ORIGINAL ARTICLE

Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms

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ABSTRACT

BACKGROUND

Approximately 50 to 60% of patients with essential thrombocythemia or primary myelofibrosis carry a mutation in the Janus kinase 2 gene (*JAK2*), and an additional 5 to 10% have activating mutations in the thrombopoietin receptor gene (*MPL*). So far, no specific molecular marker has been identified in the remaining 30 to 45% of patients.

METHODS

We performed whole-exome sequencing to identify somatically acquired mutations in six patients who had primary myelofibrosis without mutations in *JAK2* or *MPL*. Resequencing of *CALR*, encoding calreticulin, was then performed in cohorts of patients with myeloid neoplasms.

RESULTS

Somatic insertions or deletions in exon 9 of *CALR* were detected in all patients who underwent whole-exome sequencing. Resequencing in 1107 samples from patients with myeloproliferative neoplasms showed that *CALR* mutations were absent in polycythemia vera. In essential thrombocythemia and primary myelofibrosis, *CALR* mutations and *JAK2* and *MPL* mutations were mutually exclusive. Among patients with essential thrombocythemia or primary myelofibrosis with nonmutated *JAK2* or *MPL*, *CALR* mutations were detected in 67% of those with essential thrombocythemia and 88% of those with primary myelofibrosis. A total of 36 types of insertions or deletions were identified that all cause a frameshift to the same alternative reading frame and generate a novel C-terminal peptide in the mutant calreticulin. Overexpression of the most frequent *CALR* deletion caused cytokine-independent growth in vitro owing to the activation of signal transducer and activator of transcription 5 (STAT5) by means of an unknown mechanism. Patients with mutated *CALR* had a lower risk of thrombosis and longer overall survival than patients with mutated *JAK2*.

CONCLUSIONS

Most patients with essential thrombocythemia or primary myelofibrosis that was not associated with a *JAK2* or *MPL* alteration carried a somatic mutation in *CALR*. The clinical course in these patients was more indolent than that in patients with the *JAK2* V617F mutation. (Funded by the MPN Research Foundation and Associazione Italiana per la Ricerca sul Cancro.)

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PHILADELPHIA CHROMOSOME–NEGATIVE myeloproliferative neoplasms include polycythemia vera, essential thrombocythemia, and primary myelofibrosis.[1] A unique gain-of-function mutation in the Janus kinase 2 gene (*JAK2*) is found in about three quarters of patients in whom these disease entities have been diagnosed.[2,3] The valine-to-phenylalanine (V617F) alteration constitutively activates JAK2, resulting in increased phosphorylation of its substrates and leading to increased cytokine responsiveness of myeloid cells. The *JAK2* V617F mutation is present in approximately 95% of patients with polycythemia vera and in 50 to 60% of those with essential thrombocythemia or primary myelofibrosis.[4]

In addition, somatic mutations of *JAK2* exon 12 are found in polycythemia vera,[5,6] and activating mutations of the thrombopoietin receptor gene *MPL* are present in 5 to 10% of patients with essential thrombocythemia or primary myelofibrosis with nonmutated *JAK2*.[7,8] Thus, whereas somatic *JAK2* mutations are found in nearly all patients with polycythemia vera,[9] approximately one third of patients with essential thrombocythemia or primary myelofibrosis do not carry any mutation in *JAK2* or *MPL*.[7,8] Somatic mutations in other genes, such as *TET2*,[10,11] *CBL*,[12,13] *EZH2*,[14,15] *DNMT3A*,[16] and *ASXL1*,[17] are present in a proportion of cases of myeloproliferative neoplasms, but these can co-occur with *JAK2* and *MPL* mutations and are found in all types of myeloid cancers.

The natural history of Philadelphia chromosome–negative myeloproliferative neoplasms is characterized not only by the occurrence of thromboembolic complications but also by a tendency toward progression to more aggressive disease, including post–polycythemia vera myelofibrosis or post–essential thrombocythemia myelofibrosis and acute myeloid leukemia or blast-phase disease.[1] Progression of the disease is typically associated with the acquisition of somatic mutations in driver genes responsible for subclonal evolution.[18-22] However, these mutated genes do not have a primary role in the pathogenesis of essential thrombocythemia or primary myelofibrosis associated with nonmutated *JAK2* and *MPL*. We performed whole-exome sequencing to identify somatic mutations responsible for the initiation of disease in these subsets of myeloproliferative neoplasms.

METHODS

PATIENTS AND SAMPLES

We studied patients with Philadelphia chromosome–negative myeloproliferative neoplasms who were followed at the Medical University of Vienna in Austria, and Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo in Pavia, Italy. The study was approved by the ethics committee at each institution, and all the patients provided written informed consent.

Genomic DNA was obtained from bone marrow mononuclear cells, peripheral-blood granulocytes, or whole-blood samples. Germline control DNA was obtained from immunomagnetically purified circulating T cells.

WHOLE-EXOME SEQUENCING

Whole-exome sequencing was performed in six patients with primary myelofibrosis who did not have mutations in *JAK2* or *MPL*. Genomic DNA libraries were generated from peripheral-blood granulocyte DNA (tumor samples) and matched CD3+ T-lymphocyte DNA (control samples) with the use of the NEBNext DNA Sample Prep Reagent set (New England BioLabs). Whole-exome enrichment was performed with the use of the SureSelect Human All Exon kit (Agilent Technologies) according to the manufacturer's instructions. The libraries were sequenced with the use of a HiSeq 2000 sequencing system (Illumina). The data analysis is described in the Supplementary Appendix, available with the full text of this article at NEJM.org.

