"Whole exome sequencing identifies novel MPL and JAK2 mutations in triple negative myeloproliferative neoplasms."

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Abstract

Essential thrombocythemia (ET) and primary myelofibrosis (PMF) are chronic diseases characterized by clonal hematopoiesis and hyperproliferation of terminally differentiated myeloid cells. The disease is driven by somatic mutations in exon 9 of CALR, exon 10 of MPL or JAK2-V617F in >90% of the cases, while the remaining cases are termed "triple negative". We aimed to identify the disease causing mutations in the triple negative cases of ET and PMF by applying whole exome sequencing (WES) on paired tumor and control samples from 8 patients. We found evidence of clonal hematopoiesis in 5/8 studied cases based on clonality analysis and presence of somatic genetic aberrations. WES identified somatic mutations in 3/8 cases. We did not detect any novel recurrent somatic mutations. In 3 patients with clonal hematopoiesis, analyzed by WES, we identified a somatic MPL-S204P and a germline MPL-V285E mutation, as well as a germline JAK2-G571S variant. Sequencing of entire coding region of MPL a...

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Whole exome sequencing identifies novel *MPL* and *JAK2* mutations in triple negative myeloproliferative neoplasms

Jelena D. Milosevic Feenstra[1], Harini Nivarthi[1], Heinz Gisslinger[2], Emilie Leroy[3,4], Elisa Rumi[5], Ilyas Chachoua[3,4], Klaudia Bagienski[1], Blanka Kubesova[1,6], Daniela Pietra[5], Bettina Gisslinger[2], Chiara Milanesi[5], Roland Jäger[1], Doris Chen[1], Tiina Berg[1], Martin Schalling[2], Michael Schuster[1], Christoph Bock[1], Stefan N. Constantinescu[3,4], Mario Cazzola[5,7], and Robert Kralovics[1,2]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [2]Department of Internal Medicine I, Division of Hematology and Blood Coagulation, Medical University of Vienna, Vienna, Austria; [3]Ludwig Institute for Cancer Research, Brussels, Belgium; [4]de Duve Institute, Université catholique de Louvain, Brussels, Belgium; [5]Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [6]Center of Molecular Biology and Gene Therapy, Department of Internal Medicine – Hematology and Oncology, University Hospital Brno and Medical Faculty MU, Brno, Czech Republic; [7]Department of Molecular Medicine, University of Pavia, Pavia, Italy

Short title: *MPL* and *JAK2* mutations in triple negative MPN

Corresponding author: Robert Kralovics, Ph.D.; CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, AKH

BT25.3, 1090 Vienna, Austria; Tel: +4314016070027, Fax: +43140160970000; e-mail: robert.kralovics@cemm.oeaw.ac.at

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Key points:

- **Activating mutations outside exon 10 of *MPL* were identified in 10% of triple negative cases of ET and PMF.**
- ***JAK2*-V625F and *JAK2*-F556V were identified in two triple negative cases of ET and shown to activate JAK/STAT5 signaling.**

Abstract

Essential thrombocythemia (ET) and primary myelofibrosis (PMF) are chronic diseases characterized by clonal hematopoiesis and hyperproliferation of terminally differentiated myeloid cells. The disease is driven by somatic mutations in exon 9 of *CALR*, exon 10 of *MPL* or *JAK2*-V617F in >90% of the cases, while the remaining cases are termed “triple negative”. We aimed to identify the disease causing mutations in the triple negative cases of ET and PMF by applying whole exome sequencing (WES) on paired tumor and control samples from 8 patients. We found evidence of clonal hematopoiesis in 5/8 studied cases based on clonality analysis and presence of somatic genetic aberrations. WES identified somatic mutations in 3/8 cases. We did not detect any novel recurrent somatic mutations. In 3 patients with clonal hematopoiesis, analyzed by WES, we identified a somatic *MPL*-S204P and a germline *MPL*-V285E mutation, as well as a germline *JAK2*-G571S variant. Sequencing of entire coding region of *MPL* and *JAK2* was performed in additional 62 and 49 triple negative cases of ET or PMF, respectively. We detected new somatic (T119I, S204F, E230G, Y591D) and one germline (R321W) *MPL* mutation in 5/62 cases. All the mutations were gain-of-function mutations when analyzed in functional assays. *JAK2* variants were identified in 5/57 triple negative cases and 3 of them were germline. We could demonstrate that *JAK2*-V625F and *JAK2*-F556V are gain-of-function mutations. Our results suggest that triple negative cases of ET and PMF do not represent a homogenous disease entity. Cases with polyclonal hematopoiesis might represent hereditary disorders.

Introduction

Essential thrombocythemia (ET), primary myelofibrosis (PMF) and polycythemia vera (PV) are the classical *BCR-ABL* negative myeloproliferative neoplasms (MPN). They are chronic diseases, characterized by clonal expansion of differentiated myeloid cells driven by somatic mutations. Most of the cases are sporadic, however familial clustering has also been observed. In 95% of PV and 50-60% of ET and PMF cases the disease is driven by an acquired mutation in the *JAK2* gene - V617F[1-4]. *JAK2*-V617F leads to the constitutive activation of the JAK2 kinase and subsequently the JAK/STAT signaling pathway. The remaining cases of PV carry mutations in exon 12 of *JAK2*. Although *JAK2* mutations in MPN are acquired, in recent years several families with hereditary thrombocytosis (HT) have been described to have germline *JAK2* mutations[5-7]. The second most commonly mutated gene in ET and PMF is *CALR* encoding calreticulin. Mutations in exon 9 of *CALR* have been described in 25% and 35% of patients with ET and PMF, respectively[8,9]. There is over 40 different *CALR* mutation types reported in the literature and they all result in a frameshift into alternative reading frame 1 of the last exon of *CALR* gene and hence the acquisition of a novel peptide at the C-terminus of the mutant protein[8,9]. The functional consequence of *CALR* mutations is not yet well understood, but the available data suggest that similar to *JAK2* mutations, *CALR* mutations also lead to the activation of the JAK/STAT signaling pathway[8]. Besides *JAK2* and *CALR*, a small proportion (5-10%) of ET and PMF patients carry somatic mutations in exon 10 of *MPL*, encoding the thrombopoietin receptor[10,11]. The most common mutation is W515L, however W515K, W515A, W515S, and others have been reported[11,12]. The mutations cluster in the intracellular juxtamembrane domain of the receptor, which prevents its activation in the absence of the ligand[13]. Germline

mutations in *MPL* have been associated with cases of HT. *MPL* S505N in exon 10 was found in one family, although it was also reported as somatic in sporadic cases of ET[12,14]. Mutations in *JAK2*, *CALR* and *MPL* are usually mutually exclusive and account for >90% of ET and PMF cases[8]. However, in 12% of ET and 5% of PMF cases the disease driver is currently unknown and this group of patients is referred to as the triple negative. In clinical practice, the diagnostic work-up of these patients is limited to analyzing exons 14 of *JAK2*, exon 10 of *MPL* and exon 9 of *CALR* for the presence of mutations. Mutations in other genes such as *TET2*, *ASXL1*, and *CBL* have been described in all MPN patients, however they co-occur with *JAK2*, *MPL* and *CALR* mutations and are found across a variety of myeloid malignancies[15-18]. Most of these mutations were shown to be involved in establishing clonal hematopoiesis or disease progression.

