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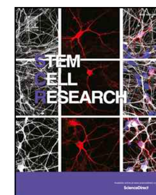

Lab resource: Stem Cell Line

# Characterization of a human induced Pluripotent Stem (iPS) cell line (INCABRi002-A) derived from a primary myelofibrosis patient harboring the 5-bp insertion in *CALR* and the p.W146X mutation in *TP53*

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## ABSTRACT

Primary myelofibrosis (PMF) is a hematological malignancy characterized by activation of the JAK/STAT pathway and risk of leukemic transformation. In this study, we generated an induced Pluripotent Stem (iPS) cell line derived from a 65-year old male PMF patient carrying the 5-pb insertion in the *CALR* gene (*CALR*$^{ins5}$) and the c.437 G > A mutation in the *TP53* gene (p.W146X). The newly derived PMF3.17 iPS cell line harbors the original mutations and was characterized as *bona fide* iPS.

Resource table

| | |
|---|---|
| Unique stem cell line identifier | INCABRi002-A |
| Alternative name(s) of stem cell line | PMF3.17 |
| Institution | Brazilian National Cancer Institute and D'Or Institute for Research and Education |
| Contact information of distributor | Martin Bonamino, PhD, mbonamino@inca.gov.br, Bárbara Monte Mór, PhD, barbara.montemor@inca.gov.br, Stevens Rehen, PhD, srehen@lance-ufrj.org |
| Type of cell line | iPS cell |
| Origin | Human |
| Additional origin info | Age: 65<br>Sex: male |
| Cell source | Peripheral blood |
| Clonality | Clonal |
| Method of reprogramming | Sendai Virus |
| Genetic modification | No |
| Type of modification | Not applicable |

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| | |
|---|---|
| Associated disease | Primary myelofibrosis |
| Gene | CALR gene: CALRins5, c.1154_1155insTTGTC, p.K385 fs*47<br>TP53 gene: c.437 G > A, p.W146X |
| Method of modification | Not applicable |
| Name of transgene or resistance | Not applicable |
| Inducible/constitutive system | Not applicable |
| Date archived/stock date | July 7th, 2015 |
| Cell line repository/bank | Not applicable |
| Ethical approval | Ethics Committee of the Brazilian National Cancer Institute (INCA) under the number 062/08. Ethics Review Board-competent authority obtained. |

## Resource utility

PMF patients are at increased risk of leukemic transformation and mutations in *TP53* have been identified in this context. The PMF3.17 iPS generated here harbor *CALR*$^{ins5}$ and *TP53* p.W146X mutations. Hematopoietic differentiation of this cell line will contribute to elucidate the role of both mutations in hematopoiesis and malignant transformation.

## Resource details

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm (MPN) derived from mutated hematopoietic stem cells. Driver somatic mutations in *JAK2*, *CALR* or *MPL* have been detected in bone marrow (BM) and peripheral blood (PB) cells of PMF patients (Vainchenker and Kralovics, 2017). Insertions and deletions (Indels) in *CALR* are mainly classified in type 1 (52-bp deletion) or type 2 (5-bp insertion) mutations (Nangalia et al., 2013; Klampfl et al., 2013) and have been shown to activate the JAK/STAT pathway. MPN patients are at increased risk of developing acute myeloid leukemia (AML) and loss-of-function mutations in *TP53* were associated with leukemic transformation (Rampal et al., 2014). Heterozygous *TP53* mutations have been found in NMP chronic phase and leukemic transformation was associated with loss of heterozygosity (Lundberg et al., 2014). Loss of *Tp53* was shown to cooperate with *JAK2V617F* to induce AML in a murine model (Rampal et al., 2014), but it is still to be established its role in combination with *CALR* mutations.

The patient was diagnosed with PMF based on the World Health Organization criteria at the Antônio Pedro University Hospital (HUAP), UFF, Brazil. A PB sample was sent to the Laboratory of Molecular Biology, Bone Marrow Transplant Center (CEMO), INCA at the time of diagnosis and patient's granulocytes were shown to carry the 5-pb insertion in *CALR* gene (*CALR*$^{ins5}$) and homozygous c.437 G > A mutation in the exon 5 of *TP53* gene, leading to a premature stop codon (p.W146X). One year later the disease progressed to AML.

