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Phospho-PTM Proteomic Discovery of Novel EPO- Modulated Kinases and Phosphatases, Including PTPN18 as a Positive Regulator of EPOR/JAK2 Signaling

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Abstract

The formation of erythroid progenitor cells depends sharply upon erythropoietin (EPO), its cell surface receptor (erythropoietin receptor, EPOR), and Janus kinase 2 (JAK2). Clinically, recombinant human EPO (rhEPO_ additionally is an important anti-anemia agent for chronic kidney disease (CKD), myelodysplastic syndrome (MDS) and chemotherapy, but induces hypertension, and can exert certain pro-tumorigenic effects. Cellular signals transduced by EPOR/JAK2 complexes, and the nature of EPO-modulated signal transduction factors, therefore are of significant interest. By employing phospho-tyrosine post-translational modification (p-Y PTM) proteomics and human EPO- dependent UT7epo cells, we have identified 22 novel kinases and phosphatases as novel EPO targets, together with their specific sites of p-Y modification. New kinases modified due to EPO include membrane palmitoylated protein 1 (MPP1) and guanylate kinase 1 (GUK1) guanylate kinases, together with the cytoskeleton remodeling kinases, pseudopodium enriched atypical kinase 1 (PEAK1) and AP2 associated kinase 1 (AAK1). Novel EPO- modified phosphatases include protein tyrosine phosphatase receptor type A (PTPRA), phosphohistidine phosphatase 1 (PHPT1), tensin 2 (TENC1), ubiquitin associated and SH3

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AUTHORSHIP CONTRIBUTIONS

All authors contributed substantively to experiment designs, execution, data acquisition, analysis, data interpretations and manuscript construction. Specific lead roles included the following: phospho-PTM proteomics – MH, MS, DMW; phosphotarget and phosphosite mining – MH, EG-A, CS; dedicated database assembly for pY- PTM data – EG-A, CS; PTPN18 constructs and cell line models – SS and DMW; cell phenotyping, SS and MH, and PTPN18 molecular mechanism studies – SS, MH, DMW; PTPN18 action models – MH, DMW. The authors also thank Ed Jachimowicz (MMCRI) and Ashley Johnson (MMCRI) for expert technical contributions with flow cytometry and western blotting.

CONFLICT OF INTEREST DISCLOSURE

No contributors or authors have conflicting interests.

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domain containing B (UBASH3B) and protein tyrosine phosphatase non-receptor type 18 (PTPN18). Based on PTPN18's high expression in hematopoietic progenitors, its novel connection to JAK kinase signaling, and a unique EPO- regulated PTPN18-pY389 motif which is modulated by JAK2 inhibitors, PTPN18's actions in UT7epo cells were investigated. Upon ectopic expression, wt-PTPN18 promoted EPO dose-dependent cell proliferation, and survival. Mechanistically, PTPN18 sustained the EPO- induced activation of not only mitogen-activated protein kinases 1 and 3 (ERK1/2), AKT serine/threonine kinase 1–3 (AKT), and signal transducer and activator of transcription 5A and 5B (STAT5), but also JAK2. Each effect further proved to depend upon PTPN18's EPO- modulated (p)Y389 site. In analyses of the EPOR and the associated adaptor protein RHEX (regulator of hemoglobinization and erythroid cell expansion), wt-PTPN18 increased high molecular weight EPOR forms, while sharply inhibiting the EPO-induced phosphorylation of RHEX-pY141. Each effect likewise depended upon PTPN18-Y389. PTPN18 thus promotes signals for EPO-dependent hematopoietic cell growth, and may represent a new druggable target for myeloproliferative neoplasms.

Keywords

EPO; EPOR; JAK2; phospho-proteomics; PTPN18; RHEX

1. INTRODUCTION

Type 1 hematopoietin receptors[1], together with their co-assembled Janus tyrosine kinases (JAKs)[2], provide insightful models for deciphering growth factor cellular and molecular signaling mechanisms. Within this receptor family, the erythropoietin receptor (EPOR) serves as an important, and tractable model as a homodimeric beta chain complex pre-assembled with paired JAK2 kinases[3,4]. EPO[5] and its receptor EPOR[6] each are essential for erythroid progenitor proliferation and survival, and consequentially, red blood cell production. rhEPO is also an important agent for the treatment of anemia associated with chronic kidney disease[7], myelodysplastic syndrome[8], and chemotherapy[9,10], but can also elicit hypertensive[11], thrombolytic[11] and certain pro-tumorigenic side effects[12], each via presently unresolved pathways. In cell signaling contexts, non-canonical EPO/EPOR effects beyond ERK1/2, AKT and STAT activation additionally have been discovered. These include EPO induction of Erythroferrone, a c1q-TNF that inhibits Hepcidin and heightens iron transport[13]; EPO suppression of fibroblast growth factor 23 (FGF23) with a resulting gain in stress erythropoiesis[14]; and erythroblast cytoprotective effects via a novel EPO/EPOR/STAT5 Spi2a (serine or cysteine peptidase inhibitor, clade A, member 3G) serpin pathway[15]. For studies of Janus kinase coupled receptors, clinical relevance also exists in the development of JAK inhibitors for the treatment of myeloproliferative neoplasms[16], myelofibrosis[17], rheumatoid arthritis[18] and atopic dermatitis[19]. Extended investigations of type 1 receptor signal transduction mechanisms therefore may further serve to identify new druggable targets that could lessen JAK inhibitor dosing, side effects[20], and drug resistance[21,22].

The latter considerations bring renewed attention to EPO's cell and molecular action mechanisms. In the human EPOR system, upstream signal transduction factors (STFs)

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include a coordinated set of molecular adaptors and associated effectors that assemble at nine human EPOR (hEPOR) cytoplasmic p-Y motifs as an EPOR/JAK2 signalosome[23–25]. Among STFs that JAK2 directly modulates, JAK2 dimers themselves first have been evidenced to cross-phosphorylate at critical p-Y sites[26]. Additional direct JAK2 targets include STAT5[27] (pY694/Y699, STAT5A/B), Histone-3 (pY41)[28], and the DNA cytosine methylase TET2 (Y1939, Y1964)[29]. For each, functional consequences of these p-Y PTMs also have been implicated[26,28,29]. Within EPOR/JAK2 coupled phospho-tyrosine signaling cascades, additional protein tyrosine kinases (PTKs) are also known to function downstream of the EPOR and JAK2. These include Lyn[30] and Fyn[31] Src kinases, Tec kinase[32], and canonical MEK/ERK and PI3K/AKT kinase signaling modules. Concomitant with the recruitment of the above positively acting STFs, EPO engages select sets of negative feedback factors. These include SH2B adaptor protein 3 (LNK)[33] and cytokine inducible SH2 containing protein (CIS)[34] adaptors that squelch JAK2 and STAT5 activation; the ubiquitin ligases suppressor of cytokine signaling (SOCS)[35], Cbl proto-oncogene (CBL)[36] and beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC)[37]; and several EPOR/JAK2 modulated phosphatases[38–46].

Known EPO/EPOR engaged phosphatases include inositol phosphatases inositol polyphosphate-5-phosphatase D (INPP5D/SHIP1)(ref.[38]) and inositol polyphosphate phosphatase like 1 (INPPL1/SHIP2)(ref.[39]), and the protein tyrosine phosphatases (PTP) PTPN6/SHP1(ref.[40]) and PTPN11/SHP2(ref.[41]). INPP5D is expressed primarily in hematopoietic cells[42] and functions predominantly as a negative regulator of hematopoietic cytokine receptor signaling[42], while INPPL1 is a more potent inhibitor of insulin signaling[39]. PTPN6 can mediate the dephosphorylation and inhibition of JAK2[43], and PTPN6-KO mice exhibit hematopoietic cell hyper-expansion[44]. The homologous SH2-PTP, PTPN11, in contrast, has been reported to act together with phosphatase and tensin homolog (PTEN) to regulate erythropoiesis[45], and GOF mutations in PTPN11 can act as drivers of juvenile monomyelocytic leukemia[46]. With one primary goal of discovering new kinases and phosphatases engaged upon EPOR ligation, we presently have applied phospho-tyrosine (p-Y) directed post-translational modification (PTM) proteomic profiling. To provide sufficient cells for effective PTM liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses, a unique EPO-dependent human erythroid progenitor UT7epo cell line model was employed[47]. This approach (together with data mining for each defined target) identified 17 novel EPO- modulated pY-modified kinases and 5 novel EPO- and pY- modulated phosphatases. Novel EPO- targeted pY-PTM modified kinases include MPP1(refs.[48–50]) and GUK1(ref.[51]) guanylate kinases; PEAK1 tyrosine pseudokinase and AAK1 S/T kinase[52–54]; and the ERK module kinases misshapen like kinase 1 (MINK1)(refs.[55,56]) and mitogen-activated protein kinase 7 (MAPK7)(refs.[57,58]). This target set implicates new signaling circuits for EPO dependent cell proliferative, stress and cytoskeletal responses.

Diverse new EPO- modulated phosphatases included the receptor PTP PTPRA[59–61]; the histidine phosphatase PHPT1(ref.[62]); the RhoA regulating phosphatase TENC1(ref.[63]); and two PTPs implicated in guiding protein ubiquitination, UBASH3B(ref.[64]) and PTPN18(ref.[65]). Among these, PTPN18(ref.[65]) is a PEST domain PTP that is highly expressed in hematopoietic progenitor cells[66], but has not previously been associated with type 1 hematopoietin receptors, or JAK kinase signaling. As we presently describe, PTPN18's

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novel EPO regulated p-Y389 site (and motif) additionally are unique among PTPs. Possible effects of ectopically expressed wt-PTPN18 and a PTPN18-Y389F construct, on UT7epo cell proliferation and survival therefore were investigated. wt-PTPN18 heightened EPO dose-dependent UT7epo cell proliferation and survival, while also sustaining the EPO activation of not only ERK1/2, AKT and STAT5, but also JAK2. wt-PTPN18 furthermore increased levels of high molecular weight EPOR forms, while limiting the EPO-induced p-Y phosphorylation of the EPOR- associated plasma membrane adaptor protein, RHEX[67]. Notably, each of these PTPN18 effects proved to depend upon intactness of PTPN18's EPO-regulated p-Y389 site. In erythroid progenitor cells, PTPN18 therefore is revealed to heighten EPO's pro-proliferative and cytoprotective effects via novel EPOR/JAK2 molecular action mechanisms.

