BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Megakaryocytes in Myeloproliferative Neoplasms Have Unique Somatic Mutations

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Myeloproliferative neoplasms (MPNs) are a group of related clonal hemopoietic stem cell disorders associated with hyperproliferation of myeloid cells. They are driven by mutations in the hemopoietic stem cell, most notably *JAK2*^{V617F}, *CALR*, and *MPL*. Clinically, they have the propensity to progress to myelofibrosis and transform to acute myeloid leukemia. Megakaryocytic hyperplasia with abnormal features are characteristic, and it is thought that these cells stimulate and drive fibrotic progression. The biological defects underpinning this remain to be explained. In this study we examined the megakaryocyte genome in 12 patients with MPNs to determine whether there are somatic variants and whether there is any association with marrow fibrosis. We performed targeted next-generation sequencing for 120 genes associated with myeloid neoplasms on megakaryocytes isolated from aspirated bone marrow. Ten of the 12 patients had genomic defects in megakaryocytes that were not present in nonmegakaryocytic hemopoietic marrow cells from the same patient. The greatest allelic burden was in patients with increased reticulin deposition. The megakaryocyte-unique mutations were predominantly in genes that regulate chromatin remodeling, chromosome alignment, and stability. These findings show that genomic abnormalities are present in megakaryocytes in MPNs and that these appear to be associated with progression to bone marrow fibrosis. (*Am J Pathol* 2017, 187: 1512–1522; <http://dx.doi.org/10.1016/j.ajpath.2017.03.009>)

Myeloproliferative neoplasms (MPNs) are a group of clonal hemopoietic stem cell disorders characterized by the aberrant proliferation of one or more mature myeloid cells.^{1,2} The major MPN entities, polycythemia vera, essential thrombocythemia, and primary myelofibrosis, are characterized by well-known driver mutations. The most common, *JAK2*^{V617F}, is present in 50% to 98% of MPN patients, depending on the underlying MPN subtype.^{2–6} Others are less common and are associated with specific entities, that is, *JAK2* exon 12 mutations in polycythemia vera, and *MPL* exon 10 mutations and *CALR* exon 9 insertions and deletions in essential thrombocythemia and primary myelofibrosis.^{3,7–12} Detection of these mutations has largely

been ascertained from the assessment of CD34⁺ cells, blood leukocytes, or purified granulocytes.^{1–3,5,7,8,10,11}

Moderate-to-marked megakaryocytic hyperplasia with variable degrees of pleiomorphism are characteristic features of all types of MPNs.¹³ Megakaryocytes mature from a

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hemopoietic progenitor by endomitosis, whereby they undergo multiple rounds of DNA replication without cell division. This results in the production of a large polyploid cell (50 to 200 μm) with a single lobated nucleus containing multiple copies of the genome (2N to 128N). Their primary function is to produce platelets, but they also mediate bone marrow angiogenesis, matrix deposition, and hemopoietic stem cell quiescence.^{14–16} In MPNs, megakaryocytes have characteristic morphologic features that are used for diagnostic classification as outlined by the World Health Organization paradigm.^{13,17–19} In essential thrombocythemia, megakaryocytes exhibit large or giant forms with nuclear hyperlobation as a consequence of dysregulated endomitosis, whereas in polycythemia vera they show greater pleiomorphism, including a higher nuclear-to-cytoplasmic ratio.²⁰ Primary myelofibrosis shows the greatest megakaryocytic abnormalities, namely marked hyperplasia leading to the formation of sheets, abnormal localization, and nuclear pyknosis. These morphologic changes are a consequence of increased megakaryocyte proliferation and impaired apoptosis, resulting in abnormally large cells and hyperploidy (up to 512N) with, presumably, an extended life span.²¹ It is postulated that these abnormal megakaryocytes drive the fibrotic process by releasing profibrogenic mediators.^{22–27} However, the precise mechanism by which this occurs and the biological defect that drives this have not been determined.

The megakaryocytes in MPNs harbor the same driver mutations as the other end-stage myeloid cells. However, because they have undergone further DNA replication during endomitosis, they have the capacity to acquire additional genomic aberrations.²¹ To date there are no data to substantiate this assertion, in part due to the inherent difficulties in acquiring megakaryocytes to study. Megakaryocytes constitute a relatively small percentage of cells in the marrow, and, because of their size, they are difficult to isolate using conventional methods (eg, flow sorting). Despite the significant megakaryocyte hyperplasia in MPNs, megakaryocytes remain a small percentage of cells because there is also commonly increased erythroid and granulocytic activity. Further, in MPNs the underlying fibrosis can make it difficult to obtain sufficient marrow. *In vitro* cultures of CD34⁺ cell-derived megakaryocytes have been used as an alternate to primary samples, although these can only be used for some studies because they are unable to achieve the high polyploidy seen in the primary counterpart and do not fully recapitulate the bone marrow milieu so are not truly representative of the *in vivo* cell.²⁸ Another approach has been to laser capture megakaryocytes from sections of bone marrow trephine biopsies; this method has been successfully applied for miRNA analysis.^{29,30}

Here we report a novel combined density gradient and laser capture method that has enabled us to obtain sufficient fresh megakaryocytes from aspirated human MPN bone marrow for mutational analysis. With the use of a targeted sequencing approach, we show for the first time that

megakaryocytes in MPNs have acquired somatic mutations. We demonstrate that these are present in the greatest allelic burden in patients with increased reticulin deposition in the marrow and are not in patient-matched nonmegakaryocytic hemopoietic cells.

Materials and Methods

Patient Samples

Bone marrow aspirate samples were obtained from the posterior iliac crest of 12 prospectively recruited MPN patients at diagnosis or at the time of reassessment of disease status. All patients gave written informed consent for the sample to be collected and used for this study. The project had ethical approval from the Sir Charles Gairdner Hospital Human Research Ethics Committee (no. 2012-094) and the University of Western Australia Human Research Ethics Committee (no. RA/4/1/6566), in accordance with the Declaration of Helsinki. Aspirated bone marrow was smeared directly onto glass for morphologic assessment after Romanovsky staining. An additional 0.5- to 1-mL aliquot was collected into EDTA anticoagulated vacutainers (Greiner Bio-One, Frickenhausen, Germany) and processed within 2 hours of collection. A bone marrow trephine biopsy specimen was collected and processed into paraffin using standard methods. Sections were cut and stained with hematoxylin and eosin and for reticulin (Gordon and Sweet method) and assessed using the World Health Organization classification criteria.³ *JAK2*^{V617F}, *MPL*, and *CALR* mutation testing was performed on blood leukocytes by allele-specific PCR.

