FLT3-Mediated p38–MAPK Activation Participates in the Control of Megakaryopoiesis in Primary Myelofibrosis

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Abstract

Primary myelofibrosis (PMF) is characterized by increased number of hematopoietic progenitors and a dysmegakaryopoiesis which supports the stromal reaction defining this disease. We showed that increased ligand (FL) levels in plasma, hematopoietic progenitors, and stromal cells from PMF patients were associated with upregulation of the cognate Flt3 receptor on megakaryocytic (MK) cells. This connection prompted us to study a functional role for the FL/Flt3 couple in PMF dysmegakaryopoiesis, as a route to reveal insights into pathobiology and therapy in this disease. Analysis of PMF CD34$^+$ and MK cell transcriptomes revealed deregulation of the mitogen-activated protein kinase (MAPK) pathway along with Flt3 expression. In PMF patients, a higher proportion of circulating Flt3$^+$CD34$^+$CD41$^+$ cells exhibited an increased MAPK effector phosphorylation independently of Jak2^{V617F} mutation. Activation of FL/Flt3 axis in PMF MK cell cultures, in response to FL, induced activation of the p38–MAPK cascade, which is known to be involved in inflammation, also increasing expression of its target genes (*NFATC4, p53, AP-1, IL-8*). Inhibiting Flt3 or MAPK or especially p38 by chemical, antibody, or silencing strategies restored megakaryopoiesis and reduced phosphorylation of Flt3 and p38 pathway effectors, confirming the involvement of Flt3 in PMF dysmegakaryopoiesis via p38 activation. In addition, in contrast to healthy donors, MK cells derived from PMF CD34$^+$ cells exhibited an FL-induced migration that could be reversed by p38 inhibition. Taken together, our results implicate the FL/Flt3 ligand–receptor complex in PMF dysmegakaryopoiesis through persistent p38–MAPK activation, with implications for therapeutic prospects to correct altered megakaryopoiesis in an inflammatory context. *Cancer Res; 71(8); 2901–15. ©2011 AACR.*

Introduction

Primary myelofibrosis (PMF) is a Ph (Philadelphia)-negative myeloproliferative neoplasm (MPN) characterized by extrame-

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dullary hematopoiesis with splenomegaly, myelofibrosis, and neoangiogenesis. The clonal myeloproliferative process is illustrated by an increased number and mobilization of hematopoietic stem cells/progenitors (HSC/HP) with a hypersensitivity to cytokines partly resulting from Jak2^{V617F} or MPL$^{515L/K}$ mutations (1–6). A prominent proliferation of megakaryocytes (MK) with a dysmegakaryopoiesis characterized by dysplastic MK with plump lobulation of nuclei and disturbance of nuclear/cytoplasmic maturation is observed in patients (7). Previous studies (8–10) have suggested that bone marrow fibrosis was secondary to fibroblast activation by inflammatory and fibrogenic growth factors including TGF-β produced by the necrotic and dysplastic MKs (11, 12). Recently, evidences have been accumulating that stromal cells also contribute to the hematopoietic clone development through specific and mutually dependent interactions with pathologic HSCs (13).

FL, the ligand for the tyrosine kinase receptor Flt3 (14), is mainly expressed in stromal cells, including fibroblasts, likely stimulated by TGF-β. FL is of paramount importance in the proliferation of primitive hematopoietic progenitors (15–17) as confirmed by the reduced myeloid and B-lymphoid progenitor content observed in FL$^{-/-}$ and Flt3$^{-/-}$ mice (18, 19).

However, role of FL on normal megakaryopoiesis appears controversial because it does not stimulate, as a single agent, MK progenitors (20), but it enhances their proliferation in association with cytokines such as thrombopoietin (TPO; ref. 21). Its cognate receptor, Flt3, is expressed by HSC and myeloid progenitors (22) in which it signals through several downstream pathways including the mitogen-activated protein kinase (MAPK) pathway (16). Alterations of Flt3 are frequently observed in leukemic cells and mutations have been detected in about 30% of patients with acute myelogenous leukemia as well as in patients with acute lymphocytic leukemia or myelodysplastic syndrome. Mutations most often involve small tandem duplications within the juxtamembrane domain of the receptor (Flt3-ITD), resulting in constitutive tyrosine kinase activity. The recent demonstration that expression of Flt3-ITD in murine marrow cells results in a lethal MPN (23) strengthens the role of FL/Flt3 deregulation in leukemia and encourages us to investigate its possible contribution to human MPN pathogenesis and especially in PMF, in which a deregulation of primitive hematopoieisis is strongly associated with profound alterations of stroma.

In this study, we evidenced that the FL plasma level is increased in PMF patients and is overexpressed by stromal and CD34$^+$ hematopoietic cells. FL augmentation is associated with an aberrant Flt3 expression in CD34$^+$ and MK cells and with an alteration of the MAPK pathway and especially of p38, in patients, independently of their Jak2 mutational status. We further showed that the persistence of Flt3 signaling, which elicits activation of MAPK, known to be involved in MK polyploidization (24), participates in the dysmegakaryopoiesis that characterizes PMF. Taken together, our results implicate the "FL/Flt3" couple in PMF MK deregulation through persistent p38–MAPK activation.

Materials and Methods

Patients

One hundred twenty-six PMF patients [Jak2^{V617F} ($n = 51$), Jak2^{WT} ($n = 45$), and Jak2 status not determined ($n = 30$)], obtained from clinicians of the French and European networks, and 90 unmobilized healthy donors (HD) were enrolled. Samples were obtained with the informed consent of subjects according to the Helsinki declaration.

Cell selection

CD34$^+$ cell selection was carried out on mononuclear cells (MNC) from peripheral blood (PB) or bone marrow (BM) samples from PMF patients and HD as previously described (purity > 97%; ref. 25). Stromal cells obtained from osteomedullar biopsies or hip surgery from PMF patients and HD, respectively, were cultivated for 3 to 4 passages in DMEM (Dulbecco's modified Eagle's medium) + 10% FCS (fetal calf serum). FL plasma level was quantified using Quantikine ELISA (R&D Systems).

Microarray analysis and quantitative RT-PCR

For microarray technique, see Supplementary Material. For quantitative reverse transcription PCR (QRT-PCR), total RNAs were subjected to RNase-free DNase and converted into cDNA by using the Reverse Transcription Kit (Applied Biosystem). cDNA (2 μL) was added to the QuantiTect SYBR Green amplification reaction (QIAGEN) in a 20 μL final volume and 10 pmol of each primer (Supplementary Table S1) were added to carry out specific amplification. *RPL38* was used as housekeeping gene and relative quantification was based on the 2$^{\Delta\Delta CT}$ method (26).

Phenotypic analysis of CD34$^+$ cells

Cells (5×10^4) were labeled with 2 μg/mL of the following monoclonal antibodies (mAb): CD38-fluorescein isothiocyanate (FITC; clone-T16) or CD41-FITC (clone-P2; Beckman Coulter) versus IgG1-FITC isotype; Flt3-PE (CD135, clone-4G8; BD Pharmingen) or CD41-PE (clone-5B12; Dako) versus IgG1-PE isotype; CD34-PerCP (clone-8G12) versus IgG1-PerCP isotype; CD41-APC (clone-386629) versus IgG1-APC isotype (BD Pharmingen). Membrane antigen fluorescence was quantified by using CellQuest software on a FACScalibur (Becton Dickinson). Live cells (5×10^3) were analyzed.

MK derived from CD34$^+$ cultures

CD34$^+$ cells (5×10^4/500 μL per well) were cultured for 10 to 14 days in MK differentiation medium [SYN.H serum-free medium containing Recombinant human stem cell factor (rhSCF): 5 ng/mL; rhIL-3: 2 ng/mL; rhIL-6: 1 ng/mL; rhIL-11: 40 ng/mL, rhTpo: 50 ng/mL; AbCys Synergie] with or without inhibitors and viability was evaluated by trypan blue. For RNA silencing, cells were cultured for 6 days and distributed in 24 well per plate per 250 μL with or without control or specific siRNA (1 μg) and a vector MISSION II (1/50; Sigma). Biological effect of siRNA was evaluated after 48-hour incubation.

Megakaryocyte ploidy measurement

DNA content was measured by incorporation of propidium iodine (PI). Megakaryocytes obtained at day 12 (D12) of culture were fixed with 70% ethanol ($-20°C$), centrifuged and treated with RNase (500 μg/mL) and PI (50 μg/mL; Sigma). Live cells (3×10^4) were analyzed and the percentage of polyploid cells (8N-256N) was determined on FACScalibur with CellQuest software. The B/S ratio (big/small MK proportion) was calculated as following: $\Sigma(64N + 128N + 256N)/\Sigma(8N + 16N + 32N) \times 100$.