TARGETED RESEQUENCING AND MUTATION SCREENING

Genomic regions of interest were amplified by means of polymerase chain reaction (PCR). PCR products were purified, and a sequencing reaction was set up with the use of the BigDye Terminator, version 3.1, Cycle Sequencing Kit (Life Technologies). Sequencing products were analyzed with the use of a 3130xl Genetic Analyzer (Applied Biosystems). To screen for insertion and deletion mutations in *CALR* exon 9, we designed PCR primers spanning exon 9 and labeled the forward primer with 6-carboxyfluorescein. After PCR, the products were sized on a 3130xl Genetic Analyzer. Details of the methods and analysis are provided in the Supplementary Appendix.

RESULTS

WHOLE-EXOME SEQUENCING IN PRIMARY MYELOFIBROSIS

Using whole-exome sequencing, we analyzed genomic DNA from granulocytes (tumor samples) and CD3+ T cells (control samples) obtained from six patients with primary myelofibrosis. Independent validation of the detected variants with the use of classic Sanger sequencing confirmed the presence of 2 to 12 somatic mutations per patient (Table S3 in the Supplementary Appendix). The only recurrently affected gene was CALR, encoding calreticulin.

Two patients had somatic deletions in exon 9 of CALR. PCR-product subcloning and sequencing revealed that Patient H_0191 had a 52-bp deletion and Patient H_0296 had a 1-bp deletion (Fig. S1 in the Supplementary Appendix). Because the 52-bp deletion in Patient H_0191 was incorrectly annotated as a 1-bp deletion coupled with a single nucleotide variant by our variant-calling analysis pipeline (Table S3 in the Supplementary Appendix), we manually reviewed the sequence alignment for this patient. We observed a misalignment of the sequence reads covering the site of mutation, owing to a repetitive element in the affected genomic region (Fig. S2 in the Supplementary Appendix).

Following up on this finding, we investigated the alignments for the remaining four patients and detected a recurrent 5-bp insertion in all four (Fig. S3 in the Supplementary Appendix). The mutations in CALR that were found by means of whole-exome sequencing were confirmed and shown to be somatic by means of Sanger sequencing of the granulocyte and matched T-lymphocyte DNA samples from all six patients.

CALR EXON 9 MUTATIONS IN MYELOPROLIFERATIVE NEOPLASMS

To estimate the prevalence of CALR mutations in myeloproliferative neoplasms, we screened a cohort of 896 patients for insertion or deletion mutations in CALR exon 9 (Fig. S4 in the Supplementary Appendix), using high-resolution sizing of fluorescent dye–labeled PCR products (Fig. S5 in the Supplementary Appendix). This cohort included 382 patients with polycythemia vera, 311 with essential thrombocythemia, and 203 with primary myelofibrosis (Table S4 in the Supplementary Appendix).

We identified 150 samples (17% of the patients) with insertions or deletions in CALR that were independently validated by means of Sanger sequencing. We did not observe CALR mutations in patients with polycythemia vera. A total of 78 patients with essential thrombocythemia (25%) and 72 with primary myelofibrosis (35%) had mutations in CALR.

All the patients were genotyped for the JAK2 V617F mutation. Patients with polycythemia vera who were negative for the JAK2 V617F mutation were tested for mutations in JAK2 exon 12. Patients with essential thrombocythemia or primary myelofibrosis who had nonmutated JAK2 were tested for mutations in MPL exon 10. The distribution of the JAK2, MPL, and CALR mutations in the three disease entities is shown in Figure 1A. All patients with mutated CALR had nonmutated JAK2 and MPL. A total of 67 patients had nonmutated JAK2 and MPL as well as nonmutated CALR exon 9. Of these 67 patients with triple-negative findings, 19 underwent Sanger sequencing for mutations in all nine exons of CALR, but no mutations were detected (data not shown).

Because CALR mutations were strongly associated with essential thrombocythemia or primary myelofibrosis with nonmutated JAK2 and MPL, we analyzed samples from an additional 211 patients in these disease categories. In the combined cohort of 1107 patients with analyzed samples, we studied 289 patients with essential thrombocythemia and nonmutated JAK2 and MPL, of whom 195 had mutated CALR (67%). Of the combined 120 patients with primary myelofibrosis and nonmutated JAK2 and MPL, 105 had a mutation in CALR (88%). In 150 patients with mutated CALR for whom we had matched T-lymphocyte DNA available, the mutations were somatic.

CALR EXON 9 MUTATIONS IN OTHER MYELOID NEOPLASMS

To investigate whether CALR mutations are present in other myeloid neoplasms, we screened 254 patients with acute myeloid leukemia, 45 with chronic myeloid leukemia, 73 with the myelodysplastic syndrome, 64 with chronic myelomonocytic leukemia, and 24 with refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T) for mutations in CALR exon 9.

A Distribution of *JAK2*, *MPL*, and *CALR* Mutations in Philadelphia Chromosome–Negative Myeloproliferative Neoplasms

B Frequency of *CALR* Mutations in Myeloid Cancers

C Distribution of *JAK2*, *MPL*, *CALR*, and *SF3B1* Mutations in RARS-T

Figure 1. Frequency of *CALR* Mutations in Myeloid Neoplasms.