In this study we aimed to identify the disease causing mutation in triple negative cases of ET and PMF by applying whole exome sequencing (WES) primarily for the purpose of identifying somatic mutations. In cases where somatic mutations could not be identified, we looked for germline mutations that might be relevant for the phenotype. Finally we aimed to assess the functional consequence of identified mutations.

Methods

Patient samples

The study included 79 patients diagnosed with ET (N=67) or PMF (N=12), followed at the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy and the Medical University of Vienna, Vienna, Austria. All patients provided written informed consent in accordance with the Declaration of Helsinki and the study was approved by local ethics committees. Diagnostic criteria were applied as previously reported, and later revised[3,19,20]. Genomic DNA was isolated according to standard procedures from peripheral blood granulocytes, whole blood samples and immunomagnetically purified circulating T cells. *JAK2*, *MPL* and *CALR* mutation analyses were performed as previously described, and presence of these mutations was excluded in all patients included in the study[8].

Microarray analysis

DNA samples were processed according to the manufacturer's instructions and hybridized to Genome-Wide Human SNP 6.0 arrays (Affymetrix). The data was analyzed using Genotyping Console version 3.0.2 software (Affymetrix), by applying previously reported criteria for annotation[21]. The data has been deposited to the ArrayExpress database under E-MTAB-3778.

Whole exome and Sanger sequencing

DNA libraries were generated from genomic DNA isolated from granulocytes and CD3+ T-lymphocytes using TruSeq DNA Sample Prep-Kit v2 (Illumina) with

TruSeq Exome Enrichment Kit (Illumina) and Nextera Rapid Capture Exome kit (Illumina) according to the manufacturer's instructions. Sequencing was performed using the Illumina HiSeq 2000 platform, the 51-bp or 100-bp paired-end configuration and Illumina v3 reagents. Data analysis is described in Supplemental Methods.

Sanger sequencing was performed as described previously[8]. Details and primer sequences are listed in the Supplemental methods.

X-inactivation based clonality assay

Clonal analysis of hematopoietic cells was performed through the study of methylation pattern and allelic expression of *HUMARA*, *PGK* and *IDS* genes on peripheral blood granulocytes, whereas T lymphocytes were used as a control tissue, as previously described[22].

Functional analysis of identified mutations

Methods used for generation of cDNA constructs, virus production and transduction, cell proliferation and viability assays performed in Ba/F3 cells and Western blotting are described in Supplemental Methods. Details of the luciferase assay performed in γ2A cells are also described in Supplemental Methods.

Results

Whole exome sequencing identifies novel mutations in triple negative PMF and ET

To identify disease driving mutations in triple negative MPN we selected 4 cases of ET and 4 cases of PMF in which mutations in *MPL* exon 10, *CALR* exon 9 and *JAK2*-V617F were excluded, as previously reported[8]. Whole exome sequencing (WES) was performed, in all 8 cases, on the DNA samples from granulocytes and T-cells, which were considered as the tumor and the control tissue, respectively. Median number of 104,713,153 unique reads per sample was obtained, which resulted in median coverage of 110x across all target regions. The variants identified in the tumor tissue were annotated for functional consequence and several filtering criteria were applied, including subtraction of variants present in the control tissue, in order to detect potential somatic mutations (Supplemental Methods). Candidate somatic mutations were validated using Sanger sequencing on samples from both tissues, to exclude false positive and insufficiently covered germline variants. Using this approach we identified somatic mutations in 3/8 patients. Two patients diagnosed with PMF carried 10 somatic mutations each, while 2 somatic mutations were found in one patient with ET (Figure 1A). In both PMF patients we identified mutations in genes known to be relevant for myeloid malignancies, *CBL*, *TET2*, *ASXL1*, *SRSF2*, including a somatic mutation affecting the thrombopoietin receptor – *MPL*-S204P. We did not detect any recurrent novel somatic mutations in the 8 studied patients.

As in 5/8 patients we did not detect a single somatic mutation by WES, we analyzed the WES data for the presence of germline mutations in genes involved in

MPN pathogenesis, by applying the same filtering criteria as described for analysis of somatic mutations, except for the subtraction of variants present in the control tissue (Supplemental Methods). In one PMF patient we identified a germline mutation in *MPL*-V285E and validated it by Sanger sequencing. In the ET patient with two identified somatic mutations we detected and validated a germline variant in *JAK2*-G571S (Figure 1A). The germline mutations in *MPL* and *JAK2* were not annotated as common variants in any of the databases we used for the analysis of the WES data.

Granulocyte DNA samples of the 8 selected patients were, in addition to WES, analyzed for acquired chromosomal copy number alterations and uniparental disomies (UPD) using genome-wide SNP microarrays. Due to DNA quality the data was obtained for 7/8 cases. We identified a somatic UPD of chromosome 6p in one patient with PMF (Supplemental Figure 1) and a gain on chromosome 4q in a patient with ET. The patient with 6p UPD carried a germline *MPL*-V285E mutation and no detectable somatic mutations, while the ET patient with 4q gain carried the 2 validated somatic mutations in *ITGAV* and *WBSCR28* (Figure 1A).

Chromosomal aberrations often target genomic regions that carry the genes relevant for the disease. We integrated the microarray data from additional 20 triple negative MPN patients, with the microarray and WES data from the 8 patients described above, however we did not identify any somatic mutations in the regions affected with chromosomal aberrations (Figure 1B). Chromosomal aberrations were detected in 5/20 cases, suggesting that the disease is clonal in at least 25% of the cases. UPD of chromosome 6p was observed in 2 cases, however the target gene remains unknown.

As 3/8 patients studied by WES and microarrays were female, we performed X-inactivation based clonality analysis. Clonal hematopoiesis was observed in one PMF case, polyclonal pattern in one ET case, while in the second case of ET the patient was not informative at the analyzed loci. Taking results of WES, microarray analysis and clonality assay in consideration we have found evidence of clonal hematopoiesis in 5/8 triple negative MPN patients (4/4 with PMF and 1/4 with ET) included in the study, suggesting that the disease might be polyclonal in the other 3 cases. Two triple negative MPN patients carried mutations affecting the extracellular domain of the thrombopoietin receptor, in contrast to the previously reported mutations, which cluster in the juxtamembrane domain encoded by exon 10 of the *MPL* gene.

Targeted sequencing of all coding exons of *MPL* and *JAK2* genes

To determine the frequency of the *MPL* mutations outside of exon 10, in triple negative MPN patients, we performed Sanger sequencing of all coding exons of *MPL* on samples from an additional cohort of 61 triple negative MPN cases (53 patients with ET and 8 patients with PMF). Variants outside exon 10 of *MPL* were identified in 5/61 cases. The control tissue was available for 4 patients and we could determine the origin of identified mutations. *MPL*-T119I, *MPL*-S204F and *MPL*-E230G were somatic mutations (Figure 2A), while *MPL*-R321W was germline (Figure 2B). We identified a second triple negative case with the *MPL*-S204P mutation and diagnosis of ET, however the control tissue was not available. The same mutation was confirmed to be somatic in the PMF case subjected to WES (Figure 2A). We also detected a variant R321Q in one case of triple negative ET,

however this variant was reported with a variant frequency of 3% in East Asian population in the 1000 Genomes project, and therefore was not considered further for functional analysis.

