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Genetic screening of triple negative thrombocytosis patients identifies germline *MPL* compound mutations and *SH2B3/LNK* truncating mutations.

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Disclosures

No conflicts of interest to disclose

Authors' contributions

BC, NM and LV designed the study and supervised the work. LL, SAT, DC and DE performed the in vitro experiments and analyzed the results. BC, DE, VE, NB, DLP and NM processed and analyzed the

molecular data. RDO, HP, LB, LD, JS, TL, LDarnige, LDrouet, LPZ, FP, LDrevon, SG and JJK collected the clinical samples and compiled the clinical information. TL, EL, RDO, LB and DLP critically reviewed the manuscript. BC, NM and LV wrote the original and revised manuscript. All authors read and approved the final manuscript.

Data-sharing statement

For access to the original data, please contact the corresponding authors.

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In addition to reactive thrombocytosis—which typically stems from iron deficiency or inflammatory conditions—primary thrombocytosis of hematopoietic origin may occur, often associated with somatic or germline mutations. Essential thrombocythemia (ET) is one of the BCR::ABL1 negative myeloproliferative neoplasms (MPN), a group of chronic disorders due to the acquisition of somatic mutations leading to the constitutive activation of the JAK2/STAT5 pathway. In ET, the driver mutations are located in the *JAK2*, *MPL* or *CALR* genes and lead to increased platelet counts. However, 10 to 20% of ET patients, the so-called "triple negative" patients, do not harbor any of these known driver mutations. Previous studies identified rare alternative somatic mutations in the *MPL* or *JAK2* genes that can be considered responsible for the thrombocytosis. MPL S204P, S204F, Y591D, Y591N, L498W, H499C, H499Y or H499_L500delinsVISLVT somatic mutations and JAK2 Y317H, H345L, S523L somatic mutations have been identified in patients with thrombocytosis and/or shown to lead to constitutive activation of the JAK2/STAT5 pathway.[1-3] Hereditary thrombocytosis (HT) are usually characterized by polyclonal proliferation of the megakaryocytic lineage. They have been associated with *MPL* germline variants shown to confer hypersensitivity to TPO (L265F or Y252H) or to result in reduced MPL expression at the cell surface (K39N or P106L), as well as in increased circulating TPO levels, leading only in the homozygous cases to thrombocytosis.[4-6] However, the characterization of patients with unexplained thrombocytosis and no molecular marker remains nowadays an issue. We screened a cohort of 154 patients with triple negative thrombocytosis followed in our institution using an NGS panel of 36 genes involved in MPN pathogenesis, in which a subgroup of patients harbored compound heterozygous germline mutations in *MPL.* Moreover, we identified an unreported subset of patients with isolated truncating *SH2B3* mutations of mainly germline origin presenting with severe thrombotic clinical features. This study was approved by our institutional review board (IDRCB 2023-A01414-41) and was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Among the 154 patients with thrombocytosis, 37 had a diagnosis of ET according to WHO criteria in whom we identified rare already described *MPL* mutations with the potential to drive MPN phenotype: p.(Ser505Asn) in 3 patients (VAF 10%, 46% and 39% respectively), p.(Ser204Pro) in 2 patients (VAF 6% and 24% respectively) and p.(Ser204Phe) in 1 patient (VAF 10%). In these cases, MPL activating mutations were considered responsible for the ET phenotype. In the remaining 117 cases a diagnosis of HT was suspected (see supplemental Table S1 and Figure S1 for patients' characteristics). Among these patients we observed four distinct molecular patterns. In 73 patients we did not identify any mutation in the 36 genes explored by the NGS panel. Fourteen patients harbored one or more non-driver mutations with allelic frequencies (VAF) below 10%, suggesting clonal hematopoiesis of indeterminate potential (CHIP). The most frequently mutated genes found in these patients were

DNMT3A (20 mutations), *TET2* (8 mutations), *ASXL1* (6 mutations), *TP53* (5 mutations), *CBL* (2 mutations) and *IDH1* (1 mutation).