Panel A shows the distribution of *JAK2*, *MPL*, and *CALR* mutations in the three classical entities of myeloproliferative neoplasms. Panel B shows the frequency of *CALR* mutations in various myeloid cancers. The total numbers of patients included in the analysis for a specific disease entity are shown. Panel C shows the distribution of mutations in *JAK2*, *MPL*, *CALR*, and *SF3B1* among 24 patients with refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T). AML denotes acute myeloid leukemia, CML chronic myeloid leukemia, CMML chronic myelomonocytic leukemia, and MDS myelodysplastic syndrome.
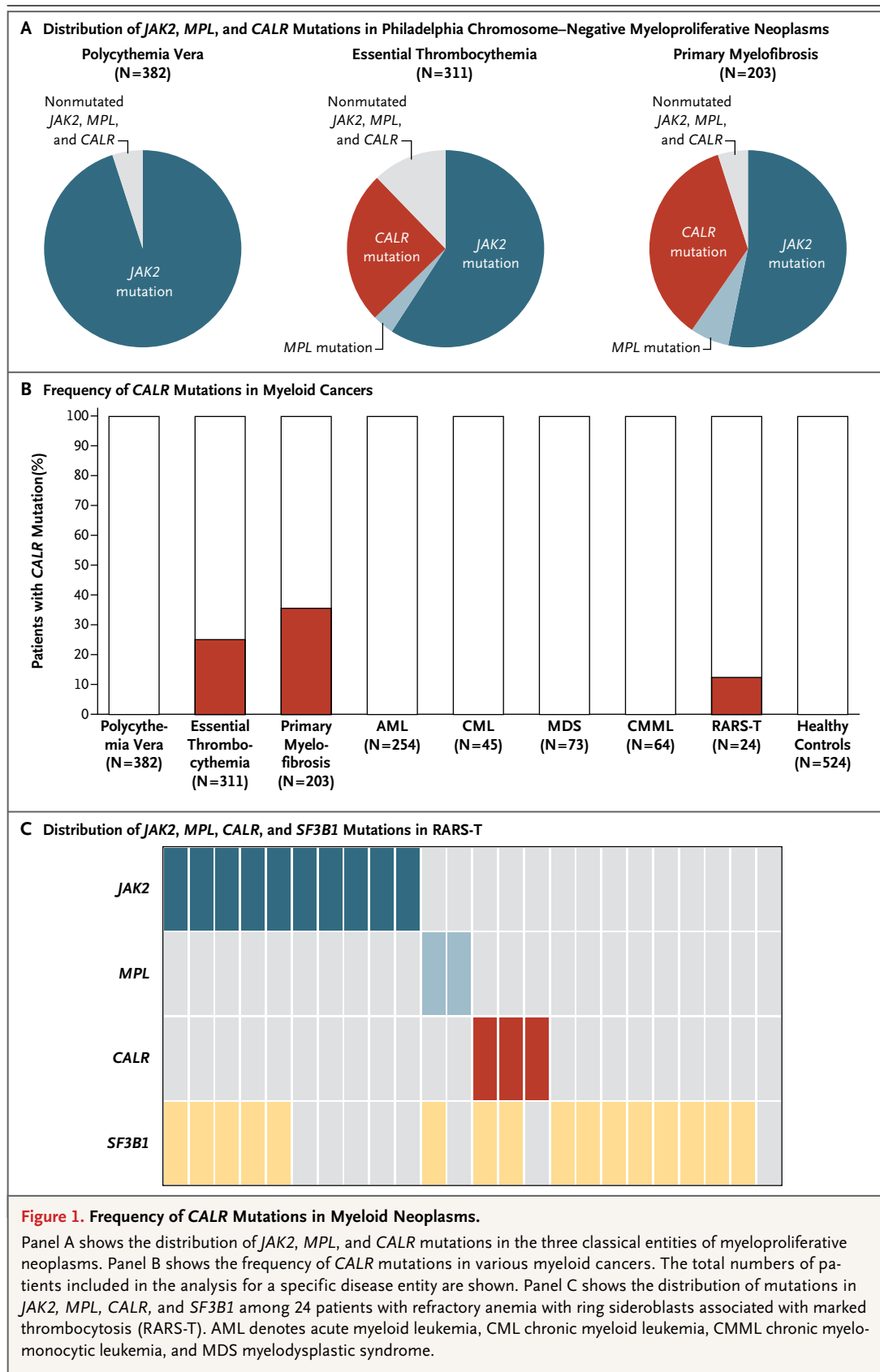
Figure 2. Mutations in *CALR* and the Generation of a Novel C-Terminal Peptide in the Calreticulin Protein.
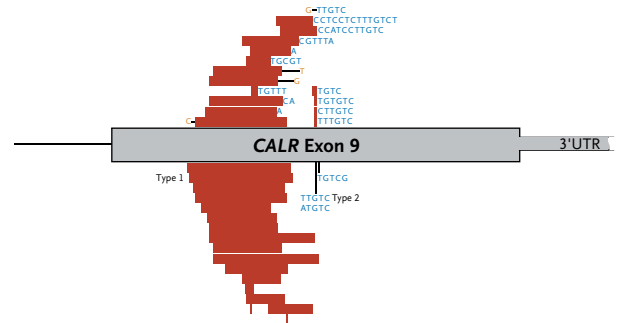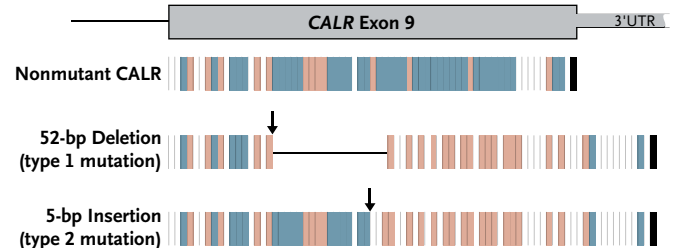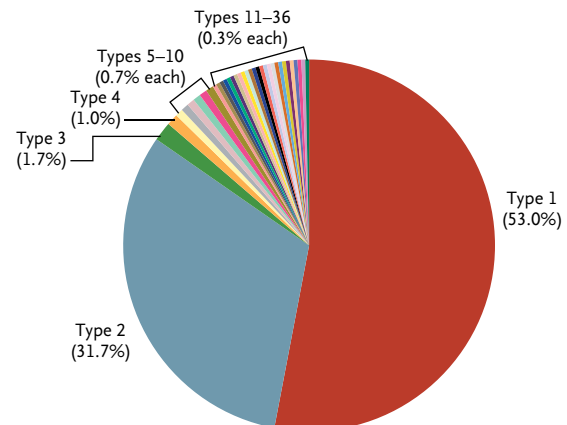
Panel A shows the genomic positions of all 36 mutation types detected in the current study. Red bars indicate deletions, blue letters inserted nucleotide sequences, and orange letters somatically acquired substitutions. Black lines connecting deletions or insertions with substitutions indicate their occurrence on the same allele. The red bars and black lines are drawn to scale. The most frequent mutations, type 1 and type 2, are marked. 3′UTR denotes 3′ untranslated region. Panel B is a schematic representation of the three alternative peptide sequences derived from the three reading frames across exon 9 of *CALR*. Each bar represents an amino acid: blue bars indicate negatively charged amino acids, red bars positively charged amino acids, and black bars stop codons. Panel C shows the specific peptide makeup of nonmutant CALR and of the two most frequently detected types of mutations. Panel D shows the relative frequencies of all 36 mutation types observed in *CALR*. Percentages do not sum to 100 because of rounding.