CD34$^+$ hematopoietic stem/progenitor cells were enriched from peripheral blood mononuclear cells (PBMC) and analyzed using flow cytometry (Fig. 1A). Erythroblasts were differentiated *in vitro* from CD34$^+$ cells and mutations in *CALR*and *TP53* were confirmedby capillary electrophoresis and Sanger sequencing, respectively. The *TP53* mutant allele burden was at 93% as calculated by next generation sequencing (Fig. S1). The PMF3.17 iPS cell line was generated from erythroblasts using the CytoTune®-iPS 1.0 Sendai Reprogramming Kit (Table 1). The mentioned mutations were confirmed to be present in the PMF3.17 iPS cell line (Fig. 1B, C), and we also confirmed the cell identity by STR analysis. The morphology of the iPS cells was compatible with embryonic stem cell morphology and confirmed by microscopy observation (Fig. 1D). The immunofluorescence staining showed a expression for the following pluripotency markers: Oct3/4, Sox2, TRA-1-81 and SSEA-4. Cell nuclei were stained with DAPI. The pluripotency marker expression and DAPI have been quantified by HCA and shown as average percentages, Sox2: 95,6%, TRA-1-81: 95,94%, Oct3/4: 99,98% and SSEA-4: 89,98%."(Fig. 1E, Table S1). Absence of mycoplasma and Sendai virus-related gene expression was confirmed in iPS cells (Table S2 and Fig. 1F). Chromosomal analysis of PMF3.17 iPS cells was performed by low-pass whole-genome sequencing. A normal ploidy also was confirmed.The values are indicated in y-axis and the chromosome number in x-axis (Fig. 1G). The three-germ layer differentiation potential was verified *in vitro* by embryoid bodies (EB) formation followed by spontaneous differentiation. Resulting cells were characterized by immunocytochemistry and RT-PCR using markers specific for the three germ layers and we confirmed the presence of: β3-Tubulin, NESTIN, GFAP (Ectoderm), SMA, CK8 and CK18 (Mesoderm) and AFP and SOX-17 (Endoderm) (Fig. 1H, I, J and Fig. S3). Therefore, we conclude that the PMF3.17 iPS is characterized as *bona fide* iPS.

## Materials and methods

### *Hematopoietic stem/progenitor cells enrichment*

CD34$^+$ cells were isolated from PBMC using the CD34 MicroBead Kit (Miltenyi Biotech, USA) and characterized by flow cytometry with anti- human CD34 antibody (Table 2) in the Accuri C6 Flow Cytometer (BD Biosciences, USA), using the FlowJo, LLC v8 software.

### *In vitro erythroblast differentiation from CD34$^+$ cells*

Erythroblasts differentiation was performed during 9 days, using *in house* prepared expansion medium (EM): 50% Iscove's Modified Dulbecco's Medium (Life Technologies®, USA), 50% Ham's F-12 Nutrient Mixture (GIBCO®, USA), supplemented with 15% BSA (Sigma-Aldrich, USA), 50 μg/mL ascorbic acid (Sigma-Aldrich), 1% Glutamine (Thermo Scientific Fisher, USA), 20 μL/mL lipids, 10% Insulin-transferrin-selenite solution (GIBCO), 25 ng/mL SCF (PeproTech, USA), $10^2$U/mL IL-3 (PeproTech), 1 U/mL EPO (Farmaceutic Biosynthetic laboratory, BR) and 100 U/mL penicillin and 100 μg/mL streptomycin

