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by Florence Pasquier, Caroline Marty, Thomas Balligand, Frédérique Verdier, Sarah Grosjean, Vitalina Gryshkova, Hana Raslova, Stefan N. Constantinescu, Nicole Casadevall, William Vainchenker, Christine Bellanné-Chantelot, and Isabelle Plo

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# New pathogenic mechanisms induced by germline erythropoietin receptor mutations in primary erythrocytosis

**Running title:** Erythropoietin receptor mutations in PFCP

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• New heterozygous frameshift *EPOR* mutation (c.1300dup) in primary familial and congenital polycythemia (PFCP)

• Nonsense and frameshift *EPOR* mutations contribute to PFCP through distinct mechanisms

**Keywords:** EPOR, JAK2, erythrocytosis

**Abstract**

Primary Familial and Congenital Polycythemia is characterized by erythropoietin hypersensitivity of erythroid progenitors due to germline nonsense or frameshift mutations in the erythropoietin receptor gene. All mutations already described lead to the truncation of the C-terminal receptor sequence that contains negative regulatory domains. Their removal is presented as sufficient to cause the erythropoietin hypersensitivity phenotype. Here, we provide evidence for a new mechanism whereby the presence of novel sequences generated by frameshift mutations are required for the phenotype rather than just extensive truncation resulting from nonsense mutations. We show that the erythropoietin hypersensitivity induced by a new erythropoietin receptor mutant, p.Gln434Profs*11, could not be explained by the loss of negative signaling and of the internalization domains, but rather by the appearance of a new C-terminal tail. The latter, by increasing erythropoietin receptor dimerization, stability and cell-surface localization causes pre-activation of erythropoietin receptor and JAK2, constitutive signaling and hypersensitivity to erythropoietin. Similar results were obtained with another mutant, p.Pro438Metfs*6, that shares the same last 5 amino acid residues (MDTVP) with erythropoietin receptor p.Gln434Profs*11, confirming the involvement of the new peptidic sequence in the erythropoietin hypersensitivity phenotype. These results suggest a new mechanism that might be common to erythropoietin receptor frameshift mutations. In summary, we show that Primary Familial and Congenital Polycythemia is more complex than expected since distinct mechanisms are involved in the erythropoietin hypersensitivity phenotype, according to the type of erythropoietin receptor mutation.

**Introduction**

Primary erythrocytosis **(**also known as Primary Familial and Congenital Polycythemia, PFCP) is a pathology of the erythroid progenitors that display hypersensitivity to erythropoietin (EPO).[1-14] This rare inherited entity is usually passed down with an autosomal dominant pattern with complete penetrance.[2-6,8-14] PFCP is characterized by an isolated primary polycythemia that associates increase of the red cell mass and subnormal serum EPO levels. It can therefore mimic the clinical presentation of a Polycythemia Vera (PV). However, the hematopoiesis is polyclonal and PFCP patients do not exhibit any risk of transformation into myelofibrosis or leukemia. Moreover, somatic *JAK2*V617F [15] and *JAK2*exon12 [16] mutations, hallmarks of PV, are not found in PFCP [12,13], which is rather characterized by the presence of germline mutations in the erythropoietin receptor (*EPOR*) gene.

EPOR is a type I cytokine receptor [17] expressed essentially by the erythroid progenitors in the hematopoietic tissue. EPO binding to its receptor at the cell surface leads to the transient activation of preformed EPOR-JAK2 complexes [18,19] and downstream signaling pathways, including Signal Transducer and Activators of Transcription (STATs) [20], PhosphatidylInositol 3 Kinase (PI3K)/Akt [21] and Mitogen-Activated Protein Kinase (MAPK) pathways. The EPO-induced signaling is crucial for the proliferation and the survival of erythroid progenitors as well as for terminal erythroid differentiation [22].

Around 20 germline heterozygous nonsense and frameshift mutations located in the exon 8 of *EPOR* have been described so far in PFCP, leading to the truncation of the C-terminal part of the receptor.[3-11,13,14,23-28] Interestingly, similar EPOR truncations have been described in BCR-ABL1-like Acute Lymphoblastic Leukemia (ALL), due to acquired rearrangements of the *EPOR* gene with immunoglobulin chain loci.[29] The C-terminal part of the receptor includes several conserved tyrosine residues that are docking-sites for positive and negative

regulators of EPOR signaling. The EPO hypersensitivity of PFCP progenitors is therefore usually explained by the disappearance of negative regulatory domains located in the truncated part of the receptor.[30,31] Few missense *EPOR* mutations have also been described, but their involvement in the PFCP phenotype is not yet clear.[4,7,13,26-28,32] Previous studies have suggested that truncated EPOR mutations might not be equivalent in term of underlying mechanisms leading to EPOR activation, [14,27] but relatively few functional studies have been carried out. We therefore investigated the mechanism of some *EPOR* mutations in PFCP.

We identified and extensively studied a new germline frameshift *EPOR* mutation, c.1300dup (p.Gln434Profs*11), responsible for a high EPO hypersensitivity as in *JAK2*V617F-positive PVs. We modeled EPOR p.Gln434Profs*11 and several other EPOR mutants already described in Ba/F3 cell line and demonstrated that different mechanisms are involved in the EPO hypersensitivity phenotype, according to the type of *EPOR* mutation, highlightening that PFCP is a more complex pathology than usually admitted.

## Methods

### Materials

Human recombinant EPO and Interleukin-3 (IL-3) were generous gifts from Amgen (Neuilly, France). Stem Cell Factor (SCF) was purchased from Biovitrum AB (Stockholm).

### Patients, cell purification and erythroblast cultures

Peripheral blood samples from the patient or healthy donors were collected from leukapheresis. Written informed consent was obtained from patient in accordance to the Declaration of Helsinki and the study approved by Ethics Committee from la Pitié-Salpétrière

Hospital. Mononuclear cells were separated over a Ficoll density gradient and $CD34^+$ cells were purified by a double-positive magnetic cell sorting system (AutoMACS; Miltenyi Biotec). $CD34^+$ cells were amplified in erythroid conditions for 7-10 days in Iscove Modified Dulbecco Medium with penicillin/streptomycin/glutamine, alpha-thioglycerol, Bovine Serum Albumin (BSA), a mixture of sonicated lipids and insulin-transferrin in the presence of recombinant human cytokines (25 ng/mL SCF, 100 U/mL IL-3, 1 U/mL EPO).

**EPOR sequencing**

Genomic DNA was extracted from blood, nails and hair using standard procedures. The exon 8 of the *EPOR* gene was amplified and sequenced in both directions. Mutations are numbered as recommended by the Human Genome Variation Society (http://www.hgvs.org/) using the reference sequence NM_000121.3. Participants gave written informed consent for the genetic study.

**Quantification of clonogenic progenitors in semi-solid cultures**

Colony assays were performed with 500 purified $CD34^+$ progenitors per culture dish in duplicate in H4100 Methocult media (StemCell) supplemented with 12% BSA, 30% or no Fetal Bovine Serum (FBS), 2-β-mercaptoethanol (1 µM), 1% L-glutamine, SCF (25 ng/mL), IL-3 (100 U/mL) and in absence or presence of increasing EPO concentrations (0.001; 0.01; 0.1 and 1 U/mL). Burst-forming units-erythroid (BFU-E)-derived colonies were counted at day 14.

**DNA manipulations, retrovirus production**

*EPOR* mutations were introduced into the pMX-HA-human EPOR WT-IRES-GFP plasmid by the QuikChange site-directed mutagenesis method using the PfuUltra high-fidelity DNA polymerase (Stratagene): c.1300dup (p.Gln434Profs*11, EPOR FS); c.1330G>T (p.Gln444*, EPOR STOP); c.1303_1304delinsGC (p.Leu435Ala, EPOR WTdiL or EPOR STOPdiL); c.1195G>T (p.Glu399*) ; c.1273G>T (p.Glu425*) ; c.1311_1312del (p.Pro438Metfs*6) ; c.1327_1329delinsTAA (p.Pro443*) ; c.[1300dup ; 1311G>C] (p.[Gln434Profs*11 ; Ala437Arg]) (Table 1). Full-length *EPOR* mutant cDNAs were verified by sequencing.

**Cell lines**

The murine Ba/F3 and human UT7 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% FBS (Stem Cell Technologies) and 5% WEHI-conditioned medium as a source of murine IL-3 or GM-CSF (10 ng/mL). Cells were transduced with pMX-IRES-GFP retrovirus to stably express the human wild-type EPOR or EPOR mutants, carrying a N-terminal HA-tag. $GFP^+$ cells were subsequently sorted by flow cytometry (Influx, Beckman-Coulter).

**Proliferation assays**

The premixed WST-1 cell proliferation assay was carried out according to the manufacturer's instructions (Takara Bio Europe, Clontech). Experiments were performed in triplicate. Dose-response curves to EPO were expressed as percentages of viability of the maximal response.

**Western blot analysis**

For signaling studies, cells were starved for 5 hours and cells were incubated with increasing EPO concentrations for 15 minutes or stimulated with 1 U/mL EPO for 15 minutes and washed 3 times in PBS 1X to remove the cytokine.

