Concomitant constitutive LNK and NFE2 mutation with loss of sumoylation in a case of hereditary thrombocythemia

Lukas Clemens Böckelmann,[1] Titiksha Basu,[1] Albert Gründer,[1] Wei Wang,[1] Jan Breucker,[2] Sandra Kaiser,[1] Andrea Pichler[2] and Heike Luise Pahl[1]

[1]Department of Medicine I, Medical Center, University of Freiburg, Faculty of Medicine, University of Freiburg and [2]Department of Epigenetics, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

Correspondence: HEIKE L. PAHL - heike.pahl@uniklinik-freiburg.de

doi:10.3324/haematol.2020.246587

Supplemental Data

Supplemental Table S1: Clinical course, platelet counts and bone marrow histopathology of patient 10579, showing constitutive NFE2-K368X and LNK-E208Q mutations.

Date	Platelet count [/nl]	Comments
10/2008	854	• **Initial diagnosis with bone marrow biopsy*** • **Symptoms: headaches and intermittent angina pectoris** • Normal serum electrophoresis • Spleen size (ultrasound): 3.7 x 8.4 cm • Hb 12.4 g/dl • Normal serum range for iron, vitamin B12, folic acid and CRP • No signs of hemorrhage or bleeding • No acute infection, no fever, no B-symptoms • Secondary diagnoses: hypertension, coronary artery disease, hypertrophic cardiomyopathy
02/2009	1393	• Start of hydroxyurea treatment (1000 mg/d) • Subsequent relieve of headaches and angina pectoris
09/2010	438	• Continued hydroxyurea treatment (1000 mg/d)
12/2010	508	
05/2011	688	
09/2011	769	
10/2011	556	
01/2012	684	
02/2012	588	

***Histopathologic evaluation of bone marrow biopsy:** Increased number of enlarged, mature megakaryocytes, irregularly distributed, without formation of clusters. No left-shift in granulopoiesis or erythropoiesis. Some singular siderophages. No ring sideroblasts. No reticulin accumulation/fibrosis in H&E or on silver staining.

Immunohistochemistry: Normal distribution of $CD20^+$ lymphocytes, $CD34^+$ blasts < 1%, $CD117^+$ progenitors 2%, increased number of $CD61^+$ enlarged megakaryocytes. DNA was extracted from the paraffin block and assessed for the JAK2V617F mutation, which revealed a wildtype gene on both alleles.

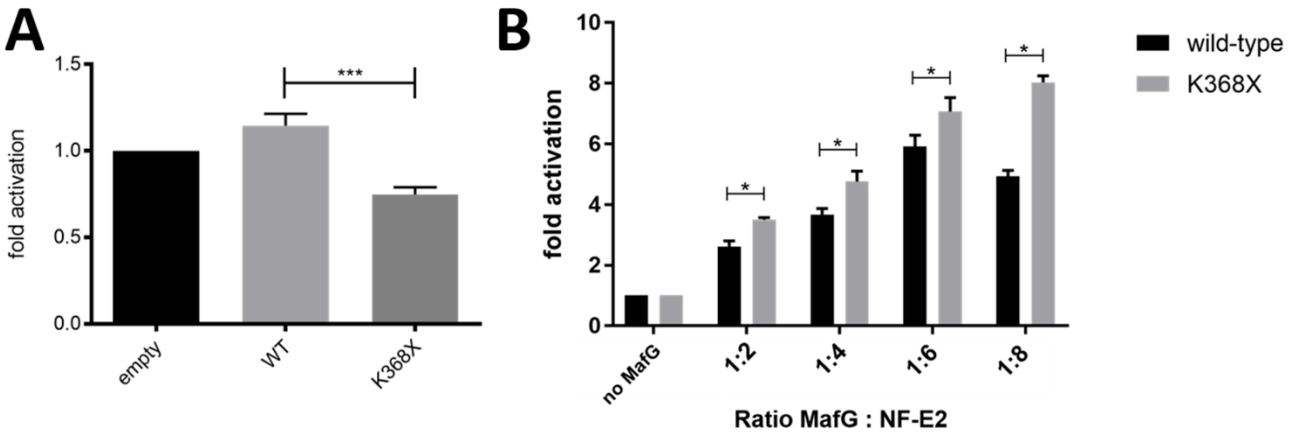
Supplemental Table S2: 34 myeloid neoplasm associated genes were sequenced using an NGS-based gene panel. Sequenced exons (E) are given in brackets. If nothing is stated, the gene was sequenced entirely.