For cyclin D3 expression, MK derived from PMF CD34$^+$ culture (D10) were labeled with a cyclin D3-FITC mAb (MOPC-21; BD Pharmingen) and analyzed by fluorescence microscopy (400 $\times$).

MAPK and effector phosphorylation analysis by flow cytometry

Cells were fixed in PBS with formalin (2%) for 1 hour and in 70% ethanol overnight (4°C). After washes in PBS–0.5% BSA–Triton 0.25× (PBT), cells were incubated with either anti-MAPK mAbs [Cell Signaling; phospho-p38 Thr180/Tyr182 (clone-12F8), phospho-p42/44 Thr202/Tyr204 (clone-20G11), phospho JNK/SAPK (c-jun N-terminal kinase/ stress-activated protein kinase) T183/Y185 (clone-98-F2)] or rabbit IgG isotype for 45 minutes (4°C). Cells were washed and incubated with a

secondary anti-rabbit antibody coupled to Alexa Fluor 488 nm (Invitrogen). Phosphorylation levels of Raf, p38 effectors, and Flt3 on gated $CD34^+CD41^+FLT3^+$ cells were determined after labeling with rabbit mAbs included in Raf Family Antibody or Phospho-p38 MAPK Pathway Sampler Kits (nos. 2330 and 9913, respectively) or Tyr591-Flt3 from Cell Signaling, followed by a secondary GAR-Alexa Fluor 633 nm antibody (Invitrogen). About 10^4 events were analyzed on a FACScalibur.

In vitro migration assay

Transwell migration assays were carried out on MK derived from $CD34^+$ cultures (D6) as previously described (27). Cells (10^5) were loaded on the top chamber and rhFL (10–100 ng/mL) was added or not to the bottom chamber, with or without rhSDF-1 (100 ng/mL), and incubated for 48 hours at 37°C in RPMI/0.5%BSA. In some experiments, Flt3 inhibitor IV (42 nmol/L; Calbiochem) or p38 inhibitors (SB203580, 1 μmol/L, SB202190, 40 nmol/L; Sigma) were added to the culture. The percentage of migrating cells was calculated after quantification of live cells in top and bottom chambers.

Western blot

Total cell lysates (10/30 μg), obtained as previously described (28), were subjected to SDS-PAGE, electrophoretically transferred into nitrocellulose membranes, and blotted using primary mAbs similar to those used for cytometric analysis or using actin antibody (Santa Cruz Biotechnology). Membranes were revealed with anti-mouse or anti-rabbit IgG horseradish peroxidase–linked antibodies and a chemiluminescence detection kit (ECL-Plus; GE-Healthcare); signals were quantified by using ImageJ software.

Patient group prediction model

Upstream p38 effectors and C-MYC expression modulation in MK derived from $CD34^+$ (D6) in response to 18-hour FL stimulation kinetics was quantified by QRT-PCR. Ratio at each time point of kinetics was normalized as compared with quantification at the starting point of stimulation. Cumulative scores of each kinetic time ratio were included in a multivariate model composed of a principal component analysis associated to a hierarchical clustering in which patient clinicobiological information were reported.

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between patients and controls were validated by unpaired t test with a significant $P < 0.05$. Statistical differences between conditions were validated by paired t test with significant $P < 0.001$ (***); $P = 0.001$ to 0.01 (**); and $P = 0.01$ to 0.05 (*). Fisher ANOVA test with 2 factors (samples and time of differentiation) was also used; a $P < 0.05$ was significant. NS, not significant.

Results

FL plasma level is increased in PMF patients and is overexpressed in $CD34^+$ hematopoietic and stromal cells

We first showed that FL plasma level was significantly increased in PMF patients as than in HDs (Fig. 1A; 130.1 ±

78.41, $n = 52$ vs. 69.84 ± 30.94, $n = 28$; $P = 0.0001$) with no significant difference between PMF patients according to their Jak2 status (181.4 ± 70.8 pg/mL, $n = 7$ vs. 128.8 ± 81.9 pg/mL, $n = 18$ for $Jak2^{V617F}$ and $Jak2^{WT}$, respectively). The increased FL level appears to be restricted to PMF patients, as it was statistically different from that of polycythemia vera (PV; $n = 17$) and essential thrombocytopenia (ET; $n = 17$) patients.

We further analyzed the nature of FL producing cells and showed that fibroblasts from PMF BM expressed higher FL level than those from HD (1.17 ± 0.4, $n = 8$ vs. 0.56 ± 0.17, $n = 6$, respectively; $P = 0.002$). As $CD34^+$ and MK cells from PMF patients are known to produce cytokines (13, 29), we analyzed whether they expressed FL. Whereas we did not detect FL transcript in PMF or HD MK cells (data not shown), a 2-fold increase in FL mRNA level was observed in PMF $CD34^+$ cells as compared with that in HD cells (Fig. 1A; 0.080 ± 0.218, $n = 22$ vs. −0.294 ± 0.242, $n = 6$; $P = 0.0006$).

Flt3 expression and phosphorylation are increased in PBMNC, MK, and $CD34^+$ cells from PMF patients

Figure 1B shows that Flt3 mRNA level was increased in mononuclear cells from peripheral (PBMNC) from PMF patients than in HD (0.69 ± 0.96, $n = 11$ vs. −0.06 ± 0.47, $n = 16$, respectively; $P < 0.006$). Similar to FL, Flt3 overexpression was restricted to PMF because Flt3 mRNA level was statistically different in PMF as compared with ET and PV. We further analyzed Flt3 expression on PBMNC by flow cytometry and showed that the percentage of cells coexpressing the $CD41^+$ MK antigen and Flt3 was higher in PMF than in HD (Fig. 1B). Western blot analysis confirmed the Flt3 increased expression and evidenced its phosphorylation in PBMNC from PMF patients (Fig. 1B). As Flt3 is reported to be expressed in HP, we analyzed its distribution on freshly purified $CD34^+$ cells. Figure 1C showed that the percentage of $CD34^+Flt3^+$ cells was increased in PMF compared with that in HD cells (17.77 ± 14.98, $n = 11$ vs. 4.72 ± 7.09, $n = 10$, respectively; $P = 0.01$). Flt3 was mainly expressed on PMF MK progenitors, as the proportion of $CD34^+Flt3^+CD41^+$ cells was significantly increased in $Jak2^{WT}$ and $Jak2^{V617F}$ patients than in HD (76.11 ± 14.49, $n = 4$ and 43.37 ± 23.86, $n = 12$ vs. 9.11 ± 10.97, $n = 11$; $P < 0.0001$ and $P = 0.0001$, respectively; Fig. 1D) with a higher proportion in $Jak2^{WT}$ patients ($P = 0.01$). To assess Flt3 activation on this cell subset, we quantified the percentage of $CD34^+CD41^+Flt3^+$ cells expressing phospho-Flt3 and showed that it was increased in PMF patients ($Jak2^{WT}$ and $Jak2^{V617F}$) versus HD (28.21 ± 22.67, $n = 5$ and 18.35 ± 24.81, $n = 4$ vs. 0.22 ± 0.34, $n = 10$; $P = 0.007$ and $P = 0.01$, respectively; Fig. 1D). In accordance with these results, Western blot analysis also evidenced the presence of the Flt3 protein and of its phosphorylated form in freshly isolated $CD34^+$ cells and in MK-derived $CD34^+$ cells from PMF patients (Fig. 1E).