Megakaryocyte Isolation

A discontinuous human albumin density gradient was used to enrich megakaryocytes from the aspirated bone marrow.³¹ Briefly, the bone marrow aspirate was mixed with Percoll (GE Health Care, Uppsala, Sweden) and 1× phosphate-buffered saline (Thermo Fisher Scientific, Scoresby, Australia) in 3% (v/v) purified human albumin (Albumex 20; CSL Behring, Broadmeadows, Australia) to achieve a final density of 1.020 g/mL. The resulting mixture was layered over a solution of Percoll and 1× phosphate-buffered saline in 0.15% (v/v) human albumin with a density of 1.050 g/mL in a 15-mL tube (Olympus, Tokyo, Japan). A third solution containing 1× phosphate-buffered saline in 0.15% (v/v) human albumin with a density of 1.006 g/mL was then layered over the bone marrow aspirate mixture, and the entire sample was centrifuged at 400 × *g* for 20 minutes at room temperature. After centrifugation the megakaryocytes were harvested from the opaque interphase and the megakaryocyte-depleted bone marrow hemopoietic cells (BM^{MK⁻}) from the pellet.

The megakaryocyte-enriched fraction was washed, resuspended, and cytocentrifuged onto a glass slide (Menzel Glaser, Braunschweig, Germany). The slide was stained

Table 1 Primers Used for Sanger Sequencing

Gene	Variant	Primer sequence	Direction
<i>ASXL1</i>	p.Ser1366Asn	5'-GCTGAGATCCCTCCAGTTTTTC-3'	Forward
<i>BCORL1</i>	p.Leu1460Pro	5'-GACCTCTCTTTGCTCATGGAGTT-3'	Forward
<i>BCORL1</i>	p.Cys817Ser	5'-GGAACCTCTCTTTGTGGAAACCCA-3'	Forward
<i>BIRC3</i>	p.Asp530Glu	5'-CTTCTGTTGCCTTGAAATGAGTATTTGG-3'	Forward
<i>BOD1L1</i>	Exon7 splice site_5	5'-TGCTTTCTGTTTTCTGTTTCTTCAAGT-3'	Forward
<i>BOD1L1</i>	p.Ser2631Ter	5'-TGTCACACACATTTTCTCAGAAGACA-3'	Forward
<i>BOD1L1</i>	p.Ala2527Gly	5'-GGGAAGGACGACTTATGAACGA-3'	Reverse
<i>BOD1L1</i>	p.Ile1818Met	5'-AGTCACAATGCCTTCATCATCACA-3'	Forward
<i>BRAF</i>	p.Gly397Ser	5'-GCTGAGGTCCTGGAGATTTCT-3'	Forward
<i>CACNA1E</i>	p.Lys2048Ter	5'-CACTATTCGGGATAAGCGTTCAAATTC-3'	Forward
<i>CACNA1E</i>	p.Pro1009His	5'-CACCAGCTCTCTCTTCTCTCT-3'	Forward
<i>CACNA1E</i>	p.Ala2255Thr	5'-CTACATCTCCGAGCCCTACTTG-3'	Forward
<i>CBL</i>	p.Ala757Thr	5'-GCTGCCCGTATTGAAATGT-3'	Forward
<i>CBLB</i>	p.Phe150Leu	5'-CAGCATCTGCTTTTGTGATACGA-3'	Forward
<i>CBLB</i>	p.Glu664Ter	5'-CCTCTCAAAACCACTTACCATATCTTG-3'	Forward
<i>CBLB</i>	p.Pro387Ser	5'-TGCTAATGATTATACCTGCCATGCC-3'	Forward
<i>CBLB</i>	p.Pro492Thr	5'-ACAGGGAGATCTAACTATGCCTTTCT-3'	Forward
<i>CREBBP</i>	p.Gln241fs	5'-GCTCTCACAATGCTACAAGCC-3'	Forward
<i>CREBBP</i>	p.Thr2337Arg	5'-GGGCTGTATCCGTGGTGAC-3'	Forward
<i>CSF1R</i>	p.Thr833Ala	5'-TCGTGAATGTAACGGGAAGTGT-3'	Reverse
<i>CSF3R</i>	p.Arg269Ser	5'-CAGCTCGAGCCCGACTTAC-3'	Forward
<i>CTCF</i>	p.Glu691fs	5'-TCGTACTACCTGGCCACTAC-3'	Reverse
<i>CTNNA1</i>	Exon12 splice site_5	5'-TCAGTGGAGTCTCTACCTGTTGA-3'	Reverse
<i>CTNNA1</i>	p.Ala6Val	5'-TTCCTGATGCAAAAGTCCCAAAGTA-3'	Forward
<i>DNMT1</i>	p.Pro595Ser	5'-GGATGTGGGCCATGCTCTAC-3'	Forward
<i>DST</i>	p.Cys1745Phe	5'-TCCAATCATGAAGGCTTCTTGGT-3'	Forward
<i>DST</i>	p.His5355Asn	5'-TGCATGATATCGTGGCAGAAC-3'	Forward
<i>DST</i>	p.Lys4331Asn	5'-AAAAGCCATTCTGATAAGTAAGCCAGT-3'	Forward
<i>DST</i>	p.Ser1720Leu	5'-TTTCCAGAGCCATGCAATCAA-3'	Forward
<i>ECT2L</i>	Exon3 splice site_3	5'-GAGTGGCTCTTATAAGTCATTGGTTTGA-3'	Forward
<i>ECT2L</i>	p.Asn503Lys	5'-AATTACAGGGCATGTGGCCTT-3'	Forward
<i>EP300</i>	p.Gln2247fs	5'-GGATGCAGCATCACATGCAA-3'	Forward
<i>EP300</i>	p.Asn656Lys	5'-ACTACCACCTTCTAGCTGAGAAAATCT-3'	Forward
<i>EP300</i>	p.Ala105Val	5'-CGATCTGGTAGTTCCTTAACCT-3'	Forward
<i>EP300</i>	p.Arg2012Ser	5'-GTATGAACCCACCTCCCATGAC-3'	Forward
<i>EP300</i>	p.Pro525Ser	5'-GTGGCTGTTGTATTTATTTCTGTCTCCT-3'	Forward
<i>EZH2</i>	p.Ser371fs	5'-GGAGCTCGAAGTTTTCATCTTTCTTCTC-3'	Forward
<i>FLT3</i>	p.Ala814Asp	5'-GCCCCTGACAACATAGTTGGA-3'	Forward
<i>GATA2</i>	p.Trp97Arg	5'-TCCCACCCGCTTTTCTAGTC-3'	Reverse
<i>GNAS</i>	p.Cys619fs	5'-CCCCAGCGCAACTTACTCC-3'	Forward
<i>GNB1</i>	p.Ala113Thr	5'-CGTTCCTCCCTCACGAGTTTTCAG-3'	Forward
<i>HPRT1</i>	p.Glu14Gln	5'-CCTGTAATGCTCTCATTTGAAACAGCTA-3'	Forward
<i>IDH1</i>	p.Ala63Ser	5'-CCCAGAATATTTCTGATGGTGCCATT-3'	Forward
<i>IDH2</i>	p.Thr146fs	5'-GGACTAGGCGTGGGATGTT-3'	Forward
<i>IL7R</i>	p.Ala76Thr	5'-TCCACTGCATACAGGAACCTCTA-3'	Forward
<i>IRF1</i>	p.Lys134Glu	5'-CTGGAATCCCCACATGACTGT-3'	Forward
<i>JAK1</i>	p.Ala449Thr	5'-TCAGACTTCTCAAAGCAGGTGAC-3'	Forward
<i>KANSL1</i>	p.Asn225Asp	5'-CAAGTTAGAGCTGGAGTCTGTACC-3'	Forward
<i>KANSL1</i>	p.Lys104Thr	5'-GTCGAAAGGGTTTCAAGGAAC-3'	Reverse
<i>KDM6A</i>	p.Ala71Ser	5'-CCTGCTACCAGCTCCCC-3'	Reverse
<i>KIT</i>	p.Ala814Val	5'-TAAATGGTTTTCTTTCTCTCCAACC-3'	Forward
<i>KIT</i>	p.Pro874Ser	5'-AGGTAAAATGATCCTTGCCAAAGACA-3'	Forward
<i>KMT2A</i>	p.Lys1219Asn	5'-CTCACTATAGACAGATGATGTTGTTGTGT-3'	Forward
<i>KMT2D</i>	p.Ser3478Asn	5'-TCGTGTCTTCTATTACACAACCGT-3'	Reverse
<i>KMT2D</i>	p.Gly2863Asp	5'-TTGTGCCGAGCTCAAT-3'	Forward
<i>KMT2D</i>	p.Ser762fs	5'-CACAGCGCATAGGCATGG-3'	Forward
<i>KMT2D</i>	p.Glu4731Asp	5'-GTAGCACATACCTGGGATGCT-3'	Forward