2. METHODS

2.1. Erythroid cell culture and phospho-PTM proteomics

In phospho-tyrosine PTM proteomic analyses, UT7epo cells were plated at 1×10^5 cells/mL in IMDM (HyClone, #SH30228.01), 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate ("P/S"), with rhEPO at 2.5 U/mL (Procrit®, Amgen). Cells were grown to log-phase (~7×10^5 cells/mL), collected (500 × g, 5 minutes), washed twice in one-half culture volumes of phosphate buffered saline (PBS, Invitrogen #07905), 2% FBS, and cultured in IMDM, 10% FBS, P/S, for 20 hours without EPO. For phospho-PTM profiling, cells were stimulated in parallel for 15 minutes with EPO (5 U/mL) or PBS (equivalent 0.002 volume). Cells (1×10^8 UT7epo cells per sample) were then transferred to an equal volume of 2°C PBS, incubated on ice for 5 minutes, and collected (2°C, 500 x g, 8 minutes). Pellets were washed twice in 50 mL 2°C PBS, flash-frozen and stored at −80°C. For phospho-PTM LC-MS/MS proteomic studies, cell pellets were lysed in 10 mL of 9M urea, 20 mM HEPES, pH 8.0 (containing 500 μM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM betaglycerophosphate), sonicated and cleared by centrifugation at 20,000 × g for 15 minutes. Extracted proteins were then reduced (4.5 mM dithiothreitol), incubated at 55°C for 30 minutes, cooled to room temperature, and alkylated with iodoacetamide (1.9 mg/mL, 15 minutes in darkness, room temperature). Reduced and alkylated proteins were then digested with trypsin. Peptides were then acidified using trifluoroacetic acid, desalted using C18 columns (Waters Corp); and eluted in 40% acetonitrile, 0.1% trifluoroacetic acid.

Phospho-tyrosine peptides next were immunoadsorbed to anti-phosphotyrosine antibody-conjugated immunobeads (anti-pY-1000, CST, #8954), and eluted from washed beads. In LC-MS/MS analyses, tandem spectra of samples were collected in a data-dependent manner with a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer. Replicate injections of each sample were run non-sequentially. Peptides were eluted via a 90-minute linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min. MS/MS spectra were evaluated using SEQUEST and Harvard University's Core platform[68–70] and files searched against the SwissProt *Homo sapiens* FASTA database. Reverse decoy databases were included for all searches to estimate false discovery rates (FDR), and filtered using a 2.5% FDR in the Linear Discriminant module of Core. A mass accuracy of +/−5

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ppm was used for precursor ions and 0.02 Da for product ions. Enzyme specificity was limited to trypsin, with at least one tryptic (K- or R-containing) terminus required per peptide and up to four mis-cleavages allowed. Cysteine carboxamidomethylation was specified as a static modification, oxidation of methionine and phosphorylation on serine, threonine, or tyrosine residues were allowed as variable modifications. Peptides were further filtered by a −/+ 5ppm mass error range and presence of a phosphorylated amino acid. Quantification was performed using Skyline V3.7 (ref.[71]), and confirmed via manual review of peaks in ion chromatogram plots for all peptides within this study.

2.2. Bioinformatics and in silico mining of EPO modulated p-Y modified kinases and phosphatases

EPO- modulated phospho-PTM peptides and parent kinase and phosphatase proteins were assembled into an overall dataset using confirmed annotations (see Supplemental Table S1). Matched phospho-peptide sequences to LC-MS/MS ion spray spectra and those modulated 2.0 fold or greater were defined as EPO targets. In addition, LC-MS/MS phospho-PTM datasets were uploaded to an upgraded ErythronDB to provide for public access, and further data mining (https://www.cbil.upenn.edu/ErythronDB_alpha/app). In assessments of connections of EPO-modulated p-Y modified kinases and phosphatases to EPOR, JAK2, and STAT5 (core EPO signaling components), STRINGdb[72] (version 10.5) was employed. Connections (edges) were defined using first-shell interactors, and the network was filtered for edges supported by “experiments” and “database curated” evidence. An added filter for edges supported by a confidence level of ≥ 0.4 was also applied. Additional known EPO-modulated phosphatases not identified by trypsin-based LC-MS/MS (PTPRC, PTPRG, PTPN1, and PTPN6) were added to this network to explore possible extended connectivities. For domain maps of individual proteins, determination of novel (or known) phosphorylated sites due to EPO dosing (or other cytokines, drugs) was performed by data mining using UniProt, GeneCards, Phosphosite-Plus, NCBI Blast, NCBI Protein, NCBI COBALT, Google, Google Scholar and PubMed resources. In the mining of data from JAK2 inhibitor studies[73], phosphatase targets modulated at phosphorylation sites ≥ 2-fold were considered, and average fold-changes +/− SE due to JAK2 inhibitors NVP-BSK805 and NPV-BBT594 were determined. In the assessment of transcript expression levels of novel EPO-modulated kinases and phosphatases in primary human erythroid cells, mining of NCBI GeoProfiles (GDS3860) was performed, including the determination of predominant expression patterns of these kinases and phosphatases at early (CFUe/proerythroblast) or late stages (mid/late erythroblast) of erythropoiesis. In primary murine cells, the ErythronDB resource (https://www.cbil.upenn.edu/ErythronDB/) was utilized to profile kinase and phosphatase expression in primitive (yolk sac), fetal liver, or adult definitive (bone marrow) cells.

2.3. Lentivirus preparations, and UT7epo transductions

In studies of effects of the ectopic expression of PTPN18 in UT7epo cells, cDNAs encoding either wild-type PTPN18, or PTPN18-Y389F were cloned at the MCS of the lentivirus template plasmid DNA pCDH-MSCVMCS- EF1α-Puro (SBI, #CD710B-1). For each, codons for a C-terminal FLAG epitope tag were included. As a negative control, empty vector (“EVEC”) lentivirus also was prepared. In transfections, early passage 293TN cells (SBI, #LV900A-1) were plated at 20% confluency in DMEM, 10% FBS on collagen-coated

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100 mm dishes. For each transfection, 60 μL of Fugene-6 (Promega, #E2692) was added dropwise to 230 μL DMEM.

Lentivirus template pDNAs (12.9 μg) were then combined with pGAG/POL (6.6 μg), pREV (3.6 μg) and pVSVg (2.7 μg) plasmids in 100 μL DMEM. Combined pDNAs were added dropwise to Fugene-6/DMEM, and incubated at room temperature for 30 minutes. Fugene-6/DMEM/pDNA preparations were then added dropwise to 293-TN cells, with gentle swirling. At 18 hours of culture, 50% of medium was removed, and replaced. At 55 hours post transfection, supernatants containing lentiviruses were recovered, brought to 4°C on ice, and cleared by stepwise centrifugation (600 × g for 10 minutes and 1800 × g for 10 minutes, 4°C). Lentiviruses were then filtered (0.45 micron filter, VWR #87006–070), and collected by centrifugation (18,000 × g for 4 hours at 2°C). Following the complete removal of supernatants, lentiviruses were reconstituted in 0.01 volume of PBS, 0.1% BSA (StemCell Technologies, #09300).

For transductions, UT7epo cells were plated in a 12-well format at 1×10^5 cells/mL in IMDM, 10% FBS, 2.5 U/mL EPO and cultured overnight. Lentiviruses were then added to cells, followed by addition of polybrene (8 μg/mL, final concentration). At 24 hours post transduction, cells were recovered, expanded for 3 days, and then replated (at 1×10^5 cells/mL) in IMDM, 10% FBS, 2.5 U/mL EPO with puromycin at 2.5 μg/mL. Cells transduced at levels providing for 10% to 30% survival (post transduction and selection) were then expanded as stably transduced sublines by selection for two weeks in puromycin (2.5 μg/mL).

2.4. Cell and molecular assays

For UT7epo cells and derived stably transduced PTPN18, PTPN18-Y389F and EVEC sublines, short-term (3 day) and long-term (21 day) cell proliferation assays were performed as follows. Exponentially growing cells (in EPO at 2 U/mL) were collected (500 × g, 8 minutes), washed twice in PBS, 1% FBS and adjusted to 1×10^5 cells/mL in IMDM, 10% FBS with EPO at 0, 0.15, 0.3, 0.6, 1.2 or 2.4 U/mL. For 3-day assays, viable and dead cell numbers were determined by staining with EthD-1 (Invitrogen, #E1169) and using MOXI Go II benchtop flow cytometer analysis (OrFlo Technologies). In extended growth assays, cells were cultured in EPO at 0.3 U/mL, and on days 4, 8, 12 and 16 were sub-cultured at matched volumes as 1.0 mL cells plus 0.5 mL medium. On day 21, viable cell numbers were determined by hemocytometer counts (0.1% trypan blue). In 24-hour EPO withdrawal assays, frequencies of apoptotic cells were assayed by Annexin-V-FITC and propidium iodide staining, and flow cytometry (MACSQuant Analyzer 10 cytometer, Miltenyi Biotec, 50,000 events).