Mutations outside exon 10 of *MPL* were identified in 7/69 cases of triple negative MPN (Figure 2A-B). All mutations affect the extracellular domain of the thrombopoietin receptor (Figure 2D).

In addition to these 61 cases, we performed targeted sequencing of all coding exons of *MPL* in a case of ET, which was initially diagnosed as *JAK2* and *MPL* negative, but developed a *JAK2*-V617F positivity 5.5 years following the diagnosis. We tested the diagnostic sample for *CALR* mutations, however it was negative. When we sequenced the entire coding region of *MPL* we identified a somatic *MPL*-Y591D mutation in the diagnostic sample (Figure 2A), which remained throughout the 5.5 years follow-up. Since the clinical data indicates that the *MPL*-Y591D mutation was sufficient to cause the phenotype at diagnosis we included this mutation for the functional validations.

As WES revealed a germline *JAK2*-G571S mutation in one of the triple negative MPN patients, we performed Sanger sequencing for all coding exons of *JAK2* on DNA samples from additional 49 triple negative MPN patients (41 patients with ET and 8 with PMF). We identified variants in *JAK2* in 4/49 analyzed cases. All patients with *JAK2* variants were diagnosed with ET. *JAK2*-G335D and *JAK2*-V625F were confirmed as germline mutations, while the control tissue was not available from patients with *JAK2*-F556V and a second case of *JAK2*-G571S (Figure 2C, E). Mutations affecting exons 8, 13 and 15 of *JAK2* gene were identified in total of 5/57 triple negative MPN cases.

Clonality analysis was performed for 23/39 female patients included for targeted sequencing of both *MPL* and *JAK2*. Clonal hematopoiesis was revealed in 3 cases, while 10 cases were polyclonal and 10 cases were either non-informative or showed an ambiguous result of the analysis. In regard to patients with germline mutations, patient carrying *JAK2*-V625F was not informative, while patient with *MPL*-R321W had clonal hematopoiesis. Other patients with germline mutations (*JAK2*-G335D, *JAK2*-G571S and *MPL*-V285E) were male. All the patients with germline *MPL* and *JAK2* mutations where considered as sporadic as the family history was either negative for presence of MPN phenotype in family members, or unavailable.

Functional analysis of identified *MPL* and *JAK2* mutations

We next performed functional assays to determine whether the identified MPL mutants were able to induce ligand-independent JAK-STAT signaling, similar to other pathogenic mutants such as S505N and W515[23]. We employed a luciferase reporter assay in JAK2-deficient gamma 2A cells where we transiently expressed the cDNAs for wild type or mutant MPL, JAK2, STAT5, the STAT5 reporter Spi-Luc and pRL-TK for transfection control[23]. As shown in Figure 3A expression of MPL mutants supported cytokine-independent JAK2-STAT5 signaling. Of interest, detection of activity required longer times (48h) than for the usual pathogenic mutants W515K or S505N, which exhibit constitutive activity at 24h post-transfection[23]. This data suggests that the identified mutations have a milder effect compared to the common W515K mutant.

To further study the functional consequence of MPL mutations we expressed the MPL wild type or mutants in the interleukin-3-dependent murine cell line Ba/F3.

To test how the expression of MPL mutants affects proliferation of Ba/F3 cells, we performed a starvation assay for 72h (Figure 3B). None of the mutants conferred cytokine independent growth of Ba/F3 cells or hypersensitivity to IL-3, except for the W515K which was used as a positive control (Figure 3B-C and Supplemental Figure 2A). Since most of the mutations clustered in the extracellular domain of the thrombopoietin receptor, we tested the cell viability in the dilution series of TPO to determine whether the mutations confer hypersensitivity to TPO (Figure 3D and Supplemental Figure 2B). We did not observe a difference between the cells expressing the mutants affecting the extracellular domain of MPL compared to the wild type. However, Ba/F3 cells expressing MPL-Y591D showed marked hypersensitivity to TPO. To confirm that the MPL-Y591D mutation gives proliferative advantage to the Ba/F3 cells, we performed a competition assay in which the cells expressing MPL wild type (pMSCV-HA-MPLwt-puro) were mixed in 4:1 ratio with cells expressing MPL-Y591D and GFP (pMx-IRES-HA-MPLY591D-GFP). We assessed the GFP positivity by flow cytometry analysis at day 0, 3 and 6. In two independent biological replicates we could show that the GFP positivity of mixed culture reaches 60% in the presence of 1ng/mL of TPO in 6 days, meaning that expression of MPL-Y591D confers proliferative advantage *in vitro* (Figure 3F and Supplemental Figure 2D). The effect on proliferation was lower than the one observed for MPL-W515K, and also absent when the cells were cultured in the presence of IL-3 (Figure 3E and Supplemental Figure 2C).

The functional data suggests that all identified MPL mutations are gain-of-function mutations, leading to the activation of JAK2-STAT5 signaling. MPL-Y591D is the only mutation which gives an obvious but mild proliferative advantage to the cells *in vitro*.

We employed the luciferase reporter assay in JAK2-deficient gamma 2A cells to assess the ligand-independent JAK-STAT5 signaling activation of identified JAK2 mutants in the presence of MPL (Figure 4A) and EPOR (Supplemental Figure 3A)[23]. Only the expression of JAK2-V625F supported cytokine-independent JAK2-STAT5 signaling in the presence of MPL, while no difference was observed for other mutants compared to the wild type. Upon expressing all the JAK2 mutants or the wild type in Ba/F3-MPL cells, we tested the cell viability in the serial dilution of IL-3 and TPO concentrations. The cells expressing JAK2-V625F showed hypersensitivity to both IL-3 and TPO (Figure 4B-C, Supplemental Figure 3B-C), while for JAK2-F556V expressing cells we could also observe hypersensitivity to TPO. In addition we examined the phosphorylation of STAT5 in the absence of cytokine in the transfected cell lines. We detected increased phosphorylation of STAT5 in the absence of cytokines in the cells expressing JAK2-V625F and JAK2-F556V (Figure 4D). Thus, increased activation of JAK-STAT signaling is likely responsible for the hypersensitivity to TPO observed for the two mutants.