The increased percentage of PMF $CD34^+CD41^+$ MK progenitors expressing Flt3 and its activated form motivated us to analyze its expression during in vitro megakaryopoiesis. We quantified by QRT-PCR the Flt3 transcript in MK derived from $CD34^+$ culture (D10) and showed that it was significantly overexpressed in PMF patients as compared with
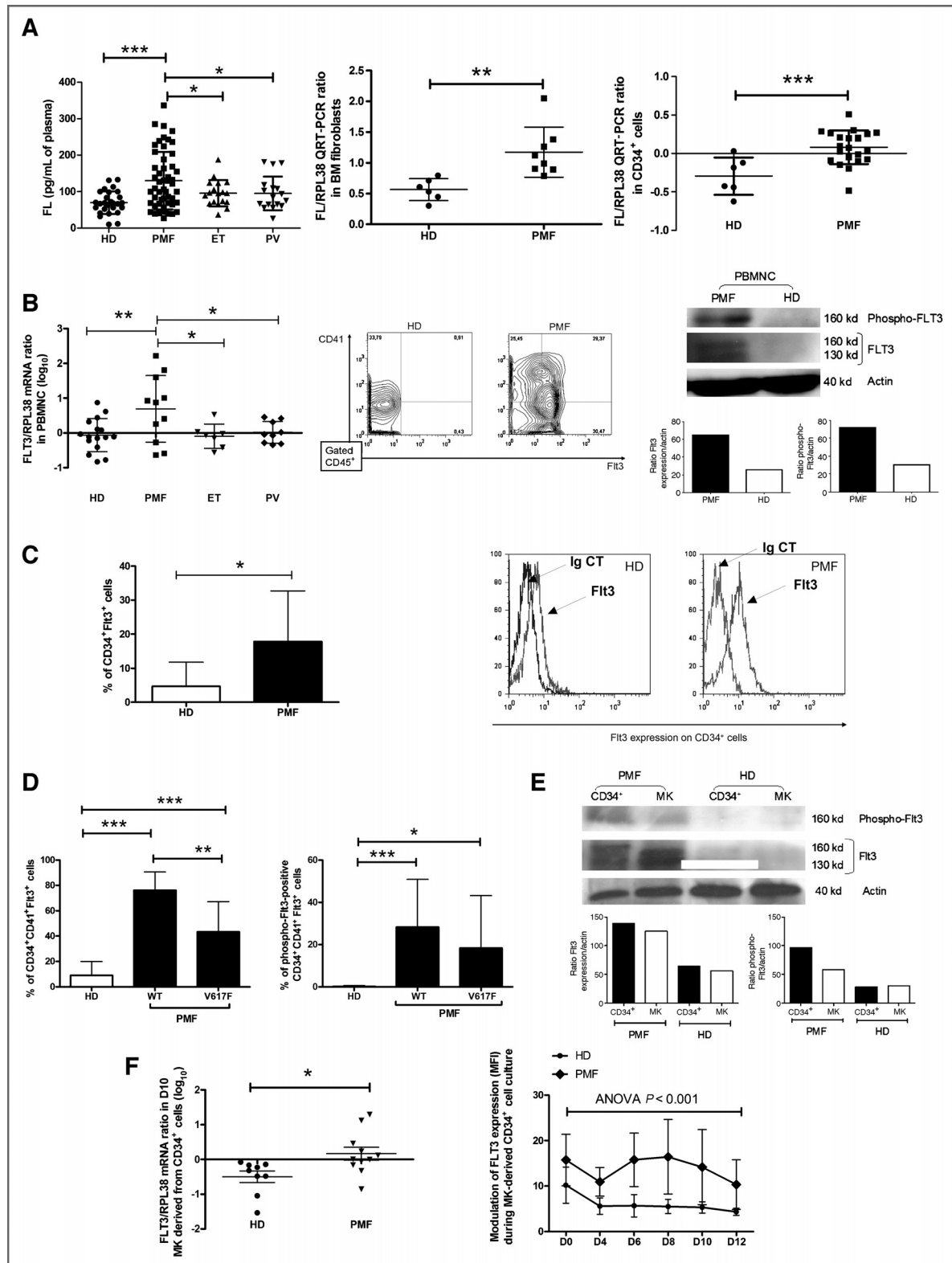
Figure 1. Increased FL level, Flt3 expression, and phosphorylation in PMF patients. A, FL plasma level in HD, PMF, ET, and PV; FL mRNA expression in BM fibroblasts and CD34$^+$ cells from HD and PMF. B, Flt3 mRNA expression in PBMNC from HD, PMF, ET, and PV, Flt3 membrane expression in CD41$^+$CD45$^+$ PBMNC and Flt3 protein expression and phosphorylation in PBMNC determined by Western blot analysis. C, percentage of circulating CD34$^+$Flt3$^+$ cells and cytogram of Flt3 expression. D, CD34$^+$CD41$^+$Flt3$^+$ cells and of phospho-Flt3–positive CD34$^+$CD41$^+$Flt3$^+$ cells in HD and PMF, according to their Jak2 status. E, Flt3 expression and phosphorylation in CD34$^+$ and MK derived from CD34$^+$ cells determined by Western blot analysis. F, Flt3 mRNA expression in MK and modulation of its membrane expression during MK-derived CD34$^+$ culture. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$.

HD (0.168 ± 0.615, $n = 11$ vs. -0.498 ± 0.488, $n = 9$; $P = 0.008$; Fig. 1F). We further analyzed the variation of Flt3 membrane expression at different time points during *in vitro* MK differentiation. In contrast to HD, Flt3 expression persisted in patients throughout the culture with a maximal expression between days 6 and 10; the ANOVA/Fisher test showed a significant difference ($P < 0.001$) between HD (BM, $n = 10$; PB, $n = 12$) and PMF patients ($n = 16$; Fig. 1F).

Altogether, these data indicate that Flt3 expression and phosphorylation are increased in PMF CD34$^+$ cells and are maintained during MK differentiation.

MAPK phosphorylation is increased in PMF CD34$^+$ and MK cells and is related to cells expressing Flt3

Binding of FL to Flt3 triggers several downstream signals mainly including PI3K and Ras/MAPK pathways. A profiling gene expression of PMF CD34$^+$ cells had allowed us to generate 2 lists of differentially expressed genes (HD vs. PMF Jak2^{WT} and HD vs. PMF Jak2^{V617F}; Gene Expression Omnibus, GEO, no. GSE12234). These lists were used to carry out a functional representation based on a pathway annotation using NIH DAVID software (http://david.abcc.ncifcrf.gov/). This analysis indicated that several genes involved in the MAPK pathway are commonly deregulated in PMF patients (Fig. 2A and Supplementary Table S2). We also evidenced that MAPK pathway gene and especially p38-dependent transcription factors involved in inflammatory process, such as AP-1 and Fos, were altered in PMF MK cells (Supplementary Table S3). We further investigated MAPK pathway deregulation in CD34$^+$ cells and during *in vitro* megakaryopoiesis in PMF patients.

Figure 2B shows that p38, Jnk, and p42/p44 phosphorylation levels were significantly increased in PMF CD34$^+$ cells, independently of their Jak2 status, as compared with that in HD cells: p38 (HD: 7.74 ± 3.67, $n = 21$ vs. PMF Jak2^{WT}: 21.08 ± 12.05, $n = 8$; $P < 0.0001$ and vs. PMF Jak2^{V617F}: 16.78 ± 8.11, $n = 12$; $P < 0.0001$), p42/p44 (HD: 6.8 ± 2.97, $n = 22$ vs. PMF Jak2^{WT}: 16.58 ± 9.84, $n = 8$; $P = 0.0001$ and vs. PMF Jak2^{V617F}: 12.11 ± 11.76, $n = 12$; $P = 0.02$), and JNK (HD: 6.17 ± 4.54, $n = 24$ vs. PMF Jak2^{WT}: 26.76 ± 19.56, $n = 8$; $P < 0.0001$ and vs. PMF Jak2^{V617F}: 13.87 ± 12.15, $n = 12$, $P = 0.005$).

We then analyzed whether MAPK activation was maintained during *in vitro* MK differentiation and showed that, similar to CD34$^+$ cells, increased phosphorylation levels of p38 and Jnk were observed in D10 MK derived from PMF CD34$^+$ culture irrespectively of their Jak2 status (p38: 18.34 ± 11.62, $n = 10$ and 18.27 ± 12.09, $n = 18$, for PMF Jak2^{WT} and Jak2^{V617F}, respectively vs. 8.99 ± 5.11, $n = 17$ for HD, $P = 0.0038$ and $P = 0.0030$, respectively; JNK: 21.95 ± 20.98, $n = 10$ and 22.76 ± 25.35, $n = 16$ for PMF Jak2^{WT} and Jak2^{V617F}, respectively vs. 6.91 ± 3.96, $n = 17$ for HD, $P = 0.0038$ and $P = 0.0079$, respectively; Fig. 2B). A modest increased p42/p44 phosphorylation was also observed in PMF MK cells.

We further studied the phosphorylation of up- and downstream p38, JNK, and p42/p44 effectors in PMF cells and whether this phosphorylation was associated with Flt3 expression. Figure 2C showed that the percentage of CD34$^+$CD41$^+$Flt3$^+$ cells expressing phospho-a/b/c-Rafs or

phosphorylated forms of p38 effectors such as MKK3-6, MAPKAPK2, and ATF2 as well as of MSK1 and HSP27 were significantly increased in PMF patients whatever their Jak2 status (Jak2^{V617F}, $n = 4$ and Jak2^{WT}, $n = 5$) as compared with HD (PB, $n = 6$ and BM, $n = 3$).

Therefore, our data showed that MAPK phosphorylation is increased in PMF CD34$^+$ and MK cells and is related to Flt3 expressing cells.

Flt3-dependent p38–MAPK pathway deregulation in PMF CD34$^+$ and MK cells

Among MAPKs, p38 is strongly responsive to stress and inflammatory mediators such as TNF-α, IL-1β, and IL-8 known to be highly expressed in PMF (30); so, we further focused our study on p38–MAPK and on its main up and downstream effectors. We confirmed that p38 was phosphorylated in CD34$^+$ and MK cells from patients by Western blot analyses (Fig. 3A), and that p38 phosphorylation was associated with MK expressing Flt3 (Fig. 3B). p38 phophorylation was associated with activation of its upstream MKK3-6 effector and with upregulation of downstream AP-1 target gene expression in both CD34$^+$ and CD41$^+$MK derived from CD34$^+$ cells (Fig. 3C and D) reinforcing the notion that p38 axis is activated in PMF cells.