In molecular signaling studies of UT7epo cells transduced with wild-type PTPN18, PTPN18-Y389F or empty vector (“EVEC”) expression constructs, exponentially growing cells were washed twice in PBS, 1% FBS and cultured at 7×10^5 cells/mL in IMDM, 10% FBS (without EPO) for 20 hours. Cells were then challenged with EPO at 1.5 U/mL for 0, 5, 15, and 45 minutes (or in certain experiments, at limiting EPO doses for 0 or 45 minutes). Total cell lysates (or recombined fractionated lysate fractions) were then prepared using 1X RIPA buffer (Cell Signaling Technology, CST, 9806) (or a cell lysis and fractionation

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system, CST, #9806). For limiting EPO dose experiments, combined cytoplasmic and membrane lysates were prepared using 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, 0.25% Triton X-100 and 5% glycerol (10 minutes, on ice). All lysate and wash buffers were supplemented with 1X protease and phosphatase inhibitors (CST, #5872), 1 mM phenylmethylsulfonyl fluoride (CST, #8553), and 200 μM activated sodium orthovanadate (New England Biolabs, #P0758). RIPA lysates (and nuclear protein fractions) were pulse sonicated on ice (6 × 1 second, 5 Watts; Dismembrator-100, Thermo Fisher). Samples were denatured in 1X SDS loading buffer (CST, #7722), reduced (30 mM dithiothreitol) and incubated at 80°C for 5 minutes. In SDS-PAGE, samples were electrophoresed (Criterion TGX gels, BioRad, #5671123) and transferred to PVDF membranes (Merck Millipore, #IPVH00010) in 20% methanol, 40 mM glycine, 48 mM Tris, pH 8.5. For assays of PTPN18, an antibody against total PTPN18 (CST, #8311) was used for western blots at 1:1000. Additional CST antibodies used (at 1:1000) were: p-JAK2 Y1007/Y1008 (#3771), total JAK2 (#3230), p-ERK1/2 T202/Y204 (#4370), total ERK1/2 (#9102), p-AKT S473 (#4060), p-STAT5 Y694 (#4322), and total STAT5 (#25656). GAPDH (#5174) was used at 1:5000. Antibodies to total and p-Y141 RHEX and the human EPOR (monoclonal antibody clone 1.1) were as previously described[67,74,75]. In ECL, anti-rabbit IgG, HRP-conjugated secondary antibodies were used at 1:2000 to 1:5000 (CST, #7074). Signals were generated using Supersignal West-Dura ECL substrate (Thermo Fisher Scientific, #34075), and imaged using Kodak BioMax MR1 film (Perkin Elmer, #8941114001), or by Bio-Rad ChemiDoc™ digital imaging (including the quantitative measurement of western blot band intensities) (BioRad, ImageLab software v. 6.0).

In flow cytometry experiments, levels of cell surface EPOR expression and internalization were assayed as follows. Lentivirus- transduced EE-PTPN18 and EVEC UT7epo cells were grown to 7×10^5 cells/mL in IMDM medium, 10% FBS, 1% P/S, 2.5 U/mL EPO, plus puromycin at 1.5 μg/mL. Cells were then cultured in this medium for 20 hours in the absence of EPO and were then challenged with 1.5 U/mL EPO (or PBS) for 45 minutes. Following +/− EPO exposure, 4×10^6 cells were collected per sample and placed in PBS on ice. Samples were then spun and washed twice with ice-cold PBS, 0.5% BSA. Fc receptors were blocked by incubating cells with human Fc fragment (1 μg/mL, 20 minutes on ice) in PBS, 0.5% BSA. Cells were then incubated with the anti- hEPOR rabbit monoclonal antibody c38.5(ref.[74]) at 0.5 μg/mL for 1.5 hours, at 4°C (nutator platform). Cells were then collected, washed twice in PBS, 0.5.% BSA, and incubated with anti-rabbit AlexaFluor488 secondary antibody (4°C, 30 minutes). Following three washes in PBS, 0.5% BSA, EPOR levels were assayed by flow cytometry (Sony SH800 cell sorter). Non-viable cells (≤ 5%) were excluded using 5 nM Sytox Red (Thermo Fisher, cat.# S34859). For all samples, at least 1×10^5 events were analyzed and doublet-discrimination was performed.

2.5. Statistics

For experiments that assessed UT7epo cell survival and proliferation, statistical analysis of the significance between mean values was performed using Student's T-test or ANOVA (GraphPad Prism, v.5). P-values are designated as *<0.05%; **<0.01; and ***<0.001.

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3. RESULTS

NOTE: Per Cellular Signaling guidelines, Figures have been incorporated into Results adjacent to related text. Stand-alone Figures also are provided as a separate PDF.

3.1. Phospho-PTM proteomic discovery of novel EPO- modulated, phosphotyrosine-modified kinases and phosphatases

EPO's effects on target cell populations are triggered upon the ligation of EPOR-JAK2 complexes, and the conformational activation of JAK2 is indicated to be required for essentially all subsequent EPO/EPOR signaling events[76]. This includes p-Y PTM signals, certain of which involve the actions of known secondarily acting protein kinases and phosphatases. With a goal of discovering novel EPO- modulated p-Y modified kinases and phosphatases, and using a unique EPO- dependent human erythroid progenitor cell line model, UT7epo[47], we applied pY- PTM proteomic profiling. Specifically (and via the workflow outlined in Figure 1A, *see next page*) EPO first was withdrawn from exponentially growing UT7epo cells to disengage EPO signaling. Cells were then challenged with EPO (or PBS) for 15 minutes, brought to 2°C in the presence of protease and phosphatase inhibitors, washed, collected and flash frozen. Total cellular proteins were then extracted (urea, HEPES), reduced, alkylated and hydrolyzed to tryptic peptides. p-Y phospho-peptides from [+] EPO and [−] EPO samples next were immunoadsorbed to anti-pY1000 immunobeads. Following elution from washed immunobeads, p-Y peptides were identified and quantitatively assayed via LC-MS/MS and spectral analysis (see Methods).

Overall, pY-PTM profiling identified 299 unique pY-phosphopeptides with ≥2.0 to 350-fold modulation due to EPO. To enable access (and certain data mining), full data sets are provided via a searchable database, ErythronDB (access URL: https://www.cbil.upenn.edu/ErythronDB_alpha/app). Gene Ontology defined diverse enriched EPO target sets (see Erythron DB Gene Ontology tool). To focus this report, we presently concentrated on the "molecular function" categories of kinases and phosphatases. For these EPO targets, p-Y phospho-PTM proteomic findings are initially summarized in Figures 1B and 1C (and Supplemental Table S-T1) and include: The identification of 35 known and novel EPO p-Y modified kinases and phosphatases [including specific isoforms, e.g., phosphoinositide-3-kinase regulatory subunits 1 and 2 (PIK3R1, PIK3R2)]; the sequences of their derived phospho- peptides; specific p-Y sites modified due to EPO; and the fold-modulation of EPO- induced p-Y phosphorylation events. For this proteomic approach at-large, it is noted that phospho-PTM LC-MS/MS profiling reports on subsets of target proteins, and depends (in part) on the protease used to generate peptide fragments. An informative sampling among known and novel targets therefore is an anticipated (and sought) result. Certain targets known to be modified at p-Y sites by EPO are represented in our datasets, and serve as validating targets. For example, EPO-induced p-Y phosphorylation at JAK2's domain J1 catalytic loop is identified by three distinct peptides that include well-studied p-Y1007 and p-Y1008 sites[77]. Such validation targets extend to additional known EPO- modulated kinases including the Srcfamily tyrosine kinases FYN and LYN, mitogen-activated protein kinase 1 (MAPK1), and tec protein tyrosine kinase (TEC) (Figure 1B), and to known phosphatase targets including PTPN11, INPPL1, and INPP5D (Figure 1C).

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EPO- modulated, p-Y modified kinases and phosphatases each were next analyzed in silico to determine their nature as novel vs. known EPO targets (and to define the position and novel versus known nature of their p-Y phosphosites within target protein subdomains – see below). Here, (via GeneCard, Prosite, Phosphosite Plus, NCBI databases and resources) it was informative to define the following sub-categories of such EPO targets (Figure 2A): (1) Kinases and phosphatases representing novel EPO targets not previously associated with EPO signaling; (2) kinase and phosphatase targets previously reported to be modified in their p-Y PTMs by other cytokines and/or drug actions, but not by EPO; and (3) known EPO modulated kinases and phosphatases previously reported to be regulated by EPO. With an initial focus on kinases, results for two known validation targets (as examples) are first illustrated as JAK2 and PI3K (Figure 2B, upper and center sub-panels). In JAK2, five EPO-modulated p-Y known sites were identified (i.e., previously reported) as p-Y1007, p-Y1008, p-Y966, p-Y570, and p-Y221 (open circles, and open squares). In addition, three novel sites of EPO- induced JAK2 modification were identified as p-Y918 (kinase domain), together with p-Y435 and p-Y462 in JAK2's SH2 domain (filled circles). In PIK3R1, EPO-induced p-Y phosphorylation of the p85 subunit was observed at p-Y508 and p-Y580 sites within PIK3R1's SH2 domain. p-Y580 is a known EPO-modulated site (open circle), while p-Y508 is a novel site of EPO- induced modification (filled circle). For phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), we unexpectedly also observed the novel 89.1 and 31.2 fold EPO- induced phosphorylation of PIK3's catalytic subunit at p-Y317 and p-Y508 sites (Figure 2A, lower panel).

Novel kinases modulated by EPO in their pY modifications include MPP1, GUK1, PEAK1, AAK1, MINK1, and MAPK7 (Figure 2C, right panels). MPP1 is a guanylate kinase originally described in erythroid cells as a cytoskeletal interacting protein[48]. MPP1 has additionally been implicated in membrane raft formation[49], and a *MPP1* gene deletion recently has been associated with hemophilia A[50]. In MPP1, EPO- induced p-Y phosphorylation (13.1-fold) occurred at a novel p-Y331 site in MPP1's catalytic domain. EPO also induced the phosphorylation of a second guanylate kinase, GUK1, at a novel p-Y motif (p-Y53, 29.6-fold modulation) homologous to that in MPP1 (Figure 2C, top panels – *see prior page*). GUK1 functions to generate GDP for nucleotide synthesis, a metabolic pathway defined as a limiting factor during stress erythropoiesis [51].