Discussion

We identified somatic and germline mutations outside exon 10 of *MPL* in 10.1% (7/69) cases of triple negative ET and PMF. Somatic mutations of MPL at positions T119I, S204F, S204P and E230G were detected in exons 3, 4 and 5, respectively, while germline mutations V285E and R321W were affecting exon 6 of *MPL*. In one additional triple negative case of ET we identified a somatic mutation in exon 12 of *MPL*, *MPL*-Y591D, at diagnosis, however the patient became *JAK2*-V617F positive in a 5.5 year follow-up. Germline mutations of the JAK2 at positions G335D, G571S and V625F, and mutation of unknown origin F556V in exons 8, 13 and 15 of *JAK2* were found in 8.8% (5/57) cases of triple negative ET and PMF. All mutations were heterozygous. Taken together, non-canonical mutations (outside of exons usually examined for diagnostic purposes) of *MPL* and *JAK2* are present in about 18.9% of triple negative MPN cases. We could demonstrate that all identified MPL mutations lead to constitutive activation of JAK/STAT5 signaling, and that MPL-Y591D provides proliferative advantage to Ba/F3 cells. We could demonstrate that *JAK2*-V625F is a gain-of-function mutation, while for F556V we found evidence of constitutive activation of JAK/STAT5 signaling by Western immunodetection. However, the other JAK2 mutants do not significantly influence the JAK/STAT5 signaling in the presence of EPOR or MPL *in vitro*.

Mutations outside exon 10 of *MPL* have previously been reported in the literature, but their origin was not determined mainly due to lack of control tissue availability. *MPL*-S204F mutation associated with chromosome 1p UPD and heterozygous *MPL*-S204P mutation have previously been identified in two *JAK2*-

V617F negative PMF cases[24,25], while *MPL*-Y591D was reported in a *JAK2*-V617F positive PV patient with chromosome 1p UPD[24]. In all three cases the control tissue was not available, and the functional relevance of the mutations was not shown. We could demonstrate that somatic *MPL*-Y591D mutation was present at diagnosis in a triple negative ET patient who during follow-up acquired *JAK2*-V617F. Hitchcock et al. have shown that MPL-Y591 is a part of the YRRL motif (Figure 2A), that through interaction with AP2 regulates surface expression and degradation of MPL upon stimulation[26]. In the same study the authors showed that cells expressing MPL-Y591F have a proliferation advantage and an increased JAK2, STAT5 and AKT activation upon stimulation[26]. It is unclear why *MPL*-Y591D is found together with *JAK2*-V617F. Further studies will be necessary to determine whether combination of these mutations has a specific effect on the patient's phenotype.

Germline *MPL* mutations have been reported in cases of HT. Clinical features of ET and HT are similar and that is why some of the HT cases could be diagnosed as sporadic ET especially if the family history is unknown or absent. The differences in the biology of the two diseases are mainly that HT exhibit polyclonal hematopoiesis, lack somatic mutations and have generally better prognosis without disease progression[27]. The first germline *MPL* mutation associated with HT was S505N in exon 10, which was also reported as somatic in sporadic ET[12,14]. *MPL*-Y252H mutation was reported in a pediatric case of ET with clonal hematopoiesis without evidence of the mutation origin[28], while P106L was reported as homozygous in several Arabic families with HT[29]. In our cohort, two activating germline mutations were identified in cases initially diagnosed as PMF (V285E) and ET (R321W). We cannot exclude the possibility that these mutations could have been acquired in common progenitors, as the only available germline control was DNA isolated from

T-cells. Both patients had clonal hematopoiesis, as detected by presence of acquired UPD of chromosome 6 in patient with PMF (Supplemental Figure 1) and X-inactivation based clonality assay in the patient with ET. We can only speculate that *MPL*-V285E and *MPL*-R321W are either not sufficient to cause the observed phenotypes and that an additional event is necessary, likely the target of 6p aberration in the case with PMF, as we did not detect any other somatic mutations in this patient, or that the 6p aberration and presence of clonal hematopoiesis are associated with disease progression.

The four somatic mutations we identified were affecting the extracellular ligand binding domain of the MPL[30,31], while the two germline mutations affected the membrane proximal extracellular domain which appears to play a role in negative regulation of receptor activation. We initially hypothesized that the mutations might affect the affinity to TPO, however, the luciferase assay we performed in gamma 2A cells showed that all identified mutants activated STAT5 induced transcription in the absence of stimulation with TPO.

Triple negative status of patients with PMF was shown to associate with inferior overall and leukemia-free survival[32], however in 2/12 triple negative PMF patients with novel mutations identified in our study, *MPL*-S204P and *MPL*-V285E, we did not observe disease progression or leukemic transformation in a 12-year and 6-year follow-up, respectively. Although the number of patients is too low to perform statistical analyses, the clinical data indicates that these patients might have a better clinical course, in accordance with our molecular and functional findings. Analysis of larger patient cohorts will be necessary to confirm the effect of non-canonical *MPL* mutations on patients' prognosis.

Germline *JAK2* mutations clustering in the pseudokinase or kinase domain have been reported in several families with HT[5-7]. In all cases, they were weak gain-of-function mutations. Here we reported 4 different *JAK2* mutations in 5 triple negative ET cases. For JAK2-V625F and JAK2-F556V we could demonstrate that they are gain-of-function mutations leading to increased phosphorylation of STAT5 and hypersensitivity to TPO. *JAK2*-G571S was found in 2 patients. In one case we had evidence for presence of somatic mutations identified by WES and a gain of chromosome 4q, indicating a clonal disease. *JAK2*-G571S was previously reported in a *CALR* positive patient with ET, and the lack of any hematological phenotype in family members carrying *JAK2*-G571S indicated that this mutation might be a polymorphism not relevant for the disease[33]. We did not find evidence for G571S and G335D mutations altering the function of JAK2. These *JAK2* variants either do not play a role in the disease pathogenesis or require additional genetic events to exert a phenotype.

We cannot exclude that some of the patients with wild type *MPL* and *JAK2*, as determined by Sanger sequencing, might carry low burden mutations in these genes, as sensitivity of this method is estimated to ≥10%. Our data indicate that inclusion of all coding exons of *JAK2* and *MPL* in the targeted sequencing panels designed for next-generation sequencing based approaches for detection of mutations, which are likely to enter the clinical practice in near future, is recommended. Analysis of bigger patient cohorts using such panels will provide more accurate estimation of the frequency of the non-canonical *MPL* and *JAK2* mutations in triple negative MPN, and their relevance for diagnostic purposes.

In the rest of the cases studied by WES we did not identify a causative mutation due to absence of recurrent mutations. Similar results were obtained by

other groups[34,35], who reported clonal hematopoiesis in 10/17 of the cases, similar to our study (evidence of clonality in 5/8 patients). The cases with polyclonal hematopoiesis are likely to be hereditary MPN-like disorders. The lack of recurrency and the fact that in one female PMF patient with clonal hematopoiesis we did not identify any somatic mutations, can be attributed to the limitations of the WES approach, which can only detect mutations in the coding exons of genes. RNA sequencing for detection of fusion oncogenes and whole genome sequencing for covering the regulatory sequences in the genome might provide an answer to which mutations drive the disease in the remaining triple negative MPN cases and whether they represent a heterogeneous disease entity.

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Author contributions

J.D.M.F. and R.K. conceived and designed the experiments and wrote the paper. J.D.M.F., H.N., E.L., I.C., K.B., B.K., D.P., C.M., R.J., T.B., Ma.S. performed the experiments. J.D.M.F., H.N., E.L., E.R., I.C., D.P., C.M., D.C., Mi.S., C.B., S.N.C. and R.K. analysed and interpreted the data. H.G., E.R., B.G., C.B., S.C.N., M.C. and R.K. contributed reagents/materials/analysis tools. All authors contributed to the final version of the manuscript.