To analyze the role of Flt3 in p38–MAPK pathway activation in PMF CD41$^+$MK derived from CD34$^+$ culture, we quantified the phosphorylation of p38–MAPK effectors after addition of a neutralizing Flt3 antibody (10 μg/mL) to PMF cells from 6 patients. We showed that such treatment reduced phosphorylation of up- and downstream targets of p38 (Fig. 2E). Finally, to ascertain the implication of Flt3 in p38–MAPK activation, we showed that silencing 50% of Flt3 protein expression (Fig. 3F) and 80% of its transcript (data not shown) in MK derived from CD34$^+$ resulted in a decreased percentage of phospho-p38$^+$ cells (Fig. 3G).

Altogether these data showed that p38–MAPK pathway effectors are activated in CD34$^+$ and MK cells from patients and that Flt3 expression/phosphorylation participates in this activation process.

In vitro FL stimulation activates p38 and modulates downstream regulator expression in PMF MK–derived CD34$^+$ cells through Flt3 axis

To further show that MAPKs and especially p38 were activated in PMF MK–derived CD34$^+$ cells in response to FL, we compared the effect of 18-hour dose-dependent FL stimulation on MAPK phosphorylation in MK precursors obtained from PMF or HD CD34$^+$ cultures (D6). Figure 4A shows that no variations were observed for p42/p44 or JNK phosphorylations either in PMF or HD cells at 50 ng/mL FL. This result was identical whatever the dose of FL added (data not shown). In contrast, 50 ng/mL FL stimulation induced a progressive increase of phospho-p38 level in PMF MK as compared with HD (ANOVA; $P = 0.002$). This increased p38 phosphorylation level in PMF MK–derived CD34$^+$ cells after FL stimulation was confirmed by Western blot analysis and implication of Flt3 activation was supported by a reduced p38 phosphorylation by addition of Flt3 inhibitor IV (Fig. 4B). To
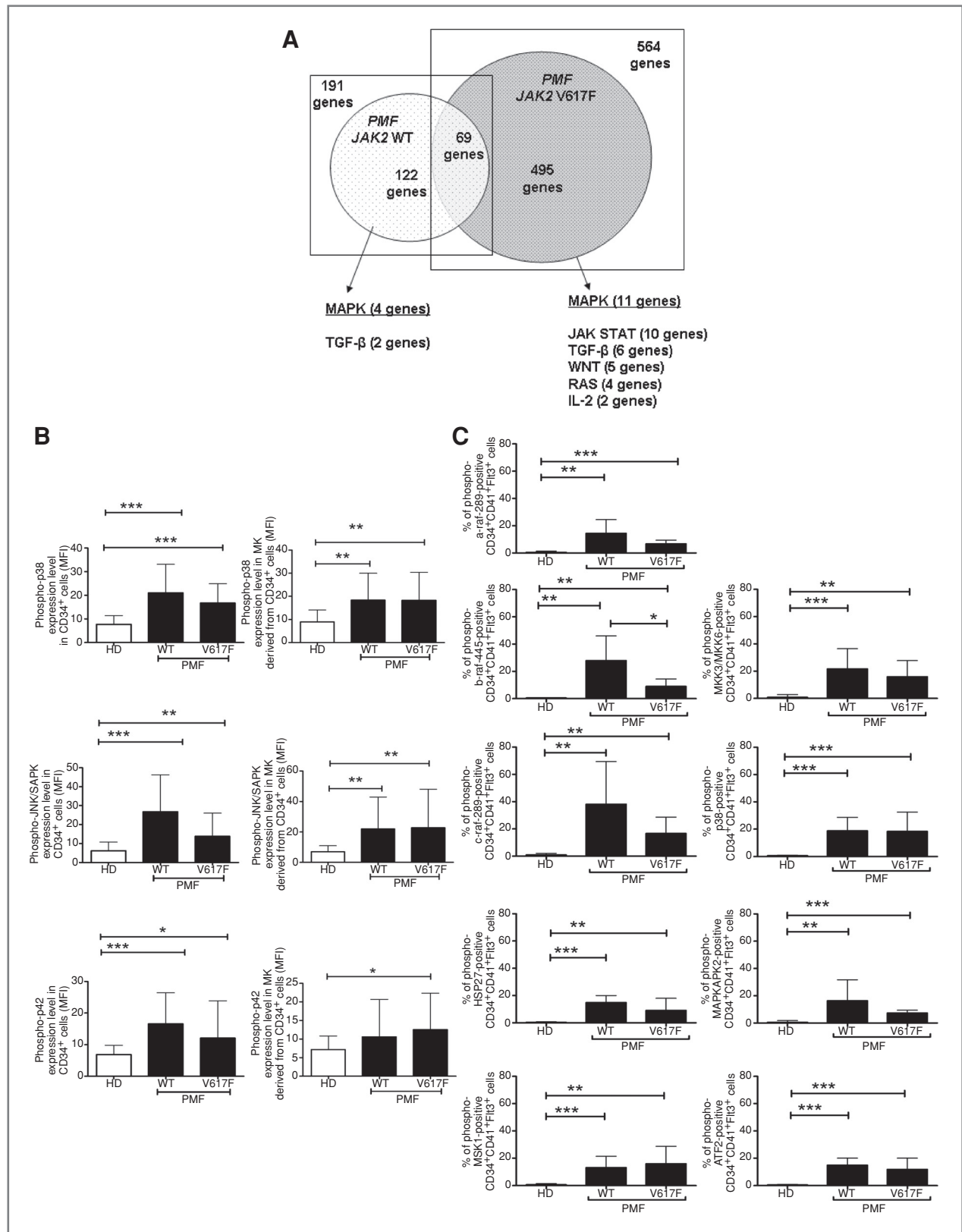
Figure 2. MAPK effector expression and phosphorylation in CD34$^+$ cells and MK cells from PMF patients. A, Venn diagram showing MAPK deregulation in PMF CD34$^+$ cell transcriptome. Phosphorylation level (MFI) of p38, JNK/SAPK, and p42/p44 in CD34$^+$ and MK derived from CD34$^+$ cells (B) of Raf (a-raf-289, b-raf-445, c-raf-289) and p38 pathway effectors (MKK3-6, MSK1, MAPKAPK2, ATF2, and HSP27) in CD34$^+$Flt3$^+$CD41$^+$ MK progenitors (C) from HD and PMF patients according to their Jak2 status. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$.
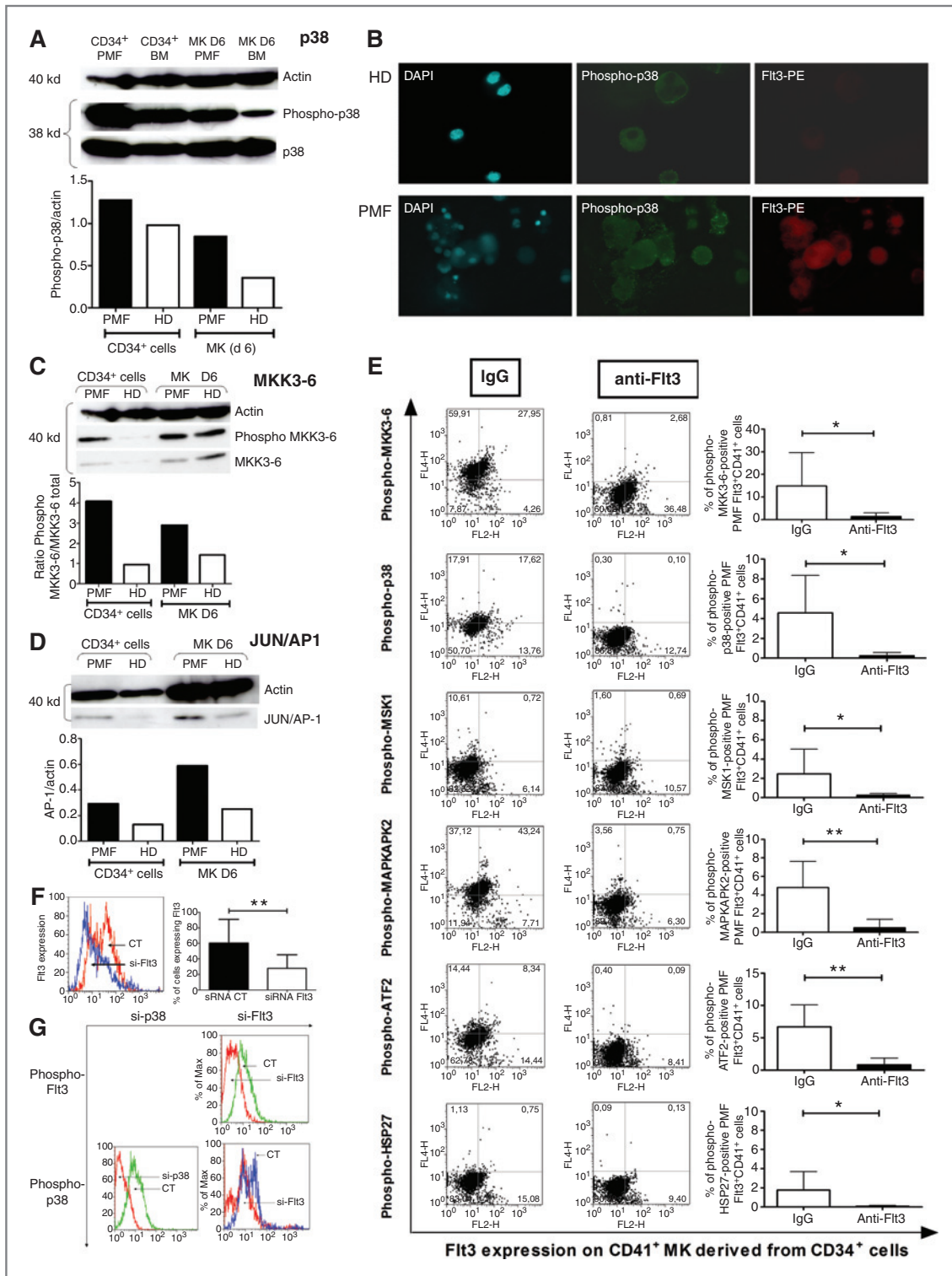
Figure 3. Flt3-dependent p38–MAPK effector phosphorylation in PMF megakaryopoiesis. A, phospho-p38 determined by Western blot analysis. B, p38 phosphorylation and Flt3 expression in MK derived from HD or PMF CD34$^+$ cells (day 10) by immunocytology (650×). C, phospho-MKK3-6 determined by Western blot analysis. D, expression level of JUN/AP-1 determined by Western blot analysis. E, effect of anti-Flt3 mAb on p38 effector phosphorylation (MKK3-6, MSK1, MAPKAPK2, ATF2, and HSP27) in Flt3$^+$CD41$^+$MK derived from PMF CD34$^+$ cells (day 10). F, Flt3 expression in MK derived from PMF CD34$^+$ cells after Flt3 silencing as determined by flow cytometric analysis. G, effect of p38 and Flt3 RNA silencing on their respective phosphorylations in MK derived from PMF CD34$^+$ cells (day 8). DAPI, 4′,6-diamidino-2-phenylindole. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01.
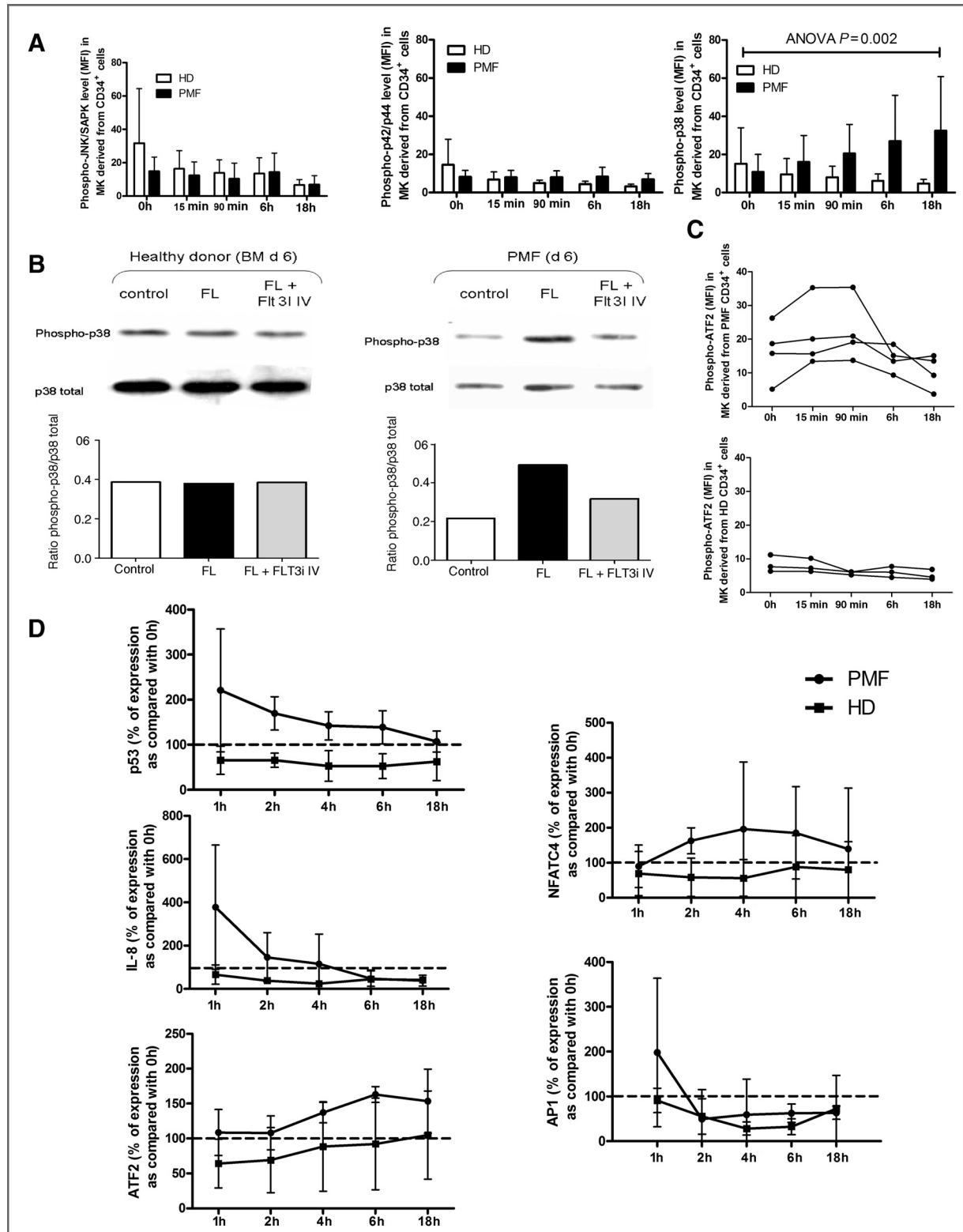
Figure 4. Modulation of p38–MAPK effector phosphorylation and MAPK downstream regulator expression in PMF MK–derived CD34$^+$ cells in response to FL stimulation. These stimulations were carried out at 50 ng/mL. A, effect of 18-hour FL kinetic on the phosphorylation level (MFI) of p-38, JNK/SAPK, and p42/p44 in PMF MK derived from CD34$^+$ cells (day 6). B, phospho-p38 in HD and PMF MK derived from CD34$^+$ cells (day 6) in response to 18-hour FL stimulation and Flt3 inhibitor treatment determined by Western blot analysis. C, modulation of ATF2 phosphorylation in response to a time-dependent FL stimulation determined by flow cytometric analysis. D, transcriptional regulation of MAPK effector (ATF2) and transcription factors as well as IL-8 after 18-hour FL kinetic in HD and PMF MK derived from CD34$^+$ cells (day 6).