Signaling studies were performed using polyclonal antibodies against the phosphorylated forms of JAK2 (Tyr 1007/1008), STAT5 (Tyr 694), ERK1/2 (Thr 202/Tyr 204) and Akt (Ser 473) and against the pan proteins (Cell Signaling Technology, Ozyme). β-actin (Sigma) was used as loading control.

For glycosidase digestion, cell lysates were incubated with either Endoglycosidase H (Endo H) or with Peptide:N-Glycosidase F (PNGase F) (New England Biolabs, 1,000 U) at 37°C for 16 hours according to manufacturer's recommendation.

**Dimerization of human EPOR monomers assessed by split Gaussia luciferase assay.** A split Gaussia subunit, Gluc1 or Gluc2, was fused to the C-terminus of each EPOR construct [33]. When an EPOR monomer with a Gluc1 fusion subunit dimerizes with another EPOR monomer with a Gluc2 fusion subunit, the two Gluc subunit proteins recombine into a catalytically active luciferase that is able to degrade coelenterazine, thus emitting light. Both EPOR constructs fused to a C-terminal Gluc1 and a Gluc2 subunit were transiently transfected at a 50:50 ratio into HEK cells using Transit-LT1 (Mirus) as a transfecting agent. The pGL3-control vector (Promega) that constitutively expresses the firefly luciferase was co-transfected in each condition as a transfection control reporter. After 48 hours, luciferase signals were read by a GloMax discover system (Promega) after addition of coelenterazine and of firefly luciferin to each well. A 530LP filter was used to discriminate the luminescence of the firefly luciferase from that of the Gaussia luciferase.

**EPOR stability assay**

Ba/F3 cells were incubated for different length of time with 50 ng/mL cycloheximide (CHX) (Sigma). After cell lysis, Western blot were performed with anti-HA antibody. Quantification of HA-EPOR was done with Image J Software, using β-actin as loading control. The half-life of the receptor was calculated using GraphPad PRISM software.

**Cell-surface localization of HA-tagged EPOR by flow cytometry**

Cells were labeled with monoclonal mouse anti-HA antibody conjugated to phycoerythrin (PE) (Miltenyi Biotec) and processed by flow cytometry (FACSCanto, Beckman-Coulter).

**EPO labeling, EPO binding and EPOR internalization studies**

EPO labeling using IODO-GEN (Pierce, Rockford, IL), EPO binding and EPOR internalization studies were performed as previously described [34-36]. Nonspecific binding was determined using a 250-fold excess of unlabeled EPO and was less than 5% in each case. All reported data represent specific binding. The number of cell-surface receptors was determined by comparing the radioactivity of Ba/F3-EPOR cells to the radioactivity of the reference UT-7 cell line [34]. For internalization experiments, after incubation with $^{125}$I-EPO, 5 x $10^6$ cells per condition were washed twice at 4°C to remove unbound ligand. An acidic wash was then performed to separate cell surface–bound from internalized EPO. Cells were incubated in 0.5 mL acidic buffer (150mM NaCl, 50mM sodium acetate, pH 3.5) for 3 minutes at 4°C. The pH was then adjusted to 7.4 using 1M Tris-HCl, pH 9 and the cell suspension was centrifuged. Radioactivity of the supernatant (cell surface–bound EPO) and of the cell pellet (internalized EPO) was determined. When $^{125}$I-EPO was bound to the cells at 4°C to inhibit EPO

internalization, more than 95% of cell-bound $^{125}$I-EPO was recovered in the acidic wash supernatant using this method. Each experiment was performed 3 times with similar results.

**Results**

**Identification of a new germline *EPOR* mutation responsible for high EPO hypersensitivity**

Primary polycythemia was diagnosed in a 28-year-old woman without history of thrombosis. She harbored high hemoglobin (21g/dL) and hematocrit (60%) values and an increased red cell mass (65%) with a low EPO level (1.2 mU/mL; laboratory standards: 5-25 mU/mL). She had no splenomegaly at physical examination. Leucocyte and platelet counts in the peripheral blood as well as bone marrow aspiration and biopsy were strictly normal. Later no *JAK2*V617F or *JAK2*exon12 mutation was found, rendering the diagnosis of PV unlikely. The search for abnormal hemoglobin affinity and for *VHL*, *PHD1/2, SH2B3 (LNK)* pathological mutations was negative. *EPOR* sequencing identified a new germline heterozygous frameshift mutation, c.1300dup (p.Gln434Profs*11), which generates a new 10 amino acid (AA) C-terminal tail and a STOP codon at position 444, leading to the truncation of 64 AA of the wild-type receptor and the loss of the 6 last conserved tyrosine residues (Table 1, Figure 1a and b). The mutation was found in neutrophils and $CD3^+$ T lymphocytes as well as in non-hematopoietic tissues like nails and hair allowing the diagnosis of PFCP. There was no familial history of polycythemia. The blood counts of the mother and two children were strictly normal. The father blood count was not available.

Erythroid progenitors displayed an autonomous growth when cultured in presence of serum (Figure 1c) probably explained by residual EPO in the serum as in the absence of serum, no spontaneous growth was observed, but a major EPO hypersensitivity (nearly a 5-fold increase) compared to control cells from a healthy donor (Figure 1d).

We performed signaling experiments with primary $CD34^+$ cells from the PFCP patient that were cultured *in vitro* for 7-10 days in presence of IL-3, SCF and EPO. Unlike control cells, PFCP erythroid progenitors exhibited a constitutive and persistent phosphorylation of JAK2 as well as a constitutive activation of STAT5 with a hypersensitive EPO dose-response (Figure 1e and f). This EPO-induced JAK2 and STAT5 activation also persisted for 4 hours after EPO removal in PFCP cells, whereas it was much more transient in control cells (Figure 1f).

Here we show that *EPOR* c.1300dup (p.Gln434Profs*11) is a strong gain-of-function mutation, which induces a major EPO hypersensitivity in primary erythroid progenitors, similar to that observed in PV patients, as well as constitutive and persistent JAK2 and STAT5 activations.

**Functional analysis of *EPOR* c.1300dup (p.Gln434Profs*11) in Ba/F3 cell line**

To study the functional impact of the new frameshift mutant, Ba/F3 cells were transduced to express different HA-tagged human EPORs: the wild-type receptor (EPOR WT), EPOR p.Gln434Profs*11 (EPOR FS), identical to the patient mutation or EPOR p.Gln444* (EPOR STOP) generating a truncated receptor at the position 444 (Table 1, Figure 2a). EPOR-FS and STOP differed by the nature of the C-terminal 10 AA residues, namely the new sequence PALASMDTVP in EPOR-FS and the natural sequence QLLRPWTLCP in EPOR-STOP. These cells expressed quite similar levels of exogenous EPOR detected with anti-HA antibody (Figure 2c). Interestingly, EPOR FS migrated slightly above EPOR STOP, suggesting differences in post-translational modification. However, the glycosylation states of EPOR WT, EPOR STOP and EPOR FS were similar after using Endo H and PGNase F (Figure S1).

MTT-like assays were performed to investigate the potential effects of EPOR STOP and EPOR FS on cell proliferation. None of these mutants was able to induce cytokine-independent cell

growth. However, EPOR FS conferred a 4- to 5-fold EPO hypersensitivity to Ba/F3 cells compared to EPOR WT. Interestingly the EPO-induced growth of EPOR STOP Ba/F3 cells was similar to that of EPOR WT cells (Figure 2b). To confirm these results in a human setting, we transduced EPOR WT, STOP and FS in the human UT7 cell line, which expresses endogenous EPOR and found similar results as in the Ba/F3 cell line (Figure S2A).

We checked the signaling pathways and observed that EPOR FS induced constitutive phosphorylation of STAT5, AKT and ERK compared to EPOR WT and at a higher extent than EPOR STOP in Ba/F3 cells (Figure 2c) and UT7 cells (Figure S2B). Semi-quantitative analysis of spontaneous STAT5 phosphorylation showed a significant increase in EPOR FS compared to EPOR WT and STOP (Figure 2d). Moreover, we observed a similar persistent STAT5 activation in both EPOR FS and EPOR STOP Ba/F3 cells compared to EPOR WT cells (4 hours, 2 hours and 30 minutes after EPO removal, respectively) (Figure 2d).

These results show that EPOR FS confers a similar EPO hypersensitivity to Ba/F3 and UT7 cells than observed in PFCP erythroid progenitors, while the truncated mutant EPOR STOP did not elicit such EPO hypersensitivity to these cells. Therefore the major EPO hypersensitivity induced by EPOR p.Gln434Profs*11 cannot be explained by the receptor truncation itself and the loss of the two SHP-1 and SOCS3 binding sites which are responsible for a persistent activation, but rather by the appearance of a new C-terminal tail that confers spontaneous signaling.