ASXL1 (E13)	DNMT3A (E07-23)	IDH2 (E04)	NPM1 (E11)	SF3B1 (E11-16)
BCOR	ETV6	JAK2 (E11-16)	NRAS (E02-03)	SRSF2
BCR-ABL1	EZH2	KIT (E08-14;E17)	PHF6	TET2
BRAF (E15)	FLT3-TKD (E20)	KLHL6	PTPN11	TP53 (E04-11)
CALR (E09)	GATA1	KRAS (E02-03)	RUNX1	U2AF1
CBL (E08-09)	GATA2	MPL (E10)	SETBP1 (E04)	WT1
CSF3R	IDH1 (E04)	NFE2	SF1	

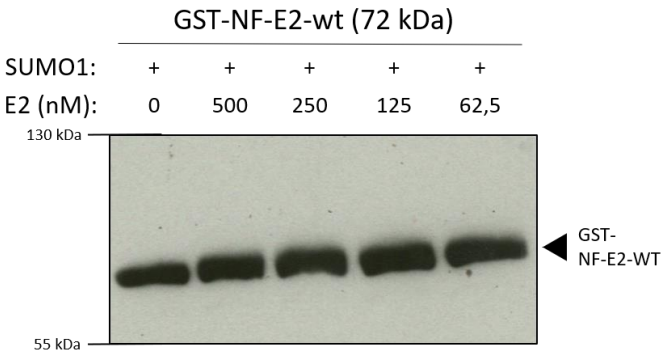
Supplemental Table S3: Primers used to amplify and sequence the LNK (SH2B3) and THPO genes.

Gene	Coding Region	Primer	
THPO	aa1-77 (+5´UTR)	Forward	GTTAAATGTTCACTCTTCTTG
		Reverse	CTTAGGGAAGCCAAGGTTAG
THPO	aa 78-134	Forward	CAGTTCTCAGCCTGTATGATTC
		Reverse	CTCAGGCCTCCCTTGTCTAAG
LNK	aa 1-244	Forward	GGTCGCGTTGGATTTCTGCTG
		Reverse	CTAGTTCCCTCTGGACACTC
LNK	aa 245-341	Forward	GATCTCAGTGTGAATGGTG
		Reverse	GCACCTGTTAGATAAGATGG
LNK	aa 342-470	Forward	CTCAGCCCAGGACATAAG
		Reverse	GTCTGCAGCAAGCCTCTAC
LNK	aa 471-577	Forward	GTCTGACCCTACTGCCCTTTG
		Reverse	CAAACTGGCACGAGCAGTTAAC

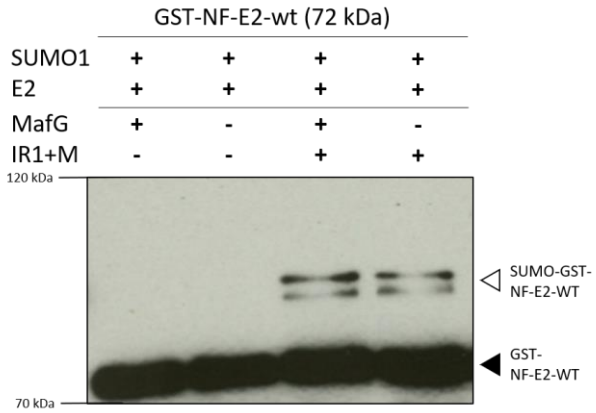
Supplemental Figure S1: Dual luciferase reporter assay with and without addition of NFE2 small subunit MafG. (A and B) The pRBGP2 plasmid containing tandemly arranged NFE2 binding sites from the chicken β-globin enhancer driving expression of the firefly luciferase reporter gene was transfected into HEK293T cells together with or without expression vectors for MafG as well as NFE2-WT or NFE2-K368X as indicated.[1] **(A)** Luciferase activity in the absence of MafG. **(B)** Luciferase activity obtained by cotransfection of NFE2-WT or NFE2-K368X, as indicated, with various ratios of MafG. As the MafG homodimer is an active repressor of transcription[2,3] the MafG:NFE2 ratio providing optimal transcriptional activation was determined. All further experiments were repeated with a ratio of 1:8 (MafG:NFE2), including those in Figure 1C. **(A and B)** Firefly luciferase activity was measured 24 h after transfection and was normalized to constitutively expressed renilla luciferase activity. Activity for transfection with an empty vector (A) or with MafG alone (B) was set as 1 and fold activity relative to this control is depicted. Experiments were carried out in duplicates in three independent assays each. Bar graphs represent the mean + SEM. Data were analyzed for statistical significance by two-tailed Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