A Genomic Position of the 36 Mutation Types Detected in *CALR*

B Peptide Sequences Derived from the Three Reading Frames across *CALR* Exon 9
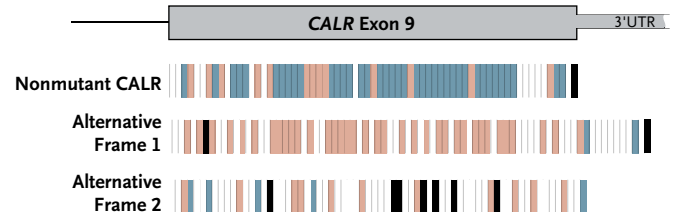
C Peptide Sequences of the Two Most Common *CALR* Mutations

D Frequency of the 36 Mutation Types Detected in *CALR*

Although most of these patients had nonmutated *CALR* exon 9, a total of 3 patients with RARS-T had mutations in *CALR* (Fig. 1B), all with nonmutated *JAK2* and *MPL* (Fig. 1C). Mutations in the gene encoding splicing factor 3B, subunit 1 (*SF3B1*) co-occurred with mutations in all three genes. Of 524 healthy study participants, 1 had a 3-bp in-frame deletion in *CALR*.

CALR MUTATIONS AND A NOVEL C-TERMINAL PEPTIDE IN MUTANT CALR

We detected a total of 36 types of somatic mutations in *CALR* (insertions and deletions) that caused a frameshift (Fig. 2A, and Table S5 in the Supplementary Appendix). Furthermore, two patients who were positive for the *JAK2* V617F mutation had in-frame germline mutations (type G1 and G2) (Table S5 in the Supplementary Appendix). All 36 types of somatic insertions or deletions resulted in a frameshift to the alternative reading frame 1 (Fig. 2B). Mutations of type 1 (52-bp deletion; c.1092_1143del) and mutations of type 2 (5-bp insertion; c.1154_1155insTTGTC) accounted for 53.0% and 31.7% of all the cases with mutated *CALR*, respectively (Fig. 2C and 2D). The other mutation types were observed at much lower frequencies, and many were detected only in a single patient (Fig. 2D, and Table S5 in the Supplementary Appendix).

Because all 36 mutation types cause a frameshift to alternative reading frame 1, the resulting mutant CALR proteins share a novel amino acid sequence at the C-terminal (Table S6 in the Sup-

plementary Appendix). The C-terminal peptide derived from alternative reading frame 1 contains a number of positively charged amino acids, whereas the nonmutant CALR C-terminal is largely negatively charged (Fig. 2B).

In addition, the nonmutant calreticulin contains the endoplasmic reticulum–retention motif at the C-terminal end (lysine, aspartic acid, glutamic acid, and leucine [KDEL] amino acid sequence). The C-terminal KDEL motif is lost in all mutant variants (Table S6 in the Supplementary Appendix). Depending on the type of mutation, the mutant proteins retain varying amounts of the negatively charged amino acids of nonmutant calreticulin. The 52-bp deletions (type 1) eliminate almost all negatively charged amino acids, whereas the 5-bp insertions (type 2) retain approximately half the negatively charged amino acids (Fig. 2C).

Given these differences, we hypothesized that type 1 and type 2 mutations may be associated with qualitatively different phenotypes. Accordingly, we found that the type 1 deletions were significantly more frequent in primary myelofibrosis than in essential thrombocythemia (P<0.001). In addition, we found that only three patients were homozygous for CALR mutations associated with uniparental disomy of chromosome 19p, and all three had a 5-bp insertion of type 2 (Fig. 3A).