**Effects of c.1300dup (p.Gln434Profs*11) mutation on EPOR stability and cell surface expression and dimerization**

To further characterize this new EPOR mutant, the cell surface expression of the wild-type receptor and both mutants was studied by flow cytometry. At similar levels of GFP

(transduction of cells with IRES-GFP retroviruses), EPOR FS was significantly more abundant at the cell surface (more than 2-fold, p=0.0002) than EPOR WT and EPOR STOP in both Ba/F3 and UT7 cell lines (Figure 3a and Figure S3). These results were further confirmed in Ba/F3 cells using radiolabeled $^{125}$I-EPO (2,412±494 receptors for EPOR FS, p=0.015, 1,018±284 receptors for EPOR STOP and 1,201±304 receptors for EPOR WT) (Figure 3b). We next investigated the stability of the receptors using treatment with the protein synthesis inhibitor cycloheximide. EPOR FS was more stable than both EPOR WT and EPOR STOP (half-life of 2 hours and 1 hour, respectively) (Figure 3c). We also studied the dimerization of human EPOR monomers by split Gaussia luciferase assay in steady state conditions in HEK cells, in the absence of EPO (Figure 3d). Close proximity between the C-terminal cytosolic domains of EPOR was increased by 4-fold with EPOR FS compared to control and EPOR STOP, as assessed by reconstitution of the split Gaussia luciferase activity (Figure 3e).

The increased stability and cell surface localization of EPOR FS may also result in a defect in the receptor internalization pattern due to the loss of specific domains located in the C-terminal part of the wild-type receptor. However, EPOR WT, STOP and FS displayed the same internalization pattern as assessed by $^{125}$I-EPO labeling experiments (Figure 4a and b). Furthermore, we noticed that a dileucine motif, known to be a potential clathrin-dependent endocytosis signal [37], was lost in the new C-terminal tail of the EPOR FS. We assumed that this particular modification could be involved in the increased cell surface expression of EPOR FS. Thus, based on EPOR WT and EPOR STOP constructs, we generated two other mutants, EPOR WT/diL and EPOR STOP/diL, where the dileucine motif was removed (Figure 4c). However its abrogation modified neither the EPO sensitivity of EPOR WT or STOP Ba/F3 cells (Figure 4d) nor the localization at cell surface of the receptors (Figure 4e). Likewise,

EPOR WT/diL and EPOR STOP/diL Ba/F3 cells displayed a similar signaling pattern than EPOR WT and EPOR STOP cells, respectively (Figure 4f).

Altogether these results show that p.Gln434Profs*11 increases EPOR stability, dimerization and localization at the cell surface without modifying internalization of the receptor.

**Different mechanisms are involved in the EPO hypersensitivity phenotype in PFCP according to the type of *EPOR* mutation**

We wondered if this model could be extended to other *EPOR* mutations already described in PFCP. We therefore investigated the impact of the frameshift *EPOR* c.1311_1312del (p.Pro438Metfs*6) mutation [25] and its designed nonsense mutant counterpart, *EPOR* c.1327_1329delinsTAA (p.Pro443*) (Figure 5a) on the proliferation rate of Ba/F3 cells. These mutants are lacking the last 65 AA of the receptor, retaining 2 of the 8 conserved tyrosine residues, Tyr-368 and -426 (Table 1, Figure 6a, c and d). Interestingly, EPOR p.Pro438Metfs*6 and EPOR p.Gln434Profs*11 (EPOR FS) are sharing the same 5 AA terminal sequence (MDTVP). The MTT-like assays showed that EPO-hypersensitivity was induced by EPOR p.Pro438Metfs*6, but not by EPOR p.Pro443* (Figure 5b), similarly to the results obtained with EPOR p.Gln434Profs*11 (EPOR FS) and EPOR p.Gln444* (EPOR STOP) respectively. We also studied two proximal nonsense mutations, *EPOR* c.1195G>T (p.Glu399*) [11] and c.1273G>T (p.Glu425*) [10] (Figure 5a) that are responsible for more extensive truncations (109 AA and 83 AA respectively) and the loss of 7 of the 8 cytoplasmic tyrosine residues, retaining only Tyr-368 (Table 1, Figure 6a and b). Interestingly, these nonsense mutants, unlike EPOR p.Pro434* and p.Gln444*, were able to confer EPO hypersensitivity to Ba/F3 cells (Figure 5c). These results show that extensive truncations, unlike shorter ones, are sufficient per se to induce the EPO hypersensitivity phenotype. In comparison, frameshift

*EPOR* mutations-induced EPO hypersensitivity is due to a distinct common mechanism based on the appearance of new AA sequences.

**Discussion**

In this study, we identified a new germline heterozygous *EPOR* mutation, c.1300dup (p.Gln434Profs*11) in a patient suffering from PFCP. Like the other mutations already described in this pathology, c.1300dup is located in the exon 8 of *EPOR*. The frameshift generates a new cytoplasmic tail of 10 AA and a premature stop codon at position 444 leading to the loss of 64 AA in the C-terminal part of the receptor. Two other mutations, c.1281dup (p.Ile428Tyrfs*17) [7] and c.1288dup (p.Asp430Glyfs*15) [4], leading to a similar truncation at position 444 had been previously reported but, as for most other EPOR mutants, no extensive functional study had been carried out. Here, we show that EPOR p.Gln434Profs*11 is a strong gain-of-function mutant that induces a major EPO hypersensitivity in primary cells as well as in transduced Ba/F3 cells, without spontaneous growth, in accordance with the diagnosis of PCFP.

Previous reports assumed that the EPO hypersensitivity phenotype observed in PFCP is due to the loss of negative regulatory domains located in the C-terminal part of the receptor, especially some conserved tyrosine residues.[30,31] Both magnitude and duration of EPOR signaling are indeed crucial for erythropoiesis and are therefore tightly regulated by several mechanisms induced as soon as EPO binds to its cognate receptor. A classic negative feedback loop is achieved by signaling inhibitors such as the tyrosine phosphatase SHP-1 [38] and the Suppressor of Cytokine Signaling (SOCS) proteins SOCS3 and CIS [39], which bind the conserved Tyr-426, -454 and -456 on the cytoplasmic tail of EPOR (Figure 6a). The rapid internalization and the degradation of the receptor induced by EPO binding [34] also

contribute to the negative regulation of EPOR signaling. Several possible cooperative and partially redundant mechanisms are involved in this process, but their relative contribution remains unclear. The C-terminal part of EPOR is degraded at the cell surface by the proteasome .[34,35] This step requires the binding of the E3-ligase βTrcp to a conserved motif Asp[461]-Ser[462]-Gly[463] of EPOR (Figure 6a).[36] The complex EPO-EPOR is then internalized in a process depending on the Tyr-454, -456 and -504 and the p85 subunit of the PI3K, and targeted to the lysosomes for degradation (Figure 6a).[30,31,35] The loss of these negative signaling regulatory and degradation domains due to EPOR truncation is usually thought to explain EPO hypersensitivity in PFCP.[6,7,14,27,29-31,36,40] In the present study, we studied two extensive EPOR truncations, p.Glu399* and p.Glu425*, which are lacking 7 of the 8 conserved tyrosine residues (Table 1, Figure 6b) and all sequences that constitute the EPOR negative regulatory domains. These mutants conferred a major EPO hypersensitivity to Ba/F3 cells compared to EPOR WT. Thus, confirming previous reports, we showed that extensive truncations lacking all EPOR negative regulatory sites are sufficient in themselves to induce the PFCP phenotype.

Yet, our results highlighted that a different mechanism is underlying EPO hypersensitivity due to frameshift *EPOR* mutations. The study of EPOR p.Gln434Profs*11 (EPOR FS) and EPOR p.Pro438Metfs*6 and their designed non-sense counterparts, EPOR p.Gln444* (EPOR STOP) and EPOR p.Pro434*, respectively, allowed us to discriminate between the effects due to the truncations in itself leading to the loss of SHP-1 and SOCS3 binding sites and to the appearance of a new cytoplasmic tail. In accordance with the loss of negative regulatory sites and previous reports [9,14,27,29], EPOR FS and EPOR STOP Ba/F3 cells displayed a persistent phosphorylation of STAT5 after EPO removal, but only EPOR FS was able to induce constitutive STAT5 phosphorylation and EPO hypersensitivity. In a similar way to EPOR

p.Gln434Profs*11 (EPOR FS), Ba/F3 cells expressing EPOR p.Pro438Metfs*6 displayed EPO hypersensitivity, unlike EPOR p.Pro443*, suggesting a common mechanism for the frameshift *EPOR* mutations in PFCP due to the presence of new partially common cytoplasmic tail (MDTVP). Of note EPOR p.Pro443* and p.Gln444* have never been identified in PFCP patients. Besides, a previously described murine mutant that is missing the last 40 AA and displays a new cytoplasmic tail, which does not include the MDTVP motif is also responsible for EPO hypersensitivity. This suggests that other sequences of AA could be involved in the EPO hypersensitivity .[41] Furthermore, another mutant, EPOR p.Trp439*, was found in a large family of erythrocytosis with a mild phenotype probably associated with an incomplete penetrance of the mutation.[3] This finding suggests that our *in vitro* cellular model may be insufficiently sensitive to unmask weak functional effect or alternatively that the wild-type WTLCP motif may act as negative regulator of the EPO sensitivity.