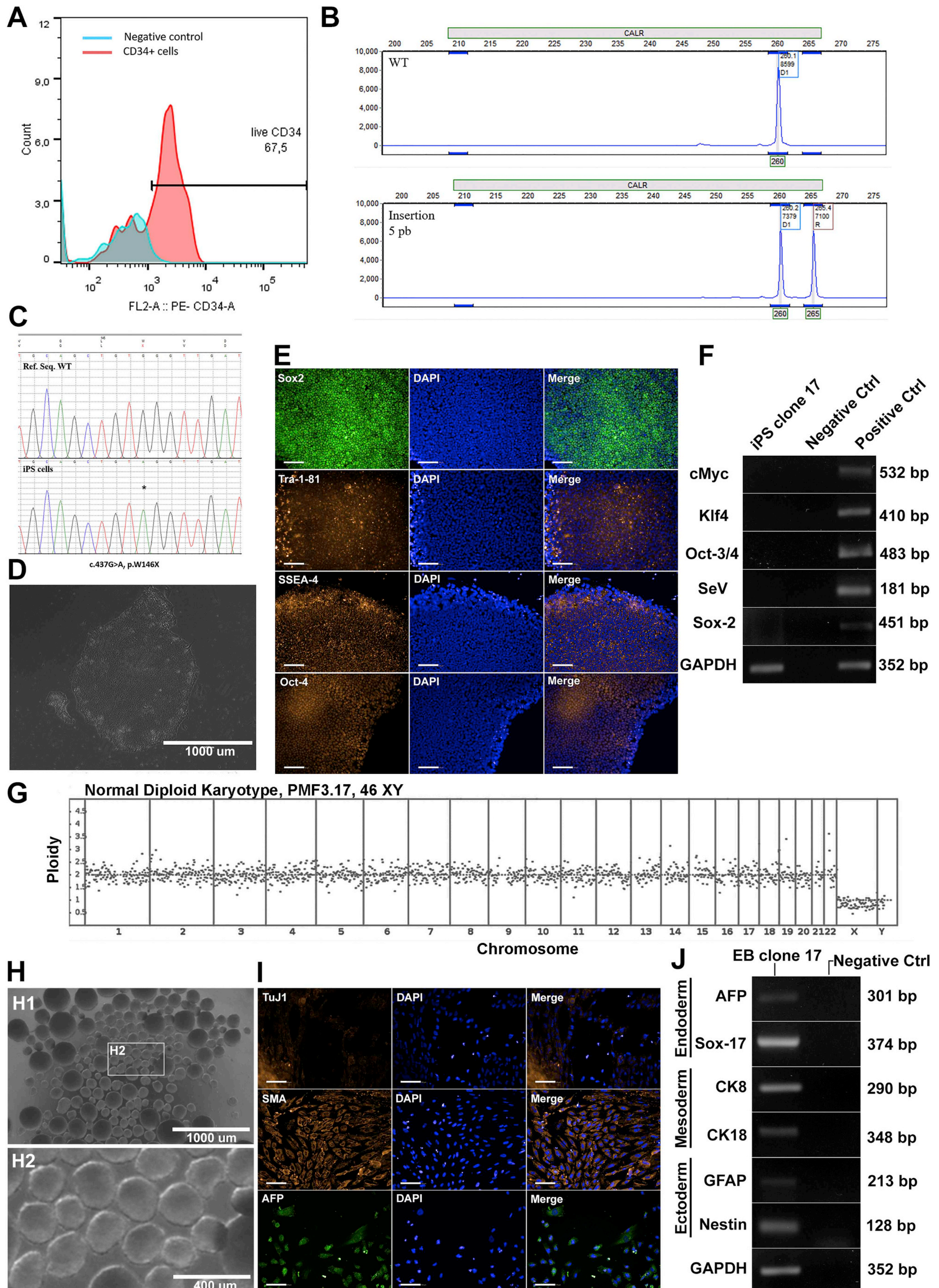


Fig. 1.

**Table 1**
Characterization and validation.

| Classification | Test | Result | Data |
|---|---|---|---|
| Morphology | Photography | Visual record of the line: normal | December 12th, 2016 Fig. 1, Panel D |
| Phenotype | Qualitative analysis: Immunocytochemisty | Assess staining/expression of pluripotency markers: Oct4, Sox2, TRA1–81, SSEA-4 | Fig. 1, Panel E |
| | Quantitative analysis: Immunocytochemistry counting: | Assess % antigen levels & cell surface markers: Sox-2: 96,23%, TRA-1-8: 87,28%, Oct3/4: 86,83%, SSEA-4: 92,09% | Table S1 Fig. S2 Fig. 1, Panel A |
| | Flow cytometry: | Assess antigen levels & cell surface markers, CD34: 67,5% | |
| Genotype | Low-pass genome whole | 46XY | Fig. 1, Panel G |
| Identity | Microsatellite PCR (mPCR) | Not analyzed | Not analyzed |
| | STR analysis | 10 STR analyzed, all matched | Supplementary file |
| Mutation analysis | Capillary gel electrophoresis and fragment analysis | *CALR*$^{ins5}$, Heterozygous, c.1154_1155insTTGTC, p. K385 fs*47 | Fig. 1, Panel B |
| | Sequencing | *TP53* gene, Homozygous, c.437 G > A, p.W146X | Fig. 1, Panel C Fig. S1 |
| Microbiology and virology | Mycoplasm | Luminescence, Negative | Table S2 |
| Differentiation potential | Embryoid body formation | Nestin, GFAP, B3-Tubulin, SMA, CK8, CK18, AFP, SOX-17 | Fig. 1, Panel H, I and J, Fig. S3 |
| Donor screening (OPTIONAL) | HIV 1 + 2 Hepatitis B, Hepatitis C | Not analyzed | Not analyzed |
| Genotype additional info (OPTIONAL) | Blood group genotyping | Not analyzed | Not analyzed |
| | HLA tissue typing | Not analyzed | Not analyzed |