In the next group of patients (N= 21), we identified *JAK2* or *MPL* mutations previously reported as germline variants. The JAK2 R1063H variant reported to confer a weak hypersensitivity to the JAK2 kinase but probably insufficient to drive thrombocytosis[7] was present as the sole genetic alteration with a heterozygous status in 3 patients. We identified 18 patients with previously reported MPL germline variants. The c.117G>T p.(Lys39Asn) (K39N) also called "Baltimore" variant is known to be responsible for HT when present in a homozygous status.[4] The MPL K39N variant was identified as homozygous in 2 patients and heterozygous in 6 patients. The heterozygous MPL K39N variant was also found in combination with another heterozygous MPL variant known to cause congenital amegakaryocytic thrombocytopenia (CAMT) in 3 patients. These variants were c.236_236del p.(Leu79Glufs*84), c.311T>C p.(Phe104Ser) and c.460T>C p.(Trp154Arg).[8] The heterozygous combination of the K39N variant with a CAMT-associated mutation has previously been reported as causative factors of thrombocytosis.[9] Another monoallelic CAMT-associated mutation, c.378del p.(Phe126Leufs*5), was found isolated in one patient who was ultimately diagnosed with a BCR::ABL1 positive chronic myeloid leukemia. The MPL variants c.754T>C p.(Tyr252His) (Y252H) and c.317C>T p.(Pro106Leu) (P106L), known to be associated with HT when homozygous,[5,6] were identified in one patient each in the homozygous state. In accordance with previous studies, these 2 patients were of African and Arabic origin, respectively. Interestingly, we identified compound heterozygous variants combining the MPL K39N variant with either the MPL Y252H or P106L variant in 3 patients (table 1). Finally, we identified the case of a 2-year-old boy with HT who carried both P106L and Y252H variants. The parents carried heterozygous P106L (mother) and Y252H (father) variants and had normal platelet counts. To our knowledge, this is the first report of HT cases with such a combinatorial association of heterozygous MPL variants previously identified as causative of thrombocytosis only when present in homozygous state. Although functional validation is lacking, the contributing role of these variant combinations is highly plausible.

In the remaining 9 patients we identified mutations *in SH2B3*/*LNK*, a negative regulator of JAK/STAT signaling in hematopoietic cells.[10] In three patients (2 heterozygous and 1 homozygous cases) we detected the p.(Glu208Gln) (E208Q) variant which has been reported to activate JAK/STAT signaling.[11] Six other patients harbored monoallelic *SH2B3* frameshift mutations as the sole detectable mutation in the 36 genes panel tested. Notably, the particular mutation c.685_691dup p.(Asp231Glyfs*39) was identified in 5 distinct patients. The analysis of DNA extracted from nails of these patients demonstrated a germline origin of the mutation in 4 cases. In the latter case, the mutation was absent from nail DNA, suggesting a somatic origin consistent with a 35% VAF in blood DNA. In one additional

case, we identified the heterozygous c.1093_1094ins31 p.(Phe365Trpfs*30) mutation with a 27% VAF in blood DNA, which was absent from nail DNA. Interestingly, five out of six patients with frameshift *SH2B3* mutations have presented episodes of severe arterial and venous thrombosis (Table 1). We interrogated an external cohort of triple negative thrombocytosis patients followed in French centers and found two patients with heterozygous p.(Asp231Glyfs*39) *SH2B3* as the sole mutation. These patients were from the same family (mother and daughter), confirming the germline origin of the mutation. They had no history of thrombotic events at the time the thrombocytosis was detected (at ages 68 and 28) and were subsequently treated with antiplatelet agents. This makes very likely that this mutation found germline in 4 unrelated and 2 related patients is causative of the thrombocytosis phenotype and also of the severe thrombotic events. In this context, loss of LNK in transgenic mice has been shown to promote thrombosis through several mechanisms, including intrinsic platelet hyperactivation[12] and NET formation,[13] which support the clinical observations in our cohort. Interestingly, the same p.(Asp231Glyfs*39) *SH2B3* mutation was reported homozygous in two young siblings presenting with autoimmunity and leukemia,[14] and more recently as a novel predisposing alteration in juvenile myelomonocytic leukemia (JMML).[15]