Conflict of Interest Disclosure: The authors have no conflict of interest to disclose.

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Figure legends

Figure 1. Overview of genetic aberrations identified in 28 patients with triple negative essential thrombocythemia or primary myelofibrosis. (A) Genetic findings from 4/8 studied patients, in which somatic genetic aberrations were identified using whole exome sequencing or microarray analysis. Somatic mutations are marked in black and germline mutations in blue. (B) Genomic overview of somatic mutations identified in 8 patients analyzed by whole exome sequencing and chromosomal aberrations found in 28 patients, detected with Affymetrix Genome-Wide Human SNP 6.0 microarrays. The position and size of the colored bars indicate the chromosomal aberrations. Red bars depict deletions, blue uniparental disomies and green gains. ET, essential thrombocythemia; PMF, primary myelofibrosis; UPD, uniparental disomy.

Figure 2. Mutations outside exon 10 of MPL and exons 12 and 14 of JAK2 identified in triple negative essential thrombocythemia (ET) and primary myelofibrosis (PMF). (A) Somatic mutations in MPL identified in 4 cases of ET and one case of PMF. A common polymorphism is marked with the blue triangle. (B) Germline mutations in MPL identified in single cases of ET and PMF. (C) Germline JAK2 mutations found in 3 cases of triple negative ET. Red boxes in (A), (B) and (C) represent the amino acid change and black arrows indicate mutated bases. PT, patient; Ref, reference; GRN, granulocytes. (D) Schematic representation of the thrombopoietin receptor and the positions of mutations identified in triple negative ET and PMF. Somatic mutations are indicated in red. (E) Schematic representation of

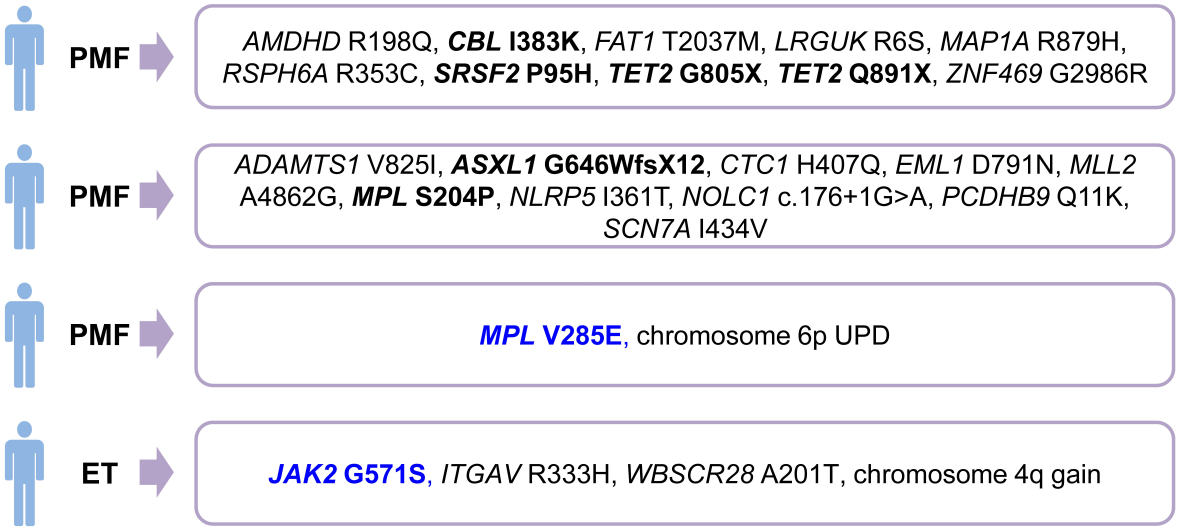
the JAK2 kinase and the mutations found in ET. Black stars in (D) and (E) indicate positions of MPL W515K and JAK2 V617F, respectively.

Figure 3. Functional analysis of MPL mutants. (A) STAT dependent transcriptional activity induced by wild type MPL or mutants of MPL. Gamma 2A cells which are JAK2-deficient were transiently transfected with wild type MPL (or mutants), JAK2, STAT5 and with Firefly STAT5 luciferase reporter spi-Luc and pRL-TK vector coding for renilla luciferase. Luminescence was measured 48 h after transfection. After transfection medium was changed after 5h and replaced either with culture medium or medium supplemented with Tpo (10 ng/mL). Shown are average units + SEM of one representative experiment performed in triplicate out of three. ** $P<.001$; * $P<.05$. (B) Number of viable Ba/F3 cells expressing wild type MPL or different MPL mutants, in the absence of interleukin-3, was measured every 24h for 72h. The data is shown as average of two biological replicates performed each in triplicate ± SEM. (C-D) The viability of Ba/F3 cells expressing wild type MPL or different mutants was assessed after 72h in the presence of increasing concentrations of interleukin-3 (C) and thrombopoietin (D). Error bars represent SEM. (E-F) Ba/F3 cells expressing wild type MPL (MPL wt-puro) but not GFP were mixed with Ba/F3 cells expressing either wild type MPL (MPL wt-GFP), MPL Y591D (MPL Y591D-GFP) or MPL W515K (MPL W515K-GFP) and GFP in the 4:1 ratio and cultured in the presence of 1ng/mL interleukin-3 (E) or 1ng/mL thrombopoietin (F). The GFP positivity of the mixed culture was assessed every 72h by flow cytometric analysis. The experiment was performed in triplicate, with error bars representing SEM. SEM, standard error of the mean.

Figure 4. Functional analysis of JAK2 mutants. (A) STAT dependent transcriptional activity induced by wild type JAK2 or mutants of JAK2. Gamma 2A cells which are JAK2-deficient were transiently transfected with wild type JAK2 (or wild type JAK2 and mutant JAK2 in 1:1 ratio to represent heterozygous condition), MPL, STAT5 and with Firefly STAT5 luciferase reporter spi-Luc and pRL-TK vector coding for renilla luciferase. Luminescence was measured 24h after transfection. After transfection medium was changed after 5h and replaced either with culture medium or medium supplemented with Tpo (10 ng/mL). Shown are means ± standard error of the mean of 3 independent experiments done in triplicate. ** $P<.001$; * $P<.05$. (B-C) The viability of Ba/F3 cells expressing wild type MPL together with wild type JAK2 or different mutants was assessed after 72h in the presence of increasing concentrations of interleukin-3 (B) and thrombopoietin (C). Error bars represent SEM. (D) The activation of STAT5 in starving condition. Ba/F3 cells expressing the wild type MPL only, or together with wild type JAK2 or JAK2 mutants were cultured for 48h on TPO (1ng/mL) and then starved for 4 hours in serum free medium without TPO. Western blot was performed on the cell lysates with antibodies against pYSTAT5, STAT5, HA, and JAK2. An antibody against Hsc70 was used as loading control.