(table continues)

Table 1 (continued)

Gene	Variant	Primer sequence	Direction
<i>KMT2D</i>	p.Asp1121Glu	5'-CCAGGCTCTGGCTGTGAA-3'	Forward
<i>KMT2D</i>	p.Gln2819Ter	5'-CCAAGTTCAGGTCCAGGAGTT-3'	Forward
<i>MEF2BNB-MEF2B</i>	p.Pro330Leu	5'-CCTATCCCCTTCCAGAATCCTTTCA-3'	Reverse
<i>MPL</i>	p.Gly313Val	5'-CTTCTTTGACTTTAGTGGCACTTGG-3'	Forward
<i>MYB</i>	p.Arg42Trp	5'-ATGACTATGATGGGCTGCTTC-3'	Forward
<i>MYCBP2</i>	p.Asn3893Ser	5'-ATTACATCAATTATCCTGTGCTAGAATATGTT-3'	Forward
<i>NBEAL1</i>	p.Glu2049Ter	5'-GCTTCTCTAGGAAGGAATGTCTAAATTGT-3'	Forward
<i>NF1</i>	p.Met760Thr	5'-TGGGAGTTAAACCTTCGGAGAA-3'	Reverse
<i>NF1</i>	p.Tyr2762Ter	5'-ACAAATTCCAGACTATGCTGAGCTTAT-3'	Forward
<i>NF1</i>	p.Ala2786Thr	5'-CCACATCTCCTTACCCTCCTG-3'	Forward
<i>NF1</i>	p.Leu2244fs	5'-AGAGTACCCGTCTATTTCTGCTATTAG-3'	Reverse
<i>NOTCH1</i>	p.Thr288Ile	5'-GTGCAGTTTAGTAAGTGGGTAGCA-3'	Forward
<i>NOTCH1</i>	p.Ser673Gly	5'-GTTGCCCGCACACTCATC-3'	Forward
<i>NOTCH1</i>	p.Glu1563Asp	5'-TGTGCGTCACGCTTGAAGA-3'	Forward
<i>PDGFRA</i>	p.Ile1076Met	5'-GTTCCAGCAGTTCCACCTTCAT-3'	Forward
<i>PDGFRB</i>	p.Gln155Lys	5'-GCAGATGTAGCTTCTGTCTCAA-3'	Forward
<i>PDGFRB</i>	p.Tyr188Ter	5'-ACTTGCTCTGCTGAGCATC-3'	Forward
<i>RAD50</i>	p.Thr43Met	5'-CGGAGTTTGGGAATAGAGGACAAAG-3'	Forward
<i>RAD50</i>	p.Val154Phe	5'-CTTGATTTTCATTTTCTGTAGGCATGGT-3'	Forward
<i>RAF1</i>	p.Cys81Tyr	5'-AACCTTTCACCCGTGTCTT-3'	Reverse
<i>RARA</i>	p.Arg137Gln	5'-AGAGTCTACGAGGATTTCTGGTTC-3'	Reverse
<i>RB1</i>	p.Ser179Thr	5'-GTCATAATGTTTTTCTTTTCAGGACATGTGA-3'	Forward
<i>RELN</i>	p.Gly153Ser	5'-TGTAATCCCATGAGAAGTCCTAAGT-3'	Forward
<i>RELN</i>	p.Arg2231Lys	5'-AGAGGCCACCGTTGAGAGAATA-3'	Forward
<i>RELN</i>	p.Ser868Ile	5'-GTGGACCATCAAGATGCCTCTAATAAG-3'	Reverse
<i>RELN</i>	p.Pro2515Gln	5'-CACTGTACTCAATTCCCTCCGT-3'	Forward
<i>SETD2</i>	p.Glu1921Lys	5'-CGTCAGCTTCTGGTTCAGATGT-3'	Forward
<i>SETD2</i>	p.Leu1160Ile	5'-GCCTAGAAGGTATTTGGCTTTTCAC-3'	Forward
<i>SETD2</i>	p.Ala224Thr	5'-TGGTTCTTTTCAGAGATCTAACTGCTACA-3'	Forward
<i>SETD2</i>	p.Ala1683Val	5'-CGTTCCTTCTTCATTTTCCCTCCTG-3'	Forward
<i>SF1</i>	p.Pro596Thr	5'-GCTGCTGCTGCTGTTGTTG-3'	Forward
<i>SF3A1</i>	p.Val661Ala	5'-GGCTGTCCTCTGTCTTCAGTT-3'	Forward
<i>SH2B3</i>	p.Arg566fs	5'-GATCTTCCACCTGGTGCCTT-3'	Forward
<i>SOCS1</i>	p.Glu18Ala	5'-GCGTGATGCGCCGGTAA-3'	Forward
<i>SUZ12</i>	p.Gly392Val	5'-CAGGAGAGACCAATGATAAATCTACGG-3'	Forward
<i>UGT1A8</i>	p.Phe302Leu	5'-ACTGAAAATTTTCTTCTGCTCTAGGAA-3'	Forward

with Mayer's hematoxylin. Morphologically identified megakaryocytes were captured by laser microdissection onto CapSure Macro LCM Caps (Thermo Fisher Scientific) using an ArcturusXT Laser Capture Microdissection System (Thermo Fisher Scientific) and stored at -80°C .