To decipher the functional consequences of these mutations, we analyzed total protein extracts from platelets isolated from patients with frameshift mutations. To distinguish the mutant from the wild-type form we used both LNK N-terminal and C-terminal directed antibodies. No specific band corresponding to the predicted truncated LNK proteins (expected at 30 and 44 kDa respectively), was observed (Figure 1 A-B). Moreover, the total amount of LNK protein was significantly reduced in platelet extracts from p.(Asp231Glyfs*39) mutated patient compared to healthy control (Figure 1B), suggesting that the frameshift-induced truncated protein synthesized from the mutant allele is unstable or the transcript degraded through nonsense-mediated decay. Flow cytometry approach using a combination of N-terminal and C-terminal fluorescent labeled antibodies on HEK cells transduced with the wild-type or mutant cDNA, did not detect the truncated LNK protein (Figure 1C). Therefore, the frameshift mutation likely leads to a defect in total LNK protein. To assess the functional consequences of mutant SH2B3/LNK, the UT7 megakaryoblastic cell line was transduced with the wild-type or the frameshift mutant and TPO-stimulated. We observed an increase in STAT5 phosphorylation after TPO stimulation in these cells (1.7-fold) compared to WT LNK-overexpressing cells (Figure 1D, 1.1-fold). Moreover, the level of STAT5 phosphorylation in platelets isolated from p.(Asp231Glyfs*39) mutated patients was significantly increased in basal conditions (2.7-fold) but also after TPO stimulation (1.8-fold) compared to platelets from healthy donors (Figure 1E). These results confirmed a hyper-signaling as a functional consequence of the p.(Asp231Glyfs*39) frameshift mutation. *Sh2b3*/*Lnk* knock-out mice have high levels of platelet counts.[11] Because the patients identified in this

study are heterozygous for this mutation and can be considered hemizygous if the mutant allele does not lead to any detectable protein, we analyzed the platelet counts in mice with one *Sh2b3*/*Lnk* allele missing. These mice also have increased platelet counts, at an intermediary level between wild-type and knock-out mice. In addition, the CFU-MK numbers are also increased in heterozygous knock-out mice (Table 2). In all, these results strongly suggest that the mutation identified is responsible for the thrombocytosis. Of note, thrombocytosis was recently reported in one patient with a biallelic frameshift *SH2B3* mutation[16] while some *SH2B3*-mutated JMML patients who experienced spontaneous resolution exhibited secondary thrombocytosis.[15,17] LNK/SH2B3 is mainly expressed in the hematopoietic lineage, but also in endothelial cells. Although further studies are still needed, enhanced intracellular signaling may promote a pro-thrombotic endothelium that could contribute to the high incidence rate of both arterial and venous thrombosis observed in the patients with germline frameshift *SH2B3* mutations.

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Table 1: Mutations in *MPL* and *SH2B3* genes identified in this study.

MPL Mutations	Patient numbers	Origin	Responsible for thrombocytosis	Thrombosis
MPL K39N homozygous	2	Germline	Yes	None
MPL K39N heterozygous	6	Germline	No	None
MPL K39N + MPL c.236_236del p.(Leu79Glufs*84)	1	Germline	Yes	None
MPL K39N + MPL c.311T>C p.(Phe104Ser)	1	Germline	Yes	None
MPL K39N + MPL c.460T>C p.(Trp154Arg)	1	Germline	Yes	None
MPL c.378del p.(Phe126Leufs*5) heterozygous	1	Germline	No but BCR::ABL1 transcript secondarily identified	None
MPL .754T>C p.(Tyr252His) (Y252H) homozygous	1	Germline	Yes	None
MPL c.317C>T p.(Pro106Leu) (P106L) homozygous	1	Germline	Yes	None
MPL K39N + MPL Y252H	2	Germline	Yes	None
MPL K39N + MPL P106L	1	Germline	Yes	None
MPL P106L + MPL Y252H	1	Germline	Yes	None

