

ETV6-PDGFRB and FIP1L1-PDGFR α stimulate human hematopoietic progenitor cell proliferation and differentiation into eosinophils: the role of nuclear factor- κ B

Carmen P. Montano-Almendras,¹ Ahmed Essaghir,¹ H el ene Schoemans,^{2,3} Inci Varis,⁴ Laura A. No el,¹ Am elie I. Velghe,¹ Dominique Latinne,⁴ Laurent Knoops,^{1,5} and Jean-Baptiste Demoulin¹

¹de Duve Institute, Universit e catholique de Louvain, Brussels; ²Hematology Department, University Hospitals Leuven, Leuven; ³Leuvense Navelstrengbloed Bank, Leuven; ⁴Cliniques Universitaires Saint-Luc, D epartement de Biologie Clinique et d'Anatomie Pathologique, Brussels; and ⁵Cliniques Universitaires Saint-Luc, Division of Hematology, Brussels, Belgium

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Supplementary Design and Methods

Constructs and lentivirus production

TP β and FP α were generated by subcloning the respective cDNA into pTM895-ires-GFP lentiviral vector, a kind gift of Prof. Thomas Michiels (Brussels, Belgium).¹ The lentiviruses were generated by transient transfection of 293T cells by calcium phosphate precipitation, as described elsewhere,² with 18 μ g of lentiviral vector, 10 μ g of pCMV-dR8.2, 6 μ g of pCMV-VSV-G and 6 μ g of pRSV-Rev. After 66 h, viral supernatants were harvested, passed through a 0.45 μ m filter, concentrated in a Centricon plus 70 (UFC703008, Millipore) and stored in liquid nitrogen.

Isolation, culture and viral infection of human CD34⁺ cells

Leukocytes were isolated from fresh cord blood by centrifugation over a Ficoll-Paque density-gradient (GE Healthcare) and CD34⁺ cells were purified using the EasySep kit (StemCell Technologies) as described previously.³ Cell purity was routinely checked by flow cytometry with an anti-CD34 antibody (Becton Dickinson) and was above 80%. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM), supplemented with 20% fetal bovine serum (FBS), 10 U/mL penicillin, 1.0 μ g/mL streptomycin, and 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine in the presence of 25 ng/mL stem cell factor and 25 ng/mL of FMS-like tyrosine kinase-3 ligand (FLT3L). One day after isolation, 5 \times 10⁵ CD34⁺ cells were resuspended in 0.5 mL of growth medium and incubated for 24 h with concentrated lentiviral particles (0.3 mL) in the presence of polybrene (8 μ g/mL). The cells were then washed and resuspended in fresh growth medium (0.5 mL), new viral supernatant (0.3 mL) and polybrene (8 μ g/mL).

Cells were centrifuged at 400 \times g for 2 h and then washed and cultured as described above. After cell transduction, the percentage of green fluorescent protein (GFP)-positive cells was routinely 95% in controls and 80% in the TP β and FP α condi-

tions, as evaluated by flow cytometry.

The κ B α super-repressor mutant (S32/36A) was a kind gift from Prof. Etienne De Plaen.^{4,5} Retroviruses were generated by transient transfection in 293T cells with 12.5 μ g of retroviral vector, 6.25 μ g of pCMV-dR8.2, 3.7 μ g of pCMV-VSV-G. After 66 h, the viral supernatant was harvested and used as described above to infect TP β -expressing CD34⁺ cells.

Flow cytometry

Intact cells were analyzed by flow cytometry after staining with anti-human IL5R α antibody (R&D Systems), and a secondary antibody coupled to phycoerythrin, as described elsewhere.⁶ Intracellular staining was performed as reported previously.⁶ Transduced CD34⁺ cells were washed and cultured in the absence of cytokines for 16 h prior to staining. The following AlexaFluor-647-labeled antibodies were used: anti-phospho-STAT1 (Y701), mouse anti-phospho-STAT5 (Y694), mouse anti-phospho-p65 NF- κ B (S529), mouse anti-phospho-STAT3 (Y705) and anti-phospho-AKT (S473) (BD Biosciences and Cell Signaling Technology). Samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson). Median fluorescence intensities were calculated using the CellQuest software 3.3 (Becton Dickinson). The background fluorescence intensity was subtracted and the average of several independent experiments is shown with SEM.