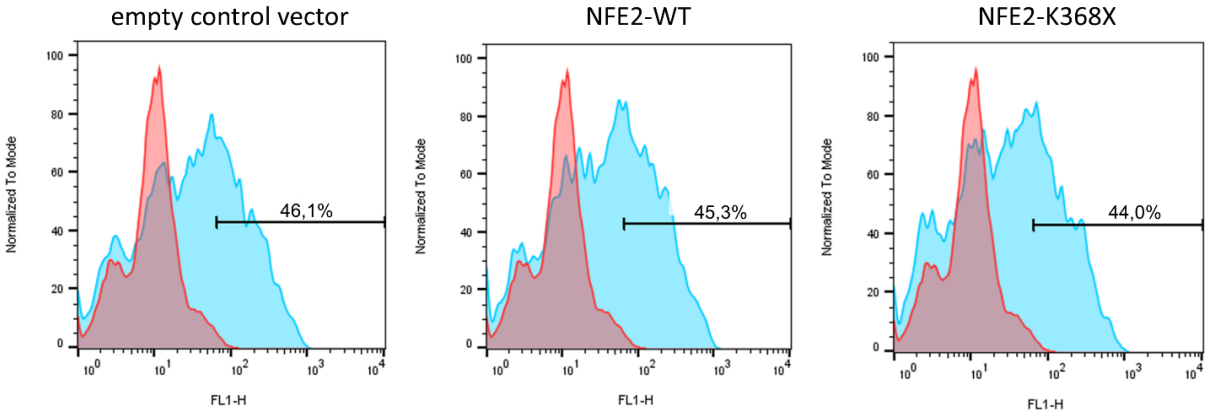
Supplemental Figure S2: In vitro sumoylation assay using different concentrations of UBC9. Recombinantly expressed and purified GST-NFE2-WT (72 kDa) was used in a concentration of 300 nM together with 100 nM E1 activating Enzyme Aos1/Uba2, 5 µM SUMO1 and different concentrations of E2 conjugating enzyme UBC9 as indicated. The reaction mixture was subjected to SDS-PAGE and immunoblotting using an anti-NFE2 antibody.



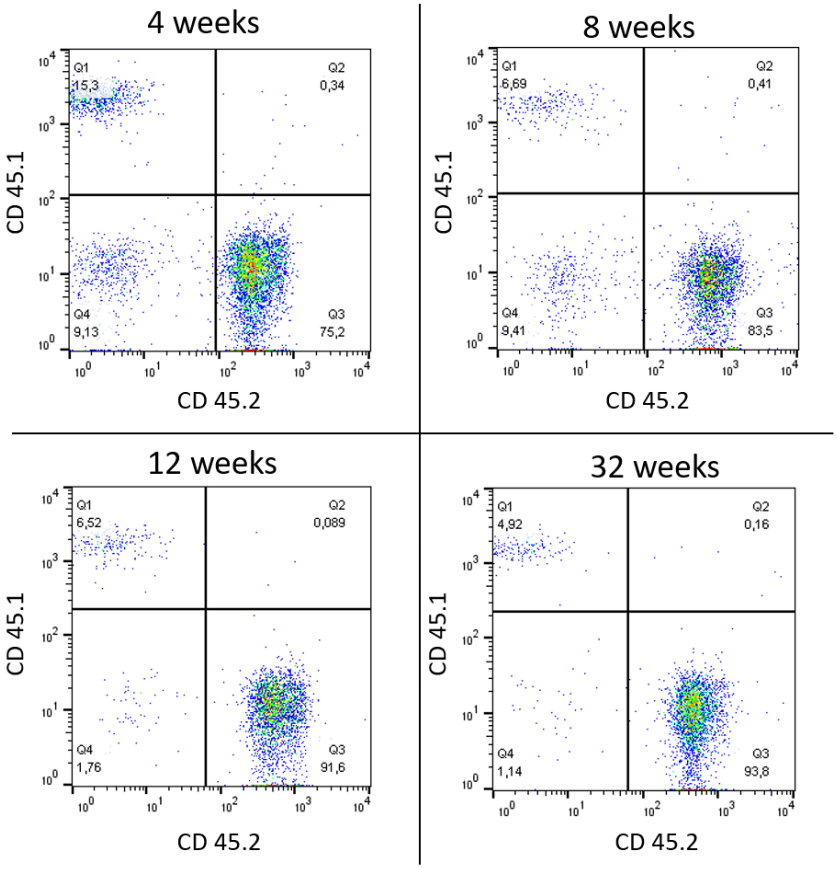
Supplemental Figure S3: In vitro sumoylation assay containing the E3 ligase IR1+M and MafG. GST-NFE2-WT (72 kDa) was incubated together with either 300 nM MafG or 200 nM IR1+M or both as indicated. UBC9 was used in a concentration of 125 nM in this and all subsequent assays. The reaction mixture was subjected to SDS-PAGE and immunoblotting using an anti-NFE2 antibody.