We also demonstrated that the increased stability and the higher localization at cell surface of EPOR FS compared to EPOR WT and EPOR STOP were not due to the loss of internalization sites located in the cytoplasmic tail of the protein as these receptors displayed similar internalization pattern. These results suggest a better addressing at the cell surface, an increased stability or a higher recycling of EPOR FS. Moreover, we found that EPOR FS increased the basal dimerization of the receptor correlating with the spontaneous activation of JAK2 and of the downstream signaling. Two recent studies have emphasized the role of the EPOR dimerization and conformation in the signal transduction. Indeed, while EPO R150Q mutant was unable to mediate a full signaling due to the decrease of EPOR dimerization and in JAK2 activation [42], alteration of EPOR conformation by diabodies was able to finely tune the receptor signaling. [43] The conformation of EPOR is indeed crucial for its optimal activation. In absence of ligand, inactive EPORs dimers are preformed at the cell

surface [44] through their transmembrane (TM) domains.[45] EPO binding to its receptor induces conformational changes like the formation of a 120° angle between the D1 domains of the two EPOR molecules [46] and the reorientation of the continuous rigid alpha helix formed by the TM domain and the cytosolic juxtamembrane (JM) region that contains a rigid hydrophobic motif, composed of residues Leu278, Ile282 and Trp283.[47-49] It has been shown that the orientation of this conserved motif is crucial for JAK2 activation and JAK2-induced EPOR phosphorylation.[47] Other active EPOR conformations have been described but this particular dimer orientation is the only one optimal for signaling.[50] As EPOR is not permissive for self-activation in terms of conformation, the appearance of a new cytoplasmic tail due to frameshift mutations might also induce a reorientation of EPOR TM and/or JM domains, leading to the pre-activation of the EPOR-JAK2 complex at the cell surface.

To our knowledge this is the first extensive functional study of *EPOR* mutations in PFCP. As suggested previously, we demonstrated here that different mechanisms, according to the type and location of *EPOR* mutations, contribute to the EPO hypersensitivity phenotype. Extensive truncations lacking all negative regulatory and degradation domains are sufficient by themselves to confer the EPO hypersensitivity, whereas more distal truncations induced by frameshift mutants confer EPO hypersensitivity that depends on the appearance of a new C-terminal tail. The latter, by increasing EPOR dimerization and stability at the cell surface, cause pre-activation of EPOR and JAK2, constitutive signaling and hypersensitivity to EPO close to that of *JAK2*V617F-positive PVs.

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**Authorship Contributions**

**IP, CBC** designed and directed the work; **FP** performed most of research and analyzed data; **CM, SG** performed research (Western blot), **FV** performed research on EPO binding experiments; **CP** co-identified EPOR mutant in the patient. **VG** performed experiments. **SNC** contributed to intellectual input, **NC** was involved in clinical part, **WV, HR,** contributed to intellectual input and all authors contributed to writing and editing

**Conflict of Interest**

The authors declare no competing financial interests.

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## Table

**Table 1. *EPOR* mutations studied in this paper and their functional consequences.**

| Mutation (DNA) | Mutation (protein) | Truncation (number of AA lost) | Number of remaining tyrosines | Reference |
|---|---|---|---|---|
| c.1195G>T | p.Glu399* | 109 | 1 | Arcasoy *et al.* [11] |
| c.1273G>T | p.Glu425* | 83 | 1 | Kralovics *et al.* [10] |
| c.1311_1312del | p.Pro438Metfs*6 | 65 | 2 | Bento *et al.* [25] |
| c.1327_1329delinsTAA | p.Pro443* | 65 | 2 | this paper |
| c.1300dup | p.Gln434Profs*11 (EPOR FS) | 64 | 2 | this paper |
| c.1330G>T | p.Gln444* (EPOR STOP) | 64 | 2 | this paper |

Abbreviations: *EPOR*: erythropoietin receptor gene (NM_000121.3), AA: amino acid

**Figure Legends**

**Figure 1. Study of the *EPOR* c.1300dup (p.Gln434Profs*11) in primary cells.**

(**A**) Electropherograms showing *EPOR* c.1300dup mutation in hematopoietic cells ($CD3^+$ T-lymphocytes and neutrophils) and in non-hematopoietic tissues (nails, hair and buccal swab). (**B**) Scheme of EPOR wild-type and of the new frameshift EPOR mutants (EPOR FS). Negative signaling regulators (on the left) and internalization/degradation sites (on the right) are indicated. The tyrosine (Y) number of the mature EPOR is also indicated in parenthesis. (**C**, **D**) Effect of EPO concentration on erythroid colony formation in presence (**c**) or absence (**D**) of fetal bovine serum. BFU-E colonies were counted at day 14. Each experiment was performed twice in duplicate. The results are expressed in percentages of the number of colonies at 1 U/mL of EPO. (**E**) Effect of EPO concentration on EPOR signaling in erythroblasts. After 7 to 10 days in culture with SCF, IL3 and EPO, $CD34^+$ cells from the patient or healthy donors were cytokine-starved for 5 hours 15-minute stimulations with increasing concentrations of EPO. JAK2 and STAT5 phosphorylations were examined by western blotting. One of three independent experiments is presented and fold activation are indicated below. (**F**) Persistence of JAK2 and STAT5 phosphorylation in erythroblasts. After 7 to 10 days in culture with SCF, IL3 and EPO, $CD34^+$ cells from the patient or healthy donors were cytokine-starved for 5 hours prior to 15 minutes of stimulation with EPO 1U/mL. Cells were then washed to remove EPO and cultured in absence of cytokine or serum. JAK2 and STAT5 phosphorylations were examined by western blotting in a time-dependent manner: after 5 hours of starvation (-), after EPO stimulation (+EPO) and at different times after EPO removal (10 min, 30 min, 1h and 4h). One out of two independent experiments is presented and fold activation are indicated below.

**Figure 2. Functional study of *EPOR* c.1300dup (p.Gln434Profs*11) in Ba/F3 cell line.**

(**A**) Ba/F3 cells were transduced with pMX-HA-huEPOR-IRES-GFP retrovirus to stably express the wild-type receptor (EPOR WT), a truncated mutant at position 444 (p.Gln444*, EPOR STOP) or the frameshift mutant *EPOR* c.1300dup (p.Gln434Profs*11, EPOR FS). (**B**) Proliferation was assessed 48 hours after culturing Ba/F3-EPOR cells in absence or in presence of increasing doses of EPO (0.01, 0.02, 0.03, 0.05, 0.1, 0.3, and 1 U/mL) by WST-1 proliferation assay. Dose-response curves are means expressed in percentages of maximum growth value ± SEM (n = 3 in triplicate). Two-tailed *t*-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (**C**) Effect of EPO concentration on EPOR signaling. Ba/F3 cells expressing different EPOR constructs were examined by western blotting for the presence and phosphorylation status of various signaling molecules. Cells were serum- and cytokine-starved for 5 hours prior to 15-minute stimulation with increasing doses of EPO (0, 0.001, 0.01, 0.1 and 1 U/mL). Expression of β-actin was used as loading control. One of three independent experiments is presented. (**D**) Phosho-STAT5/actin spontaneous phosphorylation were quantified using Image J compared to WT. Results represent the mean ± SEM (n = 3). Two-tailed *t*-test, *p<0.05, ***p<0.001, (**E**) Persistence of STAT5 phosphorylation. Ba/F3-EPOR cells were serum- and cytokine- starved for 5 hours prior to 15 minutes of stimulation with EPO 1U/ml. Cells were then washed to remove EPO and cultured in absence of cytokine or serum. STAT5 phosphorylation was examined by western blotting in a time-dependent manner: after 5 hours of starvation (-), after EPO stimulation (+EPO) and at different times after EPO removal (5 min, 30 min, 1h, 2h and 4h). One out of two independent experiments is presented.

**Figure 3. Effects of c.1300dup (p.Gln434Profs*11) mutation on EPOR stability, dimerization and cell surface expression**

(**A**) Cell-surface expression of the different EPORs was assessed by flow cytometry using PE fluorescent labeling of the extracellular HA-tag. Histogram shows the ratio of mean fluorescence intensities (MFIs) of PE-labeled cell-surface EPO on their respective MFIs of GFP. Results are the mean ± SEM of 7 independent experiments. (**B**) Cell-surface expression of the different EPORs was assessed with radiolabeled $^{125}$I-EPO. The results are expressed in cpm normalized to EPOR WT. The number of cell-surface receptors was determined by comparison between the radioactivity of transduced Ba/F3 cells and parental UT-7 cells that express 7000 receptors. (**C**) EPOR stability. Ba/F3-EPOR cells were incubated with cycloheximide during different time (0 min, 15 min, 30 min, 1h, 2h, 4h, 6h) and HA expression was studied by western blotting. A quantification of HA-EPOR and β-actin was performed using Image J software. The curves represent the HA/β-actin ratios. Three independent experiments were done. (**D**) Schematic representation of split Gaussia princeps luciferase complementation assay used to test EPOR dimerization in HEK293-derived BOSC cells. (**E**) Dimerization of human EPOR monomers was assessed by split Gaussia luciferase assay in steady-state conditions, in the absence of EPO in HEK cells. Close proximity between the C-terminal cytosolic domains of EPOR is significantly promoted by EPOR FS. The results represent the mean ± SEM from 3 independent experiments, each performed with 8 biological replicates per condition. For each experiment, raw values were normalized to the average of the EPOR WT condition before pooling the data of all experiments together. Unpaired two-tailed *t*-test with Welch's correction, **** p<0,0001.