Quantitative real time polymerase chain reaction analysis

For polymerase chain reaction (PCR) analysis, RNA samples were reverse transcribed with oligo-dT primers using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative reverse transcriptase PCR assays were performed using SYBR-green reagents from ABgene with the iCycler iQ (Bio-Rad) as described previously.^{7,8} The sequence of forward and reverse primers is provided in *Online Supplementary Table S1*.

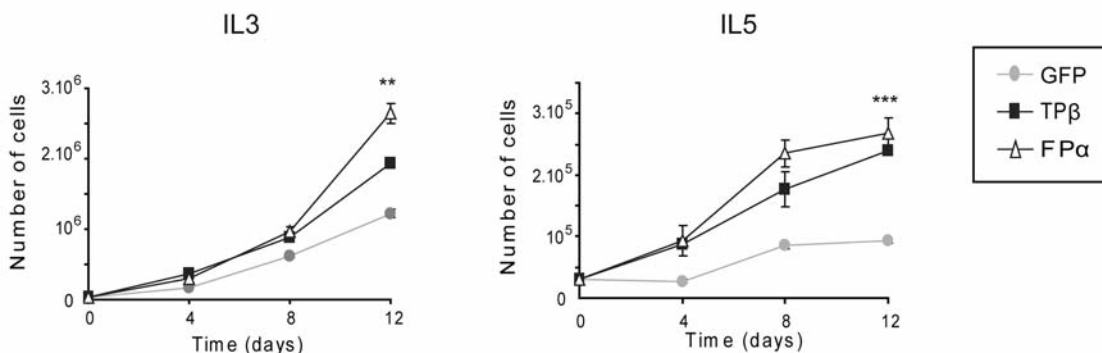
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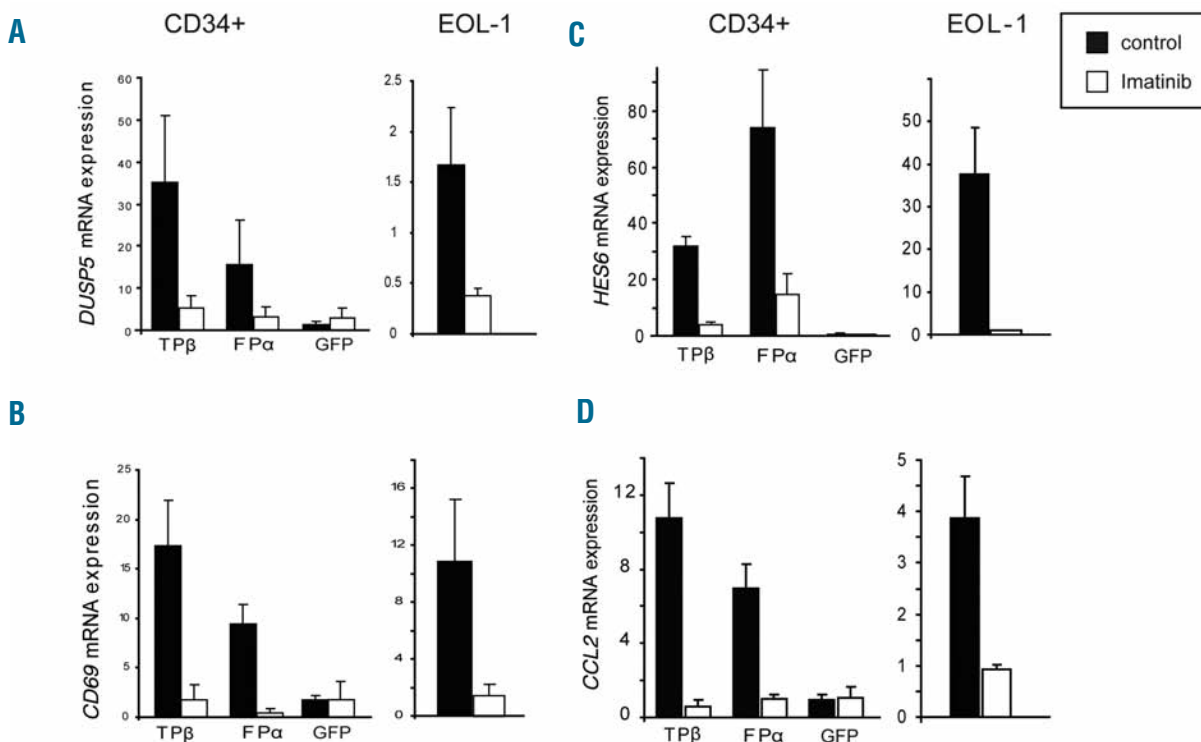
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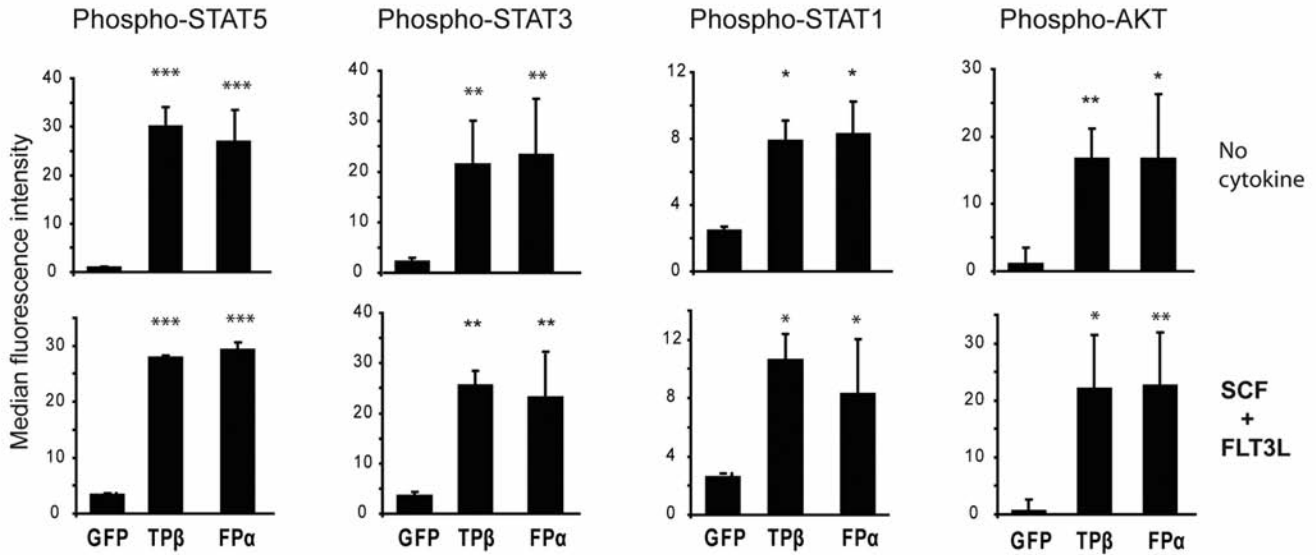