EARLY ACQUISITION OF CALR MUTATIONS AND STABLE MUTANT CLONES

To investigate whether mutations in CALR are acquired early or late in the clonal history of a patient, we analyzed hematopoietic-progenitor colonies from 2 patients for whom we had mutational profiles based on whole-exome sequencing. The clonal hierarchies for Patients H_0191 and H_0296 are shown in Figure 3B. We concluded that for these 2 patients, the mutations in CALR were acquired early in the major clones. We had follow-up samples available for 24 patients with mutated CALR; all these follow-up samples tested positive for the mutation as well.
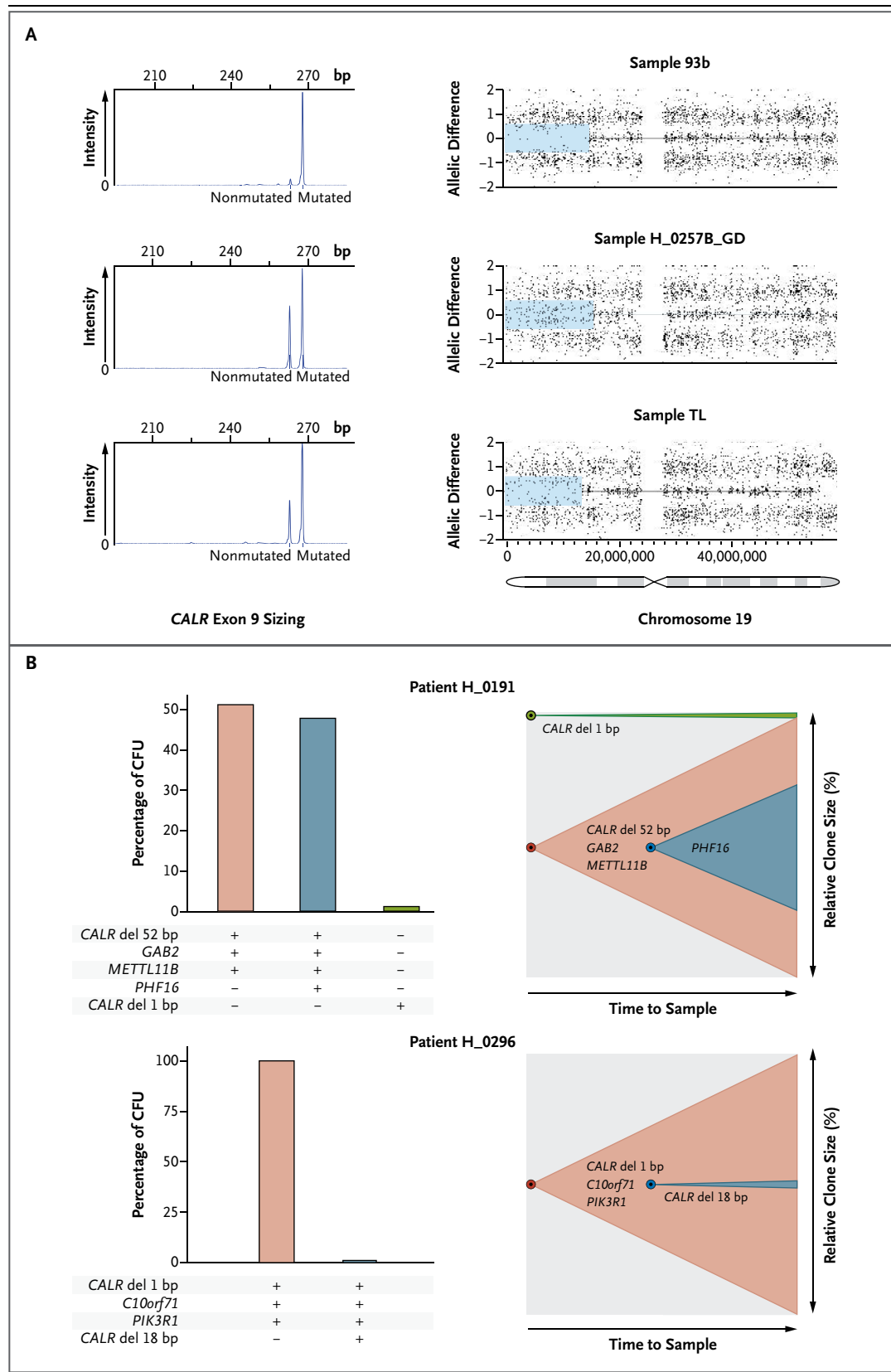
CLINICAL SIGNIFICANCE OF CALR MUTATIONS

Overall, we studied 1215 patients with essential thrombocythemia or primary myelofibrosis (Fig. S4 and Table S7 in the Supplementary Appendix). Of these patients, 63.4% carried the JAK2 V617F mutation, 4.4% carried activating mutations of MPL exon 10, 23.5% carried mutations of CALR exon 9, and only 8.8% had none of these clonal markers. Most of the patients with no clonal markers were clustered in the subgroup of patients with essential thrombocythemia.

Figure 3 (facing page). Association of *CALR* Mutations with Uniparental Disomies and Clonal Hierarchies in Patients with Multiple Somatic Mutations.

Panel A shows results from the polymerase-chain-reaction product-sizing analysis of *CALR* exon 9 in three patients with a 5-bp insertion. In the three patients, the mutated allele peak is higher than the nonmutated allele peak, indicating the presence of cells homozygous for the mutation. The plots on the right show data from Genome-Wide human single-nucleotide polymorphism (SNP) arrays, version 6.0 (Affymetrix). Each dot represents a single SNP. The x axis shows the genomic position, and the y axis the allelic status of the SNP (an allelic difference of 0 indicates heterozygous status) according to fluorescence intensity. The array data show that the three samples have clones with uniparental disomy of chromosome 19p of different sizes, which corresponds to the differences in peak heights observed in the fragment analysis of *CALR*. Taken together, the results indicate that homozygosity of mutated *CALR* is derived from duplication of a heterozygous mutation as a result of acquisition of a uniparental disomy in the three patients. Blue boxes indicate the genomic region of the uniparental disomies. Panel B shows the clonal hierarchies derived from the analysis of hematopoietic progenitor colonies. Patient H_0191 had somatic mutations in four genes. As shown in the bar chart, 51% of the colonies had mutations in *CALR, GAB2,* and *METTL11B*. The other colonies (48%) had mutations in all four genes, indicating that the mutation in the gene encoding plant homeo domain finger protein 16 (*PHF16*) was acquired later and gave rise to a subclone. In addition, one colony (1%) had a 1-bp deletion in *CALR*, in contrast to the 52-bp deletion observed in the granulocyte sample and in all other colonies from this patient. Because this colony had none of the other mutations observed in the patient, it represents an independent clone, although this conclusion is based only on a single colony. Patient H_0296 had somatic mutations in *CALR, PIK3R,* and *C10orf71*. All colonies analyzed from this patient had all three mutations. One colony showed an 18-bp deletion in *CALR* in addition to the 1-bp deletion observed in this patient. The two mutations were on the same allele. Mutated *CALR* was found in an early clone in both patients; the roles of the other mutated genes are unclear. The plots on the right side schematically depict the clonal history that gave rise to the clonal composition observed at the time of the sample. Different colors indicate the different clones, as shown in the bar charts. CFU denotes colony-forming units, and del deletion.