Two protein kinases, the pseudokinase PEAK1 and the serine/threonine (S/T) kinase AAK1, were rapidly stimulated >300-fold in their p-Y phosphorylation due to EPO (Figure 1B; Figure 2C, center panels). PEAK1 modulates cytoskeletal actin components[78], but exhibits only low level PTK activity[52], and may function predominantly as a multi-domain scaffold protein[79]. In PEAK1, EPO induced the phosphorylation of six novel p-Y sites. AAK1 was phosphorylated due to EPO at a single p-Y34 site proximal to its N-terminal kinase domain. AAK1 can be activated upon clathrin binding[80], and has been shown to support LDL receptor related protein 6 (LRP6) lipoprotein receptor endocytosis[54].

Two non-canonical ERK related kinases additionally were identified as novel EPO targets, MINK1 and MAPK7 (Figure 2C, lower panels). MINK1 is a MEK kinase kinase that can promote p38/JNK activation during cellular stress[56]. EPO induced the phosphorylation of MINK1 at a Y906 site within a RAP2A (RAP2A, member of RAS oncogene family)

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binding region that provides for RAP2A activation of mitogen-activated protein kinase 8 (JNK)[55]. For MAPK7, EPO induced the phosphorylation of p-Y221 in MAPK7's catalytic domain. MAPK7, as an atypical ERK[57], can promote cell cycle progression, but also repress ABC transporters[58]. Each of these six kinases, and their implicated functions, are novel aspects of EPO signaling.

3.2. Validation and novel EPO- modulated pY-modified phosphatases

For phosphatases modulated by EPO in their p-Y modification (Figure 3), example validation targets included INPP5D[38] and PTPN11(ref.[81]) (Figure 3B, see next page). In INPP5D, six EPO-induced sites of p-Y modification were identified. Although a validating target, none of these p-Y sites (filled circle symbols) have previously been shown to be EPO- modulated. Two, however, have been reported to be a cell signaling or drug modulated p-Y modified site (open square symbols). For PTPN11 (Figure 3B, lower panel), this PTP has previously been connected to EPO signaling[41], with two p-Y sites known to be cytokine or drug modulated (open squares), and two as novel (closed circles). Newly discovered EPO-modulated pY-modified phosphatase targets included PTPRA, TENC1, PHPT1, UBASH3B, and PTPN18 (Figure 3C). For PTPRA, a transmembrane receptor PTP, EPO induced its pY phosphorylation within a D1 PTP domain (p-Y271). D1 is PTPRA's major PTP catalytic domain[59], and the p-Y271 region (modified by EPO) is conserved among several R- PTPs (including PTPRC, PTPRG, and PTPRF)[60]. PTPRA functions in part to dephosphorylate and activate SRC and FYN kinases via Src SH2 binding at D2(ref.[61]). EPO modulation of PTPRA therefore may promote FYN activation, a co-regulated, EPO-modified PTK (see above, Figure 1B). For TENC1 (TENSIN2), EPO induced its p-Y phosphorylation at Y483, a p-Y site adjacent to a C2 domain involved in RhoA binding (Figure 3C)[82]. While not known to date to act in EPO signal transduction, TENSIN2 can bind the EPOR-related receptor MPL, and promote TPO-dependent UT7-TPO cell proliferation[83]. EPO also modulated the p-Y phosphorylation of a histidine phosphatase, PHPT1 at p-Y93 within PHPT1's substrate binding site[62]. While not well-studied, PHPT1 can dephosphorylate select G-protein beta-subunits[84] and Ca^{2+}/K^{+} flux via TRPV channels[85]. In erythroid cells, EPO has been shown to induce calcium flux via transient receptor potential cation channel subfamily C member 3 (TRPC3)(ref.[86]).

Finally, EPO modulated the p-Y modification of two PTPs implicated in ubiquitination events, UBASH3B and PTPN18 (Figure 3C, lower sub-panels). UBASH3B is an atypical p-Y PTP that can dually regulate p-Y and ubiquitin target modifications[87]. This includes negative regulation of BCR-ABL[87], and T-cell receptor signaling[88]. For PTPN18, EPO induced its p-Y phosphorylation at three novel sites (Y303, Y354, and Y389) up to 79-fold, with each site occurring within a C-terminal PEST domain[89] (Figure 3C, lower panel). In addition, the EPO-modulated p-Y389 motif in PTPN18 proved to also be represented uniquely in UBASH3B.

3.3. In silico characterization of PTPN18, a novel EPO target, and unique PEST-PTP

Among novel EPO modulated p-Y modified kinases and phosphatases, PTPN18 was selected for further study based on the following considerations. First, PTPN18 is highly expressed in hematopoietic progenitor cells, yet has not previously been associated with type

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1 cytokine receptor or JAK kinase signaling. In Figure 4A, this is illustrated based on STRING database analysis of EPO modulated p-Y PTPs (with the EPOR, JAK2, and STAT5 as STRING connectivity anchors).

Second, comparisons of PTPN18's C-terminal region with other PEST PTPs (PTPN12, PTPN22) revealed several distinguishing features (Figure 4B). Specifically, PTPN18's EPO-modulated (p)Y389 site is unique, as is its adjacent beta-TrCP binding site. Between these two motifs, an ~17 AA region represented in PTPN12 and PTPN22 (with 50+% P-E-S-T AA representation) is absent from PTPN18. In addition, regions adjacent to p-Y389 in H sapiens are not represented in mouse PTPN18 (Figure 4C). Furthermore, in a context of small molecule inhibitors to JAK2 and their effects on STF phospho-tyrosine modifications, data mining of such studies[73] identified select affected PTPs. Among these, PTPN18 and specifically its pY-389 phosphorylation were inhibited 8-fold by JAK2 inhibitors NVP-BSK805 and NVP-BBT594 (Figure 4D). These above properties focused our attention on PTPN18's possible roles during EPO- dependent UT7epo cell growth.

3.4. PTPN18 promotes EPO- dependent erythroid progenitor UT7epo cell proliferation and survival, with a requirement for PTPN18-Y389

Experiments next were performed to assess possible effects on UT7epo cell growth of the ectopic expression ("EE") of wt-PTPN18, and a PTPN18-Y389F construct (Figure 5A). Expression was via lentivirus transduction, with UT7epo cells also stably transduced with an empty vector control construct (EVEC). For PTPN18 and PTPN18-Y389F, western blotting demonstrated well-matched expression levels (Figure 5A, lower panel). When possible effects of ectopically expressed PTPN18 ("EE-PTPN18") on EPO-dependent cell growth were assessed, significant effects on increased cell numbers first were observed that proved to be essentially reversed due to Y389F mutation (Figure 5B). When effects on UT7epo cell viability were assessed (Figure 5C), frequencies of non-viable cells were lessened due to PTPN18 cytoprotection at mid-range EPO doses. This effect likewise was reversed in EE-PTPN18-Y389F cells. Experiments were additionally performed to assess whether these observed effects of PTPN18 and/or PTPN18-Y389F also impacted on long-term UT7epo cell growth. In 21d assays, in which cells were maintained by volumetrically replating every 4 days (0.5 volume media replacement, 0.3 U/mL EPO), persistent effects of ectopically expressed PTPN18 and PTPN18-Y389F on UT7epo growth were observed (Figure 5D). Finally, when cells were cultured for 24h in the absence of EPO, the heightened expression of PTPN18 (and to a lesser extent, PTPN18-Y389F), significantly protected against cell death (Figure 5E, left sub-panel). Annexin-V staining indicated this cytoprotection to involve inhibition of apoptosis (Figure 5E, right panel).

3.5. PTPN18 Y389 reinforces EPO- induced ERK1/2, AKT and STAT5 activation

Experiments were next advanced to probe molecular signaling events that underlie wt-PTPN18's heightening of EPO- dependent UT7epo cell proliferation and survival, as well as effects exerted by ectopically expressed PTPN18-Y389F. Specifically, UT7epo EE-wt-PTPN18, EE-PTPN18-Y389F and EVEC cells were expanded as exponentially growing cultures, and incubated for 20 hours in the absence of EPO. Cells were then challenged with EPO (1.5 U/mL) including a 0, 5, 15, and 45 minute time-course. At these time-points,

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signaling was halted (3 volumes of 2°C PBS on ice) and cell lysates were prepared, denatured and reduced. In analyses first of ERK1,2 and AKT activation, western blots of p-T202/p-Y204 ERK1/2 (and total ERK1/2) revealed persistent high-level activation of ERK1/2 in UT7epo cells due to EE-wt-PTPN18, as is readily observed at a 45 minute time-point, compared to control UT7epo EVEC cells (Figure 6A, upper panel – *see next page*). The specific Y389F mutation of PTPN18 essentially reversed these effects of PTPN18 on ERK1,2 activation. When effects on AKT activation were assessed (Figure 6A, lower panel) an apparent modest heightening of activated AKT was observed due to EE-wt-PTPN18 (at 15 and 45 min time points), that was likewise reversed due to Y389F mutation.

Based on observed effects of wt-PTPN18 on ERK1/2 and AKT activation, possible additional effects at an upstream JAK/STAT level were next analyzed. P-Y694 STAT5 activation proved to be sustained due to PTPN18 ectopic expression (Figure 6B, upper panel), with a similar reversal of this effect due to Y389F mutation. In keeping with this result, activated p-Y1007/1008 JAK2 levels were also sustained due to wt-PTPN18 expression, with at least partial reversal of this effect observed for EE-PTPN18-Y389F (Figure 6B, lower panel). In Figure 6C, observed effects of PTPN18 and PTPN18-Y389F expression on JAK2, STAT5, AKT and ERK1/2 signaling are summarized in the context of EPOR/JAK2 complex signaling. (Also diagrammed is the molecular adaptor protein, RHEX, which has been demonstrated to associate with the human EPOR)[67].

