

SRSF2-P95H decreases JAK/STAT signaling in hematopoietic cells and delays myelofibrosis development in mice

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Competing interests

The authors do not have any competing financial interests in relation to the work described

39 Heterozygous mutation targeting proline 95 in Serine/Arginine-rich Splicing Factor 2 (SRSF2)
40 is associated with V617F mutation in Janus Activated Kinase 2 (JAK2) in some
41 myeloproliferative neoplasms (MPNs), most commonly primary myelofibrosis. To explore
42 the interaction of *Srsf2*^{P95H} with *Jak2*^{V617F}, we generated Cre-inducible knock-in mice
43 expressing these mutants under control of the *stem cell leukemia (Scl)* gene promoter. In
44 transplantation experiments, *Srsf2*^{P95H} unexpectedly delayed myelofibrosis induced by
45 *Jak2*^{V617F} and decreased TGFβ1 serum level. *Srsf2*^{P95H} reduced the competitiveness of
46 transplanted *Jak2*^{V617F} hematopoietic stem cells while preventing their exhaustion. RNA
47 sequencing of sorted megakaryocytes identified an increased number of splicing events
48 when the two mutations were combined. Focusing on JAK/STAT pathway, *Jak2* exon 14
49 skipping was promoted by *Srsf2*^{P95H}, an event detected in patients with *JAK2*^{V617F} and *SRSF2*^{P95}
50 co-mutation. The skipping event generates a truncated inactive JAK2 protein. Accordingly,
51 *Srsf2*^{P95H} delays myelofibrosis induced by the thrombopoietin receptor agonist Romiplostim
52 in *Jak2* wildtype animals. These results unveil *JAK2* exon 14 skipping promotion as a strategy
53 to reduce JAK/STAT signaling in pathological conditions.

55 Myeloproliferative neoplasms (MPNs) are clonal diseases arising from a single, somatically
56 mutated, hematopoietic stem cell (HSC).¹ One of these diseases is Myelofibrosis (MF), which
57 is characterized by reticulin deposition and fibrosis, resulting in the progressive development
58 of ineffective hematopoiesis, and a propensity for transformation to acute myeloid
59 leukemia.² MF development involves the reprogramming of nonhematopoietic mesenchymal
60 stromal lineage cells into fibrosis-driving myofibroblasts.³⁻⁵ The release of fibrogenic soluble
61 mediators by malignant haematopoietic cells mediates this reprogramming. A master
62 soluble mediator in this crosstalk is transforming growth factor beta 1 (TGFβ1) released by
63 abnormal megakaryocytes.⁶⁻⁸

64 The most frequent driver event identified in Philadelphia negative MPN is *JAK2*^{V617F}, a gain of
65 function mutation in *JAK2*.¹ MF is typically associated with additional somatic events that
66 may arise before or after the acquisition of *JAK2*^{V617F}.⁹ Mouse models have depicted a
67 synergistic contribution of gene deletion affecting the histone methyltransferase *Ezh2*
68 (enhancer of zeste homologue 2)¹⁰⁻¹² or the chromatin modifier *Asx1* (additional sex combs-
69 like 1)¹³ or the DNA methyltransferase *Dnmt3A*¹⁴ to *Jak2*^{V617F}-driven MF development. In
70 contrast, the cooperation between somatic mutation in *Jak2*^{V617F} and the monoallelic somatic
71 mutation affecting proline 95 residue in the splicing factor SRSF2 (serine/arginine-rich
72 splicing factor 2),¹⁵ a key protein of the spliceosome machinery, remains poorly explored.
73 Among MPNs, somatic mutation in *SRSF2*^{P95} was associated with MF features and was
74 suggested to reduce survival.^{9,16,17} However, while *ASXL1* and *EZH2* mutations are enriched
75 when progressing from pre-fibrotic to fibrotic MPN, this is not observed with *SRSF2*^{P95} that
76 rather correlates with an increased risk of leukemic transformation.^{9,18}

To better explore how these two mutational events interact in hematopoietic cells, we crossed Cre-inducible *Jak2*^{VF/+}¹⁹ and *Srsf2*^{P95H,/+}²⁰ knock-in mouse models under *Scf* (*stem cell leukemia*) gene promoter. Our results reveal the attenuation of *Jak2*^{V617F}-induced phenotype by *Srsf2*^{P95H} that delays MF development while preventing *Jak2*^{V617F} HSC exhaustion. One of the pre-mRNA splicing events amplified by *Srsf2*^{P95H} is the skipping of *Jak2* exon 14 in hematopoietic stem and progenitor cells (HSPCs), generating an inactive protein that may contribute to the down-regulation of JAK-STAT signaling in these cells.

Methods

Animal models

Transgenic mice expressing a Cre-recombinase-inducible *Jak2*^{V617F}¹⁹ were crossed with transgenic mice expressing a Cre-recombinase-inducible *Srsf2*^{P95H}²⁰ to generate double mutant mice on a C57BL/6 background (**Supplemental figure 1A**). Single and double transgenic animals were crossed with animals expressing the tamoxifen-inducible Cre-ER(T) recombinase under the control of the stem cell leukemia (*Scf*) locus enhancer (herein referred to as *Scf-Cre*^{ERT} transgenic animals)²¹ to generate offsprings in which tamoxifen gavage could trigger the expression of heterozygous *Jak2*^{V617F}, heterozygous *Srsf2*^{P95H} or the two mutated alleles. In transplant experiments, recipient mice were 8-week old C57BL/6 females (Envigo Labs, www.envigo.com) intravenously engrafted with 3 x 10⁶ bone marrow cells after lethal irradiation (9.5Gy). Recombinase Cre expression was induced 5 weeks after transplantation by tamoxifen gavage (500mg/kg/day for 2 days). Romiplostim-induced myelofibrosis was generated as described.²¹ Peripheral blood samples were serially collected

99 on citrate. Sternum and spleen, as well as bone marrow cells, were collected at sacrifice.
100 Detailed analyses are described in supplemental data.