We used the Wilcoxon rank-sum test to compare hematologic values in patients carrying different mutated genes. Among patients with essential thrombocythemia, those with a CALR mutation had a lower hemoglobin level, lower white-cell count, and higher platelet count at diagnosis than patients with mutated JAK2 (P<0.001 for all comparisons). Among patients

A

CALR Exon 9 Sizing

Sample 93b

Sample H_0257B_GD

Sample TL

Chromosome 19

B

Patient H_0191

Patient H_0296

with primary myelofibrosis, those with a *CALR* mutation had a lower white-cell count (P=0.03) and a higher platelet count (P<0.001) than patients with mutated *JAK2*.

Overall survival and the risk of thrombosis were analyzed only among patients carrying a mutation in *JAK2*, *MPL*, or *CALR* (i.e., patients with a clonal marker). Assuming that mutation status did not change with time, we performed survival analyses using the date of the initial diagnosis as the start of follow-up. Because 6 patients were excluded owing to inadequate follow-up, a total of 1102 patients were examined. The median follow-up for the entire cohort of patients with any of the three mutated genes was 5.7 years (range, 0 to 31).

As shown in Figure 4A, there was a significant difference in overall survival among the three subgroups of patients with primary myelofibrosis (P<0.001). Patients with a somatic mutation of *CALR* had longer overall survival than those with a *JAK2* or *MPL* mutation (P<0.001 for both comparisons), whereas no significant difference was observed between the latter two subgroups. Among patients with essential thrombocythemia, who had much longer overall survival than those with primary myelofibrosis, there was a significant difference only between patients with a *CALR* mutation and those with a *JAK2* mutation (P=0.04) (Fig. 4B).

In a multivariate Cox regression analysis of overall survival that included type of myeloid neoplasm (essential thrombocythemia vs. primary myelofibrosis), type of mutated gene, and patient cohort (Pavia vs. Vienna) as covariates, the first two variables were found to be independent prognostic factors. As expected, primary myelofibrosis was associated with shorter overall survival, as compared with essential thrombocythemia (hazard ratio for death, 7.1; 95% confidence interval [CI], 4.9 to 10.2; P<0.001). In addition, the type of mutated gene had an independent effect on survival. As compared with patients with a *CALR* mutation, patients with a *JAK2* mutation had a higher risk of death (hazard ratio, 3.1; 95% CI, 2.0 to 4.7; P<0.001), as did those with an *MPL* mutation (hazard ratio, 3.5; 95% CI, 1.8 to 6.7; P<0.001).

The cumulative incidence of thrombosis in essential thrombocythemia was calculated with a competing-risk approach,[24] with death from any cause as a competing event; the curves are shown in Figure 4C, and the data are provided in Table S8 in the Supplementary Appendix. Among patients with essential thrombocythemia, those with a *CALR* mutation had a lower risk of thrombosis than did those with a *JAK2* mutation (P=0.003); no significant differences were found between the other subgroups. It should be noted that the subgroup of patients with an *MPL* mutation was small.

FUNCTIONAL ANALYSIS OF THE TYPE 1 *CALR* MUTATION

To study the functional effects of mutated *CALR*, we cloned the complementary DNA (cDNA) of the nonmutated *CALR* and the type 1 mutation (52-bp deletion) into a retroviral expression vector based on the murine stem-cell virus, with an internal ribosome entry site and green fluorescent protein (GFP). After retroviral production and transfection of the *CALR* cDNAs into the interleukin-3–dependent murine cell line Ba/F3, we sorted the transgene-positive cells by means of flow cytometry for GFP (Fig. S6 in the Supplementary Appendix). We then measured the interleukin-3–dependent proliferation of cells. Cells expressing the type 1 *CALR* mutation showed growth that was independent of interleukin-3 and also showed hypersensitivity to interleukin-3 (Fig. 5A). When we measured the proliferation of cells in the absence of interleukin-3, only the *CALR* type 1 mutation showed a significant accumulation of cells (Fig. 5B).

To investigate whether the interleukin-3 independence in the *CALR* type 1–mutated cells was caused by the activation of JAK–signal transducer and activator of transcription (JAK-STAT) signaling, we determined the sensitivity of cells to the JAK2 kinase inhibitor SAR302503. Cells expressing the nonmutated *CALR* or the type 1 mutation of *CALR* showed similar sensitivity to SAR302503, suggesting that the interleukin-3–independent growth of the mutated *CALR* cells depends on JAK2 or a JAK family kinase targeted by SAR302503 (Fig. S7 in the Supplementary Appendix).

To confirm this hypothesis, we examined the phosphorylation of STAT5 in the presence and absence of interleukin-3 in the control and *CALR*-transfected cell lines. We detected increased phosphorylation of STAT5 in the absence of interleukin-3 in the type 1 mutation of *CALR* and at an interleukin-3 concentration of 0.1 ng per milliliter (Fig. 5C). Thus, increased activation of JAK-STAT signaling is probably responsible for

Figure 4. Estimated Overall Survival and Cumulative Incidence of Thrombosis among Patients with *CALR*, *JAK2*, or *MPL* Mutations.