3.6. PTPN18 alters the apparent processing of the EPOR, and limits RHEX p-Y141 phosphorylation with dependency on PTPN18 Y389

The above analyses of wt-PTPN18 effects on EPO-induced ERK1/2, AKT, STAT5 and JAK2 activation implicated PTPN18 regulation of upstream EPOR/JAK2 coupled events. The EPOR is expressed at only low levels[76] and studies of EPOR expression and processing have been controversial due to the use of less-than-specific reagents and antibodies[90,91]. We have prepared and previously carefully characterized EPOR monoclonal antibodies to the human EPOR, including a c1.1 antibody that is highly specific for the hEPOR in western blotting[74]. Using this specific probe, we performed experiments to assess possible effects of ectopically expressed wt-PTPN18 and PTPN18-Y389F at the EPOR level. This included attention to the full-length EPOR, and lower molecular weight 40K, 36K and 15K EPOR peptides[74]. EPO challenge experiments revealed significant effects exerted by wt-PTPN18 on EPOR forms (Figure 7A: *see next page*). Specifically, in UT7epo EE-PTPN18 cells, a prominent EPOR-36K form was underrepresented, with substantial increases instead observed for an EPOR-40K form. An EPOR-15K fragment observed in EVEC control UT7epo cells was essentially undetectable in UT7epo EE-PTPN18 cells at 0, 5 and 15 minutes of EPO challenge (but became detectable at 45 minutes). In addition, relative to PTPN18-Y389F and EVEC cells, increases in EPOR-65K full-length EPOR were observed in EE-wt-PTPN18 cells. In contrast, in UT7epo-EE-PTPN18-Y389F cells, these effects on apparent EPOR processing were reversed to largely correspond to those observed in UT7epo-EE-EVEC controls.

The above findings prompted us to go on to assess possible effects of PTPN18 on a recently discovered EPOR associated STF, the EPO- regulated integral plasma membrane p-Y

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phosphoprotein and molecular adaptor, RHEX[67]. In parallel with wt-PTPNP18's effects on the EPOR, wt-PTPN18 proved to sharply limit the EPO- induced p-Y141 phosphorylation of RHEX (Figure 7B). Notably, the Y389F mutation in ectopically expressed PTPN18-Y389F largely reversed wt-PTPN18 inhibition of RHEX's EPO- induced pY phosphorylation (without significantly affecting total RHEX levels). These further actions of PTPN18 on EPOR and RHEX molecular signaling are summarized in Figure 7C, and are in keeping with observed positive actions of PTPN18 on erythroid progenitor cell proliferation and survival. Findings also are consistent with a possible role for RHEX as a potential mediator of EPOR turnover.

4. DISCUSSION

For the present studies, one overall goal was to uncover new candidate mediators of EPO's essential proliferative and cytoprotective effects on human erythroid progenitor cells. Specifically, a p-Y directed phospho-PTM profiling approach was applied based not only on key roles of the EPOR-coupled protein tyrosine kinase JAK2, but also on the advantages of this proteomic approach towards discovering new EPO STFs (including those for which specific antibodies and probes are unavailable). Overall, these p-Y directed PTM analyses identified a total of 207 EPO- modulated pY-modified proteins with ≥2-fold to 300+ fold modulation. To enable access (and to assist data mining), full +/− EPO p-Y PTM profiling data sets are provided via a dedicated Erythron Database platform [URL https://www.cbil.upenn.edu/ErythronDB_alpha/app].

As a focus for in silico cell and molecular analyses, our attention centered on EPO-modulated pY-modified kinases and phosphatases. In silico analyses informed on these sets of EPO modulated targets in several ways. First, 22 novel kinase and phosphatase targets were identified and were distinguished from known EPO targets, both at the STF level, and the specific nature of target p-Y modified sites and subdomains. For each new target, this includes considerations of functional implications for phospho-PTMs. In addition, ways in which our new p-Y phosphoproteomic data can be used to generate, and experimentally test, hypotheses for PTM-modified EPO target actions are illustrated for the non-SH2 PEST protein tyrosine phosphatase, PTPN18.

Among 17 novel EPO modulated kinases discovered via pY-PTM proteomics, we highlight two EPO- targeted kinases that play specific roles in cytoskeletal restructuring – a function not previously implicated for EPO – that are also relevant to EPO's reported adverse effects on breast cancer metastasis[92,93]. Specifically, the S/T kinase AAK1 and the S/T pseudokinase PEAK1 each were rapidly induced >300-fold in their p-Y phosphorylation upon EPOR ligation. AAK1 can modulate cytoskeletal Clathrin triskelion- AP2 assemblages[54,80], and is catalytically activated by Clathrin[80]. This occurs via AP2 adaptor bridging of AAK1 to Clathrin, and leads to AAK1 phosphorylation of AP2's μ2 domain[80]. These complexes can then act to recognize receptor endocytosis motifs[94]. AAK1 recently has been reported to regulate trafficking of LRP6[ref.[54]] and ERBB4 receptors[53], but has no previously described association with JAK- coupled hematopoietin receptors. In AAK1, its EPO- regulated p-Y34 site is also novel, occurs immediately adjacent to AAK1's kinase domain, and is represented in phospho-proteomic databases for Hodgkin's and acute

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lymphocytic leukemia. PEAK1, by comparison, was modified due to EPO at 7 p-Y sites (4 novel) across three sub-domains (see Figure 2C) that recently have been implicated in PEAK1's effects on focal adhesions, and cell migration[95]. Recently, PEAK1's actions have been revealed to involve dimerization- mediated inhibition of C-terminal Src Kinase (CSK)[96]. Through such CSK inhibition, SRC kinases (including FYN), thus become activated via PEAK1(ref.[96]). Our present identification of novel p-Y sites in PEAK1 modified due to EPO should help guide experiments aimed at understanding the regulation and actions of PEAK1 (an emerging new drug target for breast, colon and lung cancers, with JAK2 involvement)[97–99]. For the presently identified 22 EPO-modulated kinases and phosphatases, and in a preclinical research context, each are expressed in human, as well as murine erythroid progenitor cells. For ready reference we provide a summary of the patterned expression of transcripts for these new EPO- modulated signal transduction factors during human and murine erythropoiesis (Supplemental Figure S-1). Also see, https://www.cbil.upenn.edu/ErythronDB_alpha/app.

Among EPO- modulated pY- modified phosphatases, we identified five novel targets with first-time connections to EPO/EPOR action. Two are known to play roles in cytoskeletal restructuring, PTPRA[59] and TENC1[82]; one, PHPT1, functions as a histidine phosphatase[62]; and two, UBASH3B and PTPN18, recently have been reported to regulate receptor tyrosine kinase (RTK) and/or PTK signaling in association with effects on ubiquitination[65,87]. For the PTPRA receptor PTP, PTPRA's p-Y phosphorylation due to EPO at a major D1 catalytic domain unexpectedly implicates lateral EPOR/JAK2 signaling. While atypical, such receptor-receptor trans- regulation has previously been demonstrated for PTPRA specifically involving SRC/FAK p-Y780 phosphorylation and consequential activation of PTPRA[61]. PTPRA then becomes additionally competent to modify actin cytoskeletal restructuring[61]. For TENC1/TENSIN2 (like PTPRA) this PTP has also been shown to modulate actomyosin restructuring, including FAKs[100]. This involves the binding of RhoA at a defined motif proximal to TENC1's EPO- induced p-Y483 site (see Figure 3C). In a third novel EPO-modulated phosphatase, PHPT1, EPO induced phosphorylation at a novel Y93 site in its phosphatase domain. Globally, histidine phosphatases include only two orthologues[101], and due to a historic lack of p-His specific antibodies, are not well studied in cellular signaling. New inroads therefore are generated to investigate PHPT1's roles during hematopoietin signaling.

For the EPO- modulated PEST PTPs UBASH3B and PTPN18, in addition to their roles as PTPs, each has recently been implicated in ubiquitin modification. In myeloid leukemia contexts, and acting in part via Cbl E3 ubiquitin ligase, UBASH3B can modulate AML1-ETO[102], and can also inhibit BCR-ABL[87]. The presently observed EPO-induced phosphorylation of UBASH3B at p-Y19, adjacent to a ubiquitin association domain (see Figure 3C), may aid insight into UBASH3B's effects. This prominent EPO- modulated p-Y19 motif in UBASH3B proved to be uniquely homologous with an EPO- modulated p-Y389 motif in PTPN18 (see Figure 3C), and via a proposed ubiquitin barcoding mechanism, PTPN18 can regulate HER2 processing[65]. This further involves a specific role defined for PTPN18's binding of beta-TRCP ubiquitin ligase[65]. The presently studied EPO-modulated p-Y389 site in PTPN18 is adjacent to this beta-TrCP binding site, and as a novel EPO target and STF, PTPN18 was therefore selected for further study.

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In contrast to its effects on the apparent processing and down-regulation of erb-b2 receptor tyrosine kinase 2 (HER2) in HepG2 cells[65], wt-PTPN18 in erythroid progenitor UT7epo cells unexpectedly promoted cell proliferation and survival. Consistent with functional roles for EPO- induced PTPN18 phospho-PTM modification, these effects depended not only on EPO induced-signaling, but also upon the intactness of PTPN18's EPO- modulated p-Y site (p-)Y389. Mechanistically, these cellular phenotypes were reflected in effects of PTPN18 and PTPN18-Y389F on the EPO- dependent activation of ERK1/2, AKT, STAT5, and JAK2. Specifically, each was observed to be sustained in its EPO- induced activation by PTPN18, with each effect largely reversed due to (p)Y389 mutation (Y389F) (see Figures 5 and 6). In a related analysis, we additionally assessed these effects of ectopically expressed PTPN18-Y389F and wt-PTPN18 in an EPO dosing context. When EPO was limited to doses of 1.6, 0.8 and 0.4 U/mL, effects of PTPN18-Y389F and wt-PTPN18 on the activation of p-AKT, p-ERK and p-STAT5 continued to be exerted (see Supplemental Figure S-2). This added observation indicates significance of PTPN18 signaling at physiological EPO concentrations.