**Figure 4. Role of the diLeucine motif (diL) loss in *EPOR* c.1300dup (p.Gln434Profs*11, EPOR FS) mechanism**

(**A, B**) EPOR internalization was performed with radiolabeled $^{125}$I-EPO. Cells were incubated with $^{125}$I-EPO and washed at 4°C to remove unbound ligand. An acidic wash was then performed to separate cell surface–bound from internalized EPO. The radioactivity levels of the supernatant (cell surface–bound EPO) (**A**) and of the cell pellet (internalized EPO) (**B**) were determined. Each experiment was performed 3 times with similar results. (**C**) To study the potential role of the dileucine motif loss in the EPO hypersensitivity phenotype induced by EPOR FS, Ba/F3 cells were transduced with pMX-HA-huEPOR-IRES-GFP retrovirus to stably express EPOR WT or EPOR STOP receptor with abrogation of the dileucine motif, EPOR WT/diL and EPOR STOP/diL respectively. (**D**) Proliferation was assessed 48 hours after culturing Ba/F3-EPOR cells in absence or in presence of increasing doses of EPO (0.01, 0.02, 0.03, 0.05, 0.1, 0.3, and 1 U/mL) by WST-1 proliferation assay. Dose-response curves are means expressed in percentages of maximum growth value ± SEM (n = 3 in triplicate). Two-tailed *t*-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (**E**) Cell-surface expression of the different EPORs was assessed by flow cytometry using PE fluorescence labeling of the extracellular HA-tag. Histogram shows the ratio of mean fluorescence intensities (MFIs) of PE-labeled cell-surface EPOR on their respective MFIs of GFP. Results are the mean ± SEM of 3 independent experiments. (**F**) Effect of EPO concentration on EPOR signaling. Ba/F3 cells expressing different EPOR constructs were examined by western blotting for the presence and phosphorylation status of various signaling molecules. Cells were serum- and cytokine-starved for 5 hours prior to 15-minute stimulation with increasing doses of EPO (0, 0.001, 0.01, 0.1 and 1 U/mL). Expression of β-actin was used as loading control. One of three independent experiments is presented and fold activation are indicated below.

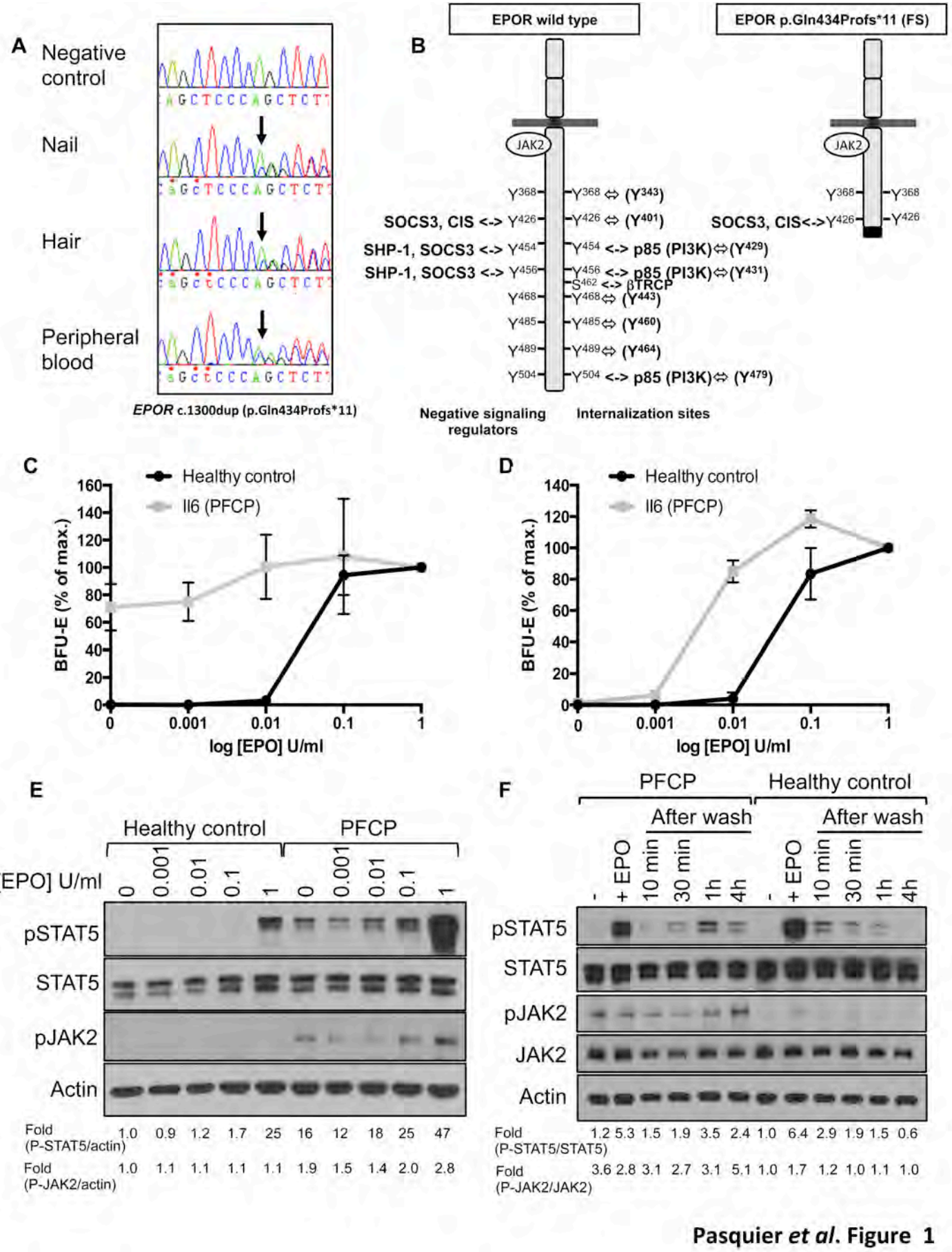
**Figure 5. Different mechanisms are involved in the EPO hypersensitivity phenotype of PFCP depending of the type of EPOR mutation.**

(**A**) Ba/F3 cells were transduced with pMX-HA-huEPOR-IRES-GFP retrovirus to stably express different kind of *EPOR* mutations already described: the frameshift *EPOR* c.1311_1312del mutant (p.Pro438Metfs*6) or its nonsense designed counterpart *EPOR* c.1327_1329delinsTAA (p.Pro443*), more proximal truncations due to the nonsense mutants *EPOR* c.1195G>T (p.Glu399*) and c.1273G>T (p.Glu425*). (**B, C**) Proliferation was assessed 48 hours after culturing EPOR p.Pro438Metfs*6 and p.Pro443* Ba/F3 cells (**B**) or EPOR p.Glu399* and p.Glu425* Ba/F3 cells (**C**) in absence or in presence of increasing doses of EPO (0.01, 0.02, 0.03, 0.05, 0.1, 0.3, and 1 U/mL) and compared to EPOR WT and EPOR FS growth by WST-1 proliferation assay. Dose-response curves are means expressed in percentages of maximum growth value ± SEM (n = 3 in triplicate). Two-tailed *t*-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

