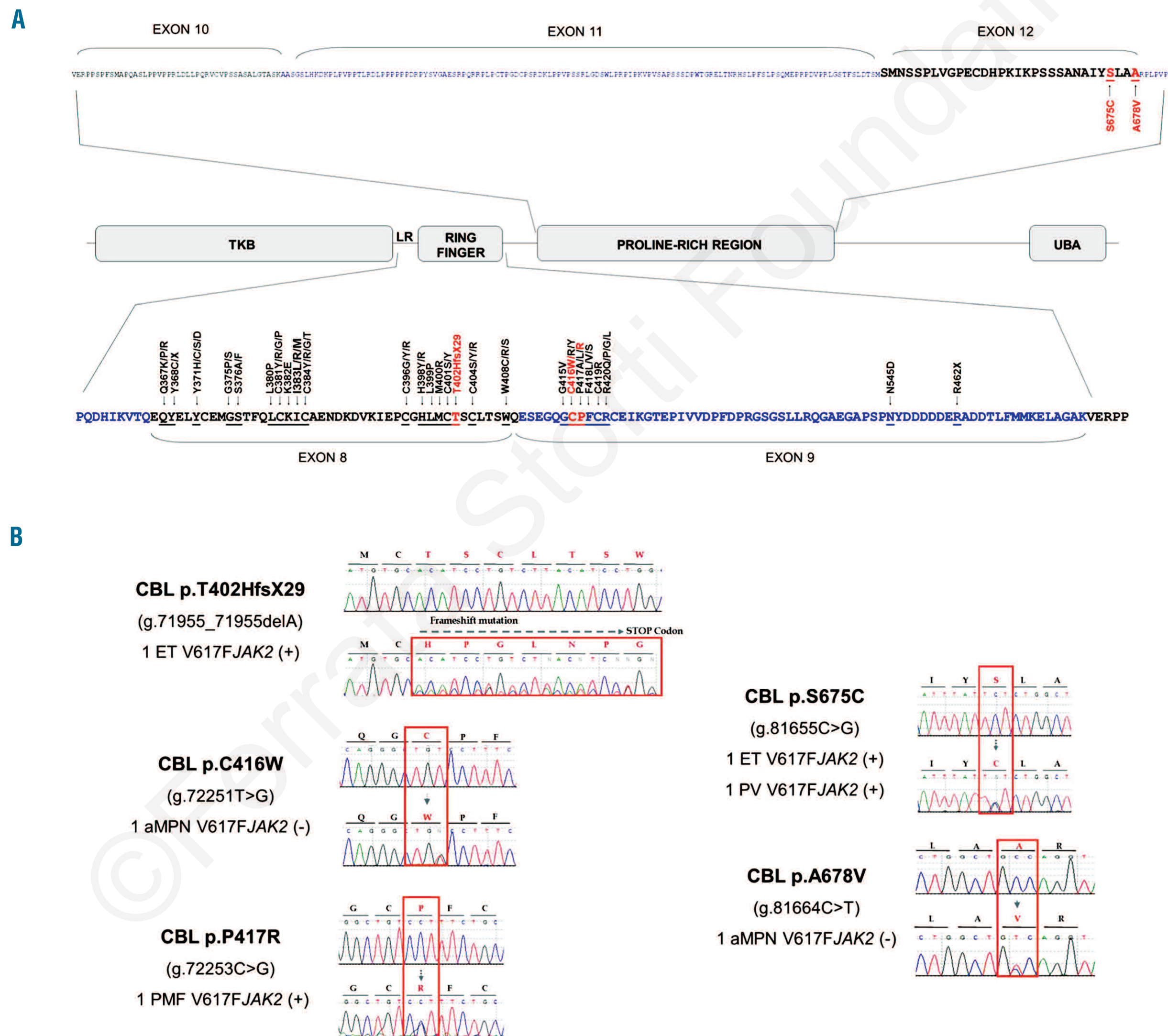

Figure 1. (A) CBL mutations described to date in myeloid malignancies. Mutations found in our analysis are colored in red. In contrast to other studies in which mutations were found in the *linker* region (LR) and the *RING finger* domain, we also found two novel changes in *CBL* exon 12 (*proline-rich* domain). (B) Sequencing results observed for each mutation. p.T402HfsX29 is a frameshift mutation (g.71955_71955delA) from the amino acid Thr402 that results in a truncated protein lacking carboxy-terminal domains. This change was identified in patient with ET. p.C416W was due