SH2B3 mutations	Patient numbers	Origin	Responsible for thrombocytosis	Thrombosis
p.(Glu208Gln) (E208Q) homozygous	1	Unknown	Possible	None
p.(Glu208Gln) (E208Q) heterozygous	2	Unknown	Possible	None
c.685_691dup p.(Asp231Glyfs*39) heterozygous	1	Germline	Yes	Splanchnic thrombosis
c.685_691dup p.(Asp231Glyfs*39) heterozygous	1	Germline	Yes	Splanchnic thrombosis
c.685_691dup p.(Asp231Glyfs*39) heterozygous	1	Germline	Yes	Several episodes of stroke
c.685_691dup p.(Asp231Glyfs*39) heterozygous	1	Somatic	Yes	Cerebral venous thrombosis and myocardial infarction
c.685_691dup p.(Asp231Glyfs*39) heterozygous	1	Germline	Yes	None
c.1093_1094ins31 p.(Phe365Trpfs*30) heterozygous	1	Somatic	Possible	Myocardial infarction

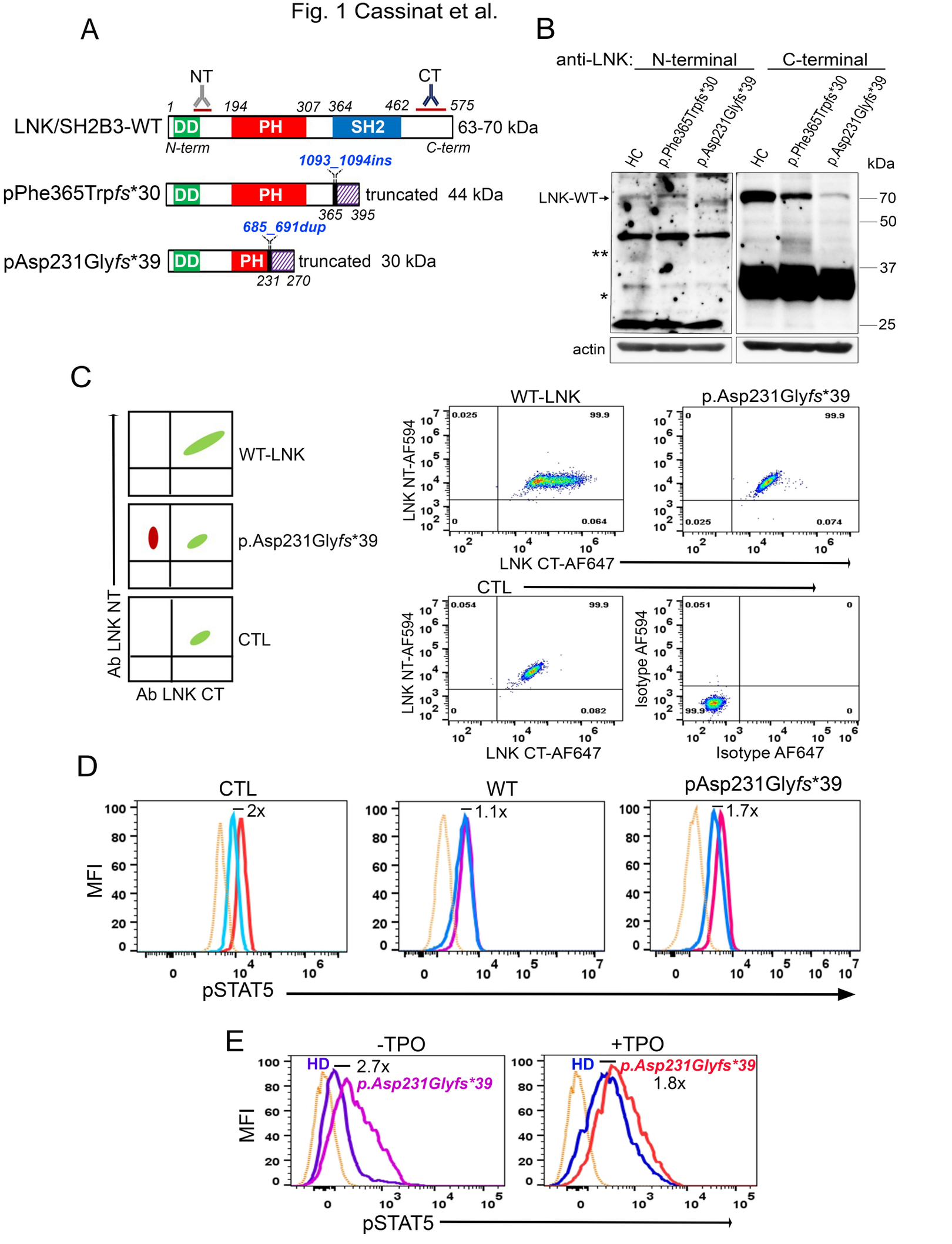
Table 2. Hematological parameters and colony-forming numbers of wild-type and *Sh2b3* transgenic mice. Peripheral blood counts were examined in wild-type, heterozygous and homozygous *Sh2b3* mice. Asterisk marks intermediate circulating platelet levels in heterozygous mice compared to wild-type and *Sh2b3* knock-out mice. WBC, white blood count; RBC, red blood count. Values represent the mean ±SD for 10 animals per determination per genotype. *In vitro* proliferative capacity of bone marrow (BM) and spleen hematopoietic progenitors from wild-type and *Sh2b3*-deficient mice was assessed in colony-forming unit assays (CFU) of megakaryocyte (CFU-MK) or mix (granulocyte, erythroid, macrophage, and megakaryocyte cells). The mean and SD of the number of colonies/10^5 cells are shown from assays using three mice of each genotype in triplicate.