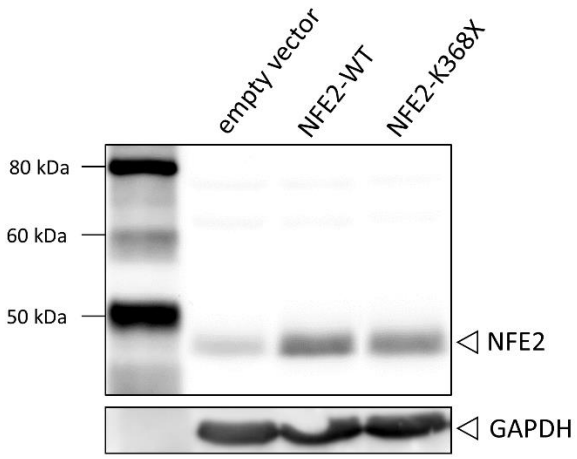
Supplemental Figure S4: Transduction efficacy of murine bone marrow following lentiviral infection. Bone marrow from WT FVB/N-45.2 donor mice was infected with either an empty control pLeGO-iG vector or with lentiviruses expressing NFE2-WT or NFE2-K368X, as indicated, and incubated *ex vivo* for 48 hours. pLeGO-iG also directs expression of the eGFP gene.[4] Transduction levels were assessed by analyzing GFP fluorescence in FACS. Representative histograms are shown for one mouse of each group. Red histograms represent untransduced murine bone marrow cells, turquois histograms represent the transduced bone marrow cells.



Supplemental Figure S5: Engraftement of transplanted CD45.2$^+$ donor bone marrow in CD45.1$^+$ acceptor mice as analyzed by FACS of peripheral blood leukocytes. Representative analysis of one mouse expressing NFE2-K368X. Peripheral blood was drawn every 4 weeks and peripheral blood leukocytes stained for CD45.1-APC and CD45.2-PE and analyzed by FACS. Engraftement of donor bone marrow exceeded 90% 12 weeks after transplantation and thereafter in all mice.



Supplemental Figure S6: NFE2 protein levels in transduced, transplanted murine bone marrow. At autopsy (16 to 18 months after transplantation), bone marrow was collected by flushing femora and tibiae of transplanted mice. Subsequently, nuclear extracts were prepared and analyzed by SDS-PAGE and western blot analysis using an anti-NFE2-Antibody. A representative blot of one mouse from each group is shown. GAPDH protein expression was determined to ensure equal protein loading.



Supplemental Materials and Methods:

Identification of mutations in MPN patients. A NGS-based gene panel diagnostic for 34 common target genes in myeloid malignancies was performed as previously described by MLL Münchner Leukämie Labor GmbH, Munich, Germany (Supplemental Table S1).[5] In addition, constitutive DNA obtained from buccal swab was subjected to PCR amplification using the primers given in Supplemental Table S2 and sequencing to determine mutations in the LNK (SH2B3) and THPO genes. All patients gave their written informed consent for scientific evaluations. The study was approved by the Internal Review Board of MLL Münchner Leukämie Labor GmbH, Munich, Germany and adhered to the tenets of the Declaration of Helsinki.

Plasmids. pRBGP2-Luciferase (pRBGP2-Luc) reporter plasmid, which contains tandemly arranged copies of the NFE2 binding site from the chicken β-globin enhancer in a TATA-luciferase reporter vector,[1] was a gift of M. Yamamoto (Tsukuba University, Tsukuba, Japan). The newly identified mutation of NFE2, c.1102A>T, was inserted into the NFE2 cDNA by site-directed mutagenesis (GeneArt Site-Directed Mutagenesis System, Invitrogen). WT and mutant NFE2 cDNA was cloned into the pLeGO-iG[4] and the pRc/CMV vector by restriction enzyme digestion. The MafG-pCMV6-XL4 expression vector was purchased from OriGene.

