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

The vast majority of patients with myeloproliferative neoplasms (MPN), polycythemia vera, essential thrombocythemia (ET), and primary myelofibrosis acquire driver mutations in the *JAK2, MPL* or *CALR* gene. Clustering of MPN is seen in select families, but in most pedigrees the MPN-predisposing change has not been determined and affected individuals somatically acquire one of the three above-mentioned driver mutations. In contrast, a small number of individuals with hereditary thrombocythemia (HT) carry constitutive alterations, e.g., in the *TPO* or the *LNK* (*SH2B3*) gene.[1-4] Acquired mutations in *LNK*, a negative regulator of JAK2 signaling, rarely occur in both sporadic and familial MPN cases.[1,2] In the latter, they do not segregate with disease phenotype and diseased individuals acquire a concomitant MPN driver mutation.[2] It, therefore, appears unlikely that mutant LNK acts as a driver in MPN.

We have shown that the transcription factor Nuclear Factor Erythroid-derived-2 (NFE2) is overexpressed in the vast majority of MPN patients, independent of other molecular aberrations.[5] In addition, we have identified *NFE2* mutations in MPN and acute myeloid leukemia (AML) patients, that enhance the activity of wild-type (WT) NFE2.[6,7] In several murine models, elevated NFE2 activity causes pathognomonic features of MPN.[6-8] The molecular mechanisms by which NFE2 mutants exert their effect remain unclear since the regulation of NFE2 transcriptional activity is poorly understood. Various post-translational modifications have been described, including ubiquitination, phosphorylation, and sumoylation but their functional role remains elusive.[9]

Here, we identified a 74-year-old female patient given the diagnosis of ET by World Health Organization criteria (*Online Supplementary Table S1*), who tested negative for the three MPN driver mutations, *JAK2, CALR*, and *MPL*. Sequencing 36 myeloid neoplasms associated genes (*Online Supplementary Tables S2 and S3*) revealed both a previously described p.E208Q point-mutation in *LNK* as well as a novel mutation in *NFE2* (c.1102A>T) that prematurely truncates the protein at lysine 368 (p.K368X, Figure 1A). Buccal swab DNA analysis determined that both mutations were heterozygously present in the germline. Because of the constitutive nature of both mutations, this patient should be designated as having hereditary thrombocythemia.

The p.K368X mutation leaves almost the entire NFE2 protein, including the bZIP domain and the N-terminal activation domain, intact. Only the terminal 4 amino acids are lost. Notably, this mutation deletes the ψKXE sumoylation consensus motif identified at lysine 368 and shown to be sumoylated by SUMO1 *in vitro* and *in vivo*.[10] The LNK p.E208Q mutation retains near-complete inhibitory capacity and did not confer significantly higher TPO-hypersensitivity in cell proliferation assays than WT-LNK, suggesting only a subtle loss of function.[3] Therefore, we hypothesized that the loss of sumoylation increases NFE2 activity, which, in co-operation with mutant LNK, drives thrombocytosis in this patient.

To test whether NFE2-K368X retains binding to its cognate DNA motif, we performed an electromobility shift assay (EMSA) using a consensus NFE2 binding site from the human PBGD promoter. DNA binding is preserved in the NFE2-K368X mutant (Figure 1B), consistent with retention of the complete DNA binding and heterodimerization domains.

We subsequently examined the ability of NFE2-K368X to transactivate transcription using a luciferase reporter assay. Heterodimerization with MafG is required for optimal NFE2 activity (*Online Supplementary Figure S1*). The NFE2-K368X mutant was more than twice as active at promoting reporter gene expression than WT-NFE2 (Figure 1C). Because transcription off a plasmid DNA template does not model intact chromatin, we used endogenous gene activation in a cell line as a second read-out. CB3 cells are devoid of NFE2 expression due to viral integration but express β-globin upon re-introduction of NFE2. We therefore lentivirally transduced CB3 cells with either WT-NFE2 or NFE2-K368X and determined β-globin expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Again, NFE2-K368X, present in the same amount, was two times more active than its WT counterpart in directing transcription, demonstrating that the mutation results in a protein with supraphysiological activity on intact chromatin (Figure 1D). NFE2-K368X thus constitutes a novel Type Ia mutation, DNA–binding and activating, according to the classification of NFE2 mutations we proposed.[7]