101 **TGFβ1 quantification**

102 Whole peripheral blood was obtained at sacrifice through cardiac puncture and centrifuged
103 at 2,200 rpm for 20 minutes before collecting the serum that was aliquoted and stored
104 at -80°C. TGF-β1 was measured in duplicates using ELISA on EnSpire Multimode Plate
105 Reader from PerkinElmer.

106 **Flow cytometry**

107 After red blood cell lysis and, when indicated, specific cell sorting, cell suspensions were
108 labeled with indicated antibodies and analyses were performed on LSR Fortessa (BD) and
109 analyzed using Kaluza Analysis software (Beckman Coulter). Ploidy was measured in CD41⁺
110 CD42⁺ cells (see Supplemental data for details).

111 **Transmission electron microscopy**

112 Bone marrow carrots were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer and were
113 postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer, embedded in Epon™ 812.
114 Polymerization before generating 1 μm semi-thin sections that were stained with standard
115 uranyl acetate and lead citrate and observed with FEI Tecnai 12 electron microscope (FEI,
116 Eindhoven, Netherlands). Digital images were taken using a SIS MegaviewIII CCD camera
117 (Olympus, Tokyo, Japan). Details are provided in Supplemental Data.

118 **RNA sequencing**

119 Bone marrow cells were collected from recipient mice 8-12 weeks after tamoxifen feeding.
120 Lin⁻ cells were enriched before collecting CD41⁺ CD42d⁺ megakaryocytes in Trizol LS (Thermo

121 Fisher, Scientific, www.thermofisher.com/fr) by using Influx 5 laser BD cell sorter. Paired end
122 sequencing of total RNA was performed on Illumina Novaseq. Analysis of differentially
123 expressed genes (DEGs) was performed after correction of batch effects and discarding of
124 low-expressed genes. Details are provided in supplemental data. Splicing defects were
125 identified with rMATS v4.1.0 (<https://www.pnas.org/content/111/51/E5593>) using the
126 Ensembl GRCm38 release 101 annotation. Reads that do not cross an exon boundary were
127 used as well as reads that span junctions. SSNG_score was calculated for each exon by the
128 sum of: +1 for each CCNG, +0.5 for each GCNG, 0.5 for each CGNG and -1 for each GGNG.
129 This is multiplied by 4 (the number of bases in the motif) and divided by the length of the
130 exon to give the SSNG fraction.

131 **Real-time quantitative polymerase chain reaction**

132 Mouse megakaryocytes RNA (100 ng) was reverse transcribed using SuperScript™ IV VILO
133 Master mix with ezDNase enzyme (Thermo Fisher Scientific). RT-qPCR was
134 performed with AmpliTaq Gold polymerase. Jak2 exon14 skipping (NM_008413)
135 was analyzed using Jak2_e13_F: 5'-CTAGATAAAGCACATAGGAACTATTCA-3' and
136 Jak2_e15_R: 5'CTTCAGGTATGTATCCAGTGATCCAA-3' primer set. In patients
137 samples, RT-qPCR was performed in sorted granulocytes using JAK2_e13_F: 5'-
138 CGGTCAACTGCATGAAACAGA-3' and JAK2_ex15_R: 5'-
139 CCAAATTTTACAAACTCCTGAACCAGAAT-3' (NM_004972) primers. Melting curve
140 profiles were used to visualize exon-14 skipping and the amplicons were separated
141 in a 2% agarose gel (More details in supplemental data).

142 **Dual luciferase transcriptional assay**

143 Full-length human *JAK2* cDNA and *JAK2*^{Δex14} cDNA were amplified by PCR and introduced into
144 pCDNA3.1 vector using TOPO Expression Kit (Invitrogen). STAT5 transcriptional activity of
145 these constructs was measured in JAK2-deficient γ-2A cells (mutant gamma1A fibrosarcoma
146 cells) by dual luciferase assay as described.²⁴ γ-2A cells were transiently transfected using
147 Lipofectamine 3000 (ThermoFisher) with cDNAs coding the SpiLuc reporter as a readout of
148 STAT5 transcriptional activation and pRLTK-Luc reporter as an internal control (Promega)
149 together with human thrombopoietin receptor MPL, murine STAT5 and pCDNA3 vectors
150 encoding or not JAK2 constructs. Medium was changed 4 hours after transfection and cells
151 were stimulated or not with 10 ng/mL thrombopoietin (Miltenyi Biotec) for 24 hours.

152 **Phosphoflow assay**

153 Mouse BM and spleen Lin⁻ cells were negatively sorted using rat antibodies targeting Ter-
154 119, B220, Gr-1, CD3 and CD11b and fixed with 1.6% formaldehyde (ThermoFisher). LSK cells
155 were stained with anti-Sca-1 (PE-Cy7) and c-Kit (PE) antibodies (BioLegend), and
156 megakaryocytes with Abs against CD41 (Alexa Fluor 700) and CD42d (APC) (BioLegend). After
157 methanol permeabilization and staining for p-STAT5 (BV421, BD Biosciences), samples were
158 analyzed by flow cytometry (LSRFortessa) with BD FACSDiva software. Data were analyzed
159 with Kaluza Analysis software (Beckman Coulter).

160 **Statistical analysis**

161 Results are shown as means ± SEM. To assess the statistical significance among individual
162 cohorts, one-way ANOVA with subsequent Bonferroni's post-hoc multiple comparison test
163 or unpaired Student's *t* test were used (Prism version 7 software; GraphPad Software). *P* ≤
164 0.05 was considered significant. In all figures, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* <
165 0.0001.