EMSA. HEK293T nuclear extracts were prepared as previously described.[6] For EMSA, 1–2 µg of nuclear extracts were added to a binding reaction mixture containing 2 µg poly dI-dC, 2 µl 10× binding buffer (10 mM Hepes, pH 7.9, 5 mM MgCl2, 30 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol), and 0.5 ng ^{32}P-labeled oligonucleotide. The oligonucleotide containing the NFE2 consensus binding site at bp -160 of the human porphobilinogen deaminase gene promoter[7] was synthesized by Eurofins MWG Operon and the NF-κB consensus oligonucleotide was purchased from Promega. For supershift control, a 100-fold excess of nonradioactive oligonucleotide, an NFE2 antibody (Sigma-Aldrich), or an NF-κB p65 antibody (Santa Cruz Biotechnologies, Inc.) were added. The reaction was incubated at room temperature for 15 min. For supershift assays, extracts and antibodies were preincubated for 10 min at room temperature before addition of the radioactive nucleotide.

Transient transfections and dual luciferase assays. HEK293T cells were transiently transfected with 0.2 µg of the pRBGP2-Luc reporter gene construct, 1.36 µg of either the pRc/CMV-NFE2-WT or the pRc/CMV-NFE2-K368X mutant expression vector and with 0.17 µg of the MafG-pCMV6-XL4 expression vector (OriGene) to obtain a MafG:NFE2 ratio of 1:8. In addition, 0.1 µg of a TK-Renilla plasmid (Promega) was cotransfected for normalization. 6

h after transfection cells were washed and supplemented with fresh medium and harvested after 18 h. The optimal MafG:NFE2 ratio was experimentally determined (Supp. Fig. S1) and a 1:8 ratio was used for all assays. Experiments were carried out in duplicates in three independent assays each. Luciferase activity was determined using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to the Renilla internal control to compensate for variations in transfection efficiency.

Lentiviral transduction. CB3 cells, a kind gift of V. Blank (McGill University, Montreal, Quebec, Canada) and E. Bresnick (University of Wisconsin, Madison, WI), were transduced with empty LeGO-iG virus particles or virus providing for the expression of NFE2-WT or NFE2-K368X mutant as previously described.[4,8] An overall MOI of 10 was used. GFP-expressing cells were FACS sorted to obtain pure populations and re-cultured for recovery.

qRT-PCR assays. RNA isolation from CB3 cells was performed using the RNeasy Mini Kit (Qiagen). For reverse transcription of total CB3 cell RNA into cDNA, the One Step TaqMan reverse transcription kit (Applied Biosystems) was used. cDNA was diluted to a final concentration of 2 ng/µl. qRT-PCR assays were performed in duplicate in a 96 well plate in an ABI PRISM 7000 Cycler and analyzed using the ABI PRISM 7000 software (Applied Biosystems). An assay on demand was used for the analysis of murine β-globin expression (Mm01611268_g1 Hbb-b1; Applied Biosystems) as well as the following primer and probe sequences for murine β2-microglobulin expression: FP: 5'-TCTTTCTGGCCTGGAGGCTATC-3'; RP: 5'- TGCTGGATGACGTGAGTAAACC-3'; TaqMan Probe: 6FAM-AGCGTACTCCAAAGAT-MGBNFQ. β-globin expression was determined relative to the expression of the β2-microglobulin house-keeping gene using the ΔΔcT method.

In vitro sumoylation reactions. GST-NFE2-WT, NFE2-K368X, MafG and the SUMO enzymes were purified from E. coli BL21-GOLD as previously described.[9] A sumoylation reaction buffer was used as previously published.[9,10] Reaction was carried out in 20 µl total volume for 30 min at 30°C and then stopped by boiling with 2x Laemmli-Buffer for 4 min and subsequently administered to SDS-PAGE. Protein concentrations: 5µM SUMO1 or SUMO 2/3; 100nM AOS1/UBA2; 125nM UBC9; 200nM E3; 5mM ATP and 300nM GST-NFE2-WT or NFE2-K368X. For each assay, GST-mst2 protein served as an internal control for proper sumoylation reaction (data not shown).

Immunoblotting. CB3 cells or HEK293T cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0,1% SDS, 0,5% sodium deoxycholate supplemented with 1× Roche Complete Protease Inhibitor Cocktail). Cell lysates and in vitro sumoylation assays were subjected to SDS-PAGE and immunoblotting. A primary antibody raised against

NFE2 was used as previously described,[11] SUMO1 and SUMO2/3 antibodies were purchased from Abcam (ab133352; ab81371). The blots were stripped and reprobed against β-actin (Clone AC-15; Sigma-Aldrich) or GAPDH (Clone 71.1; Sigma-Aldrich) to control for equal loading where applicable. Immune complexes were detected using chemiluminescence western blotting reagents (GE Healthcare).