to a g.72251T>G substitution identified in a patient with an atypical MPN. We also found a previously reported change, p.P417R (g.72253C>G) in a patient with PMF. Changes found in exon 12 were p.S675C (g.81655C>G) in two cases (one with PV and another with ET) and p.A678V (g.81664C>T) in an atypical MPN that later evolved to CMML. While p.C416W and p.A678V changes were detected in patients negative for the p.V617F*JAK2* mutation, p.T402HfsX29, p.P417R and p.S675C changes were found in patients with the p.V617F*JAK2* mutation.

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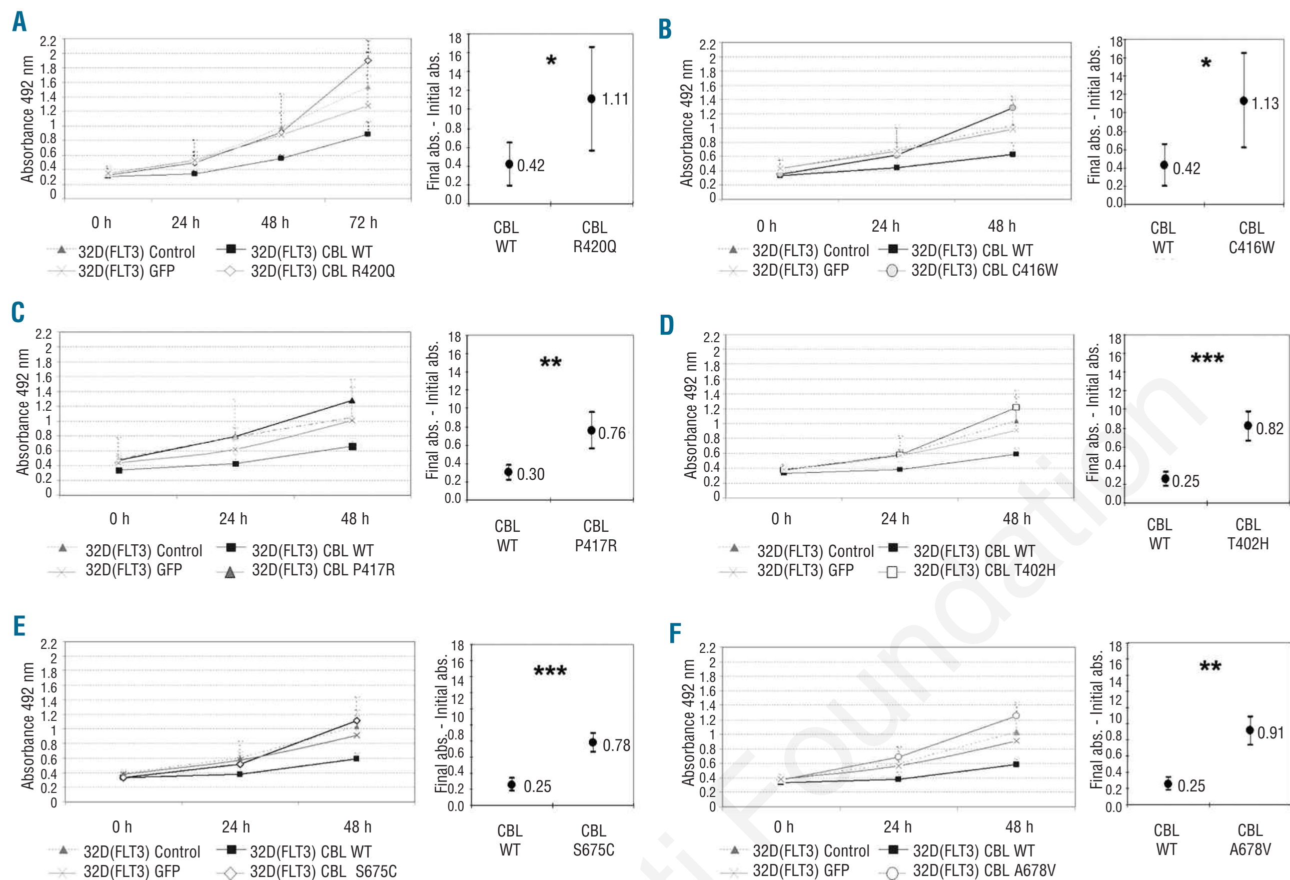