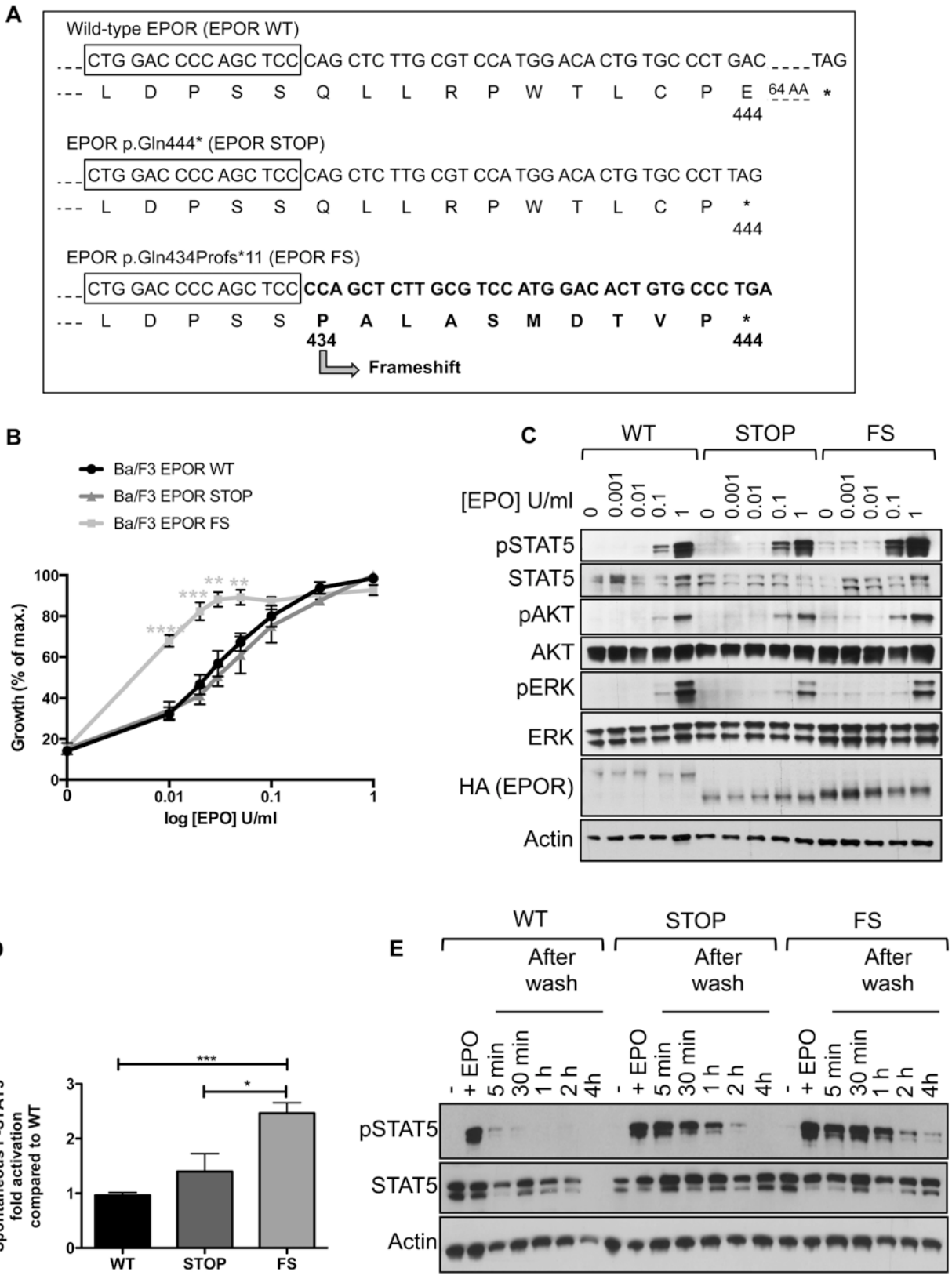
**Figure 6. Regulation of EPOR signaling and positions of the EPOR truncations.**

(**A**) Negative regulators of EPOR signaling and their binding sites in the C-terminal part of the receptor: negative signaling regulators (on the left) and internalization/degradation sites (on the right). The tyrosine (Y) number of the mature EPOR is also indicated in parenthesis. (**B**) Proximal truncations due to EPOR p.Glu399* and p.Glu425* are lacking all negative regulation sites of the receptor. (**C and D**) More distal truncations due to frameshift EPOR mutants and their designed counterparts retain the Tyr-426.