Outcome estimates in patients with essential thrombocythemia or primary myelofibrosis were stratified according to somatic mutation. Panel A shows the Kaplan–Meier analysis of overall survival among patients with primary myelofibrosis. Subgroups were compared with the use of the log-rank test. Patients with myelofibrosis and a somatic mutation of *CALR* had longer overall survival than those with the *JAK2* V617F mutation (median, 21.4 years [95% confidence interval {CI}, 17.1 to 22.9] vs. 11.0 years [95% CI, 7.8 to 14.4]; P<0.001) or the *MPL* mutation (median, 8.2 years [95% CI, 2.0 to not reached]; P<0.001). No significant difference in survival was observed between the *JAK2* V617F and *MPL* subgroups. Panel B shows the Kaplan–Meier analysis of overall survival among patients with essential thrombocythemia. Subgroups were compared with the use of the log-rank test. The median value for overall survival was not reached in any subgroup. Patients with a *CALR* mutation had longer overall survival than those with the *JAK2* V617F mutation. Overall survival at 10 years was 96.9% (95% CI, 91.7 to 98.8) in the former group, as compared with 91.1% (95% CI, 87.1 to 93.9) in the latter group (P=0.04). Panel C shows the cumulative incidence of thrombosis among patients with essential thrombocythemia. Death in the absence of the event of interest was considered a competing event, and subgroups were compared with the use of the Pepe–Mori test.[23] Patients with a *CALR* exon 9 mutation had a lower cumulative incidence of thrombosis than those with the *JAK2* V617F mutation: the cumulative incidence at 10 years was 11.0% (95% CI, 6.3 to 17.1) in the former group versus 21.0% (95% CI, 16.6 to 25.7) in the latter group (P=0.003).

A Survival in Primary Myelofibrosis

— *CALR* exon 9 mutation
— *JAK2*-V617F mutation
— *MPL* exon 10 mutation

No. at Risk

CALR mutation	98	69	38	21	9	3	1
JAK2 mutation	189	85	24	6	0	0	0
MPL mutation	18	8	2	1	1	0	0

B Survival in Essential Thrombocythemia

— *CALR* exon 9 mutation
— *JAK2*-V617F mutation
— *MPL* exon 10 mutation
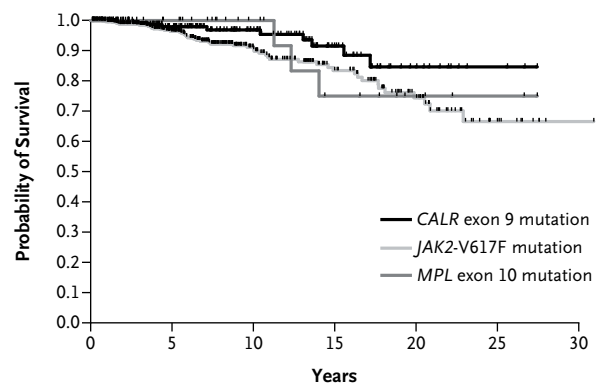
No. at Risk

CALR mutation	186	122	71	33	11	3	0
JAK2 mutation	576	310	145	83	42	10	1
MPL mutation	35	25	14	8	4	2	0

C Thrombosis in Essential Thrombocythemia

— *CALR* exon 9 mutation
— *JAK2*-V617F mutation
— *MPL* exon 10 mutation

No. at Risk

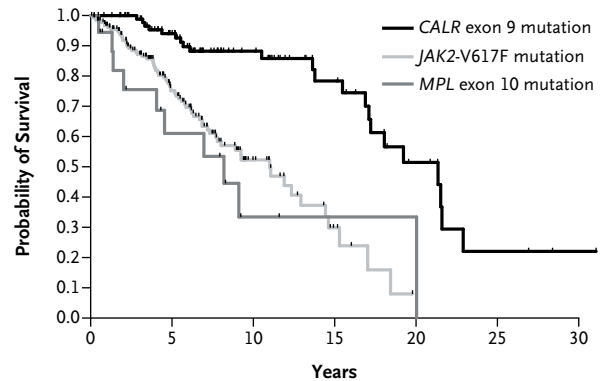|						
CALR mutation	186	115	63	27	9	3	0
JAK2 mutation	575	267	116	62	25	5	0
MPL mutation	35	21	13	8	4	2	0

the cytokine-independent growth of cells expressing the type 1 mutation of *CALR*.

Immunofluorescence microscopy was used to determine the cellular localization of nonmutated and type 1–mutant CALR. On overexpression in human embryonic kidney (HEK) cells, the nonmutant CALR colocalized with the endoplasmic reticulum (stained with calnexin). In the case of the type 1–mutant CALR, however, this colocalization was less prominent, probably because of the absence of the C-terminal KDEL sequence of the mutant CALR (Fig. 5D).

DISCUSSION

We identified somatic mutations in *CALR* in patients with primary myelofibrosis or essential thrombocythemia. *CALR* mutations are mutually exclusive with mutations in both *JAK2* and *MPL*.

Figure 5. Functional Analysis of *CALR* Type 1 Mutation.

Panel A shows the viability of cells from a murine interleukin-3–dependent pro-B cell line (Ba/F3 cells) expressing an empty vector (green fluorescent protein [GFP]), nonmutant CALR with GFP, or mutant CALR with GFP. Viability was assessed after 72 hours in the presence of an increasing concentration of interleukin-3. I bars represent standard errors. RLU denotes relative luminescence units. Panel B shows the proliferation of Ba/F3 cells expressing an empty vector, nonmutant CALR, or mutant CALR. Proliferation was assessed after 7 days in the absence of interleukin-3. Panel C shows the activation of signal transducer and activator of transcription 5 (STAT5) in response to interleukin-3. Ba/F3 cells expressing the empty vector, the nonmutant CALR, or the mutant CALR were cultured for 4 hours in serum-free medium without interleukin-3 and were then stimulated for 20 minutes with interleukin-3 at concentrations of 0.1 ng per milliliter and 1 ng per milliliter, as indicated. Western blot analysis was performed on the cell lysates with antibodies against phosphorylated STAT 5 (pSTAT5), STAT5, and CALR. An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Panel D shows immunofluorescence staining of CALR (red) and calnexin, an endoplasmic reticulum–specific marker (green) in human embryonic kidney T cells transfected with the respective constructs. The nonmutant CALR colocalizes with calnexin in the endoplasmic reticulum (merge in yellow). However, the mutant CALR is not constrained within the endoplasmic reticulum. The nucleus is stained blue (4´,6-diamidino-2-phenylidole [DAPI]).