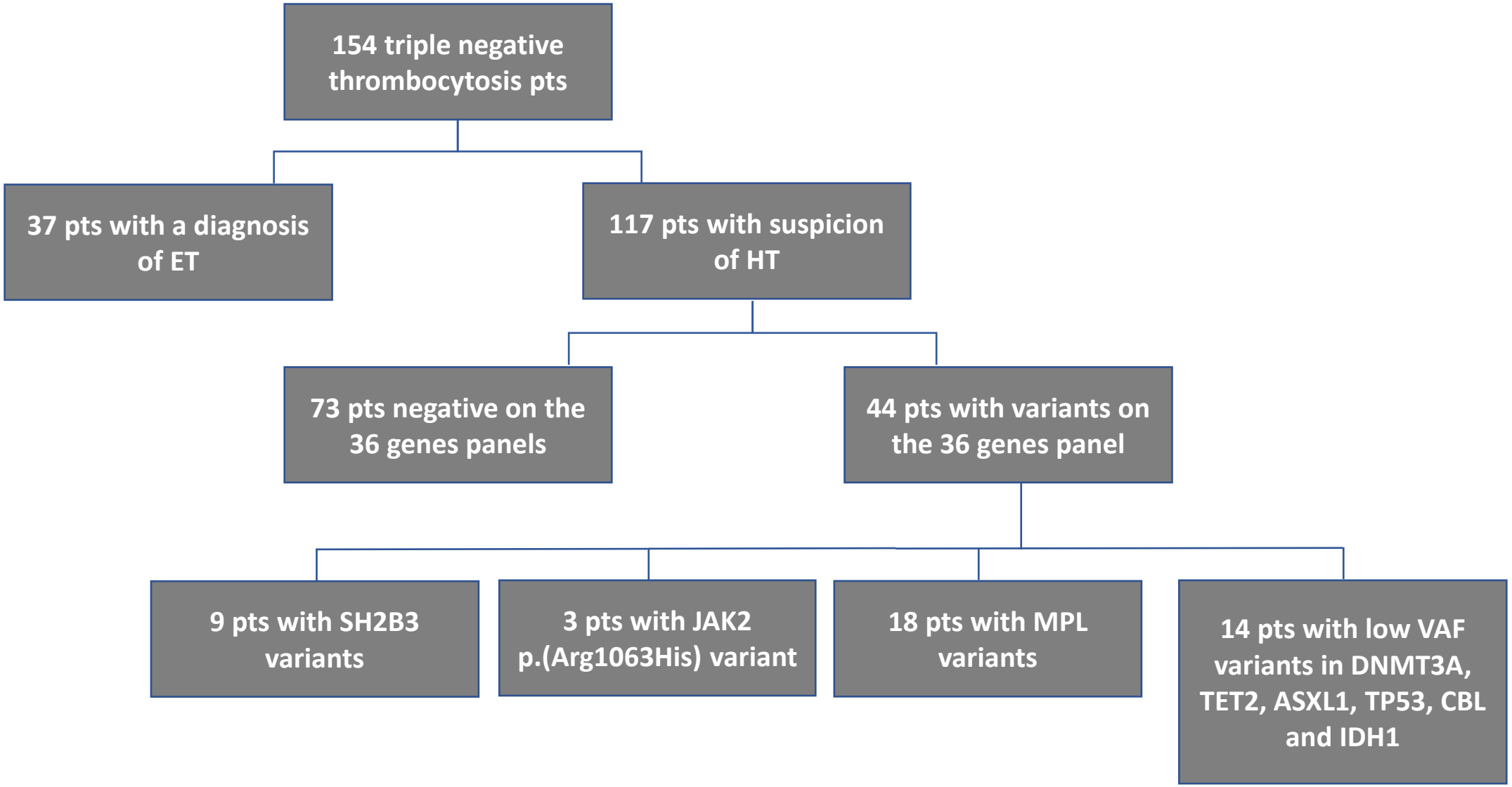
***Lnk/Sh2b3* genotype**	**+ / +**	**+ / -**	**- / -**
Platelet counts (G/L)	368 ±50	445 ±41*	1886 ±226
WBC (G/L)	8.3 ±1	8.3 ±1.9	24.6 ±5
RBC (T/L)	8.6 ±0.7	8.7 ±0.4	8.9 ±0.6
CFU-Mix (BM)	62 ±8	98 ±21	148 ±53
CFU-Mix (spleen)	6 ±4	17 ±10	46 ±22
CFU-MK (BM)	71 ±5	83 ±9	99 ±21
CFU-MK (spleen)	11 ±1	13 ±3	70 ±13