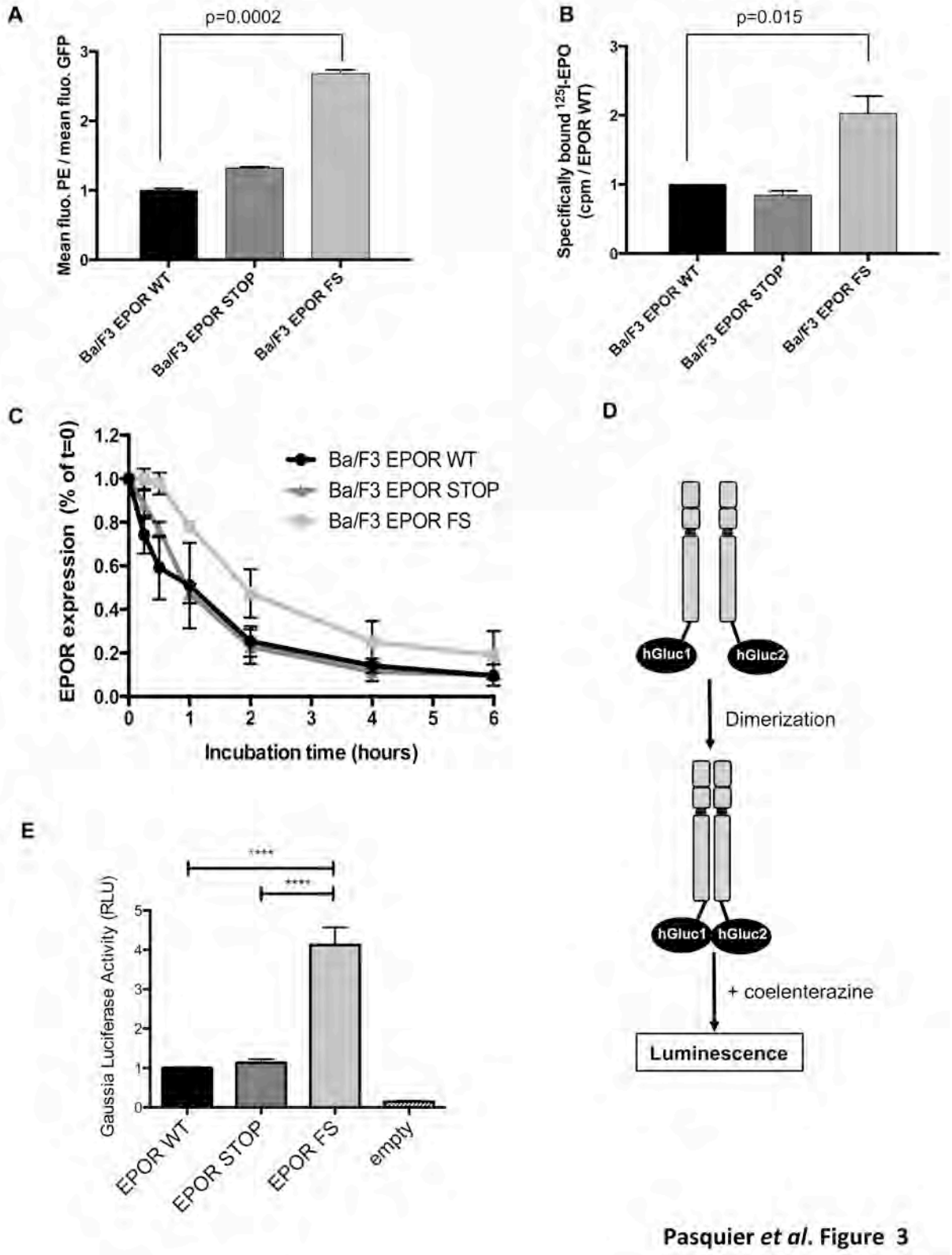
**Figures**



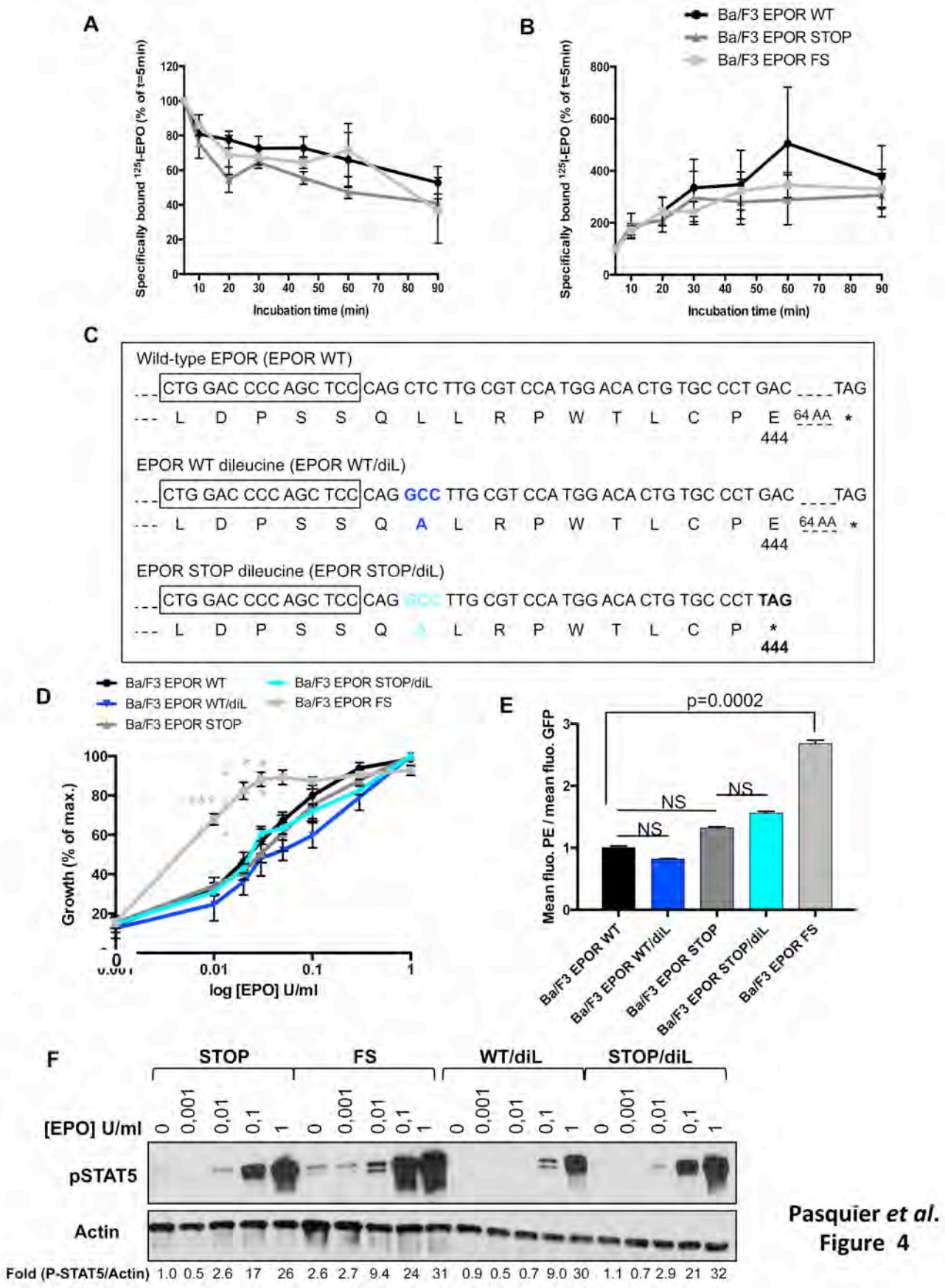

**Pasquier *et al*. Figure 1**



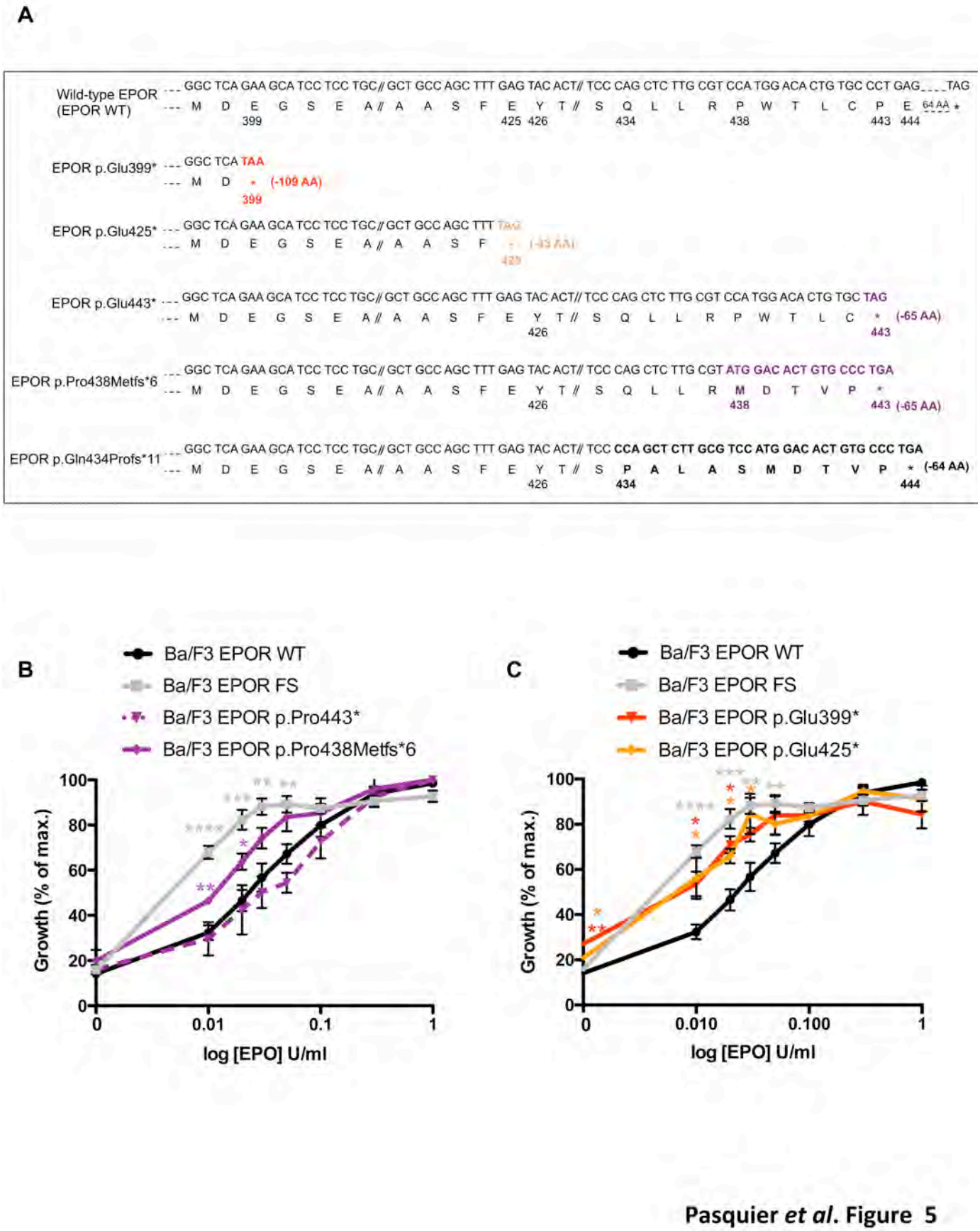

**Pasquier *et al*. Figure 2**



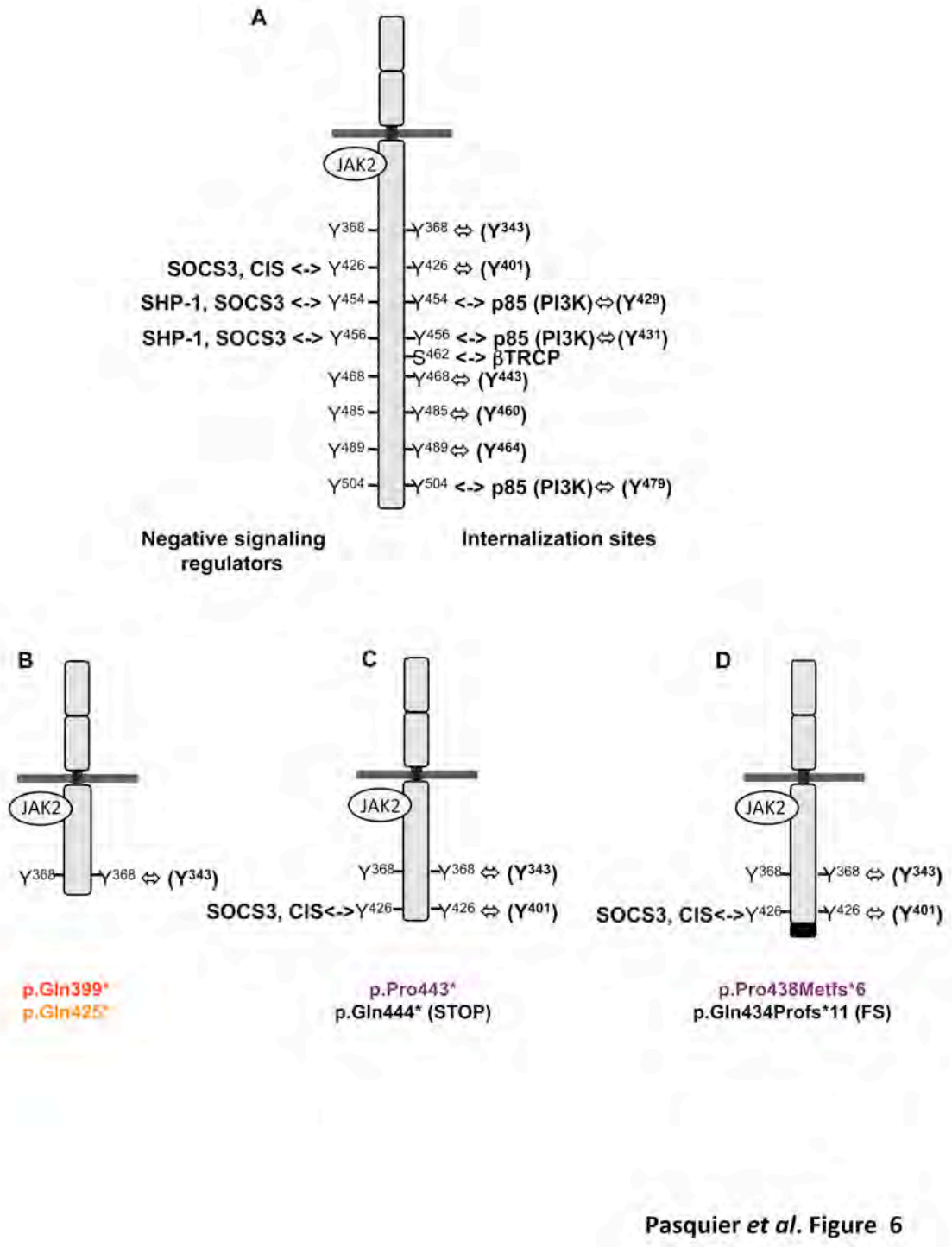

**Pasquier *et al*. Figure 3**




**Pasquier *et al.***
**Figure 4**




**Pasquier *et al*. Figure 5**




**Pasquier *et al*. Figure 6**

# New pathogenic mechanisms induced by germline erythropoietin receptor mutations in primary erythrocytosis

**Running title:** Erythropoietin receptor mutations in PFCP

**Florence Pasquier [1,2,3,4], Caroline Marty [1,2,4], Thomas Balligand [5] Frédérique Verdier [4,6], Sarah Grosjean [1,2,4], Vitalina Gryshkova [5] , Hana Raslova [1,2,4] , Stefan N. Constantinescu [5], Nicole Casadevall [1,7], William Vainchenker [1,2,4], Christine Bellanné-Chantelot [1,2,8*], Isabelle Plo [1,2,4*]**