Figure 2. (A to F) **All missense mutations detected in CBL were functionally tested. Left, results of cell proliferation assays corresponding to four different transfections for each mutation. Two transfection controls (cells mock-transfected and cells transfected with GFP control vector) were included. Right, proliferation rates (final absorbance - initial absorbance) obtained for each mutant compared to wild-type (Student's t-test) pooling data from four separate experiments. (A) Left, results of proliferation assays corresponding to the CBL p.R420Q mutant control. Seventy-two hours after the start of the assay, cells transfected with the mutant vector showed a significantly higher absorbance value than those transfected with the wild-type vector (*P*=0.034). Right, the proliferation rate of cells with p.R420Q was significantly higher than cells with wild-type (*P*=0.040). (B) Left, results of assays for CBL p.C416W mutation (*P*=0.003). Right, proliferation rate compared that of wild-type cells (*P*=0.023). (C) Left, results of assays for CBL p.P417R mutation (*P*=0.003). Right, proliferation rate compared to wild-type (*P*=0.003). (D) Left, results of assays for CBL p.T402HfsX29 mutation (*P*=0.001). Right, proliferation rate compared to wild-type (*P*<0.001). (E) Left, results of assays for CBL p.S675C mutation (*P*<0.001). Right, proliferation rate compared to wild-type (*P*<0.001). (F) Left, results of assays for CBL p.A678V mutation (*P*<0.001). Right, proliferation rate compared to wild-type (*P*=0.001).**

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with V617F*JAK2*-negative PV, which has been described as a polymorphism (rs66944506).

None of the cell lines included in these analyses (HEL, SET-2, UKE-1 and M07e) showed any *CBL*, *CBLB* or *CBLC* mutation.

CBL *mutations promote hypersensitivity to interleukin-3 in 32D(FLT3) cells*

A significantly higher number of cells was observed in 32D(FLT3) cells transfected with *CBL* mutants than with wild-type *CBL* (*P*<0.05, Figure 2), grouping data from four independent cell proliferation assays. In addition, cells transfected with mutant vectors showed significantly higher proliferation rates in all cases (*P*<0.05, Figure 2) and with stronger effects than those observed for the p.R420Q control mutation.

By contrast, assays for p.R462W in *CBLB* and for p.Q419PfsX81, p.E392K and p.P435S in *CBLC* (*Online Supplementary Figure S1*) showed no significant differences (*P*>0.05) in proliferation rates.

Discussion

In the last few years the detection of regions with acquired loss of heterozygosity in some patients, mainly caused by acquired uniparental disomy, has allowed the identification of candidate genes that may be mutated in myeloid neoplasms. One of these genes is *CBL*, which codes for an E3-ubiquitin ligase protein. Cbl family proteins (CBL, CBLB and CBLC) play an important role as regulators of several signaling pathways promoting the ubiquitination and degradation of some RTK and CTK,[44] many of which are involved in these diseases.[4,45,46]

The first *CBL* mutation identified was p.R420Q, affecting the *RING finger* domain in a patient with AML.[28] Subsequently, other mutations have been reported with variable frequencies in myeloid neoplasms, affecting not only the *RING finger* domain but also the *linker* region (Figure 1). These events have been observed in 1-33% cases of secondary AML, 1-7% of MPN and 2-33% of MDS/MPN and AML,[18-23,28-39] but their frequencies could be as high as 85-