166 **Study approval**

167 Animal experiments were conducted in Gustave Roussy animal facility and approved by the
168 ethical review board (#2016–104-7171). All mice were kept under specific pathogen–free
169 conditions with free access to food and water, housed under 12-hour light/12-hour dark
170 cycles, with monitored ambient temperature ($21\pm1^{\circ}\text{C}$) and humidity range (20–70%), in
171 accordance with French laws for animal welfare. Patient samples were collected samples
172 with informed consent and all authorizations (IRB 00003835- Protocol 2015/59-NICB; CNIL
173 #915663).

Results

***Srsf2*^{P95H} delays *Jak2*^{V617F}-induced myelofibrosis.**

Since *Scl*-promoter driven genes are expressed in both hematopoietic and endothelial cells, we engrafted bone marrow cells collected from *Scl-Cre^{ERT}-Srsf2^{P95H}*, *Scl-Cre^{ERT}-Jak2^{V617F}*, *Scl-Cre^{ERT}-Jak2^{V617F} / Srsf2^{P95H}*, and *Scl-Cre^{ERT}* (control) animals into lethally irradiated congenic recipient animals and tamoxifen was given five weeks after transplantation, which allowed to specifically explore gene expression impact on hematopoietic cells (**Supplemental figure 1A and Figure 1A**). Sequential peripheral blood analyses showed an increase in hemoglobin level (**Supplemental figure 1B**), white blood cell count (**Supplemental figure 1C**) and platelet count (**Figure 1B**) two weeks after tamoxifen gavage in *Jak2^{V617F}* expressing animals. The increase in hemoglobin level and white blood cell count was initially slowed down by *Srsf2^{P95H}* co-mutation (**Supplemental figure 1B and 1C**), with *Srsf2^{P95H}* mostly decreasing B220⁺ B-cell number among white blood cells (**Supplemental figure 1D**), as previously described in *Srsf2^{P95H/+}* animals.²⁰ Within the first 8 weeks after tamoxifen gavage, *Jak2^{V617F}*-induced thrombocytosis was not affected by *Srsf2^{P95H}* (**Figure 1B**). At later time points, the platelet count decreased in *Jak2^{V617F}* but continued to rise in animals co-expressing *Srsf2^{P95H}* (**Figure 1C**).

BM histopathology detected an increase in reticulin fibrosis in animals expressing *Jak2^{V617F}* in hematopoietic cells, eight weeks after initiation of tamoxifen gavage (**supplemental Figure 1E**), which further increased with time (**Figure 1D**). Fibrosis was significantly delayed by *Srsf2^{P95H}* co-expression (**Figure 1D and 1E**). At 8 and 24 weeks after tamoxifen gavage, *Srsf2^{P95H}* also reduced *Jak2^{V617F}*-induced splenomegaly and spleen fibrosis (**Supplemental Figure 1F**) as well as the two-fold increase in the serum level of TGFβ1 induced by *Jak2^{V617F}* (**Figure 1G**). Together, these experiments provided the unexpected observation that the co-

197 expression of *Srsf2*^{P95H} in mouse hematopoietic cells delays rather than promotes *Jak2*^{V617F}
198 induced myelofibrosis.

199 ***Srsf2*^{P95H} delays exhaustion of *Jak2*^{V617F} stem cells in stressful conditions.** _ _

200 We analyzed HSPCs in the BM and spleen of transplanted mice between 16 and 24 weeks
201 after tamoxifen gavage. We observed an increase in the fraction of LSK (Lin⁻ Sca-1⁺ Kit⁺), MPP
202 (LSK CD48⁺ CD150⁻) and SLAM (LSK CD48⁻ CD150⁺) cells in animals engrafted with either
203 *Jak2*^{V617F} or *Jak2*^{V617F}/*Srsf2*^{P95H} cells when compared to *Scl-Cre* and *Srsf2*^{P95H}, without significant
204 difference between *Jak2*^{V617F} or *Jak2*^{V617F}/*Srsf2*^{P95H} engrafted animals (**Figure 2A**).

205 In competitive experiments, CD45.1 recipient mice were first engrafted with an equal
206 fraction of CD45.2 *Jak2*^{V617F} cells expressing the green fluorescent protein (GFP) and CD45.2
207 *Jak2*^{V617F}/*Srsf2*^{P95H} GFP-negative cells (**Supplemental Figure 2A**). Double mutant cells
208 demonstrated a decreased competitiveness attested by the reduced fraction of GFP-
209 negative SLAM in the BM (**Supplemental Figure 2B**) and the time-dependent decrease in
210 GFP-negative peripheral blood cells (**Supplemental Figure 2C**).

211 We also transplanted CD45.1 recipient animals with 30% genetically modified cells mixed
212 with 70% wild-type cells expressing the GFP (**Figure 2B**). In accordance with previous
213 report,¹⁹ *Srsf2*^{P95H} alone decreased cell competitiveness in transplanted animals, as indicated
214 by the rapid loss of GFP-negative Ter119⁺ cells in the peripheral blood. When combined with
215 *Jak2*^{V617F}, *Srsf2*^{P95H} slightly delayed the rapid increase in Ter119⁺ cells induced by *Jak2*^{V617F}
216 alone (**Supplemental Figure 2D**). Analysis of GFP-negative SLAM cells in the BM collected 24
217 weeks after tamoxifen gavage demonstrated a decrease in the fraction of *Srsf2*^{P95H} cells while
218 *Jak2*^{V617F} cells almost completely invaded the SLAM compartment. *Srsf2*^{P95H} co-mutation
219 significantly reduced *Jak2*^{V617F} SLAM cell expansion (**Figure 2C**).