Whole Genome Amplification of Megakaryocytes

Isolated megakaryocytes were thawed and lysed, and the DNA underwent whole-genome amplification (WGA) using the lysis and denaturation protocols from an illustra GenomiPhi V2 DNA Amplification Kit (GE Health Care). When >120 megakaryocytes were captured, the lysate was split into three aliquots for WGA, and equal amounts of amplified DNA were pooled for ensuing analyses.³² When <120 megakaryocytes were captured, the entire lysate was used for WGA in a single triple-volume reaction. Splitting the lysates with <120 megakaryocytes significantly reduced

the amplification efficiency (data not shown). Positive controls (supplied in the kit) and negative controls (nuclease-free water) were included with all WGA reactions.

The $\text{BM}^{\text{MK-}}$ cells collected from the pellet after centrifugation were stored at -80°C . Genomic DNA was extracted from the $\text{BM}^{\text{MK-}}$ cells using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and quantified using a dsDNA High Sensitivity Kit (Thermo Fisher Scientific) on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). All DNA were stored at -20°C . $\text{BM}^{\text{MK-}}$ cell DNA (1 ng) also underwent WGA as an internal patient-specific control. Amplified DNA was purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) with a 1:1 ratio of beads to DNA. Purified WGA DNA was quantitated using a dsDNA High Sensitivity Kit and assessed by gel electrophoresis using 1% E-Gel EX Agarose Gels (Thermo Fisher Scientific).

Library Preparation and Sequencing

Amplicon libraries were prepared using an Ion AmpliSeq custom panel (Thermo Fisher Scientific) to amplify the exons of 120 genes associated with myeloid malignancies (Supplemental Table S1). The panel includes *JAK2*, which was used as both a positive and negative control because the *JAK2*^{V617F} status had been established by allele-specific PCR. Target regions were amplified from 10 ng of DNA in each of the two multiplex PCR reactions using an Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific).³³ The completed libraries were quantified and visualized on a 2100 Bioanalyser (Agilent Technologies, Mulgrave, Australia) using a High Sensitivity DNA Kit (Agilent Technologies). Final libraries were stored in DNA LoBind tubes (Eppendorf, Hamburg, Germany) at −20°C.

Sixteen uniquely barcoded sample libraries were diluted in low Tris-EDTA (Thermo Fisher Scientific) to reach a final concentration of 150 pmol/L, and equal volumes of each were pooled. The pooled libraries then underwent template preparation (emulsion PCR, recovery of template-positive Ion Sphere Particles, and loading onto an Ion PI v3 Chip) on an Ion Chef System (Thermo Fisher Scientific) using an Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific). Semiconductor sequencing was performed on an Ion Proton Sequencer (Thermo Fisher Scientific) using an Ion PI Hi-Q Sequencing Kit (Thermo Fisher Scientific).

Torrent Suite software version 4.4 (Thermo Fisher Scientific) was used to process barcoded reads, perform base calling, align reads to the reference genome (human genome build 19; hg 19), generate run metrics, analyze coverage, and identify variants. Ion Reporter software version 4.4 (Thermo Fisher Scientific) was used to annotate variants and predict amino acid changes and clinical significance. The filtered megakaryocyte-unique variants were also assessed using MutationTaster2.³⁴

Variant Validation

Amplicons were generated from 10 ng of DNA using MyTaq DNA Polymerase (Bioline, Alexandria, Australia) and custom primers (Thermo Fisher Scientific). The amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter), then quantified using a dsDNA High Sensitivity Kit (Thermo Fisher Scientific). Purity was assessed using a 1% E-Gel EX Agarose Gels (Thermo Fisher Scientific) or a High Sensitivity DNA Kit (Agilent Technologies), depending on the quantity of DNA available. Purified amplicons and primers (Integrated DNA Technologies, Singapore Science Park II, Singapore) were submitted for Sanger Sequencing at the Australia Genome Research Facility (Perth, Australia). Primer sequences are listed in Table 1. Chromatograms were visualized using FinchTV version 1.4.0 (Geospiza Inc., Seattle, WA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 6.0.1 (GraphPad Software Inc., La Jolla, CA). Linear regression goodness of fit analyses were performed to evaluate the significance of variant allele frequency concordance between unamplified and amplified samples. Statistical significance was set at *P* < 0.05.

Results

Megakaryocyte Isolation and DNA Extraction

Marrow was aspirable from all patients, even when there was pathologic reticulिन (grade 2 to 3). Table 2 shows the patient demographic and marrow morphologic features of the patients studied. Six of the 12 patients had increased reticulिन content (grade ≥2). Nine patients were *JAK2*^{V617F}-positive and three were triple-negative (*JAK2*^{V617F}, *CALR*,

Table 2 Patient Age and Bone Marrow Morphologic Details of the Patients Studied

Patient	Age	Megakaryocyte morphologic details	MPN subtype	Reticulिन grade	<i>JAK2</i> ^{V617F} status
1	80	Megakaryocytic hyperplasia. Large forms with hyperlobated nuclei	ET	0	− (<i>CALR</i> and <i>MPL</i> negative)
2	40	Megakaryocytic hyperplasia. Large with nuclear hyperlobation	ET	0	− (<i>CALR</i> and <i>MPL</i> negative)
3	64	Megakaryocytic hyperplasia with essentially normal structure	ET	0	− (<i>CALR</i> and <i>MPL</i> negative)
4	37	Megakaryocytic hyperplasia. Pleiomorphism and large and pyknotic forms	PV	1	+
5	74	Megakaryocytic pleiomorphism. Pyknotic forms	ET	2	+
6	64	Large megakaryocytes with hyperlobated nuclei	ET	2	+
7	57	Normal megakaryocyte structure	ET	1	+
8	63	Marked megakaryocytic hyperplasia including large and giant forms with hyperlobated nuclei	ET	0	+
9	61	Megakaryocytic hyperplasia in sheets. Pleiomorphic and pyknotic forms	MF	3	+
10	75	Megakaryocytic hyperplasia in sheets. Pleiomorphism	MF	3	+
11	69	Megakaryocytic atypia including large with hyperlobated nuclei, pyknotic and bare nuclei	MF	2	+
12	81	Megakaryocyte clusters and sheets. Pleiomorphism and pyknotic nuclei	MF	3	+

ET, essential thrombocythemia; MF, myelofibrosis; MPN, myeloproliferative neoplasm; PV, polycythemia vera.