To investigate sumoylation of the NFE2-K368X mutant, we conducted *in vitro* sumoylation assays using recombinantly expressed proteins. SUMO is attached to proteins by hierarchical action of the E1-activating enzyme Aos1/Uba2, the E2-conjugating enzyme UBC9, and a substrate-specific E3-ligase. Sumoylation of WT-NFE2 with SUMO1 has been shown in assays that contained Aos1/Uba2 and UBC9 but lacked an E3-ligase.[10] Under these conditions, we could not detect SUMO1 modification of NFE2 (*Online Supplementary Figure S2*). Substrate recognition is accomplished by UBC9, but E1 and E2 enzymes have poor transfer efficiency, which is stimulated by E3-ligases. Addition of the E3-ligases IR1+M,[11] PIAS1,[12] or ZNF451-N,[13] led to sumoylation of GST-NFE2-WT but not the NFE2-K368X mutant (Figure 2, top).

IR1+M, the catalytic core domain of the E3-enzyme RanBP2, has high SUMO ligase activity but low substrate specificity.[11] Unphysiological *in vitro* conditions can facilitate sumoylation of non-canonical lysine residues, which may facilitate both the observed IR1+M mediated sumoylation of WT-NFE2 (Figure 2, lane 1, marked*) as well as unspecific modification of the NFE2-K368X mutant (Figure 2, lane 6, marked**). The RanBP2 fragment RanBP2ΔFG has a higher substrate specificity,[11] and did not sumoylate either GST-NFE2-WT or NFE2-K368X (Figure 2, lanes 2 and 7). These data suggest that while NFE2 sumoylation is facilitated by the minimal catalytic activity of the IR1+M fragment,[11] NFE2 is not a substrate of the RanBP2 E3-ligase itself. Presence of the small subunit MafG does not influence the sumoylation efficacy of NFE2 by IR1+M with SUMO1 (*Online Supplementary Figure S3*).

Two additional E3-ligases modified NFE2: PIAS1 and ZNF451-N, both with either SUMO1 or SUMO2/3 (Figure 2, top and bottom, lanes 3 and 4). The ZNF451-N ligase has been described as specific for SUMO2/3,[13] but modified GST-NFE2-WT with SUMO1 in our study. This activity may result from the high concentration of the components and the unphysiological conditions *in vitro*. PIAS1 is a member of the PIAS family class of E3-ligases and was described to facilitate MafG sumoylation by

SUMO2/3.[14] PIAS1 is also involved in the negative regulation of the JAK/STAT signaling pathway,[12] which is aberrantly upregulated in MPN patients and constitutes a central molecular hallmark of MPN pathogenesis.

Negative regulation of transcription factors by sumoylation has previously been described and can occur through several distinct mechanisms.[15] For example, sumoylation can compete with acetylation or phosphorylation of nearby residues. While the acetylated or phosphorylated transcription factor is active, the sumoylated form is not. NFE2 activity is regulated by phosphorylation, but the precise molecular mechanisms have not been defined.[9] We, therefore, propose that sumoylation may negatively regulate NFE2 by interfering with other post-translational modifications, such as acetylation, methylation or phosphorylation, required for full activity.

Alternatively, rather than controlling the placement of other post-transcriptional modifications, NFE2 sumoylation may regulate nuclear localization. PIAS1 and ZNF451-N sumoylate the tumor suppressor PML, the main component of PML nuclear bodies (PML-NBs).[12] PML-NB control gene expression by sequestering transcriptions factors in a SUMO-dependent manner.[15] WT-NFE2 and PML co-localize in the nucleus of K562 cells, while sumoylation-deficient NFE2 did not.[10] Sumoylated WT-NFE2 might, therefore, become