(Thermo Scientific Fisher).

*Generation of iPS cells*

We transduced $7{,}5 \times 10^4$ erythroblasts with CytoTune®-iPS1.0 Sendai Reprogramming Kit (Thermo Fischer Scientific), MOI = 6. The EM was used until day 5, when transition to hESC medium began. Selected colonies were expanded on Matrigel®-coated culture dishes using the mTeSR1™ medium and passaged with Accutase® (all from Stem Cell Technologies, USA). Cell culture media was tested for the presence of mycoplasma using the Mycoalert™ PLUS (Lonza, USA).

*Mutation detection*

Genomic DNA (gDNA) and total RNA were obtained using AllPrep DNA/RNA MiniKit (Qiagen®, USA). Indels in *CALR* were identified by PCR using one primer labeled with 6′carboxilfluorescein (6-FAM) followed by capillary electrophoresis (Table 2). Mutation in *TP53* was detected by direct Sanger sequencing using the BigDye® Terminator v3.1 cycle Sequencing Kit (Applied Biosystems, USA) (Table 2).The results were analyzed using the Chimer Marker software (Soft Genetics, USA) or the Mutation Surveyor 3.1 (Soft Genetic LLC).

*Embryonic bodies (EBs) assay*

EBs were formed from iPS cells dissociated with Accutase® during 5 min at 37 °C. Cell clumps were maintained in differentiation medium (DMEM/F12, 20% KOSR, non-essential amino acids. L-Glutamine, penicillin/streptomycin – all from Thermo Fisher Scientific) on horizontal shaker. The first day the cells were cultured in presence of ROCK inhibitor (Merck, Millipore, USA). On day 6, EBs were transferred to gelatin-coated plates and kept in culture for additional 10 days.

*Immunofluorescence assays*

Pluripotency markers and germ-layer specific markers were analyzed by immunofluorescence staining, in iPS cell and EB, respectively. Briefly, samples were fixed in 4% paraformaldehyde solution, permeabilized in 0.3% Triton-X and blocked with 3% BSA. Then, cells were incubated with appropriate primary and secondary antibodies (Table 2) and nuclei were stained with DAPI. All images were acquired using the Operetta®High-Content Imaging System (PerkinElmer, USA), magnification at 20× high numerical apertures objective (NA). For cell quantification, images from 32 fields in each well were analyzed using the Harmony High-Content Screening platform software (PerkinElmer). Each image was segmented in each channel for automatic element detection and quantification. A specific fluorescence threshold was set and applied for all subsequent experiments to determine positive and negative cells for each marker. Negative controls were imaged to make sure of antibodies specificity (Fig. S2).

*Gene expression analysis*

Total RNA was treated with DnaseI (Invitrogen, USA) and cDNA was synthesized using the M-MLV reverse Transcriptase (Invitrogen). RT-PCR using specific primers was performed to analyze expression of SV-derived genes in the iPS cells and of germ-layer specific markers in EB derived cells (Table 2).

*Aneuploidy analysis*

Low-pass whole-genome sequencing was used to evaluate ploidy in the iPS cells (Thermo Fisher Scientific). Genomic DNA was enzymatically fragmented using the Ion Shear™ Plus enzyme cocktail. Fragments were ligated to sequencing adapters and barcoded using the Ion Xpress™ Plus Fragment Library Kit. To ensure library size uniformity, ligated fragments were electrophoretically separated on preparative 2% agarose gels. The library was amplified using universal primers and Platinum™ PCR SuperMix High Fidelity. Sequencing templates were prepared and enriched by emulsion PCR using the Ion PGM™ Hi-Q™ View OT2 chemistry on an Ion OneTouch™ 2 System. Enriched templates were quantitated and equal amounts of each were mixed into a single pool and applied to an Ion 316™ v2 Chip. Sequencing was carried out on an Ion PGM System using the Ion PGM™ Hi-Q™ View chemistry. Ploidy analysis was performed using the Ion Reporter software and the Low-pass whole-genome aneuploidy r.0 v5.0 automated analysis workflow.