confirm the triggering effect of FL/Flt3 on p38 pathway, we further analyzed by flow cytometry the activation of ATF2, a key cross-talk molecule for p38 transcriptional activity, in response to FL stimulation. Figure 4C shows that ATF2 phophorylation level is increased in MK derived from PMF CD34^{+} in response to FL in a time-dependent manner. In contrast, there is no ATF2 phosphorylation in response to FL stimulation in HDs.

We further analyzed whether the expression of MAPK-associated downstream transcription factors was modulated in response to FL. Figure 4D shows that p38-associated p53, NFATc4/NFAT3, AP-1, and ATF2 transcripts are upregulated after FL stimulation in PMF but not in HD samples. In contrast, MSK2, PARK, MK3, MNK1, MNK2, and MSK1 transcripts that are Erk (extracellular signal–regulated kinase)-dependent transcriptional factors were not affected by FL stimulation either in HD or in PMF (Supplementary Table S4). Interestingly, the transcript of IL-8, known to be involved in PMF dysmegakaryopoiesis (30) and to be stabilized by p38 (31), showed a rapid increase in response to FL in PMF cells (Fig. 4D). This IL-8 overexpression was reduced after Flt3 or p38 silencing (data not shown) strengthening the role of the Flt3/p38 signaling in IL-8 regulation.

Altogether our data confirm that FL *in vitro* activates p38 cascade and increases its target gene expression through Flt3 in PMF MK–derived CD34^{+} cells.