No *CALR* mutations were found in patients with polycythemia vera, a myeloproliferative neoplasm that is specifically associated with *JAK2* mutations. *CALR* mutations are the second most frequent mutation after *JAK2* in myeloproliferative neoplasms. We also studied patients with other myeloid neoplasms and found *CALR* mutations only in a few patients with RARS-T, a myeloid neoplasm with both myelodysplastic and myeloproliferative features.[25] These observations strong-

ly support a causal relationship between *CALR* mutations and excessive platelet production.

Because *CALR* mutations are found in approximately 73% of patients who do not have mutations of *JAK2* and *MPL*, we believe they fill in the current molecular diagnostic gap in myeloproliferative neoplasms. Altogether, less than 10% of our patients with essential thrombocythemia or primary myelofibrosis did not have a somatic mutation of *JAK2*, *MPL*, or *CALR*. In some of these patients, the mutated clone might have been too small to be detected with the current approaches. Rare mutated driver genes may play a role in some patients, whereas other patients might not have a clonal disease at all. This is particularly true for patients with a clinical diagnosis of essential thrombocythemia, because the differential diagnosis between clonal and reactive thrombocytosis may be difficult without a clonal marker.[26] Assessment for *CALR* mutations markedly improves the current diagnostic approach for essential thrombocythemia and primary myelofibrosis and may be a useful addition to the World Health Organization criteria for these disorders.[1]

All the mutations of *CALR* we identified are insertion or deletion mutations in the last exon encoding the C-terminal amino acids of the protein. Most of these mutations are present in a heterozygous state and cause a frameshift to a specific alternative reading frame. This frameshift results in the replacement of the C-terminal negatively charged amino acids of calreticulin by a positively charged polypeptide rich in arginine and methionine. The last four amino acids of calreticulin (KDEL) contain the endoplasmic reticulum–retention signal. This signal is absent in mutant calreticulin. Consequently, mutant calreticulin has an altered subcellular localization. Because the negatively charged C-terminal domain of calreticulin is the low-affinity, high-capacity, Ca^{2+}-binding domain, the Ca^{2+}-binding function of the mutant protein may be impaired. The presence of the peptide sequence derived from the alternative reading frame at the C-terminal domain of mutated CALR offers an opportunity for immunologic targeting because it represents a cancer-specific epitope.

To analyze the oncogenic capability of the mutant calreticulin, we generated Ba/F3 cells with overexpression of the nonmutant and the type 1 mutant CALR (52-bp deletion). The Ba/F3 cells expressing the 52-bp deletion showed cytokine-independent proliferation. However, the growth of Ba/F3 cells expressing nonmutant and mutant calreticulin was suppressed equally on treatment with a JAK2 kinase inhibitor, suggesting that the JAK-STAT pathway is required for cytokine-independent proliferation induced by mutant calreticulin.

In accordance with this finding, we detected increased phosphorylation of STAT5 in the Ba/F3 cells with the 52-bp deletion, both in the absence and in the presence of stimulation by interleukin-3. Calreticulin, Ca^{2+}, and calmodulin have previously been shown to modulate the activity of Stats. The calreticulin complex with endoplasmic reticulum protein 57, in the endoplasmic reticulum, suppresses the phosphorylation and transcriptional activity of Stat3 in mouse embryonic fibroblasts.[27] Moreover, inhibition of the calcium-calmodulin–dependent protein kinase II γ results in reduced levels of phosphorylated Stat1, Stat3, and Stat5.[28] Overexpression of calreticulin attenuates interferon α–induced Stat1 phosphorylation, resulting in interferon resistance.[29]

Studies are required to elucidate the mechanism for activation of the JAK-STAT pathway by the mutant calreticulin in myeloid cells. The involvement of the JAK-STAT signaling pathway in patients who are positive for a *CALR* mutation may also explain the effectiveness of JAK2-inhibitor therapy for primary myelofibrosis. However, our results indicate that the JAK2 inhibitors may not be selective for cells expressing the mutated *CALR*, as compared with the *CALR* nonmutated cells.

Although our analyses of clinical outcome are retrospective, they strongly suggest that *CALR*-positive myeloproliferative neoplasms have a more benign clinical course than the corresponding disorders associated with *JAK2* or *MPL* mutations. Owing to the small number of patients with an *MPL* mutation, the more reliable comparisons are those between patients with a *JAK2* mutation and those with a *CALR* mutation. Our results clearly show that patients with a *CALR* mutation have a lower risk of thrombosis and have longer overall survival than those with a *JAK2* mutation. The lower incidence of thromboembolic complications might be related to our observation that patients with a *CALR* mutation had lower hemoglobin levels and lower white-cell counts than those with the *JAK2* mutation. Longer overall survival was observed among patients with pri-

mary myelofibrosis or essential thrombocythemia carrying a *CALR* mutation than among those with a *JAK2* mutation; in particular, it was much more relevant in primary myelofibrosis, which confirms previous findings in patients with and those without a *JAK2* mutation.[8,30]

From a practical point of view, the different effects of mutated genes might be incorporated into existing prognostic scoring systems for primary myelofibrosis and essential thrombocythemia[31,32] and might also guide therapeutic decision making. *CALR* molecular characterization may become a key component of the clinical management of essential thrombocythemia and primary myelofibrosis.

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