Figure 1.

A


PMF
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PMF
ADAMTS1 V825I, ***ASXL1* G646WfsX12**, *CTC1* H407Q, *EML1* D791N, *MLL2* A4862G, ***MPL* S204P**, *NLRP5* I361T, *NOLC1* c.176+1G>A, *PCDHB9* Q11K, *SCN7A* I434V
PMF
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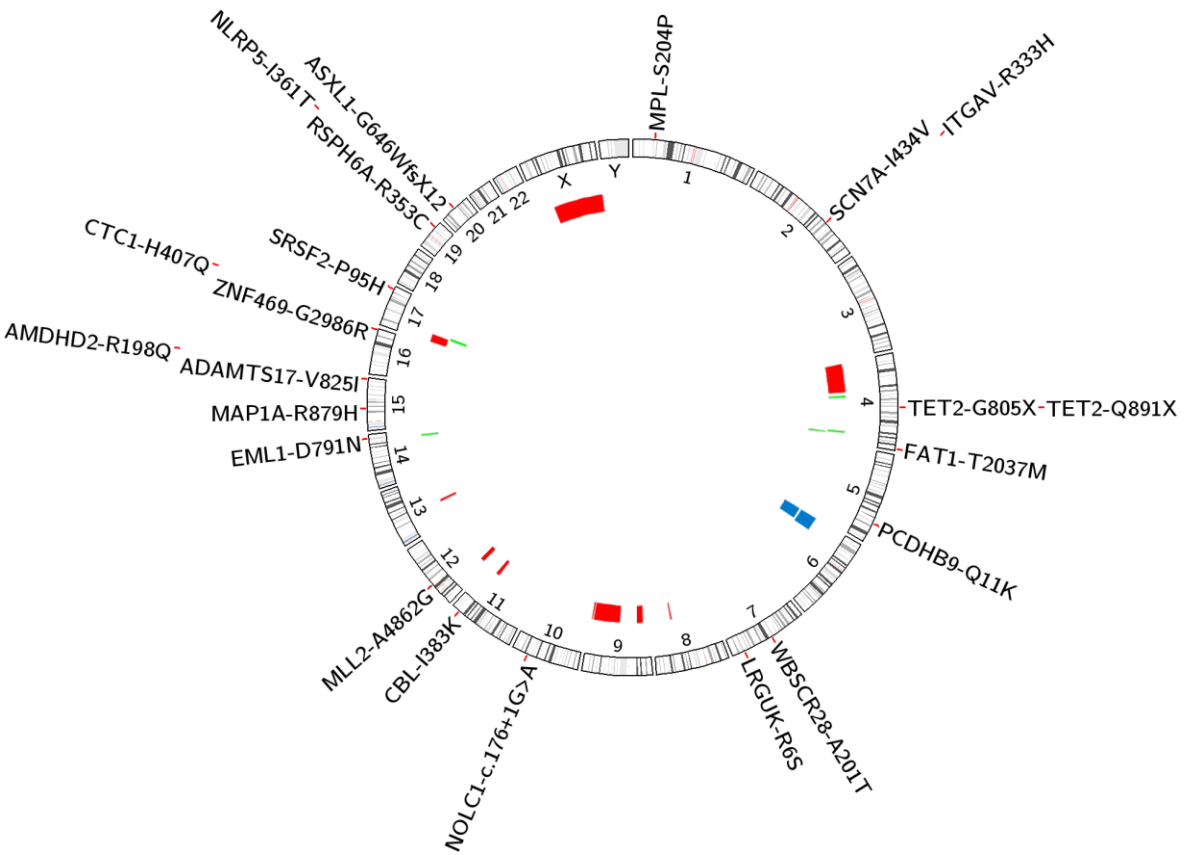
ET
***JAK2* G571S**, *ITGAV* R333H, *WBSCR28* A201T, chromosome 4q gain

B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ITGAV-R333H
SCN7A-I434V
TET2-G805X
TET2-Q891X
FAT1-T2037M
PCDHB9-Q11K
WBSCR28-A201T
LRGUK-R6S
NOLC1-c.176+1G>A
CBL-I383K
MLL2-A4862G
EML1-D791N
MAP1A-R879H
ADAMTS17-V825I
AMDHD2-R198Q
ZNF469-G2986R
CTC1-H407Q
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RSPH6A-R353C
ASXL1-G646WfsX12
NLRP5-I361T

Figure 2.