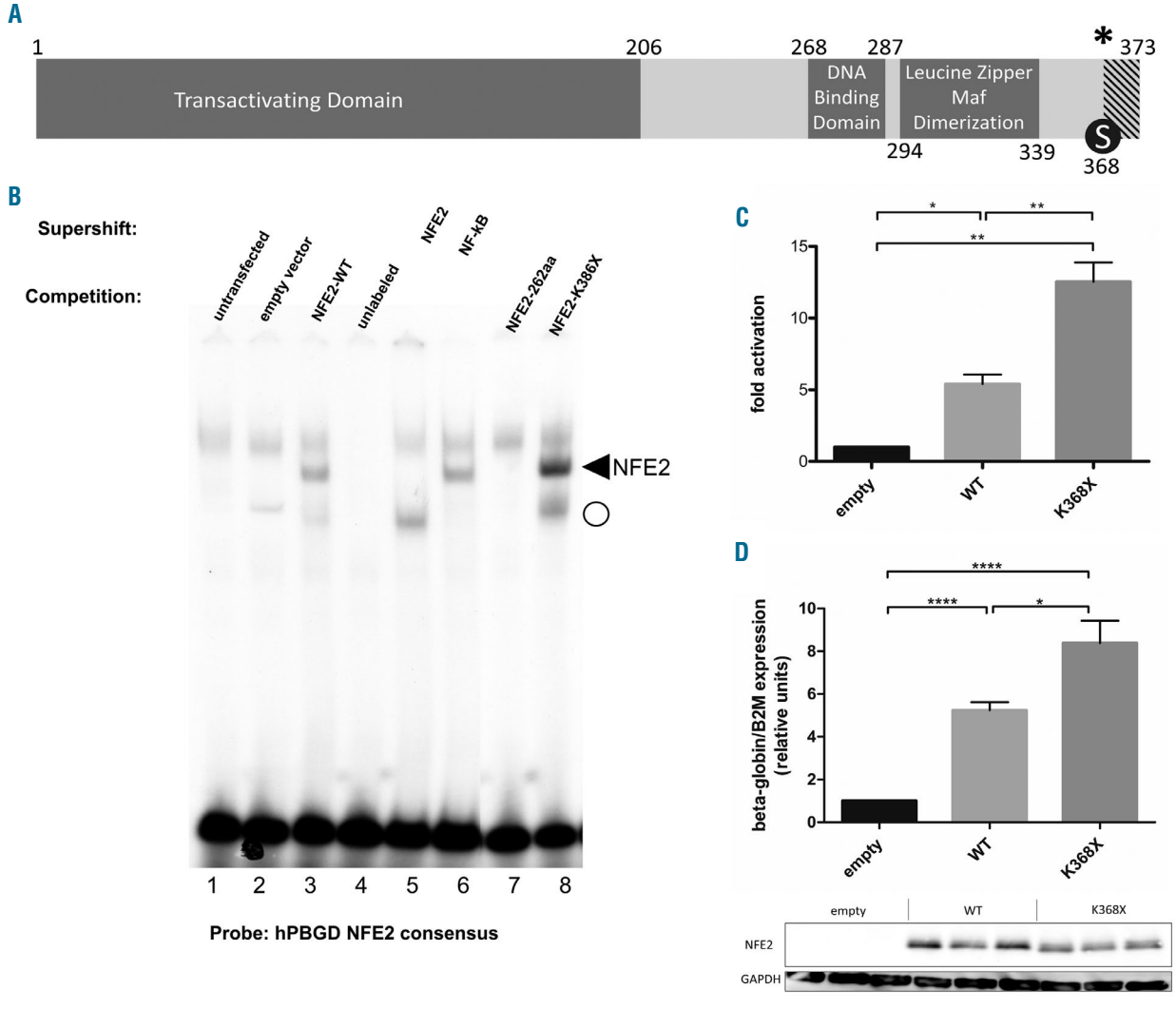

Figure 1. DNA binding affinity and transcriptional activity of the NFE2-K368X mutant. (A) Schematic representation of the NFE2 protein. The location of the K368X mutation is indicated by an asterisk, the sumoylation site at K368 is marked by a circle. (B) EMSA of wild-type (WT) NFE2 and the NFE2-K368X mutant. Nuclear extracts from HEK293T cells transduced with expression vectors encoding MafG as well as NFE2-WT (lane 3), NFE2-K368X (lane 8), or the NFE2-262aa truncation mutant (lane 7) were incubated with a ^{32}P-labeled oligonucleotide containing an NFE2 binding site. In lane 4, a 100x excess of a nonradioactive oligonucleotide was added. Alternatively, an antibody to NFE2 (lane 5) or a control NF-κB antibody (lane 6) was added. The NFE2-262aa truncation mutant, lacking the bZIP domain with consecutive loss of DNA binding, serves as a negative control (lane 7).[6] The arrowhead points to the position of NFE2/DNA complexes on the gel, the open circle indicates nonspecific bands. (C) Dual luciferase reporter assay. HEK293T cells were co-transfected with a pRBGP2-luciferase reporter construct that contains tandemly arranged NFE2 binding sites driving a minimal chicken β-globin promoter together with expression vectors for NFE2, either WT or the K368X mutant, and MafG as indicated. Experiments were carried out with a ratio of 1:8 MafG:NFE2. Firefly luciferase activity was measured 24 h after transfection and was normalized to constitutively expressed renilla luciferase activity. Activity for transfection with MafG alone was set as one and fold activity relative to this control is depicted. Bar graphs represent the mean + SEM. (D) Rescue of β-globin expression. CB3 cells were infected with lentiviral (pLeGO-iG) constructs encoding NFE2-WT, NFE2-K368X, or an empty control virus as indicated. 72 h after infection, RNA was harvested and assayed for β-globin and β2-microglobulin housekeeping gene mRNA expression by qRT-PCR. Results represent the mean + SEM of three independent experiments and are reported as relative expression levels setting β-globin expression for the empty virus as 1. Protein expression in transduced CB3 cells was assessed by western blot. Whole cell extracts, prepared from each of the three independent experiments, were probed for NFE2 and stripped blots reprobed for GAPDH as a loading control. All data were analyzed for statistical significance by two-tailed Student's *t*-test. **P*<0.05; ***P*<0.01; *****P*<0.0001.