Modulation of upstream MAPK linker expression in response to FL allows definition of PMF patient group prediction

We further searched whether p38–MAPK upstream linker expression was modified in response to FL in PMF MK–derived CD34^{+} cells and whether modulation of their expression could predict exaggerated FL/MAPK axis response. We have principally selected upstream MAPK linkers proximal to Flt3, minimizing the influence of other pathways such as PI3K and JAK/STAT (Fig. 5A) and quantified their mRNA expression at different time points of FL kinetics, by QRT-PCR. Data (Supplementary Table S5) were used to draw a gene neuronal network that shows a good correlation between genes selected and validates their choice for the predictive model (Fig. 5B). A three-dimensional (3D) projection plot of principal component multivariate analysis (PCA) showed that HD samples (BM, PB) are closely related in the centre of the plot. The PMF4 patient, who is *de novo* diagnosed, is close to the HD aggregate, in contrast to the 3 other patients (PMF1, PMF2, and PMF3) who were earlier diagnosed (Fig. 5C). These PCA dispersion data incited us to build a hierarchical classification plot correlating this clustering to clinicobiological data (Fig. 5D). This classification allowed to distinguish HD and PMF groups and to identify PMF subgroups correlated with clinical data such as Jak2 mutation status, myeloproliferation, and Dupriez score.

This predictive model confirms the implication of Flt3/MAPK axis activation in PMF megakaryopoiesis and establishes a link with patient clinicobiological parameters.

Flt3/MAPK axis is involved in PMF dysmegakaryopoiesis

Our results showing that Flt3/MAPK axis was altered in PMF megakaryopoiesis raise the question of whether it is involved in this pathologic process (30). Therefore, we assessed the effect of p38, p42/p44, Jnk, and Flt3 inhibitors (refs. 32, 33; Supplementary Table S6) or of anti-Flt3 mAb (10 μg/mL) on PMF MK–derived CD34^{+} cultures. Inhibition of either MAPK or Flt3 significantly reduced the proliferation of MK derived from PMF CD34^{+} cells (Fig. 6A), without affecting their viability (Fig. 6B). Figure 6C showed that such treatment increased the percentage of polyploid MK derived from PMF CD34^{+} cells, especially of cells with a ploidy more than 64N (Supplementary Table S6) and induced a nuclear localization of cyclin D3, known to promote endomitosis. It also stimulated MK differentiation as shown by an increased expression of the CD41 MK differentiation marker (Fig. 6D). A similar effect on CD41 expression was observed after p38 or Flt3 silencing, confirming their participation in the differentiation process (Fig. 6D and Supplementary Fig. S1). Furthermore, Flt3 inhibitors restored the formation of proplatelets (Fig. 6D). Participation of the Flt3/p38-mediated pathway in the control of PMF megakaryopoieis was further confirmed by results showing an upregulation of GATA-1, FOG-1, FLI-1, NFE2 MK transcriptional factors, of Aurora B endomitotic regulator and of TGFB1, PF4, and CD9 MK marker gene expression after Flt3 or p38 silencing (Fig. 6E).

FL has been reported to be a mobilization factor (27). Therefore, we analyzed whether Flt3/p38 axis participates in PMF MK migration in response to FL. We first determined FL doses that induced a maximal MK migration (data not shown). In contrast to HD, in PMF patients, addition of 10 ng/mL FL induced a significant MK precursor migration as compared with untreated cells (46.85% ± 10.09% vs. 25.88% ± 5.29%; $n = 3$, $P = 0.03$; Fig. 6F). FL-mediated migration was inhibited by addition of Flt3 inhibitor (Fig. 6F), confirming the role of Flt3 in the PMF FL–induced migration. Addition of p38 inhibitors also totally inhibited the FL-mediated migration (Fig. 6F) showing that it was dependent on p38 activation. As expected, addition of SDF-1 alone used as control, stimulated the migration of MK precursors from either PMF patients or HD (Fig. 6F). However, when added to FL, SDF-1 did not increase the PMF FL–induced migration (Fig. 6F), suggesting that both processes are differentially regulated in patients.

Altogether, our data support the notion for a role for the Flt3/p38-MAPK axis in PMF dysmegakaryopoiesis.

Discussion

PMF is characterized by a clonal amplification of HSC, an increased circulating CD34^{+} cell number, and a prominent MK proliferation with altered maturation and dysplastic features in the bone marrow. This myeloproliferation is associated with marked changes in the BM stroma characterized by myelofibrosis, osteosclerosis, and neoangiogenesis consequent to an overproduction of fibrogenic and inflammatory cytokines by hematopoietic cells and especially by MKs (34). Reciprocally, studies from our group have shown the role of stromal cells from PMF patients in the myeloproliferation and especially in
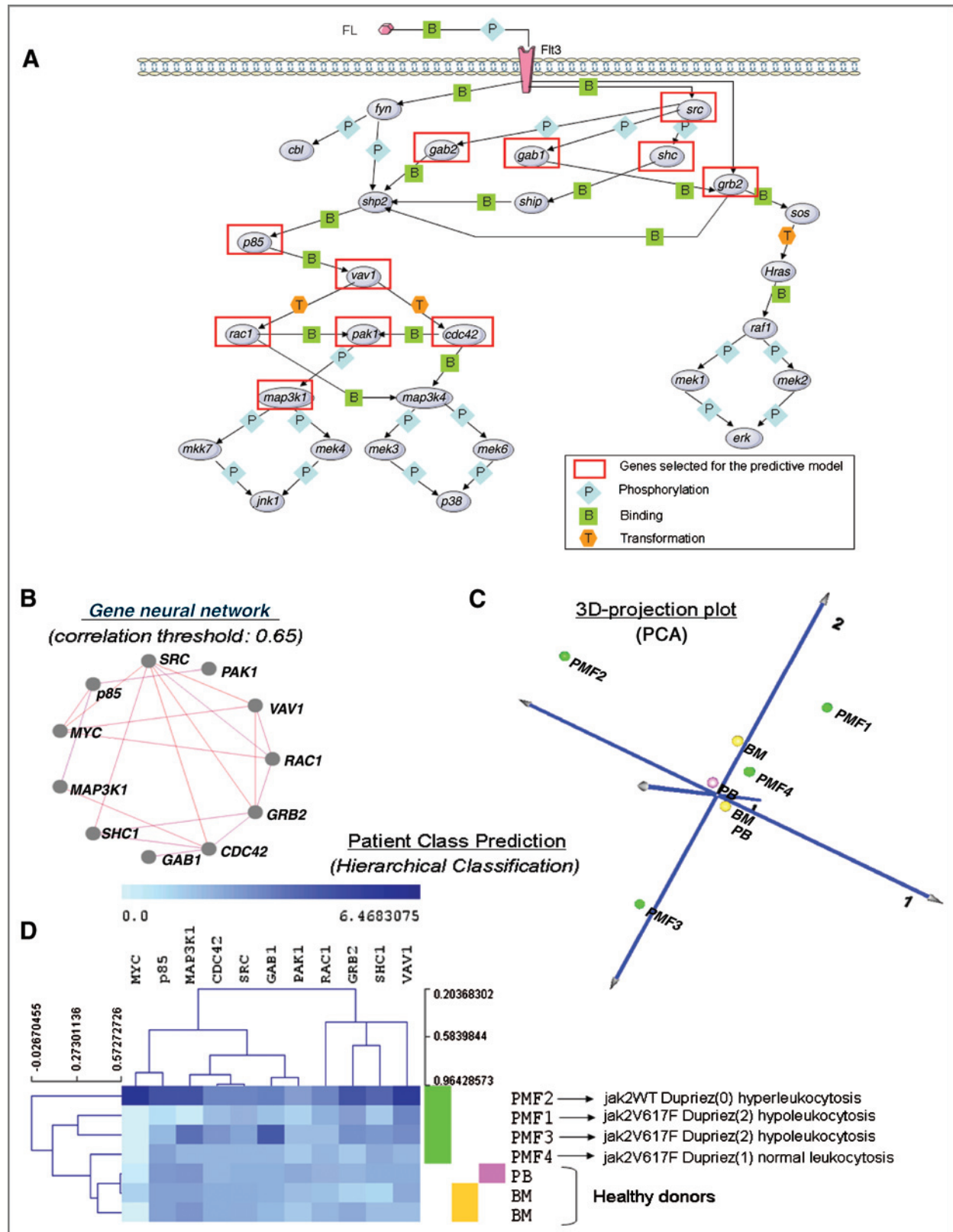
Figure 5. PMF patient group prediction model according to modulation of upstream MAPK linker and effector expression in response to FL stimulation. A, scheme of genes involved in Flt3/MAPK axis drawn from www.genego.com. Red frame genes were selected for conducting a predictive model of PMF patient groups according to FL-dependent MAPK response (B–D). Predictive model: (B) neural network with selected genes, (C) principal component analysis, (D) hierarchical clustering with correlation of clinicobiological data.
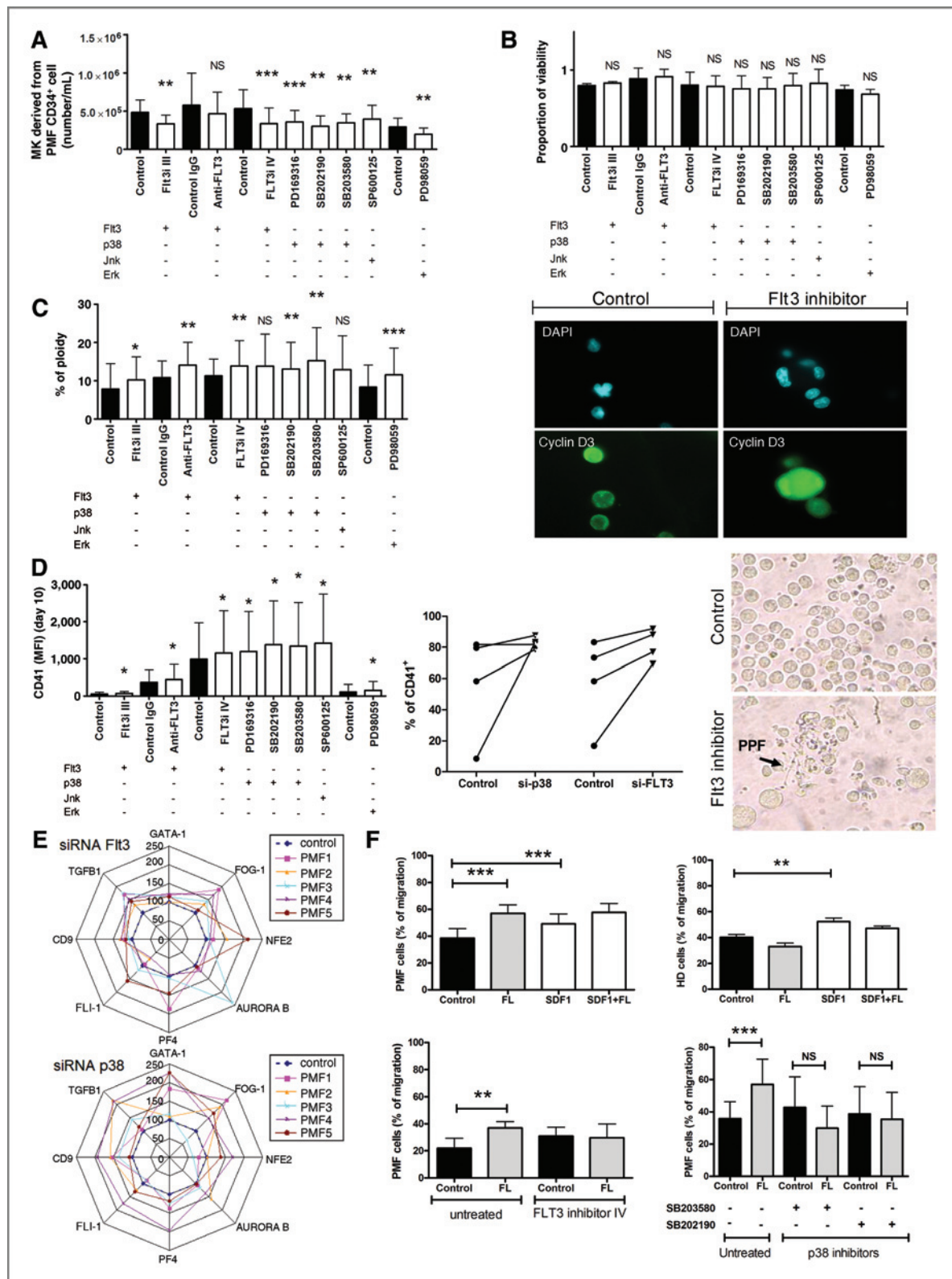
Figure 6. Flt3/MAPK axis is involved in PMF dysmegakaryopoiesis. Effect of Flt3 or MAPK inhibitors on proliferation (A) and viability (B) of PMF MK derived from CD34+ culture (D10), polyploidy cell percentage, cyclin D3 expression and cytologic maturation (400×; C). D, effect of inhibitors and siRNA on CD41 expression and proplatelet formation. E, effect of Flt3 and p38 silencing on gene expression involved in MK regulation quantified by QRT-PCR. F, percentage of migration of MK derived from CD34+ cells purified from HD or PMF patients in response to FL (10 ng/mL) in the presence or absence of SDF-1α (100 ng/mL) and effect of Flt3 and p38 inhibitors on PMF MK derived from CD34+ cell migration in response to FL (10 ng/mL). *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$; ns, not signficant.