FIGURE LEGEND

Fig. 1. *SH2B3* frameshift variants display differential expression and function of the LNK adaptor protein. (A) Schematic representation of the wild-type LNK protein (LNK-WT), showing its main functional domains: DD (Dimerization Domain) in green, PH (Pleckstrin Homology) in red, and SH2 (Src Homology 2) in blue. The structure of *SH2B3* mutations identified in triple negative (TN) thrombocytosis patients is also shown. The p.(Phe365Trpfs*30) frameshift mutation eliminates the SH2 domain, while the p.(Asp231Glyfs*39) mutation disrupts the protein at the PH domain. The epitopes recognized by the N-terminal (NT) and C-terminal (CT) antibodies are also indicated. **(B)** Total platelet extracts from TN thrombocytosis patients or healthy controls (HC) were immunoblotted with LNK-specific antibodies directed against N-terminal (AP22321a, Abcepta) or C-terminal (A-12, Santa Cruz Biotechnologies) epitopes (as shown in A). Actin is shown as loading control. Asterisks mark expected positions of p.(Asp231Glyfs*39) (*) or p.(Phe365Trpfs*30) (**) truncated forms. **(C)** Schematic representation of our flow cytometry strategy (left panels). Right panels: analysis of HEK293T cells expressing LNK wild-type (WT), mutant form and control vector (CTL) stained with anti-LNK antibodies: the C-terminal antibody (LNK CT) conjugated to Alexa Fluor 647 fluorochrome (Santa Cruz Biotechnologies; x-axis) and the N-terminal antibody conjugated to Alexa Fluor 594 fluorochrome (goat anti-rabbit IgG Alexa Fluor 594 secondary antibody; y-axis). The absence of N-terminal specific signal demonstrates that the mutant allele does not give rise to any detectable protein. Numbers indicate percentage of positive cells in each quadrant. Isotype labeling is shown as control of specific labeling. **(D)** Flow cytometry analysis of JAK/STAT pathway activation in UT7 cells transduced with control (CTL), WT or p.(Asp231Glyfs*39) vectors, stimulated or not with TPO for 10 minutes and stained with a pSTAT5 antibody conjugated to PE-CF594 (BD biosciences; x-axis). The change fold of the Mean fluorescent Intensities (MFI) between non-stimulated (blue) and TPO-stimulated (orange in control; purple in WT, 1.1 ±0.14 and pink in p.Asp231Glyfs*39, 1.7 ±0.22) peaks is indicated. Plots in (C) and (D) are representative of independent experiments repeated at least three times. **(E)** Platelets isolated from healthy control (HC) and p.Asp231Glyfs*39 patient were examined by FACS analysis for STAT5 activation. The MFI fold-change before (2.7 ±0.75) and after TPO stimulation (1.8 ±0.23) for 10 minutes between HD and *SH2B3* variant is indicated. Plots are representative of triplicates from independent experiments repeated twice. Dashed yellow peak marks unstained cells.

Fig. 1 Cassinat et al.





Supplemental Figure 1: Distribution of patients

Pts: patients; ET: essential thrombocythemia ; HT: hereditary thrombocytosis

Supplementary Table 1: Patients characteristics	
	All patients with HT (n = 117)
Age at MPN diagnosis (years), median (I	44 [2;57]
Female, nb	
No	29 (24.8%)
Yes	88 (75.2%)
Hb (g/dL), median (IQR)	13.05 [12.5;14.1]
Hte (%), median (IQR)	39.8 [38.0;42.1]
Platelets (G/L), median (IQR)	636 [546;814]
WBC (G/L), median (IQR)	8.0 [6.7;11.1]
ANC (G/L), median (IQR)	4.8 [3.9;7.0]
Immature myeloid cells, nb (%)	
< 2%	97 (82.9%)
≥ 2%	3 (2.6%)
Peripheral Blasts, nb (%)	
No	95 (81.2%)
≥ 1%	1 (0.85%)
Elevated LDH, nb (%)	
No	48 (41.0%)
Yes	8 (6.8%)
Performance status, nb (%)	
0	65 (55.6%)
1	4 (3.4%)
2	0 (0%)
3	0 (0%)
Constitutional symptoms, nb (%)	
No	75 (64.1%)
Yes	1 (0.9%)
Microvascular symptoms, nb (%)	
No	70 (59.8%)
Yes	8 (6.8%)
Splenomegaly, nb (%)	
No	78 (66.7%)
Yes	4 (3.4%)
Number of thrombotic events, median	0 [0;0]
History of thrombotic events at diagnosis, nb (%)	
No	103 (88.0%)
Yes	14 (12.0%)
Number of hemorrhagic events, mediar	0 [0;0]
History of hemorrhagic events at diagnosis, nb (%)	
No	112 (95.7%)
Yes	5 (4.3%)
Evolution to myelofibrosis, nb (%)	
No	114 (97.4)
Yes	1 (0.85%)
Evolution to AML/MDS, nb (%)	
No	117 (100%)
Yes	0 (0%)
Death, nb (%)	
No	113 (96.6%)
Yes	4 (3.4%)
Karyotype, nb (%)	
Normal	20 (17.1%)
Not normal excluding complex/monosomal	1 (0.9%)
Complex/monosomal	0 (0%)