Table 3 Number of Megakaryocytes Captured and Number of Megakaryocytic Variants Detected

Patient	No. of MK captured (% recovery)	Total no. of MK variants	Filtered MK variants, no.	MK-unique variants, no.
1	645 (90)	295	46	5
2	275 (50)	324	68	26
3	25 (50)	332	46	10
4	120 (70)	287	33	0
5	136 (80)	324	60	15
6	315 (80)	273	32	14
7	120 (80)	313	41	0
8	120 (80)	348	63	21
9	80 (100)	304	61	19
10	6 (100)	333	66	23
11	80 (100)	360	75	20
12	80 (100)	329	69	19
Mean	167	319	55	14
Range	6–645	273–360	32–75	0–26

MK, megakaryocyte.

and *MPL* negative). Megakaryocytes were successfully captured by laser capture microdissection from all patients with yields ranging from 6 to 645 megakaryocytes (Table 3), the lowest being from a patient with grade 3 reticulatin. Figure 1 illustrates the purity of megakaryocytes achieved. WGA was then used to ensure a sufficient quantity of high-quality megakaryocyte DNA was available (Supplemental Figure S1). The samples were successfully sequenced with an average on target percentage of 97.7% and mean depth of 870 (Supplemental Table S2).

To ensure no bias was introduced into variant detection, we compared allele frequencies in matched unamplified and amplified cells. When performed on BM^{MK-} cells, the variant allele dropout rate was $4.8\% \pm 0.4\%$ (Supplemental Table S3). Of the variants present in the amplified samples, $3.9\% \pm 0.6\%$ were absent from the matched unamplified samples. These were a combination of possible WGA artifacts as well as true variants that were present at low frequency ($<5\%$, below the threshold for variant calling).

The allele frequency of variants detected in unamplified and amplified samples maintained a high level of concordance (average $R^2 = 0.9322$, $P < 0.0001$) (Supplemental Figure S2). We were unable to perform this analysis on the megakaryocytes because of insufficient DNA. Having established that WGA did not significantly affect our ability to detect variants or accurately quantify their allele frequencies, we analyzed DNA from both amplified megakaryocytes and BM^{MK-} cells.

Genomic Characteristics of the Megakaryocytes

There was 100% concordance between *JAK2*^{V617F} status from allele-specific PCR and sequencing of the BM^{MK-} cells. Approximately 300 variants were detected in each sample (Table 3). After filtering to exclude intronic variants, variants with known allele frequencies $\geq 5\%$ in the general population (1000 genomes project data) and systematic artifacts (based on data accumulated from the application of this panel to other studies), there was an average of 55 megakaryocytic variants (range, 32 to 75). We then matched the variants in megakaryocytes and BM^{MK-} cells for each patient to identify variants that were unique to the megakaryocytes (Supplemental Tables S4 and S5). Of these, we validated the variants that were likely to alter protein function and had a variant allele frequency of $>10\%$ using Sanger sequencing; 90.1% of the variants were validated. This resulted in the identification of an average of 14 megakaryocyte-unique variants per MPN patient (range, 0 to 26) (Table 3).

Megakaryocyte-Unique Variants in MPNs

There were 172 megakaryocyte-unique variants detected in 80 genes (Supplemental Table S5); 131 of these were likely to affect protein function (nonsynonymous and putative splice-site variants). Two of the 12 patients (Patients 4 and 7) did not have any megakaryocyte-unique variants. The 25 most frequently mutated genes are shown in Figure 2. Five of these 25 genes were mutated in ≥ 5 of the 10 patients

Figure 1 A and B: Representative images showing megakaryocyte enrichment from aspirated bone marrow after density gradient centrifugation isolation. Cytocentrifuged preparations stained with Mayer hematoxylin (A) and Wright-Giemsa (B). Original magnification, $\times 1000$.

Figure 2 The 25 most frequently mutated genes containing megakaryocyte-unique variants showing the number of patient samples with each.

who had megakaryocyte-unique mutations. One variant was present in more than one patient: *KMT2D*^{S762_L770del} (Patients 3 and 8 at 15.5% and 30.4% allele frequency, respectively). There was only one megakaryocyte-unique variant (*EZH2*^{Y733H} in Patient 12 at 84.3% allele frequency) that had previously been reported in association with MPNs, having been detected in blood granulocytes.^{35,36}

Allele Frequency Distribution of Megakaryocyte-Unique Variants

The megakaryocyte-specific variants showed a range of allele frequencies, with most being $\leq 20\%$. However, high-allelic burdens with frequencies of $>50\%$ were also present. The six patients with the greatest allelic burden all had increased and pathologic reticulिन (grade ≥ 2) (Figure 3). The genes containing the megakaryocyte-unique variants with high-allele frequency included chromatin remodeling genes (*SETD2*, *KANSL1*, *RARA*, *BCORL1*, *BOD1L1*, *EZH2*, and

JARID2), cell signaling genes (*JAK1*, *JAK2*, *RAF1*, *SH2B3*, *BRAF*, *FGFR1*, and *TLR4*), and cell adhesion gene (*DST*). Two of these genes were mutated in more than one patient (*SETD2* and *KANSL1*).

Discussion

Megakaryocytes differ from other (diploid) hemopoietic cells in that they undergo multiple rounds of endomitosis leading to a polyploid cell. In MPNs, this normally well-controlled replication is deregulated as a consequence of driver mutations. This results in up-regulated proliferation and impaired apoptosis leading to megakaryocytic hyperplasia with hyperploid nuclei.²¹ Here, we demonstrate that these abnormal megakaryocytes in MPNs have the propensity to acquire additional somatic mutations. These are not present in the patient-matched nonmegakaryocytic cells. We also noted that these megakaryocyte-unique somatic variants had the highest allele burden in the presence of pathologic reticulिन. These are novel findings demonstrating that

Figure 3 Allele frequency distributions of megakaryocyte-unique variants. The mean number of variants in each patient is shown by the solid line and the dotted line indicates 50% allele frequency. Clusters are seen at $\leq 20\%$ and $\geq 50\%$. The reticulिन grades for each patient are indicated in boxes along the top.

genomic aberrations occur in megakaryocytes in MPNs and that they are most prevalent in the setting of marrow fibrosis.