**Table 2**
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry

| | Antibody | Dilution | Company Cat # and RRID |
|---|---|---|---|
| Hematopoietic Marker | Mouse anti-CD34 | 1:10 | BD Biosciences Cat# 348057, RRID: AB_400371 |
| Pluripotency Markers | Mouse anti-OCT4 | 1:100 | Santa Cruz Biotechnologies Cat# SC5279, RRID: AB_628051 |
| | Rabbit anti-SOX2 | 1:100 | Merck Millipore Ca# MAB5603, AB_2286686 |
| | Mouse anti-TRA1–81 | 1:100 | Merck Millipore Ca# MAB4381, AB_177638 |
| | Mouse anti-SSEA-4 | 1:100 | Merck Millipore Ca# MAB4303, RRID:AB_177628 |
| Differentiation Markers | Mouse anti-SMA | 1:300 | Sigma-Aldrich, Ca#A2547, RRID: AB_476701 |
| | Mouse anti-βIII-Tubulin | 1:250 | Merck Milipore Ca# MAB1637, AB_2210524 |
| | Rabbit anti-AFP | 1:100 | Santa Cruz Biotechnology Ca# SC15375, RRID: AB_2223935 |
| Secondary antibodies | CF488A Goat anti-rabbit IgG | 1:400 | Thermo Fisher Scientific Ca#A11008, RRID: AB_143165 |
| | CF546A Goat anti-mouse IgG | 1:400 | Thermo Fisher Scientific Cat# A-11003, RRID: AB_2534071 |

Primers

| | Target | Forward/Reverse primer (5′-3′) |
|---|---|---|
| Genotyping | CALR | GGCAAGGCCCTGAGGTGT/GGCCTCAGTCCAGCCCTTG |
| | TP53 | TGTTCAGTTGTGCCCTGACT/TCTCTGGGAGGAGGGGTTAA |
| Sendai Virus Markers (RT-PCR) | SeV | GGATCACTAGGTGATATCGAGCA/ACCAGACAAGAGTTTAAGAGATATGTATCA |
| | Oct-3/4 | CCCGAAAGAGAAAGCGAACCAG/AATGTATCGAAGGTGCTCAA |
| | Sox-2 | ATGTCACTAGATATCGAGC/AATGTATCGAAGGTGCTCAA |
| | Klf4 | TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAAG |
| | c-Myc | TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG |
| | GAPDH | TTCGACAGTCAGCCGCATC/GACTCCACGACGTACTCAGC |
| Differentiation Markers (RT-PCR) | CK18 | CACACAGTCTGCTGAGGTTGGAG/CTGCTGTCCAAGGCATCACCA |
| | CK8 | AGCAGCAGAAGACGGCTCGAA/GAAGTTGATCTCGTCGGTCAGC |
| | NESTIN | AGCCCTGACCACTCCAGTTTAG/CCCTCTATGGCTGTTTCTTTCTCT |
| | GFAP | CCCTGGACATCGAGATCGC/TGTGCTCCTGCTTGGACTC |
| | β3-Tubulin | GGCCAAGGGTCACTACACG/GCAGTCGCAGTTTTCACACTC |
| | AFP | AGAGTTGCTAAAGGATACCAGGA/AGGCCAATAGTTTGTCCTCAC |
| | SOX-17 | GTGAATCTCCCCGACAGC/TGTTTTGGGACACATTCAAAGC |

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.09.012.

## References

Klampfl, T., et al., 2013. Somatic mutations of calreticulin in myeloproliferative neoplasms. N. Engl. J. Med. 369 (25), 2379–2390.
Lundberg, P., et al., 2014. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. Blood 123 (14), 2220–2228.
Nangalia, J., et al., 2013. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N. Eng. J. Med. 2391–2392.
Rampal, R., et al., 2014. Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. Proc. Natl. Acad. Sci. U. S. A. 111 (50), E5401–E5410.
Vainchenker, W., Kralovics, R., 2017. Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. Blood 129 (6), 667–679 (Feb 9).