BM cells isolated from these animals were used to perform serial transplantations in recipient CD45.1 animals (**Figure 2D**). The third transplantation was associated with a significant decrease in the survival of animals engrafted with *Jak2*^{V617F} compared to *Jak2*^{V617F}/*Srsf2*^{P95H} containing BM cells (**Figure 2E**). After this third transplantation, *Srsf2*^{P95H} or *Jak2*^{V617F} SLAM cells had almost disappeared from the BM, contrasting with the expansion of *Jak2*^{V617F}/*Srsf2*^{P95H} double mutant cells (**Figure 2F**), amplifying an effect already observed after the second transplantation (**Supplemental Figure 2E**). Finally, monitoring of peripheral blood cells after the third transplantation showed significantly higher white blood cell and platelet counts in mice engrafted with BM cells containing *Jak2*^{V617F}/*Srsf2*^{P95H} compared to *Jak2*^{V617F} alone cells (**Figure 2G**). Together, *Srsf2*^{P95H} co-mutation preserved *Jak2*^{V617F} SLAMs from exhaustion in stressful conditions provoked by serial transplantations.

***Srsf2*^{P95H} interferes with *Jak2*^{V617F}-induced megakaryocyte phenotype**

Given their acknowledged role in TGFβ synthesis and MF development, we explored the phenotype of megakaryocytes in transgenic animals. Initial analysis were performed 8 weeks post tamoxifen. We quantified megakaryocytes by von Willebrand factor (vWF) staining of BM sections. *Jak2*^{V617F} induced an increase in the number of BM megakaryocytes as compared to *Scl-Cre* and *Srsf2*^{P95H}-transplanted animals, without significant impact of concomitant *Srsf2*^{P95H} mutation (**Figure 3A**). Similarly, electron microscopy did not detect ultrastructural modifications of megakaryocytes according to their mutational status (**Supplemental Figure 3A**). *Srsf2*^{P95H} alone or co-expressed with *Jak2*^{V617F} induced a slight decrease in the size of megakaryocytes (**Figure 3B**). In agreement with decreased megakaryocyte size, *Srsf2*^{P95H} co-expression with *Jak2*^{V617F} was associated also with a slight decrease in cell ploidy compared to *Jak2*^{V617F} megakaryocytes (**Figure 3C and supplemental**

Figure 3B). The decreased expression of the thrombopoietin receptor MPL typically observed at the surface of *Jak2*^{V617F} cells²³ was also prevented by *Srsf2*^{P95H} co-mutation (**Figure 3D**).

At later time points, 16-24 weeks post tamoxifen, the number of megakaryocytes measured in vWF-stained BM remained high in both *Jak2*^{V617F} and *Jak2*^{V617F} / *Srsf2*^{P95H} animals, yet this number had decreased in *Jak2*^{V617F} mice as compared to earlier time points and was still increasing in double-mutant mice (**Figure 3E and supplemental Figure 3C**). This evolution correlated with platelet count (**Figure 1C**), with megakaryocyte erythroid progenitor (MEP) and megakaryocyte progenitor (MKP) fractions in the BM (**supplemental Figure 3D**), and with megakaryocyte ploidy (**Figure 3F**). Together, *Srsf2*^{P95H} on its own only slightly decreased megakaryocyte size while it negatively interfered with *Jak2*^{V617F}-induced longitudinal changes in BM megakaryocyte number and maturation.

***Srsf2*^{P95H} down-regulates cell signaling in megakaryocytes**

We sequenced RNA of megakaryocytes enriched from *Scl-Cre*, *Srsf2*^{P95H}, *Jak2*^{V617F} and *Jak2*^{V617F} / *Srsf2*^{P95H} mouse BM samples. Dimensionality reduction through principal component analysis (PCA) separated megakaryocytes from *Jak2*^{WT} and *Jak2*^{V617F} animals, regardless of the *Srsf2* status (**supplemental figure 4A**). Pairwise differential analysis consistently showed a high impact of *Jak2*^{V617F} and a more limited impact of *Srsf2*^{P95H} on gene expression (as defined by a Padj <0.05) in megakaryocytes. For example, compared to *Scl-Cre* megakaryocytes, the *Srsf2*^{P95H} mutation changed the expression of 75 genes while *Jak2*^{V617F} mutation, with or without *Srsf2*^{P95H}, altered the expression of about 3,000 genes (**supplemental figure 4B**). The upset plot showed that, among the six pairwise analysis, the

largest intersection between differentially expressed genes (DEG) was across samples with *Jak2*^{V617F} mutation (**supplemental figure 4C**). Megakaryocytes collected from double-mutant *Jak2*^{V617F} / *Srsf2*^{P95H} mice clustered separately from single mutant *Jak2*^{V617F} cells (**Figure 4A**), with 71 DEG between these two conditions (**Figure 4B**). Gene set enrichment analysis (GSEA) indicated the down regulation of multiple signaling pathways, including JAK-STAT, TNF and TGFβ signaling pathways, in *Jak2*^{V617F}/*Srsf2*^{P95H} compared to *Jak2*^{V617F} cells (**Figure 4C**). Comparison of enrichment scores further confirmed the upregulation of signaling pathways in *Jak2*^{V617F} megakaryocytes and their down-regulation in cells expressing *Srsf2*^{P95H}, either alone or with *Jak2*^{V617F} (**Figure 4D**). Together, these results indicate that *Srsf2*^{P95H} reduces the enrichment in signaling pathways observed in *Jak2*^{V617F} megakaryocytes.