Because PTPN18-Y389 affects multiple STF modules (STAT5, ERK1/2, AKT) we also initially investigate possible effects of PTPN18 at the EPOR level. wt-PTPN18 substantially increased levels of an EPOR- 40kDa species, while decreasing EPOR- 36kDa and EPOR-15kDa forms, and modestly increasing an EPOR- 65kDa full-length EPOR species (see Figure 7B). For a low molecular weight (MW) EPOR- 15kDa form, PTPN18 delayed the formation of the low MW product. Notably, each effect on apparent EPOR processing was essentially reversed due to Y389F mutation in PTPN18. Y389 resides in PTPN18's distal PEST domain, and is well separated from an N-terminal PTP domain. This does not discount possible effects on PTP activity, but at least suggests mechanisms involving co-acting STFs, and upstream EPOR-JAK2 complex modulation. In one possible model for PTPN18's observed impact on EPO signaling, this might include effects on EPOR levels and/or turnover. In UT7epo cells, ectopic expression of PTPN18 (versus EVEC cells), however, did not detectably affect either the levels of unligated EPORs, or EPO- induced internalization (see Supplemental Figure S-3). This observation points to alternate action mechanisms for PTPN18 during EPO-activated cellular signaling. Here, PTPN18's actions on barcoding ubiquitination of STFs[65] might come into play if its targets were negative regulators within EPOR complexes. Two rational candidates for this hypothesized PTPN18 mechanism are LNK/SH2B3, an adaptor that inhibits JAK2 activation[33] and SOCS3, an alternate negative regulator that in addition is an E3 ubiquitin ligase[103].

Based on recent progress in the drugging of PTPs[104], and on roles for elevated JAK2 signaling in MPN[105] and myelofibrosis[106], PTPN18 may additionally represent a novel rational target for small molecule inhibition. Based on apparent consequences of wt-PTPN18 on EPOR processing, we also investigated possible PTPN18 effects on a recently discovered molecular adaptor protein, RHEX, that associates with human EPOR complexes, and modulates late erythroblast development[67]. Unexpectedly, PTPN18 markedly limited levels of EPO- induced RHEX-pY141 phosphorylation, an effect that was reversed due to PTPN18-Y389F mutation (see Figure 7B). These findings suggest that RHEX may negatively affect EPOR signaling in human erythroid progenitor cells, and that PTPN18-(p)Y389 may counter such effects.

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Supplementary Material

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HIGHLIGHTS

- Phospho-PTM proteomics reveals 22 novel EPO/EPOR/JAK2 p-Y modulated kinases and phosphatases.
- Major new EPO- modulated kinases include AAK1 and PEAK1, and the pseudokinases GUK1 and MPP1.
- Diverse EPO- modulated phosphatases include His-PHPT1, PTPR-A and PTPN18.
- In GOF studies, PTPN18-(p)Y389 sustains EPO activation of ERK1/2, STAT5 and JAK2.
- PTPN18-(p)Y389 mutation alters EPOR processing, while limiting p-Y RHEX phosphorylation.

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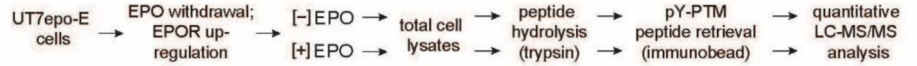
A



B

EPO MODIFIED KINASES AND p-Y PTM SITES

gene name	EPO, fold-change	site(s)	peptide	protein full name
PEAK1	397.0	531	SSAIRY*QEVWTSSTSPR	Pseudopodium-enriched atypical kinase 1
AAK1	387.5	34	REQGGSGLGSGSSGGGGSTSGLGSGY*IGR	AP2-associated protein kinase 1
JAK2	214.3	1007, 1008	VLPQDKEY*Y*KVKEPGESPIFWYAPESLTESK	Tyrosine-protein kinase JAK2
MAPK7	116.8	221	GLCTSPAEHQYFM#TEY*VATR	Mitogen-activated protein kinase 7
PIK3CA	89.1	317	RISTATPY*M#NGETSTK	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
MAPK1	74.6	187	VADPDHDHTGFLTEY*VATR	Mitogen-activated protein kinase 1
PEAK1	48.8	475	VVNLEQPLCKPY*TVVDVSAAM#ASEHLEGPVNSPK	Pseudopodium-enriched atypical kinase 1
PEAK1	39.5	1108	GCTSVTTALSLPELEREDGKEDISDPM#DPNPCSATYS*NLGQSR	Pseudopodium-enriched atypical kinase 1
PIK3CA	31.2	508	EAGFSY*SHAGLSNR	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
JAK2	30.0	1007	VLPQDKEY*YK	Tyrosine-protein kinase JAK2
GUK1	29.6	53	NPRPGEENGKDY*YFVTR	Guanylate kinase
JAK2	26.4	462	HCLITKNENEEY*NLSGTKK	Tyrosine-protein kinase JAK2
TEC	25.1	329	HAFGSIPEIIEY*HKHNAAGLVTR	Tyrosine-protein kinase Tec
JAK2	22.9	1008	VLPQDKEYY*KVKEPGESPIFWYAPESLTESK	Tyrosine-protein kinase JAK2
SLK	21.3	1225	ISKFY*PIPSLHSTGS	STE20-like serine/threonine-protein kinase
PEAK1	20.9	635	NAIKVPIVNPNAY*DNLAIYK	Pseudopodium-enriched atypical kinase 1
JAK2	16.5	918	YKGVCY*SAGRR	Tyrosine-protein kinase JAK2
JAK2	16.1	966	GMEY*LGTKR	Tyrosine-protein kinase JAK2
MARK3	13.7	432	YSDHAGPAIPSVVAY*PKR	MAP/microtubule affinity-regulating kinase 3
MPP1	13.1	331	KSEEDGKEY*HFISTEEMTR	55 kDa erythrocyte membrane protein
TYK2	12.6	1054	AVPEGHEY*YR	Non-receptor tyrosine-protein kinase TYK2
LYN	11.9	460	TNADVMTALSQGY*RMPR	Tyrosine-protein kinase Lyn
TEC	10.9	228	ARDKYGNEGY*IPSNYVTGK	Tyrosine-protein kinase Tec
PEAK1	9.8	1107	GCTSVTTALSLPELEREDGKEDISDPMDPNPCSATY*SNLGQSR	Pseudopodium-enriched atypical kinase 1
PIK3CB	9.5	425	TINPSKY*QTIRK	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform
BCR	8.5	177	KGHGQPGADAEKPFY*VNVEFHHER	Breakpoint cluster region protein
FYN	8.3	28	DGSLNQSSGY*R	Tyrosine-protein kinase Fyn
MINK1	6.8	906	NLLHADSNGY*TNLPDVVQPSHSPTENSK	Misshapen-like kinase 1
JAK2	6.8	221	AKIQDY*HILTR	Tyrosine-protein kinase JAK2
PI4KA	6.6	470	YHSQY*HTVAGNDIK	Phosphatidylinositol 4-kinase alpha
PEAK1	5.6	1372	ESQQYY*HSLAVR	Pseudopodium-enriched atypical kinase 1
PEAK1	5.5	462	IAVQTEKEESKASTDVAGQAVTINLVPTEEQAKPY*R	Pseudopodium-enriched atypical kinase 1
PIK3R1	4.3	508	YSKEY*IEK	Phosphatidylinositol 3-kinase regulatory subunit alpha
CDC42BPB	4.1	1638	NKPY*ISWPSSGSEPSVTVPLR	Serine/threonine-protein kinase MRCK beta
JAK2	3.7	570	REVGDY*GQLHETEVLLK	Tyrosine-protein kinase JAK2
PIK3R2	3.6	505	CSKEY*LER	Phosphatidylinositol 3-kinase regulatory subunit beta

C

EPO MODIFIED PHOSPHATASES AND p-Y PTM SITES

gene name	EPO, fold-change	site(s)	peptide	protein full name
INPPL1	347.8	986, 1003	NSFNNPAY*YVLEGVPHQLLPPEPPS*PARAPVPSATK	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2
PTPN11	294.3	584, 595	EDSARVY*ENVGLMQQQKS*FR	Tyrosine-protein phosphatase non-receptor type 11
PTPN11	219.4	546	KGHEY*TNIK	Tyrosine-protein phosphatase non-receptor type 11
INPPL1	98.1	185	SGSTSISAPTGPSSPLPAPETPTAPAAESAPNGLSTVSHDY*LK	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2
PTPN18	78.7	303	FLYHTVAQMFCSTLQNASPHY*QNIKENCAPLYDDALFLR	Tyrosine-protein phosphatase non-receptor type 18
PTPN11	55.0	547	KGHEYT*NIKYSLADQTSGDQSPLPPCTPTPPCAEMR	Tyrosine-protein phosphatase non-receptor type 11
INPP5D	51.3	1161	GRDY*RDNTELPHHGK	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
INPP5D	50.6	556	NQNY*MNILR	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
INPP5D	43.4	915	APPCSGSSITEIINPNY*M#GVGPFGPPMPLHVK	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
INPPL1	30.6	986	NSFNNPAY*YVLEGVPHQLLPPEPPSPAR	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2
PTPN18	29.5	354	SISVPGSPGHAMADTY*AVVQKR	Tyrosine-protein phosphatase non-receptor type 18
INPPL1	14.2	1162	GLPSDY*GRPLSFPPPR	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2
INPP5D	10.4	737	CY*ATLK	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
PTPN11	8.4	584	EDSARVY*ENVGLMQQQK	Tyrosine-protein phosphatase non-receptor type 11
PTPN18	7.6	389	SAEEAPLY*SKVTPR	Tyrosine-protein phosphatase non-receptor type 18
PTPRA	7.4	271	Y*VNILPYDHSR	Receptor-type tyrosine-protein phosphatase alpha
PHPT1	7.3	93	IHVYGY*SM#AYGPAQHAISTEK	14 kDa phosphohistidine phosphatase
TENC1	5.7	483	GPLDGSPY*AQVQRPPR	Tensin-like C1 domain-containing phosphatase
UBASH3B	2.6	19	EELY*SKVTPR	Ubiquitin-associated and SH3 domain-containing protein B
INPP5D	2.6	1022	NAGDTLPQEDLPLTKPEMFENPLY*GSLSSFPKPAPR	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
ENSA	2.5	41	AKY*PSLGQKPGGSDFLM#K	Alpha-endosulfine
INPP5D	2.3	373	ILRKEY*VFADSK	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1

Figure 1. Novel, and validating EPO- modulated pY- modified kinases and phosphatases identified via phospho-PTM proteomics.