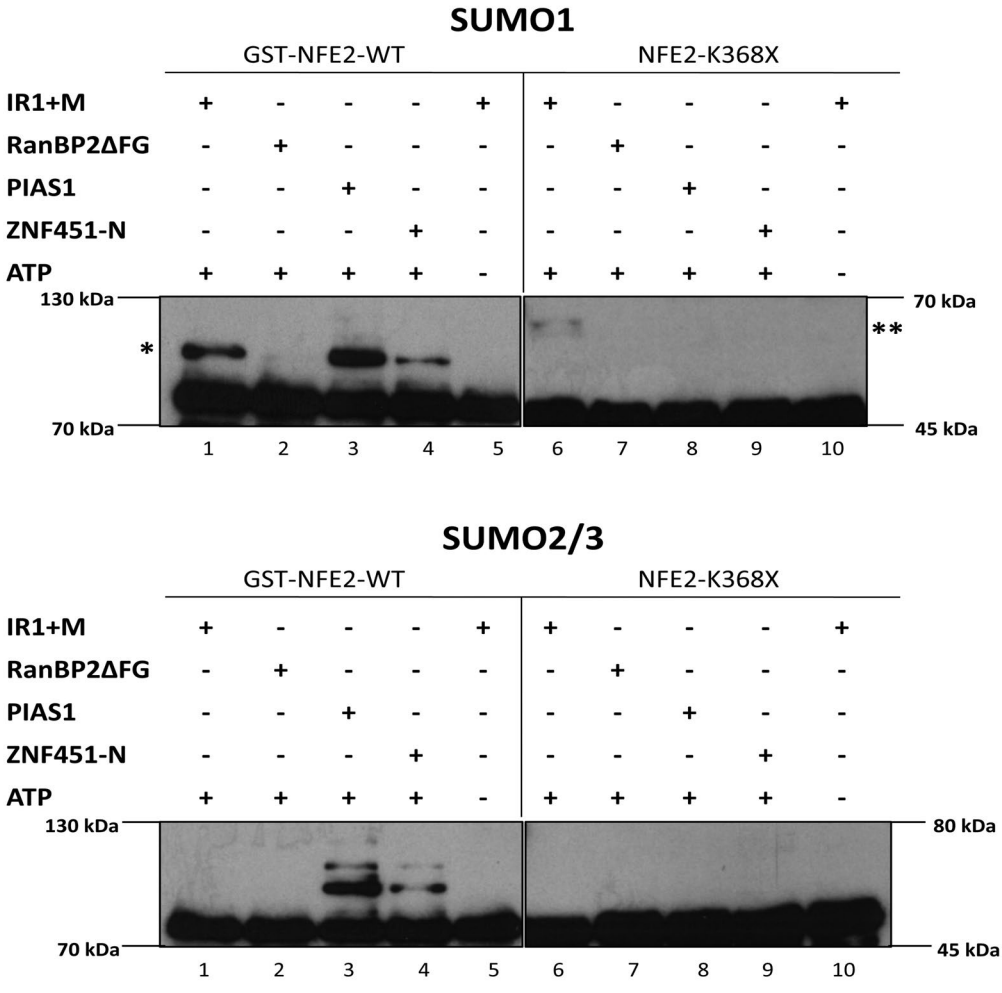

Figure 2. *In vitro* sumoylation assay of NFE2-WT and the NFE2-K368X mutant. Recombinantly expressed GST-NFE2-WT (WT, wild-type) (72 kDa) and NFE2-K368X (45 kDa) were tested for modification with the SUMO1 (top) or the SUMO2/3 (bottom) peptide by different E3 ligases, as indicated. The GST tag was proteolytically cleaved from NFE2-K368X to exclude any potential influence on protein conformation and overall charge. The reaction mixture was subjected to SDS-PAGE and immunoblotting using an anti-NFE2 antibody. Following modification with SUMO2/3, two bands appear as SUMO2/3 is capable of forming chains by self-sumoylation. *The IR1+M mediated modification of GST-NFE2-WT with SUMO1. **The IR1+M mediated modification of NFE2-K368X with SUMO1.

sequestered in PML nuclear bodies, leaving non-sumoylatable NFE2-K368X available to exert the observed, unphysiologically high transcriptional activity.

To test the physiological effect of the K368X mutation, we employed a murine bone marrow (BM) transplantation model. FVB/N-45.2 donor BM was lentivirally infected to express either NFE2-K368X or NFE2-WT and transplanted into FVB/N-45.1 recipient mice. No significant difference in CBC between mice expressing NFE2-K368X, NFE2-WT, or an empty control construct was observed, although a trend towards an elevated thrombocyte count at older age was noted in mice expressing NFE2-K368X (Figure 3A). This observation is consistent with the very late disease onset in our patient, who only manifested clinical signs of thrombocytosis at the age of 74, despite having carried the *LNK* and *NFE2* mutations since birth.

Because of the mild platelet phenotype observed, we morphologically evaluated megakaryopoiesis in histological BM sections of the transplanted mice. We defined three size categories for megakaryocytes (large, middle and small) and enumerated them in five high power fields each of 5 mice of each genotype. The total number of megakaryocytes was increased 50% in NFE2-K368X transplanted mice compared to WT controls (Figure 3B). Moreover, the fraction of small and large megakaryocytes was significantly increased compared to WT, while the fraction of middle-sized megakaryocytes decreased, suggesting alterations in megakaryocyte maturation (Figure 3B). Polymorphic and pleomorphic megakaryocytes, visible in our murine BM histologies (Figure 3C and D), are also typically observed in BM sections of MPN patients.

In conclusion, this report constitutes the first description of a constitutional *NFE2* mutation. Since in a murine model, the NFE2-K368X mutant is sufficient to cause thrombocytosis with a long latency similar to that observed in our patient, and since the LNK-E208Q-mutation was hypothesized to require co-operation with an MPN-driver mutation to produce an MPN phenotype, we now propose that the NFE2-K368X mutation constitutes such an MPN-driver. Our findings provide a rationale for investigating possible NFE2 mutations in triple-negative MPN patients. The observation that the NFE2-K368X mutation abrogates NFE2 sumoylation by either SUMO1