This is the first report that megakaryocytes in MPNs can acquire unique somatic variants. The results were obtained using a targeted panel of 120 genes in which mutations are known to occur in myeloid malignancies, and from this we were able to identify megakaryocyte-specific variants in 10 of 12 patients studied. Overall, there were mutations in 80 genes, some of which have previously been implicated in MPNs (eg, *ASXL1*, *CBL*, *TET2*, *EZH2*, and *IDH1/2*) where they have been detected in CD34⁺ cells, blood granulocytes, leukocytes, and whole bone marrow.^{35–44} However, the variants we identified differed from those in the other hemopoietic cells types. We also identified mutations in genes not previously associated with MPNs. These include genes that regulate chromatin remodeling, chromosome alignment, and stability, such as *BOD1L1*, *CREBBP*, *KMT2D*, *EP300*, and *SETD2*. Mutations in these genes have the capacity to affect genome stability, which is important because these cells undergo further rounds of endomitotic reduplication. *BOD1L1* is one of the most frequently mutated genes and was mutated in 6 of the 12 patients studied (including five of the six patients with pathologic reticulín). Loss of *BOD1L1* has been shown to lead to deleterious nucleolytic resection of damaged replication forks, compromising repair of the replication fork and resulting in genome instability.⁴⁵ Mutations in these genes could generate an error-prone environment leading to the establishment of additional mutations. *RELN* and *DST* were also frequently mutated, and they encode proteins that regulate actin filament alignment around the nucleus and modulation of cell adhesion, respectively. Mutations in these genes may be significant for the abnormal megakaryocytic nuclear morphologic features and formation of megakaryocyte clusters or sheets, which are characteristic of MPNs and especially primary myelofibrosis or after fibrotic progression of essential thrombocythemia and polycythemia vera. Taken together, these results suggest that genome instability could form the basis on which additional mutations are acquired and subsequently lead to fibrotic progression.

Because we identified variants in megakaryocytes that were not in the nonmegakaryocytic cell population, these are likely to have been acquired through the additional rounds of DNA replication in endomitotic reduplication during megakaryopoiesis.^{21,46} The predominance of low frequency (<20%) megakaryocyte-unique variants suggests they were acquired late in the endomitotic process close to full maturation. Alternatively, they could be present in a subpopulation of megakaryocytes, or they may be present in a subset of the chromosomes within these highly polyploid cells. We also detected variants with high-allele frequencies (ie, 80% to 100%), indicating that these were in every allele. These high-frequency variants are likely to have arisen in a clonal megakaryocyte-primed progenitor cell.⁴⁷ Phylogenetic studies of hemopoietic stem cells and lineage-committed megakaryocyte progenitors throughout megakaryopoiesis would be required to confirm this hypothesis.

There are many reports that have implied that megakaryocytes in MPNs drive fibrotic progression.^{22–27} In the present study we observed an association between megakaryocyte genomic aberrations and fibrosis. Variants were present at higher frequency in patients with pathologic reticulín throughout the marrow (Patients 5, 6, and 9 to 12). Specifically, variants with high-allele burden were in signaling cascade genes (*JAK1*, *JAK2*, *SH2B3*, *BRAF*, *RAF1*, *DST*, *TLR4*, and *FGFR1*). Mutations in these genes could potentially lead to aberrant downstream production and release of cytokines and chemokines, creating a proinflammatory environment that is likely to stimulate fibroblast activation and subsequent reticulín deposition. This concept is supported by a mouse model in which a steady leak of α -granule contents (ie, cytokines, chemokines, and growth factors) from megakaryocytes into the bone marrow creates a proinflammatory environment that drives the development of fibrosis.¹⁸ In this study we detected a missense mutation in a gene that regulates α -granule secretion, *TLR4* (*TLR4*^{W746C}). *TLR4* is present on platelets and regulates secretion of cytokines and chemokines from platelets on activation.^{48–50} It also plays a role in proinflammatory and profibrogenic signaling in transforming growth factor- β -mediated hepatic fibrosis through the mitogen-activated protein kinase pathway.⁵¹ Mutations in megakaryocytic *TLR4* could therefore be significant in driving marrow fibrosis in MPNs. Other potentially significant mutations in driving proinflammatory signaling were in *BRAF* and *RAF1*, also through the mitogen-activated protein kinase signaling cascade.^{52,53}

It was of interest that in 2 of the 12 patients (Patients 4 and 7) no megakaryocyte-specific variants were detected in the 120 genes analyzed. Both patients had the *JAK2*^{V617F} mutation but did not show pathologic reticulín or evidence of progression to fibrosis. Because the *JAK2*^{V617F} driver mutation was detected in the megakaryocytes (as well as the nonmegakaryocytic cells), it is evidence that the megakaryocytes extracted and sequenced were of the neoplastic clone. Variants may have been present in other genes and whole-exome analysis or WGA would be required to verify this.

To generate these data we used a novel approach to obtain pure megakaryocytes from which we could extract high-quality DNA. This required a combination of freshly aspirated bone marrow, density gradient centrifugation, morphologic identification, and laser capture microdissection. Attempts to use bone marrow trephines instead of aspirate produced inconsistent results, and megakaryocytes were frequently unable to be obtained. Alternative staining methods such as methyl green, neutral red, and CD61 immunohistochemistry similarly resulted in samples with poor quality and were thus inadequate for subsequent molecular analyses. The method presented here allows efficient isolation of megakaryocytes from patient samples and maximizes sample quantity without compromising quality. This was achievable even when only small volumes of marrow were obtained from extensively fibrotic marrows. Although it was necessary to use WGA to

generate sufficient DNA, we demonstrated that this produced low variant allele dropout rates ($4.8\% \pm 0.4\%$). Although the captured cells were all megakaryocytes, they were not all necessarily clonal. This could have affected the allele frequency in the megakaryocyte population. However, this does not differ from studying other cell types such as blood granulocytes in MPNs in which both clonal and normal cells are present in the blood.

Conclusions

We have demonstrated that megakaryocytes in MPNs have somatic mutations that are likely to have been acquired during ongoing DNA replication in the endomitotic process. The variants we have detected using a targeted sequencing approach and confirmed by Sanger sequencing were in genes associated with chromatin remodeling, chromosome alignment, and chromosome stability. As such, they could have an important role in regulating megakaryocyte ploidy and potentially the cell morphologic features. Mutations were also found in genes encoding signaling proteins with potential downstream proinflammatory and chemotactic effects leading to fibroblastic activity. This is significant because the greatest allelic burden was seen in patients with pathologic reticulins, thereby suggesting that there is an association between the megakaryocytic mutations, the allelic burden, and fibrotic potential.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.03.009>.