(A) *p-Y directed phospho-PTM profiling workflow:* Exponentially growing EPO-dependent human erythroid progenitor UT7epo cells were washed, and cultured for 20 hours in the absence of EPO. Cells were then stimulated with EPO (4 U/mL for 15 minutes vs. PBS), (+/− EPO), immediately brought to 2°C (in PBS on salted ice), washed at 4°C in PBS (with protease and phosphatase inhibitors), and processed as flash-frozen cell pellets (−75°C). Total cellular proteins w ere extracted in HEPES buffered 8M urea (5×10^7 cells per sample), alkylated, and reduced. Tryptic peptides were then generated, and p-Y phosphopeptides were adsorbed to pY-1000 antibody-coupled immunobeads. Following elution from washed beads, peptides were analyzed by LC-MS/MS for their identities, p-Y PTMs, and relative quantitative levels ([+] vs [-] EPO). **(B)** *EPO- modulated, pY-modified kinases identified by phospho-PTM proteomics*: Among EPO- modulated kinases modified

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in their p-Y phosphorylation ≥ 2-fold (to > 300-fold) due to EPO, ten correspond to known, validating EPO targets, and seventeen to novel EPO targets (for full listing, see Supplemental Table S-T1). Data provided (in columns left-to-right) include the protein/gene name; fold-increase in peptide p-Y phosphorylation due to EPO; modified phospho residue(s); peptide sequences; and protein full name. Known validation targets include JAK2, MAPK1, TEC, LYN and FYN. Novel EPO modulated pY-modified kinases include PEAK1, AAK1, MAPK7, MAPK12, PIK3CA, GUK1, MPP1, TYK2, and MINK1. **(C)** *EPO-modulated pY-modified phosphatases identified by phospho-PTM proteomics:* Known (validating) EPO modulated phosphatases included INPPL1, INPP5D, and PTPN11. Novel phosphatase targets included PTPN18, PTPRA, PHPT1, TENC1 and UBASH3B.

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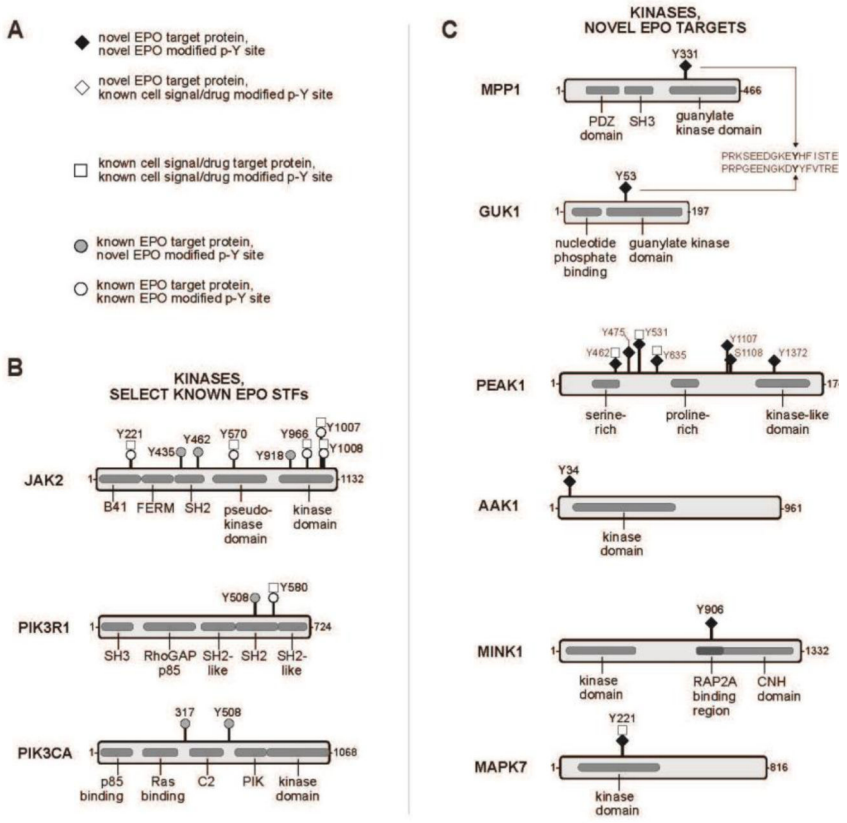

Figure 2. EPO-modulated kinases: In silico mapping of EPO- modified phospho- peptides and p-Y sites to parent proteins and their functional subdomains.

(A) *Defining of novel, and known (validating) EPO modulated, p-Y modified kinases*: In mining EPO phospho-PTM datasets (and individual modified kinases), the following categories of novel vs known kinase targets were defined. *Novel* targets are those not previously associated with EPO signaling, including those modified at novel p-Y sites (filled diamonds), or at sites reported to be regulated by factors other than EPO (open diamonds). A second related category included kinases not previously associated with EPO signaling, but previously identified in cellular signaling contexts (open squares). *Known* EPO targets are defined as those previously associated with EPO signaling, including targets for which EPO-induced pY phosphorylation is identified at either previously unreported p-Y sites (filled circles), or at established EPO- modulated sites (open circles). **(B)** *Example known EPO modulated kinases (phospho-PTM validation targets):* Using JAK2 as one validation example, pY-directed phospho-PTM identified 5 previously reported EPO-modified p-Y sites (open circles), and 3 p-Y sites not previously associated with EPO signaling (filled circles). In PIK3R1, a well-studied pY580 was represented as an EPO modulated site in this p85 regulatory subunit (open circle, square), together with a pY508 site not previously reported to be EPO- modulated (filled circle). Another validation target, PIK3CA, was also phosphorylated at unreported novel sites Y317 and Y508 (filled circles) due to EPO exposure. **(C)** *Novel EPO-modulated pY-modified kinases*: Six novel EPO targeted kinases are highlighted, and include the guanylate kinases MPP1 and GUK1 with catalytic domain p-Y modifications (filled diamonds, upper sub-panels); the (pseudo)PTK PEAK1 with 7

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novel sites of EPO-induced pY modifications; the S/T kinase AAK1 (center sub-panels); and two atypical ERK-module kinases MINK1/MAP4K6, and MAPK7/ERK5 (lower sub-panels).

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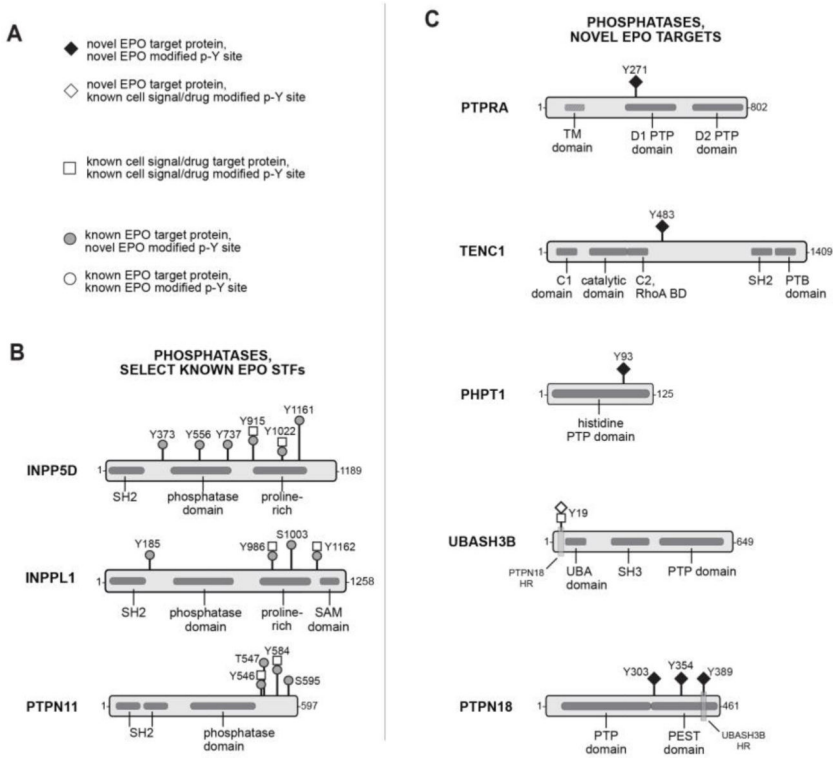

Figure 3. EPO- modulated phosphatases: In silico mapping of EPO- modified phospho- peptides and pY sites to parent proteins and their functional subdomains.

(A) *Key to diagrammed novel vs known EPO- modulated targets and modified p-Y sites:* As in Figure 2A, this key is provided as a guide to known vs. novel EPO- modulated target proteins and domain specific phosphosites. **(B)** *Example known EPO- modulated phosphatases (phospho-PTM validation targets)*: With INPP5D serving as one example, among six EPO modulated p-Y sites in this known EPO target, 2 have been previously reported (open circles) and 4 are established phospho-PTMs not previously associated with EPO signaling (filled circles). INPPL1 phosphatase was also phosphorylated due to EPO at four sites (filled circles). Among these, two are known to be phosphorylated via other cell signaling events (open squares). In the known EPO STF, PTPN11, EPO induced the phosphorylation of four PTM pY sites, with two previously reported (open squares) but none previously reported as EPO target sites (filled circles). **(C)** *Novel EPO- modulated pY-modified phosphatases*: Novel EPO-modified protein phosphatases included: Two modified at p-Y sites in their catalytic domains as the receptor PTP PTPRA and the histidine phosphatase PHPT1 (top and middle sub-panels); the tyrosine phosphatase TENC1 (second from top sub-panel); and two PTPs that are associated with substrate ubiquitination events, UBASH3B and PTPN18 PTPs (lower sub-panels). In each, their EPO- modified sub domains and specific sites of p-Y PTM are defined.