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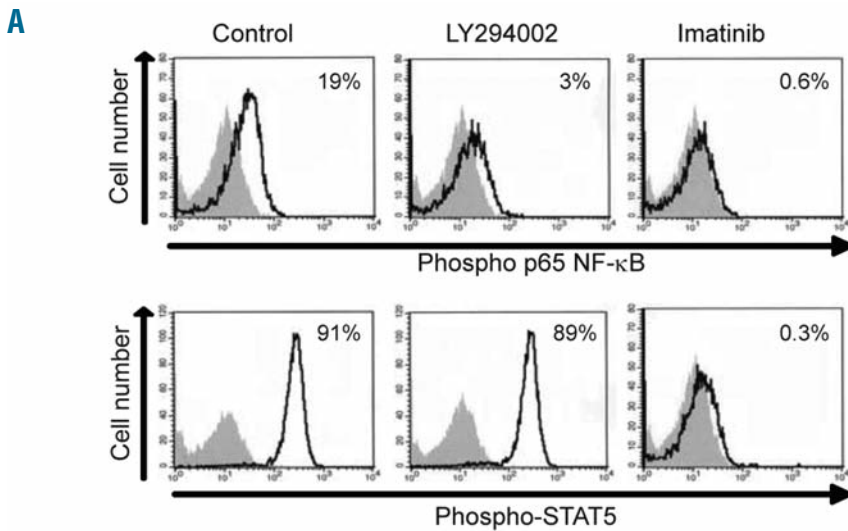
Online Supplementary Figure S1 (complement to Figure 1). Effect of TP β and FP α on cell proliferation in the presence of IL5 or IL3. Transduced CD34⁺ cells were seeded at 3x10⁴ cells/well in the presence of IL3 (left panel) or IL5 (right panel) in duplicate. Viable cells were counted in the presence of trypan blue every 4 days. The difference between cells expressing GFP (solid gray circles) and TP β (solid black squares) or FP α (open triangles) was significant in all cases (ANOVA). Results of one representative experiment are shown.