References

1. Tefferi A, Vainchenker W: Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. *J Clin Oncol* 2011, 29:573–582
2. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR; Cancer Genome Project: Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 2005, 365:1054–1061
3. Tefferi A, Vardiman JW: Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia* 2008, 22:14–22
4. Tefferi A, Thiele J, Orazi A, Kvasnicka HM, Barbui T, Hanson CA, Barosi G, Verstovsek S, Birgegard G, Mesa R, Reilly JT, Gisslinger H, Vannucchi AM, Cervantes F, Finazzi G, Hoffman R, Gilliland DG, Bloomfield CD, Vardiman JW: Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. *Blood* 2007, 110:1092–1097
5. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC: A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005, 352:1779–1790
6. Abdel-Wahab O: Genetics of the myeloproliferative neoplasms. *Curr Opin Hematol* 2011, 18:117–123
7. Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M, Steensma DP, Elliott MA, Wolanskyj AP, Hogan WJ, McClure RF, Litzow MR, Gilliland DG, Tefferi A: MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood* 2006, 108:3472–3476
8. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, Cuker A, Wernig G, Moore S, Galinsky I, DeAngelo DJ, Clark JJ, Lee SJ, Golub TR, Wadleigh M, Gilliland DG, Levine RL: MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med* 2006, 3:e270
9. Chaligne R, Tonetti C, Besancenot R, Roy L, Marty C, Mossuz P, Kiladjian JJ, Socie G, Bordessoule D, Le Bousse-Kerdiles MC, Vainchenker W, Giraudier S: New mutations of MPL in primitive myelofibrosis: only the MPL W515 mutations promote a G1/S-phase transition. *Leukemia* 2008, 22:1557–1566
10. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, Them NC, Berg T, Gisslinger B, Pietra D, Chen D, Vladimer GI, Bagienski K, Milanese C, Casetti IC, Sant'Antonio E, Ferretti V, Elena C, Schischlik F, Cleary C, Six M, Schalling M, Schonegger A, Bock C, Malcovati L, Pascutto C, Superti-Furga G, Cazzola M, Kralovics R: Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013, 369:2379–2390
11. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, et al: Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med* 2013, 369:2391–2405
12. Broseus J, Park JH, Carillo S, Hermouet S, Girodon F: Presence of calreticulin mutations in JAK2-negative polycythemia vera. *Blood* 2014, 124:3964–3966
13. Vytrva N, Stacher E, Regitnig P, Zinke-Cerwenka W, Hojas S, Hubmann E, Porwit A, Bjorkholm M, Hoefler G, Beham-Schmid C: Megakaryocytic morphology and clinical parameters in essential thrombocythemia, polycythemia vera, and primary myelofibrosis with and without JAK2 V617F. *Arch Pathol Lab Med* 2014, 138:1203–1209
14. Machlus KR, Italiano JE Jr: The incredible journey: from megakaryocyte development to platelet formation. *J Cell Biol* 2013, 201:785–796
15. Malara A, Abbonante V, Di Buduo CA, Tozzi L, Currao M, Balduini A: The secret life of a megakaryocyte: emerging roles in bone marrow homeostasis control. *Cell Mol Life Sci* 2015, 72:1517–1536
16. Bruns I, Lucas D, Pinho S, Ahmed J, Lambert MP, Kunisaki Y, Scheiermann C, Schiff L, Poncz M, Bergman A, Frenette PS: Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat Med* 2014, 20:1315–1320
17. Breton-Gorius J, Vainchenker W, Nurden A, Levy-Toledano S, Caen J: Defective alpha-granule production in megakaryocytes from gray platelet syndrome: ultrastructural studies of bone marrow cells and megakaryocytes growing in culture from blood precursors. *Am J Pathol* 1981, 102:10–19
18. Guerrero JA, Bennett C, van der Weyden L, McKinney H, Chin M, Nurden P, McIntyre Z, Cambridge EL, Estabel J, Wardle-Jones H, Speak AO, Erber WN, Rendon A, Ouwehand WH, Ghevaert C: Gray platelet syndrome: proinflammatory megakaryocytes and alpha-granule