***Srsf2*^{P95H}-induced *Jak2* exon 14 skipping.**

SRSF2 splicing factor recognizes both CCNG and GGNG sequences.²⁵ Using RNA sequences collected from sorted megakaryocytes, we compared the relative enrichment of all four SSNG variants, where S represents C or G, in cassette exons that were differentially spliced upon expression of mutant *Srsf2*^{P95H}, in the context of either wild-type or mutated *Jak2*. Megakaryocytes expressing *Srsf2*^{P95H} either alone or in combination with *Jak2*^{V617F} exhibited an enrichment for CCNG while the other sequences, especially GGNG, were depleted in exons that were included versus excluded, respectively (**Figure 5A**). Based on a False Discovery Rate (FDR) < 0.05, we detected a total number of 1,729 differential splicing events in mutated compared to *Scf-Cre* megakaryocytes. We detected 666 differential splicing events in *Srsf2*^{P95H} cells. We also identified 587 differential splicing events in *Jak2*^{V617F} cells, which may reflect the ability of mutant JAK2 to phosphorylate proteins involved in mRNA

288 processing.²⁶ The highest number (n=1,241) was observed in double mutant
 289 *Jak2*^{V617F}/*Srsf2*^{P95H} megakaryocytes. One hundred thirty four events (7.7% of total) were
 290 common to the three genetically modified cell populations (**Figure 5B**).

 291 Given the importance of the JAK/STAT pathway activation in MFs, we focused on differential
 292 splicing events that affect genes of this pathway. Of the 147 genes listed in
 293 KEGG_JAK_STAT_SIGNALING_PATHWAY, 16 showed abnormal splicing events, with only one
 294 common to the three cell types (*il15ra* gene). One of the two abnormally spliced genes
 295 common to *Srsf2*^{P95H} and *Jak2*^{V617F} / *Srsf2*^{P95H} cells, but not detected in *Jak2*^{V617F} cells, was *Jak2*
 296 (**Figure 5C, supplemental table 1**).

 297 In *Srsf2*^{P95H} expressing cells, an abnormal skipping of *Jak2* exon 14 (*Jak2*^{Δex14}), which encodes
 298 the pseudokinase domain including Valine 617, results in a frameshift (**Figure 5D**). Using an
 299 isoform-specific RT-qPCR, *Jak2*^{Δex14} was detected in LSK (Lin⁻, Sca⁺, Kit⁺) cells, erythroid cells
 300 (CD71⁺, Ter119⁺) and megakaryocytes sorted from the BM of *Srsf2*^{P95H} and *Jak2*^{V617F} / *Srsf2*^{P95H}
 301 animals (**Figure 5D, Supplemental Figure 5A and 5B**). This alternatively spliced *Jak2* was no
 302 more detected in megakaryocytes when these cells were generated by *ex vivo*
 303 differentiation of LSK in the presence of TPO, suggesting a counter-selection of cells
 304 expressing *Jak2*^{Δex14} isoform in these culture conditions (**Figure 5D**). Importantly, a *JAK2*^{Δex14}
 305 isoform could be detected also in peripheral blood granulocytes collected from two *JAK2*^{V617F}
 306 MF patients with a co-existing *SRSF2*^{P95} mutation, while not being detected in the cells of two
 307 healthy donors and three *JAK2*^{V617F} MF patients without *SRSF2* mutation (**Figure 5E**).

 308 The presence of a *Jak2*^{Δex14} isoform correlated with a decrease in the level of phosphorylated
 309 STAT5 measured by flow cytometry in sorted LSK cells (**Figure 5F and supplemental 5C**).
 310 When transfected in γ-2A cells, *Jak2*^{Δex14} isoform was translated into a shortened protein at

the expected 66kDa molecular weight (**Figure 5G**). Using a dual luciferase assay in γ -2A cells expressing the thrombopoietin receptor MPL, we observed that the shortened protein encoded by JAK2 Δ ex14 (**Figure 5G**) did not transduce a P-STAT5 signal in response to thrombopoietin (**Figure 5G and 5H**). When JAK2^{V617F} was co-expressed with JAK2 Δ ex14 at various ratio in γ -2A cells, JAK2 Δ ex14 isoform behave like the empty vector, in the absence or presence of thrombopoietin (**Figure 5I**). These results suggested a loss of function of JAK2 Δ ex14 that may explain the weaker phenotype observed when the Srsf2^{P95H} mutation is combined with Jak2^{V617F} through a quantitative decrease in the functional JAK2^{V617F} (**Figure 5D and supplemental 5A**).

***Srsf2*^{P95H} down-regulates romiplostim-induced MF**

Since Srsf2^{P95H} induces the abnormal skipping of JAK2 exon 14, generating a truncated and inactive protein, we wanted to further validate Srsf2^{P95H} mutation effect on JAK/STAT signaling. Therefore, we used a previously described mouse model of MF induced by subcutaneous administration of high dose romiplostim, a thrombopoietin receptor agonist that generates MPL-mediated hypersignalling. Animals were engrafted with either *Scl-Cre* or *Srsf2*^{P95H} bone marrow cells. Three weeks after tamoxifen gavage, they received three weekly administration of high dose (1 mg/kg/day) romiplostim (**Figure 6A**). This treatment induced a rapid increase in the peripheral blood platelet count, which was significantly less important in *Srsf2*^{P95H} engrafted animals (**Figure 6B**). All the *Scl-Cre* engrafted animals developed a MF, which was reduced in *Srsf2*^{P95H} engrafted animals (**Figure 6C and 6D**), an effect that was also observed in the spleen (**Figure 6E**). Together, these results validated that the *Srsf2*^{P95H} genotype negatively interfered with MF induction upon JAK/STAT hypersignaling, either induced by Jak2 mutation or MPL stimulation.