the dysmegakaryopoiesis that characterizes the disease (35). Among cytokines secreted by stromal cells, FL (36) has been reported to play a role in HSC proliferation and survival (37). Its receptor, Flt3, is frequently altered in leukemia through gene rearrangements such as ITD that induces a myeloproliferative syndrome in knockin mice model (23). Evidence for such a myeloproliferative phenotype incited us to study the potential involvement of the FL/Flt3 couple in PMF pathogenesis.

Our present data show that the FL plasma level is specifically increased in PMF patients, in whom it is produced by stromal and hematopoietic cells. Furthermore, in contrast to HD in whom Flt3 expression is restricted to HSC/HP and granulomacrophagic progenitors (22), in PMF patients, Flt3 expression and phosphorylation are associated with MK differentiation, as shown by an increased percentage of circulating $CD34^+Flt3^+$ cells expressing the CD41 MK antigen as well as by the increased Flt3 expression on $CD41^+$MK cells. Flt3 overexpression is maintained during $CD41^+$MK derived from PMF $CD34^+$ cell culture, consistent with sustained Flt3 expression along the MK lineage in patients.

Whereas we have shown that Flt3 overexpression is $Jak2^{V617F}$ independent, its mechanism is still unknown. Actually, we did not find any mutations of the *Flt3* gene sequence that could lead to the receptor activation, being compatible with a recent study in PMF patients (38). Among other hypotheses, mutations in the *c-Cbl* gene, an adapter protein that regulates the ubiquitination of receptor protein tyrosine kinases, recently reported in PMF (39), could participate in maintaining Flt3 membrane expression. Flt3 deregulation could be also secondary to other signals including epigenetic modifications, as already shown for CXCR4 in PMF $CD34^+$ cells (40).

Evidence for specific alterations of the FL/Flt3 couple in PMF patients encouraged us to study its potential role in the dysmegakaryopoiesis that characterizes the disease. Among the different mechanisms proposed to be involved, NF-κB (41) activation and IL-8 (30) overexpression are reported to participate in this dystrophic process. $Jak2^{V617F}$ and $MPL^{W515L/K}$ mutations that induce enhanced STAT signaling (42), are suggested to indirectly activate MAPK pathways, known to be important in MK differentiation and especially in endomitosis (24). Our present data, showing in PMF MK cells (i) an increased expression of Flt3 and its phosphorylation, (ii) a modulation of gene profiling involved in MAPK signaling, (iii) an increased phosphorylation of p38, p42/44, and JNK MAPKs, (iv) an increased phosphorylation of p38 pathway effectors, and (v) an activation of Flt3/p38-MAPK axis and an increase of p38 target genes in response to FL, establish a link between Flt3 and MAPK activation, and especially p38, in PMF MKs.

Participation of Flt3 signaling in the PMF dysmegakaryopoiesis through MAPK pathways is suggested by our results showing that treatment with MAPK inhibitors or p38 RNA silencing reverses MAPK phosphorylation and restores MK differentiation. Our hypothesis that a sustained MAPK pathway activation participates in MK maturation block observed in PMF is also supported by our data showing that FL stimulation of PMF MK precursors provokes an increase of p38, recently reported to play a role in PMA (phorbol-12-myristate-13-acetate)-induced MK differentiation of K562

cells (43). The presence of phospho-p38 in the cytoplasm of polyploid MKs (Fig. 3B) strongly suggests its activation in those cells (44). It is also consistent with the stabilization of transcripts with 3′UTR (untranslated regions) AU-rich element-motif (ARE-motif; ref. 31) like IL-8, shown to be upregulated in response to FL (Fig. 4D) and to participate in PMF dysmegakaryopoiesis (30). The p38 phosphorylation recently reported in BM sections from MPN patients (45) confirms our data obtained in culture and supports our hypothesis on p38 activation in PMF.

As expected, FL-induced MAPK phosphorylation is not observed in HD MK precursors which no longer express membrane Flt3. In contrast, in PMF patients, we suggest that an elevated FL level maintains MAPK activation in MK precursors which abnormally expressed Flt3. Our data, showing that Flt3 inhibition by either antibodies or gene silencing improves the megakaryopoiesis in PMF $CD34^+$ cultures and reduces the p38–MAPK effector phosphorylation, reinforce the role of Flt3/p38 axis in PMF dysmegakaryopoiesis. As IL-8 transcripts are suggested to be upregulated by FL stimulation via p38 activation, it can be speculated that phospho-p38 may participate in inflammation observed in PMF by stabilizing transcripts for cytokines/chemokines participating in this process (31). AP-1 and NFATc4 that are upregulated during megakaryopoiesis (46), are overexpressed in response to FL in MK-derived PMF $CD34^+$ cells. Being partners in transcription, they likely cooperate to induce transcription of genes involved in such inflammatory process (47).