- loss cause myelofibrosis and confer metastasis resistance in mice. *Blood* 2014, 124:3624–3635
19. Balduini A, Badalucco S, Pugliano MT, Baev D, De Silvestri A, Cattaneo M, Rosti V, Barosi G: In vitro megakaryocyte differentiation and proplatelet formation in Ph-negative classical myeloproliferative neoplasms: distinct patterns in the different clinical phenotypes. *PLoS One* 2011, 6:e21015
 20. Mazur EM, Lindquist DL, de Alarcon PA, Cohen JL: Evaluation of bone marrow megakaryocyte ploidy distributions in persons with normal and abnormal platelet counts. *J Lab Clin Med* 1988, 111: 194–202
 21. Malherbe JA, Fuller KA, Arshad A, Nangalia J, Romeo G, Hall SL, Meehan KS, Guo B, Howman R, Erber WN: Megakaryocytic hyperplasia in myeloproliferative neoplasms is driven by disordered proliferative, apoptotic and epigenetic mechanisms. *J Clin Pathol* 2016, 69:155–163
 22. Villeval JL, Cohen-Solal K, Tulliez M, Giraudier S, Guichard J, Burstein SA, Cramer EM, Vainchenker W, Wendling F: High thrombopoietin production by hematopoietic cells induces a fatal myeloproliferative syndrome in mice. *Blood* 1997, 90:4369–4383
 23. Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH: A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* 1997, 16:3965–3973
 24. Vannucchi AM, Bianchi L, Cellai C, Paoletti F, Rana RA, Lorenzini R, Migliaccio G, Migliaccio AR: Development of myelofibrosis in mice genetically impaired for GATA-1 expression (GATA-1^{low} mice). *Blood* 2002, 100:1123–1132
 25. Ciurea SO, Merchant D, Mahmud N, Ishii T, Zhao Y, Hu W, Bruno E, Barosi G, Xu M, Hoffman R: Pivotal contributions of megakaryocytes to the biology of idiopathic myelofibrosis. *Blood* 2007, 110:986–993
 26. Li J, Spensberger D, Ahn JS, Anand S, Beer PA, Ghevaert C, Chen E, Forrai A, Scott LM, Ferreira R, Campbell PJ, Watson SP, Liu P, Erber WN, Huntly BJ, Ottersbach K, Green AR: JAK2 V617F impairs hematopoietic stem cell function in a conditional knock-in mouse model of JAK2 V617F-positive essential thrombocythemia. *Blood* 2010, 116:1528–1538
 27. Koschmieder S, Mughal TI, Hasselbalch HC, Barosi G, Valent P, Kiladjian JJ, Jeryczynski G, Gisslinger H, Jutzi JS, Pahl HL, Hehlmann R, Maria Vannucchi A, Cervantes F, Silver RT, Barbui T: Myeloproliferative neoplasms and inflammation: whether to target the malignant clone or the inflammatory process or both. *Leukemia* 2016, 30:1018–1024
 28. Rondina MT, Weyrich AS: Regulation of the genetic code in megakaryocytes and platelets. *J Thromb Haemost* 2015, 13(Suppl 1): S26–S32
 29. Hussein K, Theophile K, Dralle W, Wiese B, Kreipe H, Bock O: MicroRNA expression profiling of megakaryocytes in primary myelofibrosis and essential thrombocythemia. *Platelets* 2009, 20:391–400
 30. Hussein K, Dralle W, Theophile K, Kreipe H, Bock O: Megakaryocytic expression of miRNA 10a, 17-5p, 20a and 126 in Philadelphia chromosome-negative myeloproliferative neoplasm. *Ann Hematol* 2009, 88:325–332
 31. Castro-Malaspina H, Rabellino EM, Yen A, Nachman RL, Moore MA: Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. *Blood* 1981, 57:781–787
 32. Rook MS, Delach SM, Deyneko G, Worlock A, Wolfe JL: Whole genome amplification of DNA from laser capture-microdissected tissue for high-throughput single nucleotide polymorphism and short tandem repeat genotyping. *Am J Pathol* 2004, 164:23–33
 33. Tabone T, Abuhusain HJ, Nowak AK; Australian Genomics and Clinical Outcome of Glioma (AGOG) Network, Erber WN, McDonald KL: Multigene profiling to identify alternative treatment options for glioblastoma: a pilot study. *J Clin Pathol* 2014, 67:550–555
 34. Schwarz JM, Cooper DN, Schuelke M, Seelow D: MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods* 2014, 11:361–362
 35. Guglielmelli P, Biamonte F, Score J, Hidalgo-Curtis C, Cervantes F, Maffioli M, Fanelli T, Ernst T, Winkelman N, Jones AV, Zoi K, Reiter A, Duncombe A, Villani L, Bosi A, Barosi G, Cross NC, Vannucchi AM: EZH2 mutational status predicts poor survival in myelofibrosis. *Blood* 2011, 118:5227–5234
 36. Vannucchi AM, Lasho TL, Guglielmelli P, Biamonte F, Pardanani A, Pereira A, Finke C, Score J, Gangat N, Mannarelli C, Ketterling RP, Rotunno G, Knudson RA, Susini MC, Laborde RR, Spolverini A, Pancrazzi A, Pieri L, Manfredini R, Tagliafico E, Zini R, Jones A, Zoi K, Reiter A, Duncombe A, Pietra D, Rumi E, Cervantes F, Barosi G, Cazzola M, Cross NC, Tefferi A: Mutations and prognosis in primary myelofibrosis. *Leukemia* 2013, 27:1861–1869
 37. Carbuccia N, Murati A, Trouplin V, Brecqueville M, Adelaide J, Rey J, Vainchenker W, Bernard OA, Chaffanet M, Vey N, Birnbaum D, Mozziconacci MJ: Mutations of ASXL1 gene in myeloproliferative neoplasms. *Leukemia* 2009, 23:2183–2186
 38. Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, Cervera N, Gelsi-Boyer V, Arnoulet C, Gisserot O, Verrot D, Slama B, Vey N, Mozziconacci MJ, Birnbaum D, Murati A: Mutation analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in myeloproliferative neoplasms. *Genes Chromosomes Cancer* 2012, 51:743–755
 39. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Gangat N, Finke CM, Schwager S, Mullally A, Li CY, Hanson CA, Mesa R, Bernard O, Delhommeau F, Vainchenker W, Gilliland DG, Levine RL: TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. *Leukemia* 2009, 23:905–911
 40. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, Waghorn K, Zoi K, Ross FM, Reiter A, Hochhaus A, Drexler HG, Duncombe A, Cervantes F, Oscier D, Boultonwood J, Grand FH, Cross NC: Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet* 2010, 42:722–726
 41. Pardanani A, Lasho TL, Finke CM, Mai M, McClure RF, Tefferi A: IDH1 and IDH2 mutation analysis in chronic- and blast-phase myeloproliferative neoplasms. *Leukemia* 2010, 24:1146–1151
 42. Tefferi A, Lasho TL, Abdel-Wahab O, Guglielmelli P, Patel J, Caramazza D, Pieri L, Finke CM, Kilpivaara O, Wadleigh M, Mai M, McClure RF, Gilliland DG, Levine RL, Pardanani A, Vannucchi AM: IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic- or blast-phase essential thrombocythemia, polycythemia vera or myelofibrosis. *Leukemia* 2010, 24:1302–1309
 43. Lundberg P, Karow A, Nienhold R, Looser R, Hao-Shen H, Nissen I, Girsberger S, Lehmann T, Passweg J, Stern M, Beisel C, Kralovics R, Skoda RC: Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood* 2014, 123: 2220–2228
 44. Magor GW, Tallack MR, Klose NM, Taylor D, Korb D, Mollee P, Trau M, Perkins AC: Rapid molecular profiling of myeloproliferative neoplasms using targeted exon resequencing of 86 genes involved in JAK-STAT signaling and epigenetic regulation. *J Mol Diagn* 2016, 18:707–718
 45. Higgs MR, Reynolds JJ, Winczura A, Blackford AN, Borel V, Miller ES, Zlatanou A, Nieminszczy J, Ryan EL, Davies NJ, Stankovic T, Boulton SJ, Niedzwiedz W, Stewart GS: BOD1L is required to suppress deleterious resection of stressed replication forks. *Mol Cell* 2015, 59:462–477
 46. Vogelstein B, Kinzler KW: The path to cancer—three strikes and you're out. *N Engl J Med* 2015, 373:1895–1898
 47. Sanjuan-Pla A, Macaulay IC, Jensen CT, Woll PS, Luis TC, Mead A, Moore S, Carella C, Matsuoka S, Bouriez Jones T, Chowdhury O, Stenson L, Lutteropp M, Green JC, Facchini R, Boukarabila H, Grover A, Gambardella A, Thongjuea S, Carrelha J, Tarrant P, Atkinson D, Clark SA, Nerlov C, Jacobsen SE: Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* 2013, 502:232–236

48. Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C, Garraud O: Evidence of Toll-like receptor molecules on human platelets. *Immunol Cell Biol* 2005, 83:196–198
49. Garraud O, Cognasse F: Platelet Toll-like receptor expression: the link between “danger” ligands and inflammation. *Inflamm Allergy Drug Targets* 2010, 9:322–333
50. Cognasse F, Hamzeh-Cognasse H, Lafarge S, Delezay O, Pozzetto B, McNicol A, Garraud O: Toll-like receptor 4 ligand can differentially modulate the release of cytokines by human platelets. *Br J Haematol* 2008, 141:84–91
51. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF: TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007, 13:1324–1332
52. Kaminska B, Gozdz A, Zawadzka M, Ellert-Miklaszewska A, Lipko M: MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target. *Anat Rec (Hoboken)* 2009, 292: 1902–1913
53. Thalhamer T, McGrath MA, Harnett MM: MAPKs and their relevance to arthritis and inflammation. *Rheumatology (Oxford)* 2008, 47:409–414