The present study demonstrates that *Srsf2*^{P95H} delays myelofibrosis when associated with *Jak2*^{V617F} in mouse hematopoietic cells. *Srsf2*^{P95H} promotes *JAK2* exon 14 skipping in mouse and human hematopoietic cells. This event generates a shortened, non-functional protein that may contribute to decreasing JAK/STAT signaling. These results contrast with the cooperative effect of deletion of *Ezh2*, *Asx1* and *Dnmt3a* with *Jak2*^{V617F} to exacerbate MF in mice.¹⁰⁻¹⁴

Several knock-in murine models of *Srsf2* mutation have been generated.^{20,27,28} They recapitulated human diseases to varying extents, with a common engraftment defect of mutated cells contrasting with the positive selection of *SRSF2*-mutant cells in humans.²⁹ However, this poor competitive engraftment of *Srsf2*^{P95H} mouse BM cells disappears when competitor cells and microenvironment are matched for age.^{28,29} Since the clonal selection of *SRSF2*^{P95H} hematopoietic cells is also associated with aging in humans,³⁰⁻³² cell and tissue aging may be required for the mutated splicing factor to accelerate clonal growth³² and generate a full-blown disease phenotype. Alternatively, additional mutational events in the context of *JAK2* and *SRSF2* co-mutation could account for the severe outcome associated with this molecular background.¹⁷

The abnormal maturation of megakaryocytes is a characteristic feature of *Jak2*^{V617F} expression. These abnormal megakaryocytes are critical drivers of BM fibrosis, *i.e.* they reprogram hematopoiesis-supporting cells to fibrosis-driving cells.³³ Accordingly, targeting megakaryocytes ameliorates MF in preclinical models and early phase clinical studies.³⁴⁻³⁶ Mouse models of MPNs expressing *Jak2*^{V617F} were shown to demonstrate an increased proportion of megakaryocyte-biased CD41^{high} HSPCs,³⁷ and recent investigation at the single

357 cell level validated the aberrant differentiation of HSPCs with an expansion of
 358 megakaryocyte progenitors exhibiting an abnormal gene signature associated with fibrosis.³⁸
 359 At the molecular level, aberrant megakaryocyte number, differentiation, location and
 360 function were associated with the decreased expression of GATA1 protein,³⁹ the
 361 overexpression of the anti-apoptotic protein Bcl-X_L,^{40,41} and irregular production of fibrogenic
 362 cytokines including TGFβ1.^{42,43} In the mouse model described here, *Srsf2*^{P95H} does not restore
 363 a wildtype megakaryocyte phenotype but attenuates the phenotypic alterations induced by
 364 *Jak2*^{V617F} and reduces TGFβ1 level .

365 STAT3 and STAT5 are commonly activated in human MPN, downstream of MPL and JAK2.⁴⁴⁻⁴⁶
 366 Some differences can be detected in signaling output between genotype subtypes.⁴⁷
 367 Transgenic expression of a dominant negative STAT3 mutant delays platelet recovery after
 368 myelosuppression⁴⁸ but, in the context of *Jak2*^{V617F}, *Stat3* deletion enhances thrombocytosis
 369 and shortens survival of mutated animals.⁴⁹ STAT5 plays a central role in mouse models of
 370 *Jak2*^{V617F}-induced MPN,^{50,51} animals deficient for STAT5 are thrombocytopenic,⁵² and STAT5
 371 target genes are up-regulated in *Jak2*^{V617F} megakaryocytes.⁵³ The decreased activation of
 372 STAT5 detected in LSK cells when *Srsf2*^{P95H} is co-expressed with *Jak2*^{V617F} may therefore
 373 contribute to the delayed phenotype observed in double-mutated animals.

374 Modulation of JAK/STAT pathway activity by alternative splicing of constitutive genes was
 375 described in Hodgkin lymphoma cells in which an alternative isoform of protein-tyrosine
 376 phosphatase 1B enhances JAK/STAT activity.⁵⁴ Mutation of the Proline 95 residue in SRSF2,
 377 which alters its RNA binding specificity, generates a number of mis-spliced transcripts in
 378 hematopoietic cells.^{20,27-29,55-57} Here, we identify the ability of *SRSF2*^{P95H} to promote *JAK2* exon
 379 14 skipping. This exon encodes the JAK-homology 2 (JH2) domain, a pseudokinase domain in

380 which the valine residue 617 is located. This domain both suppresses basal JAK2 activity in
381 the absence of cytokine stimulation and is required for induction of maximal JAK2 activation,
382 *i.e.* to render JAK2 responsive to cytokine stimulation with increased activity.^{58,59} Deletion of
383 *JAK2* exon 14 was already detected as a minor splice variant of *JAK2* (~12% of *JAK2* mRNA) in
384 the granulocytes of patients with MF, without analysis of *SRSF2* sequence.^{60,61} *JAK2* exon 14
385 skipping induced by *Srsf2*^{P95H} generates a frameshift with the synthesis of a truncated protein
386 without kinase domain and thus unable to induce downstream signaling. This isoform does
387 not generate a dominant negative protein, thus may decrease JAK/STAT signaling by
388 decreasing the amount of functional full-length JAK2 protein, which, on the long term, may
389 contribute to delay myelofibrosis development.

390 The decreased expression of the thrombopoietin receptor MPL at the surface of
391 megakaryocytes and platelets is an established feature of MF.^{24,62} There is evidence that this
392 decrease is related both to the loss of the chaperone activity of JAK2 by *JAK2*^{V617F} and to
393 increased MPL activation and internalization with reduced recycling. Our finding that
394 *Srsf2*^{P95H} partially restores the expression of MPL at the surface of megakaryocytes further
395 argues for an altered activity of *Jak2*^{V617F} protein in mouse cells. The delayed induction of
396 thrombocytosis and MF by romiplostim^{22,63} observed in mice expressing *Srsf2*^{P95H} in a *Jak2*
397 wildtype background, further supports a negative impact of *Srsf2*^{P95H} on megakaryocyte
398 signaling, downstream of MPL, which may involve *Jak2* exon 14 skipping.