In PMF, $CD34^+$ cells egress from the bone marrow to circulate and invade spleen and liver where an extramedullary hematopoiesis is developing. Several mechanisms, including disturbance of SDF-1α-CXCR4 axis (48) and increased extracellular matrix proteolytic activity reducing HP adhesion to BM stroma (49), can explain this migration. Besides its role in primitive hematopoiesis, the FL/Flt3 couple has been reported to regulate cell migration (27). Our data showing that FL stimulates the *in vitro* migration of PMF MK precursors support the hypothesis that the FL/Flt3 couple also participates in the abnormal migration of MKs. In our experimental conditions, the FL-dependent migration PMF MKs is not modified by addition of SDF-1α, suggesting that both processes are differentially regulated in those cells. This FL-dependent migration process is p38 dependent, leading to the assumption that targeting p38 could reduce Flt3-expressing cell mobilization in patients.

Up to now, the general concept in PMF is that stromal changes are secondary to the proliferation of hematopoietic cells and especially of MKs. Recently, we have proposed that an abnormal dialogue between hematopoietic and stromal cells, resulting from microenvironmental niche alterations, participates in the hematopoietic deregulation that characterizes PMF (13). Our present demonstration that FL, overproduced by stromal cells, participates in the altered megakaryopoiesis, supports this hypothesis (35).

In conclusion, our results suggest that activation of the Flt3/MAPK pathway and especially of p38–MAPK participate in PMF dysmegakaryopoiesis, including alterations of proliferation/differentiation and migration processes within an inflammatory context (Fig. 7).
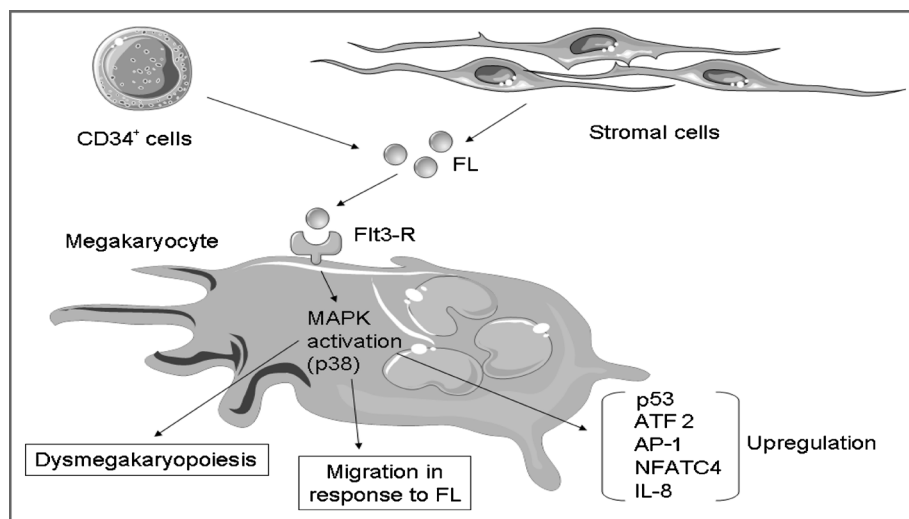
Figure 7. Potential role of Flt3/MAPK axis deregulation in PMF pathogenesis. Pathophysiologic model integrating the "FL/Flt3" couple in PMF MK deregulation through persistent p38–MAPK activation.

The recent therapeutic strategies for PMF mainly target the Jak2 kinase and some of the Jak2 inhibitors also inhibit the Flt3 kinase (50). The clinical efficacy of these inhibitors has been ascribed to a general downregulation of inflammatory cytokine production and signaling (29). Our data suggest that this anti-inflammatory effect could be mediated, at least partially, by the FL/Flt3/p38 axis. In this context, our patient group predictive model, based on FL/MAPK response in MKs and including clinicobiological data, could be powerful in the therapeutic decision to use inhibitors also targeting the Flt3 kinase.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interest.

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Correction: FLT3-Mediated p38–MAPK Activation Participates in the Control of Megakaryopoiesis in Primary Myelofibrosis

In this article (Cancer Res 2011;71:2901–15), which was published in the April 15, 2011 issue of *Cancer Research* (1), Figure 1E contains an error. A corrected version of Figure 1 appears below.
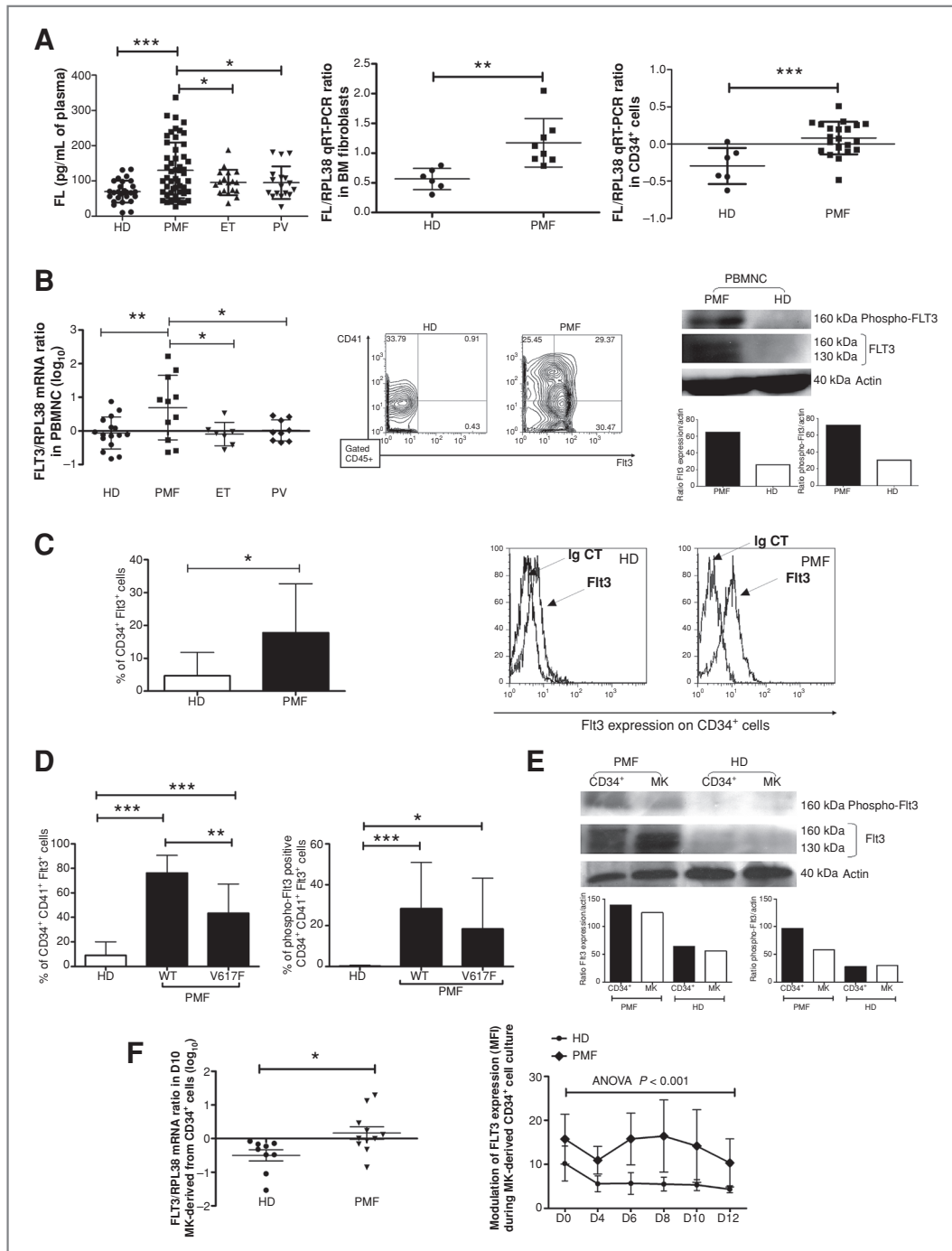

Figure 1.

Also, the name of the 20th author appeared incorrectly on the online journal. The correct surname of this author is Le Bousse-Kerdilès. This error has been corrected.

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FLT3-Mediated p38–MAPK Activation Participates in the Control of Megakaryopoiesis in Primary Myelofibrosis

Christophe Desterke, Chrystele Bilhou-Nabéra, Bernadette Guerton, et al.

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