**Supplemental figures**

Figure S1

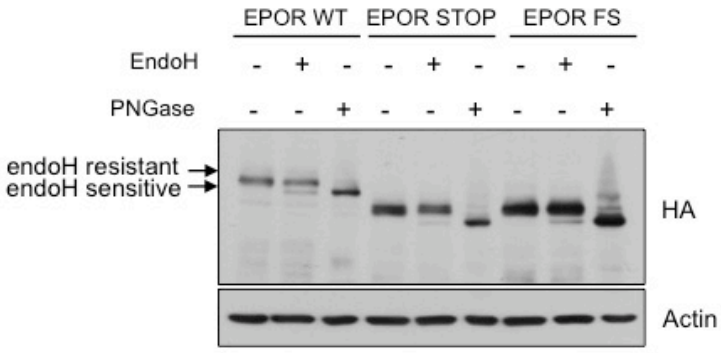
The glycosylation state of the HA EPOR WT, HA-EPOR STOP, HA-EPOR FS was investigated in Ba/F3 cells. Cell lysates were treated with either Endo H or PGNase glycosidase overnight at 37°C and then were analyzed by Western blot. Endo H-resistant HA-EPOR and Endo H-sensitive HA-EPOR are indicated by arrows.

Figure S2

(**a**) UT7 cells were transduced with pMX-HA-huEPOR-IRES-GFP retrovirus to stably express the wild-type receptor (EPOR WT), a truncated mutant at position 444 (p.Gln444*, EPOR STOP) or the frameshift mutant *EPOR* c.1300dup (p.Gln434Profs*11, EPOR FS). Proliferation was assessed 72 hours after culturing UT7-EPOR cells in absence or in presence of increasing doses of EPO (0.01, 0.02, 0.05, 0.1, 0.3, and 1 U/mL) by proliferation assay. Dose-response curves are means expressed in percentages of maximum growth value ± SD (n = 2). (**b**) Effect of increasing EPO concentration on EPOR signaling. UT7 cells expressing different EPOR constructs were examined by western blotting for the presence and phosphorylation status of various signaling molecules. Cells were serum- and cytokine-starved for 5 hours prior to 15 minute stimulation with increasing doses of EPO (0, 0.01, 0.1 and 1 U/mL). Expression of β-actin was used as loading control. One out of two independent experiments is presented.

Figure S3

Cell-surface expression of the different EPORs was assessed by flow cytometry using PE fluorescent labeling of the extracellular HA-tag. Representative histograms are shown in (**a**) Ba/F3 and (**b**) UT7 cell lines.

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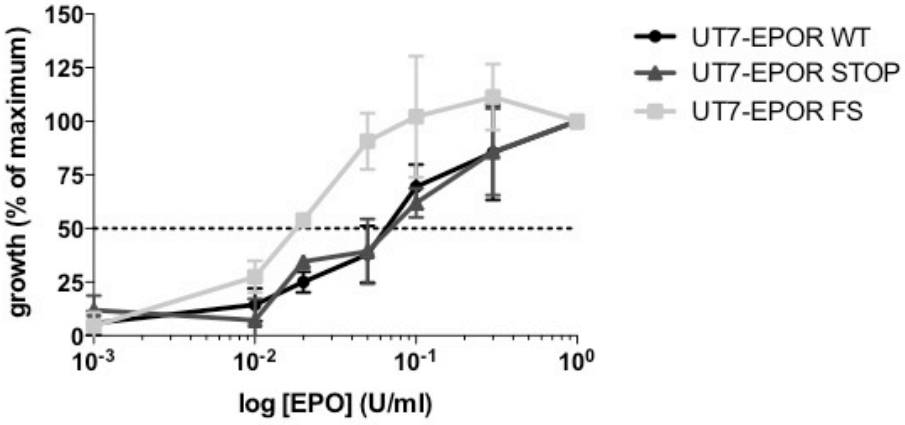
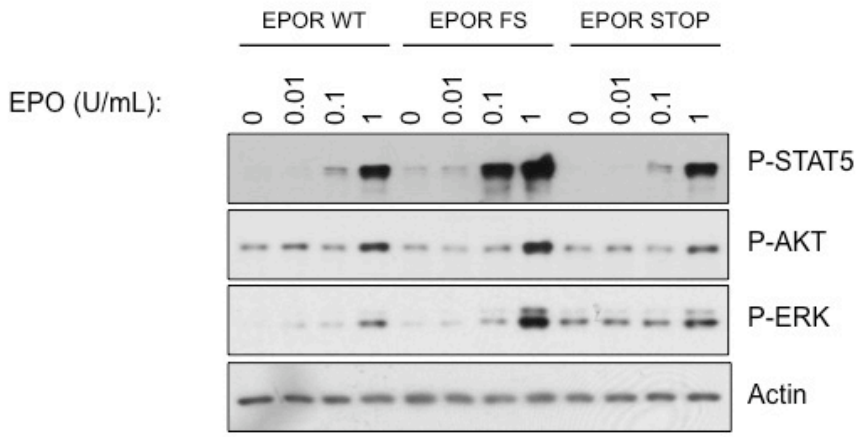
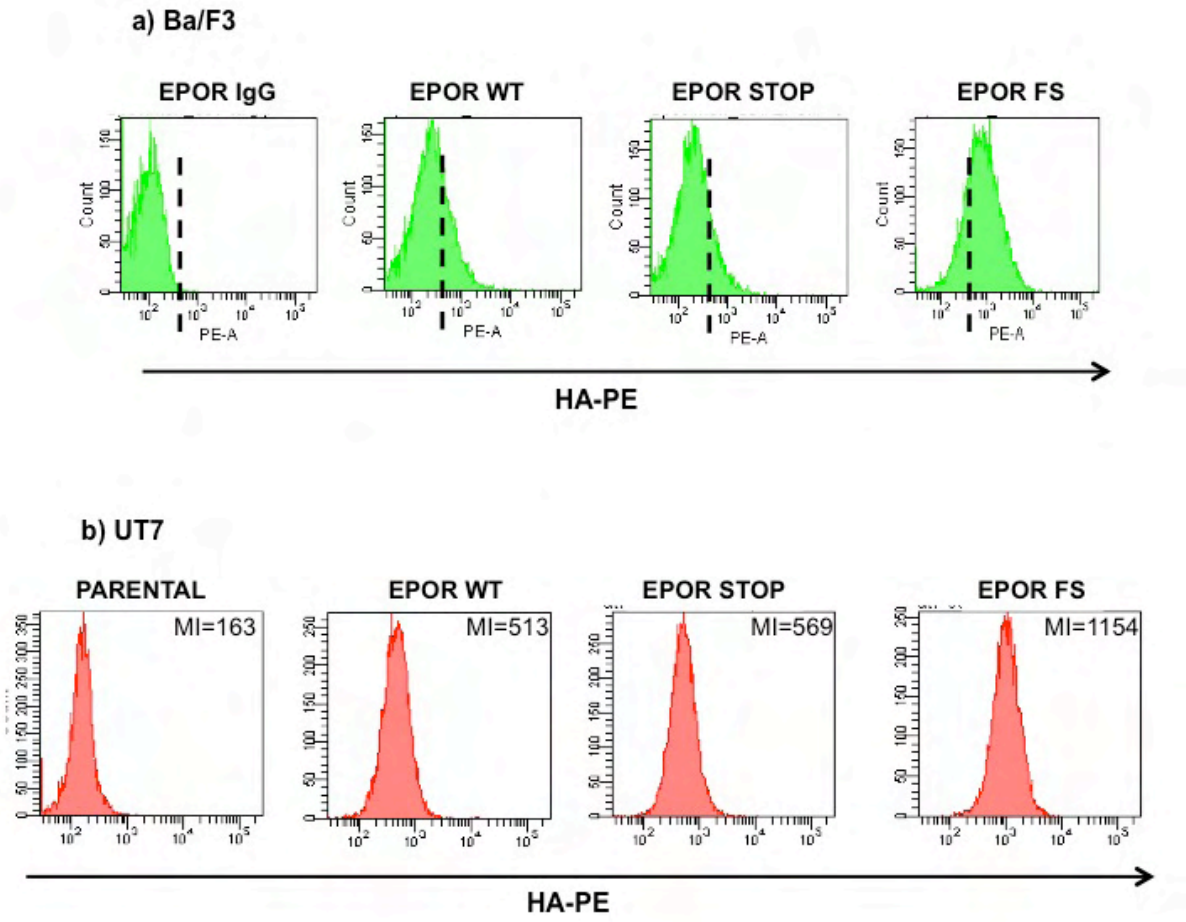
a




b




**Pasquier *et al*. Figure S2**




**Pasquier *et al*. Figure S3**