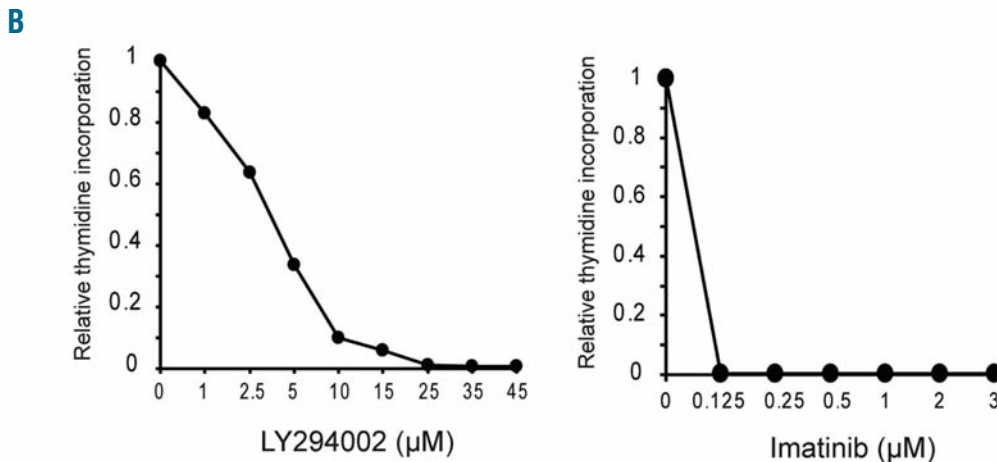
Online Supplementary Figure S2 (complement to Figure 3). FP α and TP β induce the expression of *DUSP5*, *CD69*, *HES6* and *CCL2*. CD34⁺ cells transduced with TP β , FP α or GFP (left panel) or EOL-1 cells, which express FP α (right panels), were treated with or without imatinib for 4 h. RNA was extracted. The expression of *DUSP5* (A), *CD69* (B), *HES6* (C) and *CCL2* (D) was analyzed by quantitative PCR. Results were normalized using the expression of RPLP0 as a control. Results of one representative experiment are shown.

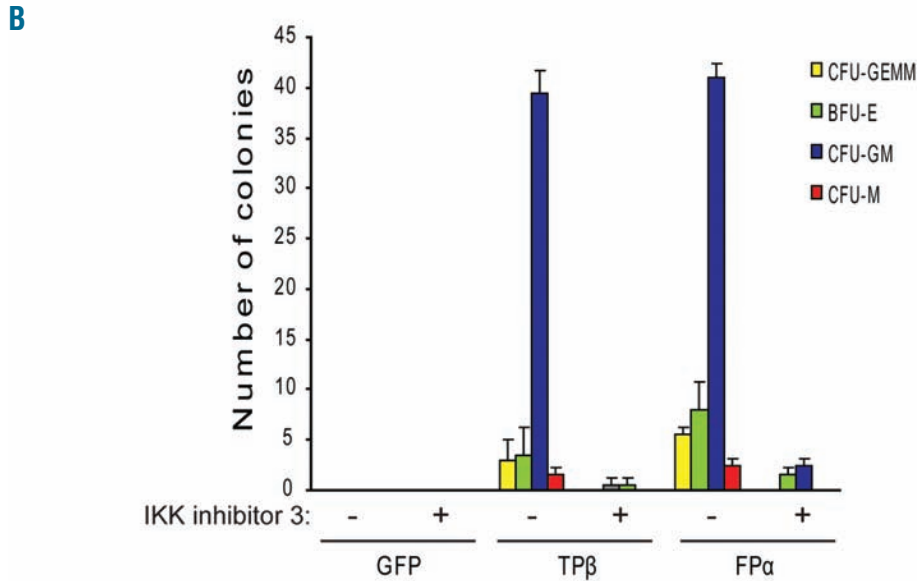
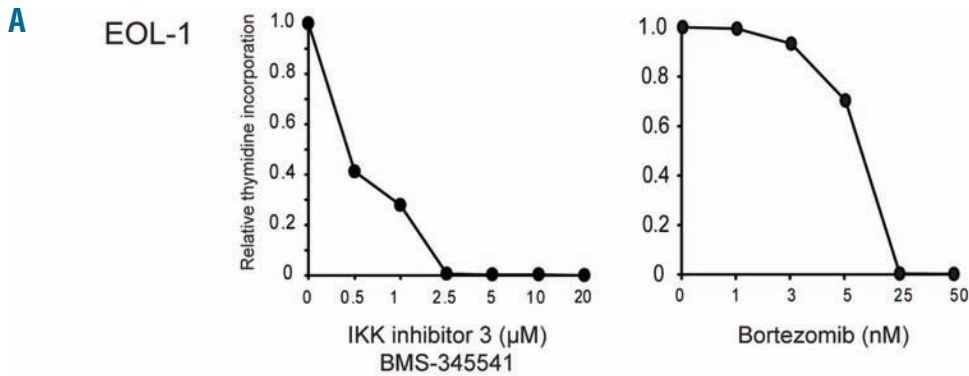