399 Taken together, we have depicted an unexpected interplay between *Jak2*^{V617F} and *Srsf2*^{P95H}
400 when co-expressed in hematopoietic cells. *Jak2* exon 14 skipping promoted by mutated
401 *SRSF2*, which is detected also in patients with a *Jak2*^{V617F} / *Srsf2*^{P95H} myelofibrosis, generates a
402 truncated, inactive JAK2. Additional events, which could be related with ageing of HSCPs and

403 bone marrow niche cells, must overcome the decrease in JAK/STAT signaling in patients with
404 *Jak2*^{V617F} / *Srsf2*^{P95H} co-mutation to promote clonal expansion. Regardless of these
405 mechanisms, our results suggest that understanding the molecular mechanisms involved in
406 *JAK2* alternative splicing could potentially drive innovative strategies to reduce JAK/STAT
407 signaling in pathological conditions.

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Figure legends

Figure 1. Co-mutation of *Srsf2*^{P95H} delays *Jak2*^{V617F}-induced myelofibrosis. **A.** Bone marrow cells (3×10^6) from *Scl-Cre* mice, either crossed or not with knock-in mice for *Srsf2*^{P95H}, *Jak2*^{V617F} or the two mutated alleles, were engrafted into lethally irradiated congenic recipients, 5 weeks before tamoxifen gavage. Phlebotomies (180μL) were performed every 2 weeks for up to 14 weeks to prevent premature death of *Jak2*^{V617F} animals. **B,C.** Peripheral blood platelet count at indicated time points after tamoxifen gavage (left panels) and at 8 weeks post-tamoxifen (right panels); **B**, three independent experiments are pooled; mean \pm SEM of *Scl-Cre* (n=25), *Srsf2*^{P95H} (n=26), *Jak2*^{V617F} (n=27), *Jak2*^{V617F}/*Srsf2*^{P95H} (n=26); **C**, one experiment, mean \pm SEM of *Scl-Cre* (n=5), *Srsf2*^{P95H} (n=6), *Jak2*^{V617F} (n=5), *Jak2*^{V617F}/*Srsf2*^{P95H} (n=4). **D.** Bone marrow reticulin fibrosis quantified by computer-assisted imaging, 8 and 16-24 weeks after tamoxifen gavage. Results collected from 2 independent experiments, mean \pm SEM of *Scl-Cre* (n=10), *Srsf2*^{P95H/+} (n=10), *Jak2*^{V617F} (n=11), *Jak2*^{V617F}/*Srsf2*^{P95H/+} (n=10) per group; **E.** Representative reticulin fiber staining (Gordon and Sweet's and von Willebrand Factor staining) in bone marrow examined 24 weeks after tamoxifen from indicated genotypes (magnification x 25). **F.** Serum concentration of TGFβ1 measured by ELISA 8 weeks (upper panel) and 24 weeks (lower panel) after tamoxifen. *Scl-Cre* (n=11), *Srsf2*^{P95H/+} (n=13), *Jak2*^{V617F} (n=7), *Jak2*^{V617F}/*Srsf2*^{P95H/+} (n=12). Statistical analyses, unpaired t-test, * P<0.05, ** P < 0.01, **** P<0.0001, NS not significant.

Figure 2: *Srsf2*^{P95H} co-mutation reduces *Jak2*^{V617F} cell competitiveness.

A. Bone marrow cells were engrafted as in Figure 1A; flow cytometry measurement of LSK (Lin⁻, Sca1⁺, cKit⁺), SLAM (LSK, CD48⁺, CD150⁺) and MPP (LSK, CD48⁺, CD150⁻) fractions in bone marrow and spleen collected from indicated mouse strains (black *Scl-Cre*, gray *Srsf2*^{P95H}, red *Jak2*^{V617F}, orange *Jak2*^{V617F}/*Srsf2*^{P95H}), 16-24 weeks after tamoxifen gavage; pooled analysis of 2 experiments : *Scl-Cre* (n=13), *Srsf2*^{P95H} (n=13), *Jak2*^{V617F} (n=13), *Jak2*^{V617F}/*Srsf2*^{P95H} (n=14); **B.** A mixture of whole bone marrow cells collected from one of the indicated CD45.2 mouse strains (0.9×10^6 ; 30%) and CD45.2 *Ubi*-GFP transgenic mice (2.1×10^6 , 70%) were engrafted into lethally irradiated CD45.1 recipients, 5 weeks before tamoxifen gavage (10 mg/day, 2 days); **C.** Flow cytometry measurement of bone marrow GFP⁺ (in green) and GFP⁻ (colors indicate strains as in panel A) CD45.2⁺ SLAM repartition in the bone marrow, 24 weeks after tamoxifen gavage; n=7 mice per group, mean \pm SEM; **D.** Following a first transplantation as

in panel B, bone marrow cells (3×10^6) were serially transplanted into CD45.1 mice (second transplantation, 24 weeks after tamoxifen gavage; third transplantation 16 weeks after transplantation); **E**. Overall survival of mice after the third transplantation with *Jak2*^{V617F} (in red) and *Jak2*^{V617F}/*Srsf2*^{P95H} (in orange) containing bone marrow cells. *Jak2*^{V617F} (n=8), *Jak2*^{V617F}/*Srsf2*^{P95H} (n=8); **F**. Flow cytometry measurement of bone marrow GFP⁺ (shown in green) and GFP⁻, CD45.2⁺ SLAM fraction among bone marrow cells in sacrificed animals; **G**. Peripheral blood white blood cell count, platelet count and hemoglobin level following the third transplantation, 16 weeks after transplantation (except *Jak2*, last blood cell analysis before death); *Scl-Cre* (n=7), *Srsf2*^{P95H} (n=7), *Jak2*^{V617F} (n=5), *Jak2*^{V617F}/*Srsf2*^{P95H} (n=8); mean \pm SEM; All panel, unpaired t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NS, not significant.