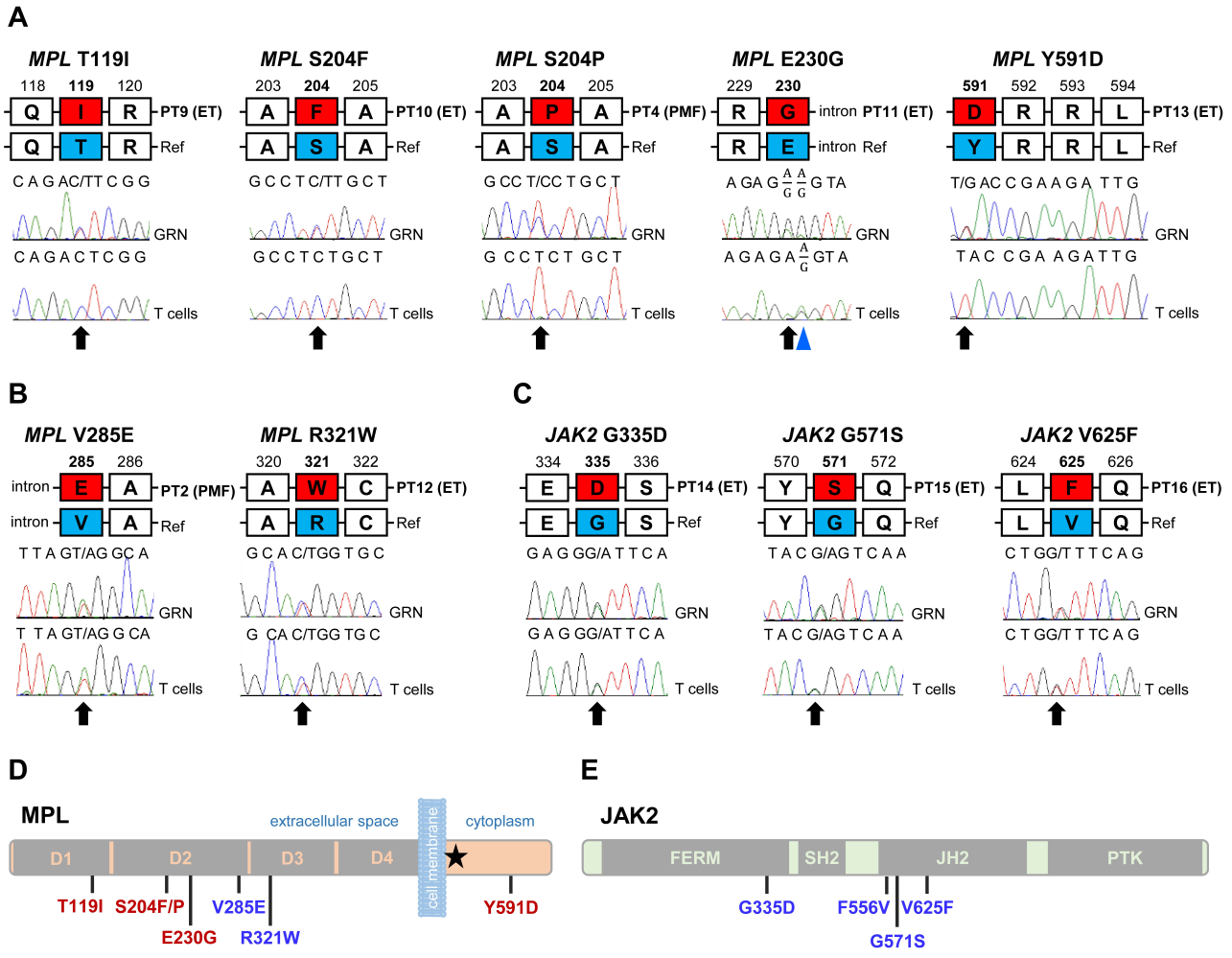
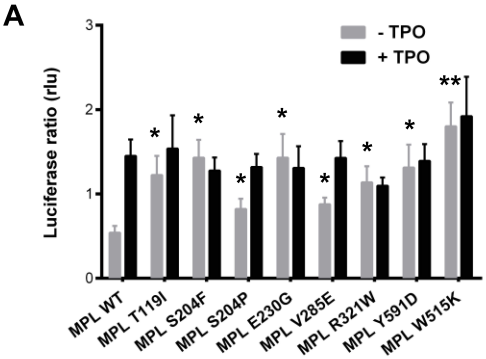
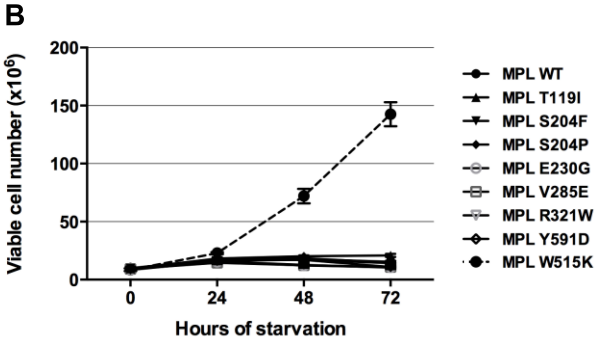
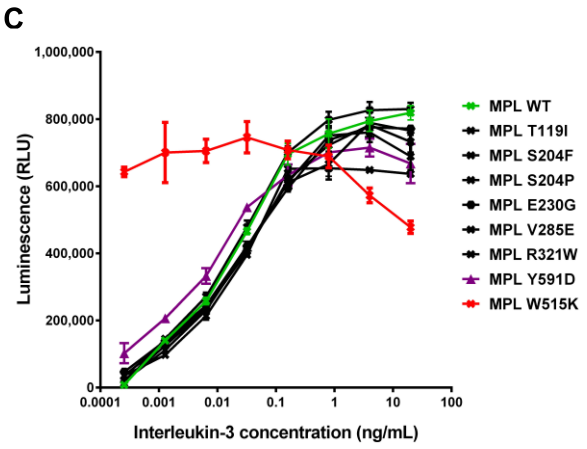
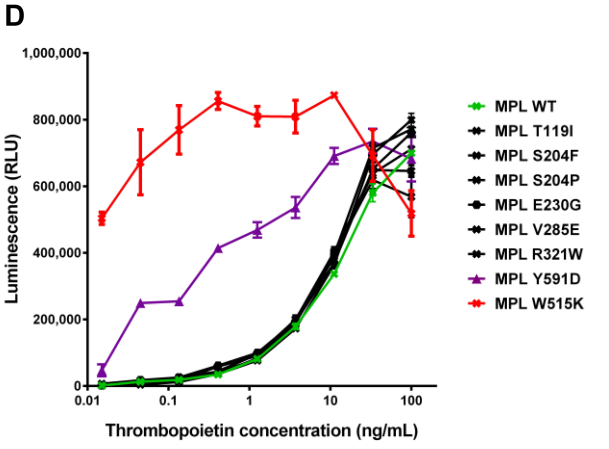

A
MPL T119I
118 119 120
Q I R PT9 (ET)
Q T R Ref
C A G AC/TT C G G
GRN
C A G A C T C G G
T cells
MPL S204F
203 204 205
A F A PT10 (ET)
A S A Ref
G C C T C/TT G C T
GRN
G C C T C T G C T
T cells
MPL S204P
203 204 205
A P A PT4 (PMF)
A S A Ref
G CC T/CC T G C T
GRN
G C C T C T G C T
T cells
MPL E230G
229 230
R G intron PT11 (ET)
R E intron Ref
A GA G A/G A/G G TA
GRN
A G A G A A/G G T A
T cells
MPL Y591D
591 592 593 594
D R R L PT13 (ET)
Y R R L Ref
T/G AC C G A A G A T T G
GRN
T A C C G A A G A T T G
T cells
B
MPL V285E
285 286
intron E A PT2 (PMF)
intron V A Ref
T T A GT/AG GC A
GRN
T T A GT/AG G CA
T cells
MPL R321W
320 321 322
A W C PT12 (ET)
A R C Ref
G C A C/TGG T G C
GRN
G CA C/TGG TG C
T cells
C
JAK2 G335D
334 335 336
E D S PT14 (ET)
E G S Ref
G A G GG/A T T C A
GRN
G A G GG/AT T C A
T cells
JAK2 G571S
570 571 572
Y S Q PT15 (ET)
Y G Q Ref
T A C G/AG T C A A
GRN
T A C G/AG T C A A
T cells
JAK2 V625F
624 625 626
L F Q PT16 (ET)
L V Q Ref
C T GG/T T T C A G
GRN
C T GG/T T TC A G
T cells
D
MPL
extracellular space
cytoplasm
cell membrane
D1
D2
D3
D4
T119I
S204F/P
E230G
V285E
R321W
Y591D
E
JAK2
FERM
SH2
JH2
PTK
G335D
F556V
V625F
G571S

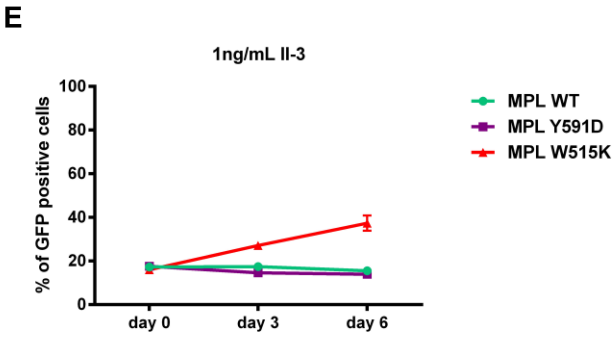
Figure 3.