Murine bone marrow transplantation. Mice were kept under specific pathogen-free conditions at the Center for Clinical Research mouse facility of the University Medical Center Freiburg. Breeding and experiments were performed in accordance with committee-approved animal protocols (by the Federal Ministry for Nature, Environment and Consumer Protection of the state Baden-Württemberg, Germany). FVB/N-45.2 donor mice[12] were treated with 150 mg/kg body weight 5-fluorouracil (5-FU) 4 days before bone marrow harvest. As previously described,[4,8] donor bone marrow was either infected with empty lentiviral particles (pLeGO-iG) or with lentiviruses providing for the expression of WT NFE2 or NFE2-K368X mutant at an MOI of 10. Sublethally irradiated FVB/N-45.1 recipient mice were transplanted intrafemorally with lentiviral transduced FVB/N-45.2 bone marrow. Engraftement of transplanted bone marrow was evaluated by FACS analysis. Peripheral blood was drawn monthly by retro-orbital puncture and was analyzed on an Advia 120 system (Siemens) to obtain a complete blood count.

Histology. Organs were fixed in 4% formalin, femurs subsequently decalcified in 10% buffered ethylene-diamine tetra-acetic acid (EDTA), pH 7.2, and all organs paraffin embedded. Sections were routinely stained with H&E for histological analysis. Megakaryocytes were identified morphologically and assigned to three size categories, large, middle and small, and enumerated for 5 mice of each genotype in 5 High Power Fields (400x magnification).

Data analysis. Paired or unpaired Student's t-tests (two-sided or one-sided) or two-way ANOVA with Bonferroni posttests were used to determine whether a significant ($p < 0.05$) difference existed between two groups. These analyses were performed using Prism 5.0 (GraphPad Software) software.

References

1. Igarashi K, Kataokat K, Itoh K, Hayashi N, Nishizawa M, Yamamoto M. Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. Nature 1994;367(6463):568–572.

2. Blank V, Kim MJ, Andrews NC. Human MafG Is a Functional Partner for p45 NF-E2 in Activating Globin Gene Expression. Blood 1997;89(11):3925–3935.

3. Motohashi H, Katsuoka F, Miyoshi C, et al. MafG Sumoylation Is Required for Active Transcriptional Repression. Mol Cell Biol 2006;26(12):4652–4663.

4. Weber K, Bartsch U, Stocking C, Fehse B. A Multicolor Panel of Novel Lentiviral “Gene Ontology” (LeGO) Vectors for Functional Gene Analysis. Mol Ther 2008;16(4):698–706.

5. Delic S, Rose D, Kern W, et al. Application of an NGS-based 28-gene panel in myeloproliferative neoplasms reveals distinct mutation patterns in essential thrombocythaemia, primary myelofibrosis and polycythaemia vera. Br J Haematol 2016;175(3):419–426.

6. Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with “mini-extracts”, prepared from a small number of cells. Nucleic Acids Res 1989;17(15):6419.

7. Mignotte V, Eleouet JF, Raich N, Romeo PH. Cis- and trans-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase gene. Proc Natl Acad Sci U S A 1989;86(17):6548–6552.

8. Jutzi JS, Bogeska R, Nikoloski G, et al. MPN patients harbor recurrent truncating mutations in transcription factor NF-E2. J Exp Med 2013;210(5):1003–1019.

9. Pichler A, Gast A, Seeler JS, Dejean A, Melchior F. The Nucleoporin RanBP2 Has SUMO1 E3 Ligase Activity. Cell 2002;108(1):109–120.

10. Pichler A, Knipscheer P, Saitoh H, Sixma TK, Melchior F. The RanBP2 SUMO E3 ligase is neither HECT- nor RING-type. Nat Struct Mol Biol 2004;11(10):984–991.

11. Goerttler PS, Kreutz C, Donauer J, et al. Gene expression profiling in polycythaemia vera: overexpression of transcription factor NF-E2. Br J Haematol 2005;129(1):138–150.

12. Guibal FC, Alberich-Jorda M, Hirai H, et al. Identification of a myeloid committed progenitor as the cancer-initiating cell in acute promyelocytic leukemia. Blood 2009;114(27):5415–5425.