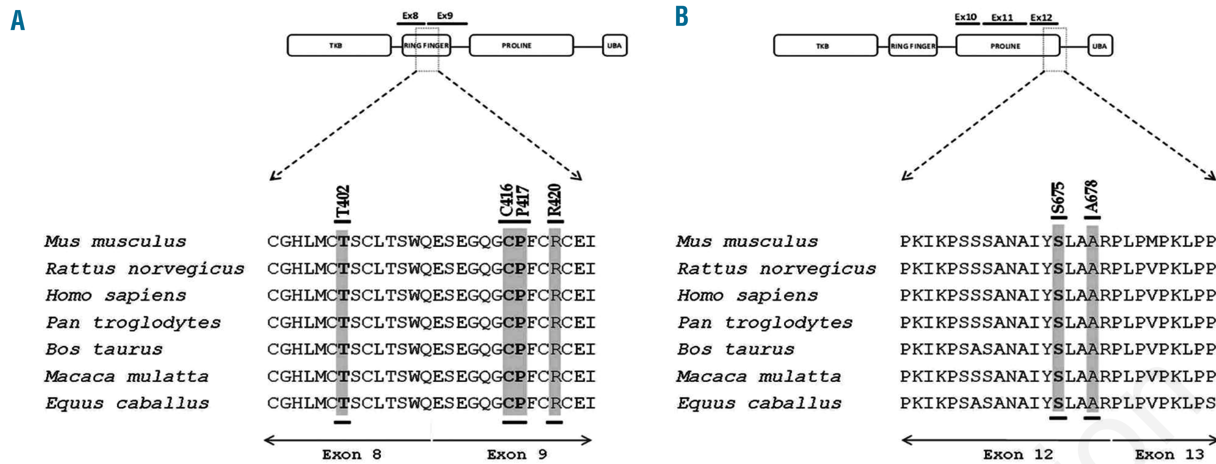

Figure 3. Evolutionary conservation of the *CBL* regions in which mutations have been detected. Sequence comparison between humans (*Homo sapiens*) and other mammals (*Mus musculus, Rattus norvegicus, Pan troglodytes, Bos taurus, Macaca mulatta and Equus caballus*) in the *RING finger* domain (A) and in the *proline-rich* domain (B). Both regions are highly conserved, suggesting an important functional role.

90% in patients with loss of heterozygosity in 11q.[18-20,22,23,33] Some reports have also described that 7% of patients with non-small cell lung cancer have *CBL* mutations, so this gene can be mutated in other types of tumor.[47] *CBLB* mutations and *CBLC* missense polymorphisms affecting the *RING finger* domain have also been described in myeloid neoplasms but at a lower frequency and with unknown effects.[22,23,29]

In this study we searched for mutations in *CBL, CBLB* and *CBLC* in a cohort of 404 V617F*JAK2*-negative and -positive MPN patients not selected for the presence of loss of heterozygosity in 11q. Our results show that *CBL* is mutated in V617F*JAK2*-negative MPN at a frequency similar to that previously reported (p.C416W and p.A678V; 1.2%, 2/172, Table 1).[49] Both patients with mutations were initially diagnosed as having atypical MPN (2/21; 9.5%), although in one of them the disease evolved to CMML due to the development of dysplastic features. CMML is the disease with the highest frequency of *CBL* mutations reported to date.[18-20,22,34] None of these mutations had been previously described and, notably, p.A678V was located in the *proline-rich* domain. In V617*JAK2*-positive MPN we found two mutations affecting the *RING finger* domain (p.T402HfsX29 in a patient with ET and p.P417R in a patient with PMF) and a recurrent change in the *proline-rich* domain (p.S675C in a patient with ET and in another one with PV) of CBL. Although *TET2*, *ASXL1* and *JAK2* mutations have been found concurrently,[50] *CBL* mutations and V617F*JAK2* seemed to be mutually exclusive events.[13,21,26,51] However, we have found a similar frequency of *CBL* mutations in both V617F*JAK2*-positive and V617F*JAK2*-negative patients (Table 1), suggesting that the prevalence of *CBL* mutations could increase if V617F*JAK2*-positive patients were also included in *CBL* mutational studies. Unfortunately, we cannot know whether both mutations are in the same or in different clones or whether they are monoallelic or biallelic because of the type of sample available. None of the V617F*JAK2*-positive cell lines analyzed (HEL, SET-2 and UKE-1) showed *CBL* mutations that might help us to elucidate how both events could cooperate to drive the disease.[43]

In order to determine the effect of all these mutations on cell proliferation, *in vitro* functional assays were performed. All *CBL* mutations induced a hyperproliferative response to interleukin-3 in the 32D(FLT3) model, similar to that induced by the well-characterized p.R420Q mutation.[28,31] This effect was not observed for the mutations detected in *CBLB* and *CBLC*. *CBL* was initially described as a putative tumor suppressor gene because of its negative regulatory function as an E3 ubiquitin ligase. Most of the mutations reported are located in conserved residues of the *linker* region and *RING finger* domain and could impair this regulatory function.[44,46,52] *RING finger* domain mutations p.T402HfsX29, p.C416W and p.P417R described in this work also affect conserved residues of the protein (Figure 3) with a similar effect on the loss of activity of CBL.