Online Supplementary Figure S3. Signal transduction by TPβ and FPα. CD34⁺ cells were transduced with TPβ, FPα or GFP alone. Cells were cultured for 7 days either in the absence of cytokine (upper panels) or with SCF and FLT3L (lower panels). In the latter case, cells were washed and left in cytokine-free medium for 16 h prior to staining. Cells were permeabilized and the phosphorylation of STAT5, STAT3, STAT1 and AKT was evaluated by flow cytometry using labeled phospho-specific antibodies. The median fluorescence intensity is shown after subtraction of background fluorescence.



Online Supplementary Figure S4. Imatinib and LY294002 inhibit EOL-1 cell proliferation and NF-κB phosphorylation. (A) EOL-1 cells were treated with the PI3K inhibitor LY294002 (25 μM) or imatinib (0.5 μM) for 4 h. Cells were permeabilized and stained with anti-phospho-p65 or anti-phospho-STAT5 antibodies. Cells were analyzed by flow cytometry. The percentage of positive cells is indicated in the corner of each graph. (B) EOL-1 cells were seeded in a microtiter plate in the presence of imatinib or LY294002 for 24 h. Tritiated thymidine was added for 4 h. Radioactivity incorporated into DNA was quantified as described elsewhere.^{5,8} Results of one representative experiment out of three are shown. The effect of both drugs was highly significant (ANOVA).





Online Supplementary Figure S5. Inhibitors of the NF-κB pathway block EOL-1 cell proliferation and colony formation from CD34⁺ cells. (A) EOL-1 cells were seeded in a microtiter plate in the presence of BMS-345541 (IKK inhibitor 3, Calbiochen) or bortezomib for 24 h. Tritiated thymidine was added for 4 h. Radioactivity incorporated into DNA was quantified as described elsewhere.^{5,8} Results of one representative experiment are shown. Both inhibitors blocked NF-κB activation (data not shown). (B) CD34⁺ cells were transduced with GFP, TPβ and FPα. Ten thousand cells were plated in methylcellulose in the presence of IKK inhibitor 3 (2.5 μM) without cytokines. Colonies were counted after 12 days as in Figure 1. Results of one representative experiment out of two are shown.

Online Supplementary Table S1. The sequences of primer sets used for quantitative PCR.

Gene	Forward primer	Reverse primer
PDGFRB	5'GACCCCAAACCCGAGGTT 3'	5'ATGGTTGAGGAGGTGTTGACTTC 3'
PDGFRA	5'CGTGTGGACTCAGACAATGCA 3'	5'GGCAGAGGAATGATGTAGCCA 3'
HES6	5'AGCAGGAGCCTGACTCAGTT 3'	5'AGTCCTGAACCATCTGCTC 3'
CCL2	5'GCTTCTTTGGGACACTTGCT 3'	5'CCCAGTCACCTGCTGTTAT 3'
lB	5'GCTGCAGGTTGTTCTGGAAG 3'	5'GTTCCCTGCACTTGCCATC 3'
IL5RA	5'ATGAAGGAAGCTGCCTGAGA 3'	5'GTAATACATGCGCCACGATG 3'
EPX	5'CACGGTTTCAAGGGACATC 3'	5'CTTTTCTTGCCTGGGGTG 3'
DUSP5	5'CGGAATATCCTGAGTGTTCGCTGG3'	5'TCCACTGGCTGATGAGGGCT3'
CD69	5'CTGATAAGGCAATGAGAGCTATG3'	5'AGTCCCATTCTCAACACG3'
RPLP0	5'TCTATCATCAACGGGTACAACGA3'	5'GGCCTTGACCTTTTCAGCAA3'

Primers used to amplify *IL5RA* and *EPX* were from Mori et al.¹ *RPLP0* primers were described previously.² All the primers were produced by Eurogentec (Liège, Belgium).

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