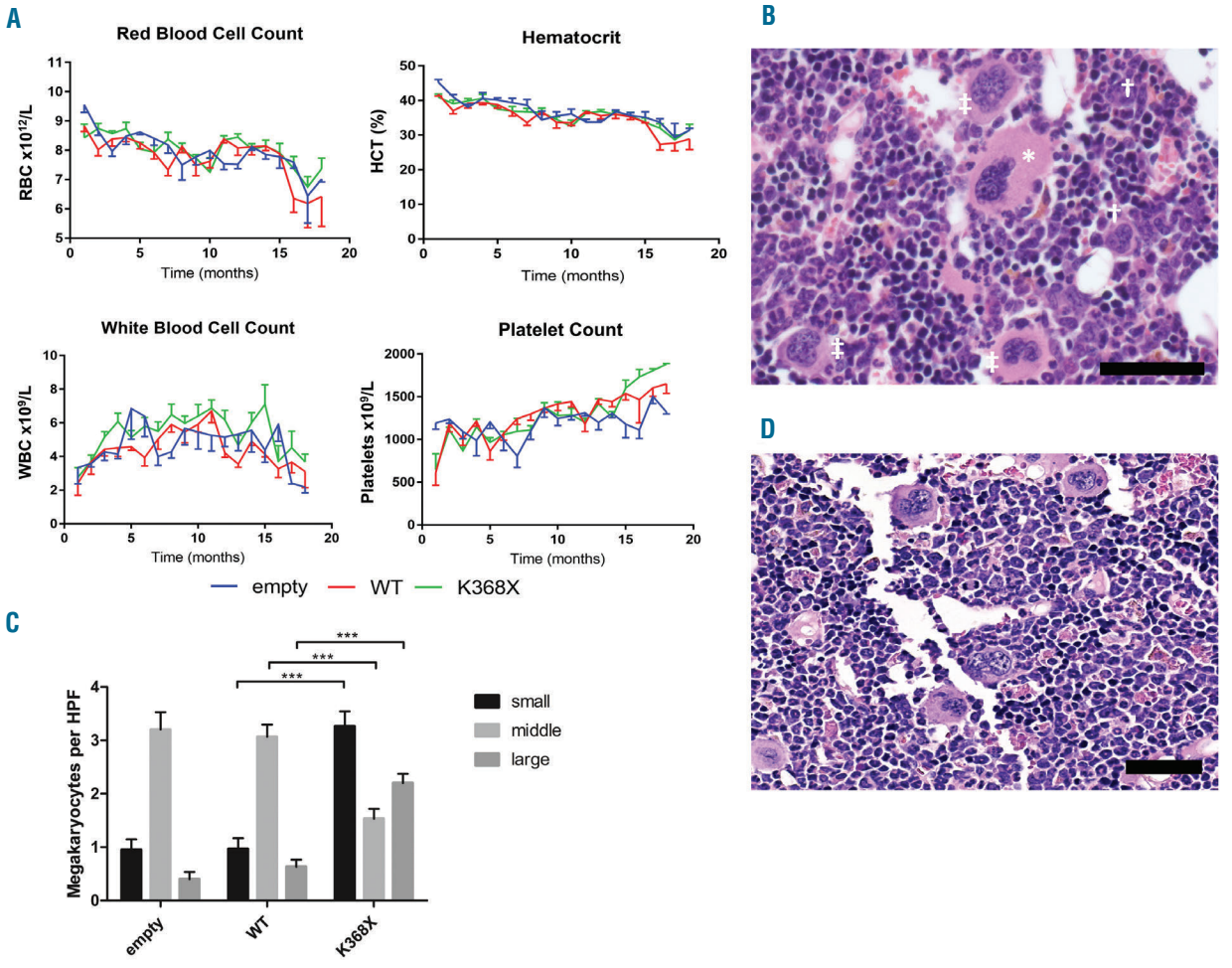

Figure 3. Effect of NFE2-K368X expression on megakaryopoiesis *in vivo*. FVB/N-45.1 acceptor mice were lethally irradiated (2x5 Gy given 4 hours apart) and transplanted with bone marrow (BM) from FVB/N-45.2 donor mice, lentivirally transduced either with an empty vector (n=5), or with a vector expressing wildtype (WT) NFE2 (n=6), or the NFE2-K368X mutant (n=6) (*Online Supplementary Figure S4*). Twelve weeks after the transplantation, engraftment exceeded 90% in all cases (*Online Supplementary Figure S5*). The expression level of NFE2 was doubled in transplanted BM compared to the endogenous level (*Online Supplementary Figure S6*). (A) Complete blood count. Mean±standard error of mean (SEM) are shown. (B) Representative Hematoxylin & Eosin (H&E) stained BM section of a mouse expressing the NFE2-K368X mutation, demonstrating the high variability in megakaryocyte size. *Large size; †middle size; ‡small size. Scale bar=50 μm. (C) H&E stained BM sections morphologically evaluated for megakaryopoiesis and enumerated for five mice of each genotype. Results represent the mean+SEM per five HPF per mouse (400x magnification). Grouped data were analyzed for statistical significance by two-way ANOVA with Bonferroni post tests. ***P<0.001. (D) Representative BM section (H&E) of a mouse expressing NFE2-WT. Scale bar=50 μm.

or SUMO2/3 suggests a previously unrecognized function of sumoylation in negatively regulating NFE2 function.

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