Notably, we found two additional, novel mutations (one of them recurrent) affecting conserved residues in the *proline-rich* region (p.A678V and p.S675C, Figure 3) which also promote cell proliferation. In fact, we found similar frequencies of mutations in *RING finger* and *proline-rich* domains. The *proline-rich* region is essential for the interaction of CBL with the adaptor proteins (such as Grb2 and FRs2α) needed to maintain a stable attachment between CBL and its substrate,[52] with proteins involved in the endocytosis of target receptors (such as SH3KBP1)[46] and with several signaling proteins (such as the Src family).[46]

As in previous studies, we observed that mock-transfected cells showed greater growth than cells transfected with wild-type *CBL*, but less than cells transfected with mutant *CBL*. This fact is concordant with the proposal by some authors of a dominant negative effect of CBL mutant forms on endogenous wild-type CBL, making it unable to perform its negative regulatory function and promoting intracellular signaling and higher cell proliferation rates.[18,26,27] However, *in vivo* studies have shown that the presence of gain-of-function mutants with a dominant effect over endogenous CBL is not enough to develop a myeloproliferative disease.[18,26,27] A possible explanation for this phenomenon could be the activity of wild-type CBL as a positive regulator of cell growth contributing to the activation of pathways such as PI3K, Ras/MAPK and Src.[52,53] Under normal conditions,

the negative regulatory activity of CBL could mask its activity as a positive regulator, but the lack of E3-ubiquitin ligase activity could reveal its signaling enhancing activity.[27] This could be the reason for the non-transforming effect of the p.R462W CBLB mutant in 32D(FLT3) cells. CBLB does not show the positive regulatory effects of CBL[52] and perhaps the loss of its E3-ubiquitin ligase activity is not enough to promote cell proliferation. In fact, although CBLB activity seems to be similar to that of CBL in hematologic cells, very few cases of myeloid neoplasms with CBLB mutations have been reported.[25,29]

Finally, the results obtained in *CBLC* suggest that missense single nucleotide polymorphisms do not increase cell proliferation in our model. Wild-type *CBLC* induced higher proliferation rates in 32D(FLT3) cells than wild-type *CBL* in all assays (*Online Supplementary Figure S1*) possibly due to the absence of an inhibitory role of CBLC in hematologic cells.[25,54,55]

In conclusion, we have identified mutations in the *proline-rich* region of *CBL* in patients with MPN and also in V617F*JAK2*-positive patients. Although the entire *CBL* coding sequence has been investigated in some studies (by sequencing, not by dHPLC),[18,19,22,32] most research in recent years has focused only on exons coding for the *linker* region and *RING finger* domain[20,21,23,28,30,31,33-36,38] and in patients without other frequent genetic aberrations, such as mutations in *JAK2*.[26] *Proline-rich* domain mutations (p.S675C and p.A678V) confer hypersensitivity to cytokines in the 32D(FLT3) model in a similar way to *RING finger* domain mutations (p.T402HfsX29, p.C416W and p.P427R), suggesting that they should also be considered in analyses of *CBL*. Although these events seem to be rare in MPN, our data highlight the importance of reevaluating the prevalence of *CBL* mutations in other regions of the gene in myeloid neoplasms. This could be of special interest in MDS/MPN because of the high incidence of *CBL* mutations in these diseases. In addition, further functional analyses of these genetic events could help us to understand the cellular functions of CBL and the role of the different protein domains. It is well known that CBL activity is mediated by the activation of different RTK, so the use of tyrosine kinase inhibitors (such as anti-FLT3) or other signal transduction inhibitors could also be effective in the treatment of patients with *CBL* mutations.[27,32,56]

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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