Figure 3: Impact of *Srsf2*^{P95H} on *Jak2*^{V617F}-induced megakaryocyte phenotypic modifications. **A-C**; Analysis of bone marrow collected 8 weeks post-tamoxifen; **A**. Number of vWF-stained megakaryocytes per field of bone marrow sections in indicated mice (mean \pm SEM); **B**. Mean size of megakaryocytes according to the genotype (mean \pm SEM); **C**. Modal ploidy measured in megakaryocytes (mean \pm SEM). **D, E**. Analysis of bone marrow collected 16-24 weeks post-tamoxifen; **D**. Number of vWF-stained megakaryocytes per field of bone marrow sections in indicated mice (mean \pm SEM); **E**. Mean ploidy measured in megakaryocytes (mean \pm SEM). All figures were analyzed using unpaired t-test. , * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant.

Figure 4. *Srsf2*^{P95H} co-mutation with *Jak2*^{V617F} down-regulates genes involved in signaling pathways. Megakaryocytes sorted from mouse bone marrow engrafted as in Figure 1A and sacrificed 8 weeks post tamoxifen were analyzed by bulk RNA sequencing (metadata in Supplementary Table 3). **A**. Principal component analysis (PCA) of *Jak2*^{V617F} (red, VF) and *Jak2*^{V617F}/*Srsf2*^{P95H} (orange, P95VF) mouse megakaryocytes; **B**. Volcanoplot of differentially expressed genes (DEGs) between *Jak2*^{V617F} and *Jak2*^{V617F}/*Srsf2*^{P95H} megakaryocytes, numbers indicate down-regulated (n=52) and up-regulated (n=19) genes in *Jak2*^{V617F}/*Srsf2*^{P95H} cells; **C**. Hallmark pathway analysis of the comparison between *Jak2*^{V617F} and *Jak2*^{V617F}/*Srsf2*^{P95H} megakaryocytes; **D**. Enrichment score of signaling pathways among mouse models (up-regulated in red, down-regulated in blue, not significantly modified in grey).

Figure 5. *Srsf2*^{P95H} promotes *Jak2* exon 14 skipping. **A.** Relative enrichment of all four SSNG variants, where S represents C or G, in cassette exons that are differentially spliced in BM megakaryocytes sorted from *Jak2*^{V617F}, *Srsf2*^{P95H} and *Jak2*^{V617F} / *Srsf2*^{P95H} animals compared to *Scl-Cre* megakaryocytes (n=4 per group). **B.** Venn diagram indicating the number of splicing events (False Discovery Rate (FDR) < 0.05), in mutated compared to *Scl-Cre* megakaryocytes. **C.** Venn diagram of differential splicing events (FDR<0.05) among the 147 genes listed in KEGG_JAK_STAT_SIGNALING_PATHWAY in mutated compared to *Scl-Cre* megakaryocytes. **D.** RT-PCR analysis of alternatively spliced isoforms of mouse *Jak2*. Upper panel, scheme of the detected 171 bp (exon 14 included) and 83 bp (exon 14 excluded) *Jak2* cDNA fragments; RT-qPCR dissociation curve showing the 2 amplicons (higher peak, 171 bp, exon 14 included; lower peak, 83 bp, exon 14 excluded / arrows). Upper line: sorted megakaryocytes; middle line: sorted BM LSK; lower line, megakaryocytes generated *ex vivo* by culturing LSK with TPO. **E.** RT-PCR analysis of alternatively spliced isoforms of human *JAK2*: gel migration of RT-qPCR products in granulocytes of 2 healthy donors, 4 patients with *JAK2* but no *SRSF2* mutation and 2 patients with both *SRSF2* and *JAK2* mutations. Lower panel: Image J quantification. **F.** Flow cytometry analysis of P-STAT5 in sorted BM LSK cells from indicated mice (3 per group). **G.** Immunoblot detection of JAK2 full length with or without V617F mutation (~130 kDa) and JAK2^{ΔEx14} (~68 kDa) expressed in γ-2A cells. **H.** STAT5 transcriptional activity measured in γ2a cells transduced with TPO receptor and indicated JAK2 constructs without (dotted blue) and with (full blue) stimulation with TPO (10 ng/ml). Mean ± SD of 3 independent experiments performed in triplicates. **I.** STAT5 transcriptional activity measured in γ2a cells transduced with various ratio of JAK2 V617F and JAK2^{ΔEx14} or an empty vector, without (left) or with (right) Tpo stimulation (10ng/ml). Mean ± SD of 3 independent experiments performed in triplicates. **G-H.** Statistics: two-ways ANOVA followed by SIDAK multiple comparison test. **** p: < 0.0001, **: p<0.01, ns: non-significant.

Figure 6. *Srsf2*^{P95H} delays romiplostim-induced myelofibrosis; **A.** Mice engrafted with 3 x 10⁶ *Scl-Cre* or *Srsf2*^{P95H} bone marrow cells were treated with tamoxifen as in Figure 1A. Three weeks later, they received subcutaneous injections of romiplostim (1 mg/kg) every week for 3 weeks and peripheral blood was collected for blood cell count. Animals were sacrificed at day 21. **A.** Evolution of platelet count in *Scl-Cre* or *Srsf2*^{P95H} transplanted animals upon romiplostim treatment; **B.** Bone marrow section

717 after Gordon and Sweet's staining of *Scl-Cre* or *Srsf2*^{P95H} transplanted animals at day
718 21; **C.** Myelofibrosis in romiplostim-treated, *Scl-Cre* or *Srsf2*^{P95H} transplanted mice at
719 day 21, evaluated by either grading (left panel) or image J quantification of reticulin
720 fibers (right panel); **D.** Spleen fibrosis was evaluated by grading in the same animals.
721 Kruskal-Wallis test, * $P < 0.05$; ** $P < 0.01$; NS, non-significant.