A
- TPO
+ TPO
Luciferase ratio (rlu)
MPL WT
MPL T119I
MPL S204F
MPL S204P
MPL E230G
MPL V285E
MPL R321W
MPL Y591D
MPL W515K


B
Viable cell number (x10^6)
Hours of starvation
MPL WT
MPL T119I
MPL S204F
MPL S204P
MPL E230G
MPL V285E
MPL R321W
MPL Y591D
MPL W515K


C
Luminescence (RLU)
Interleukin-3 concentration (ng/mL)
MPL WT
MPL T119I
MPL S204F
MPL S204P
MPL E230G
MPL V285E
MPL R321W
MPL Y591D
MPL W515K


D
Luminescence (RLU)
Thrombopoietin concentration (ng/mL)
MPL WT
MPL T119I
MPL S204F
MPL S204P
MPL E230G
MPL V285E
MPL R321W
MPL Y591D
MPL W515K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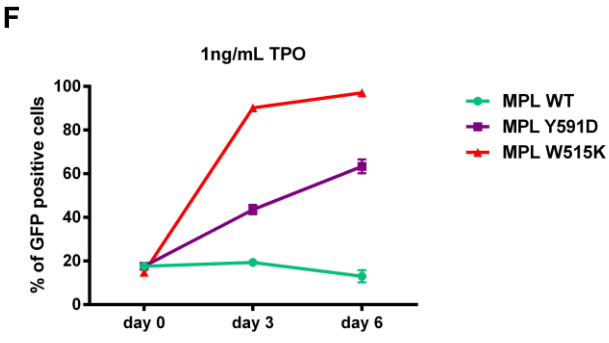
1ng/mL Il-3
% of GFP positive cells
day 0
day 3
day 6
MPL WT
MPL Y591D
MPL W515K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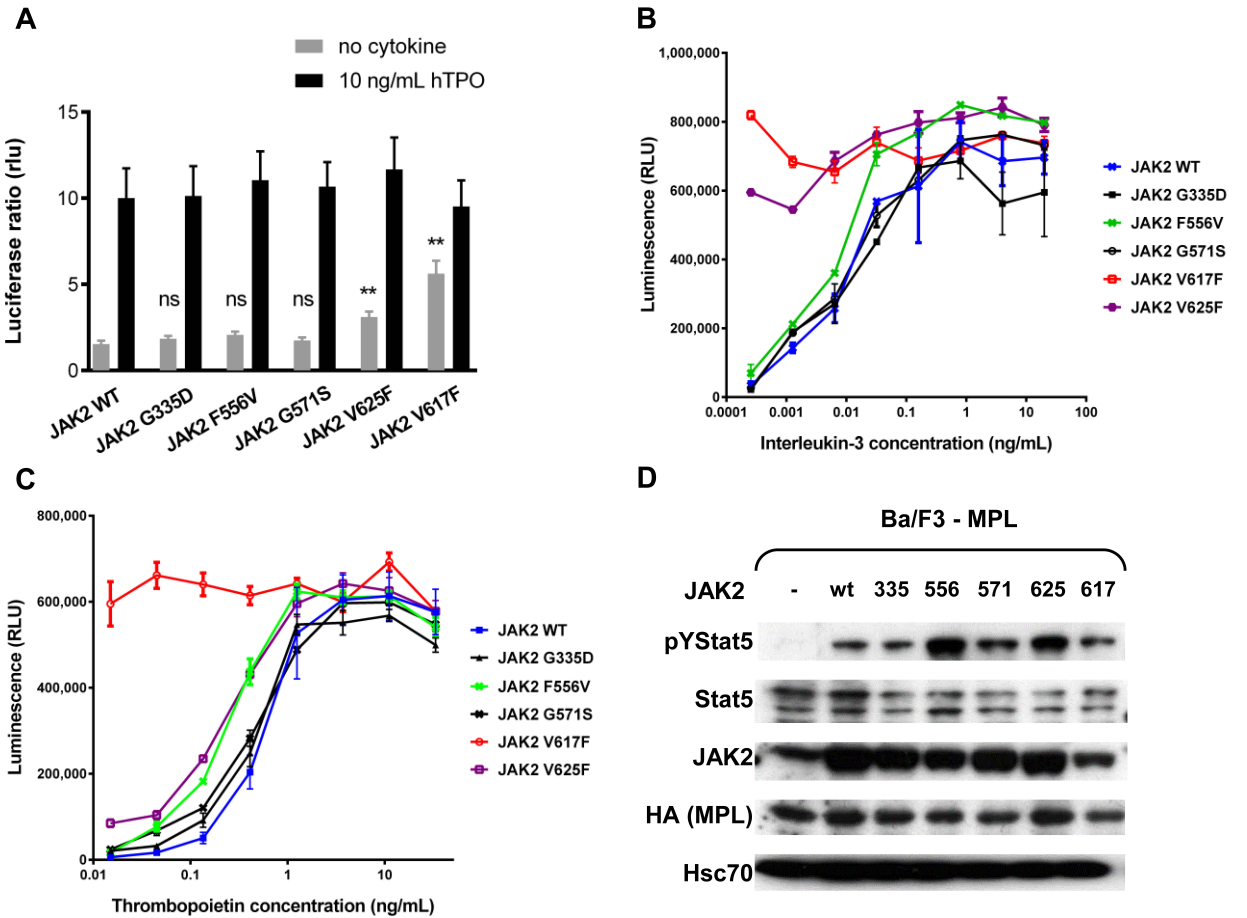
% of GFP positive cells
day 0
day 3
day 6
MPL WT
MPL Y591D
MPL W515K

Figure 4.


A
no cytokine
10 ng/mL hTPO
Luciferase ratio (rlu)
15
10
5
0
ns
ns
ns
**
**
JAK2 WT
JAK2 G335D
JAK2 F556V
JAK2 G571S
JAK2 V625F
JAK2 V617F
B
Luminescence (RLU)
1,000,000
800,000
600,000
400,000
200,000
0
0.0001
0.001
0.01
0.1
1
10
100
Interleukin-3 concentration (ng/mL)
JAK2 WT
JAK2 G335D
JAK2 F556V
JAK2 G571S
JAK2 V617F
JAK2 V625F
C
Luminescence (RLU)
800,000
600,000
400,000
200,000
0
0.01
0.1
1
10
Thrombopoietin concentration (ng/mL)
JAK2 WT
JAK2 G335D
JAK2 F556V
JAK2 G571S
JAK2 V617F
JAK2 V625F
D
Ba/F3 - MPL
JAK2
-
wt
335
556
571
625
617
pYStat5
Stat5
JAK2
HA (MPL)
Hsc70

blood

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Whole exome sequencing identifies novel *MPL* and *JAK2* mutations in triple negative myeloproliferative neoplasms

Jelena D. Milosevic Feenstra, Harini Nivarthi, Heinz Gisslinger, Emilie Leroy, Elisa Rumi, Ilyas Chachoua, Klaudia Bagienski, Blanka Kubesova, Daniela Pietra, Bettina Gisslinger, Chiara Milanesi, Roland Jäger, Doris Chen, Tiina Berg, Martin Schalling, Michael Schuster, Christoph Bock, Stefan N. Constantinescu, Mario Cazzola and Robert Kralovics