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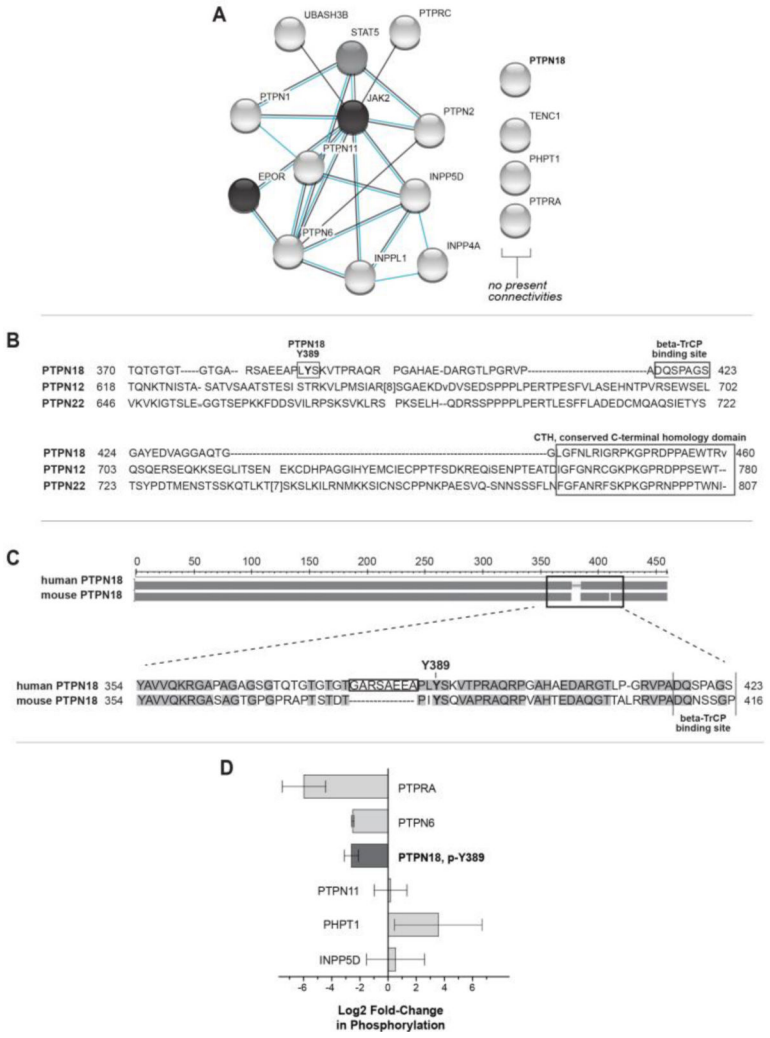

FIGURE 4. PTPN18 as a unique EPO target and pY- modified PEST PTP.

(A) *STRING database analyses of EPOR/JAK2 connectivities to phosphatase signaling:* STRING DB analyses were performed for the present set of p-Y proteomic identified EPO-modulated phosphatases (together with the known EPO associated phosphatases PTPN6, PTPN1, PTPN2, and PTPRC). EPOR, JAK2 and STAT5 served as STRING DB anchors. Connectivities are experimentally determined (black lines), or via curated datasets (blue lines). **(B)** *Unique features of PTPN18's PEST domain*: Among PEST PTPs (PTPs −12, −22, −18), the EPO- modulated pY389 site in PTPN18 is unique, as is PTPN18's beta-TrCP binding site and an adjacent gapped P-E-S-T sequence region. Comparisons were via COBALT, with a conserved CTH domain also indicated. **(C)** *Residues flanking pY389 in human PTPN18 are not represented in mouse (or rat) PTPN18:* Alignments compare human PTPN18 vs mouse PTPN18. Regions flanking human (p)Y389 are also absent in rat and Danio PTPN18s (data not shown). **(D)** *PTPN18, and pY389, are targets of JAK2 inhibitors*: Data mining of phospho-PTM studies of JAK2 inhibitor action (NVP-BSK805, NVP-BBT594) identified PTPN18 and its p-Y389 site among top modified targets. Graphed values are means (+/− SE) for JAK2 inhibitor modulation of phosphatase phosphorylation.

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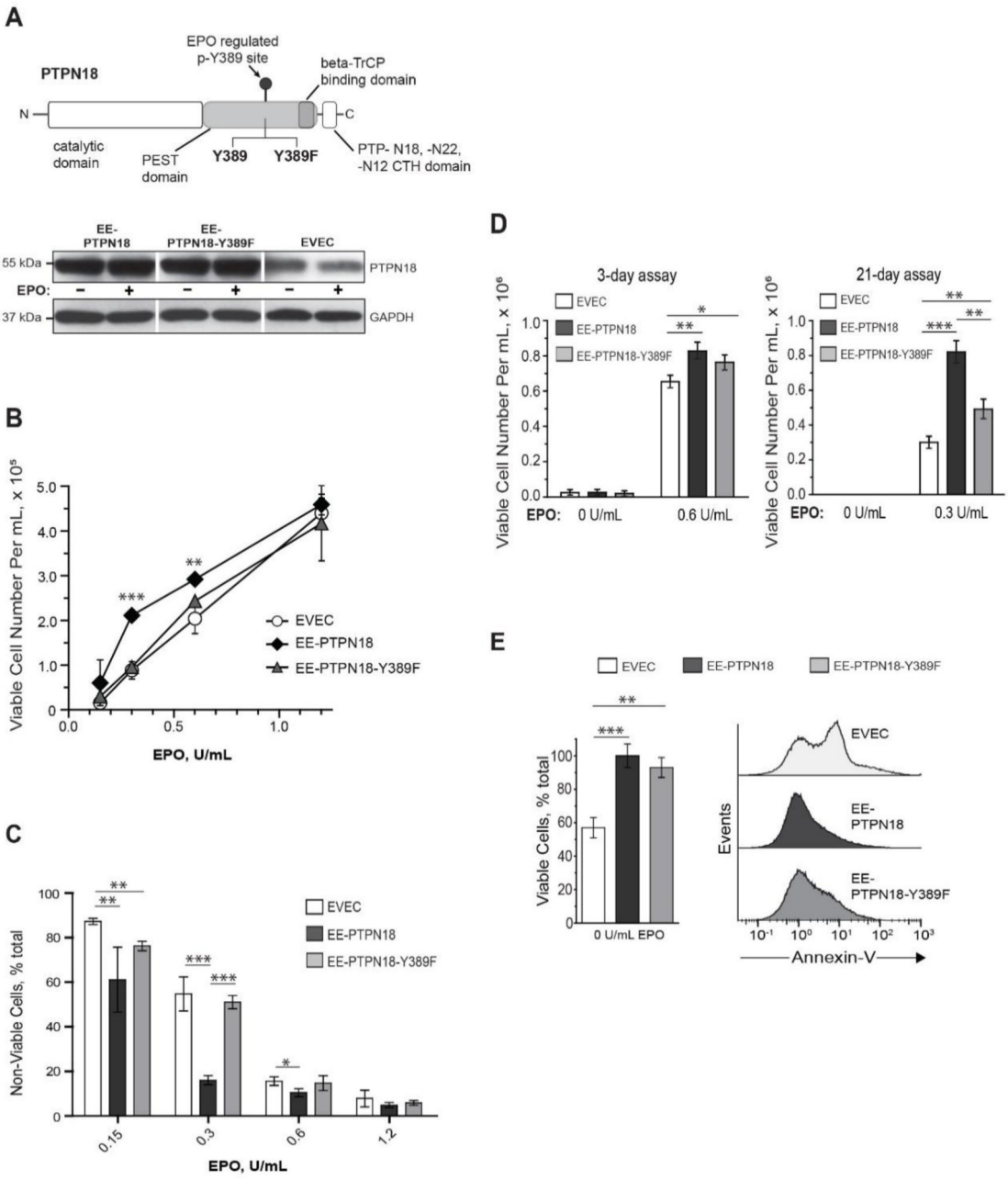

FIGURE 5. PTPN18 supports EPO dose- dependent UT7epo cell proliferation and survival.

(A) *Wild-type PTPN18 and PTPN18-Y389F constructs, and matched ectopic expression levels in stably transduced UT7epo cells:* UT7epo cells were stably transduced with lentiviruses encoding wild-type PTPN18, PTPN18-Y389F or an empty vector negative control construct (EVEC). Levels of ectopic expression ("EE") of PTPN18 and PTPN18-Y389F constructs in derived cell lines were assessed by western blotting (lower sub-panel). **(B,C)** *Ectopically expressed PTPN18 promotes UT7epo cell proliferation and survival with dependency on EPO- dose, and PTPN18-Y389F:* In cell growth assays, exponentially growing UT7epo-EE-PTPN18, EE-PTPN18-Y389F and EE-EVEC cells (each at 5 to 7×10^5 cells/mL) were washed and plated (at 1×10^5 cells/mL) in EPO at 0.15, 0.3, 0.6, or 1.2 U/mL.

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At day 3.5, viable cell numbers (B), and frequencies of non-viable cells were determined (C). In B and C, graphed values are means +/− SE (n=3). **(D)** *PTPN18, and PTPN18-Y389F effects on UT7epo cell growth are persistent:* UT7epo- EE-PTPN18, EE-PTPN18-Y389F and EE-EVEC cells were plated as in panel B. At 4-day intervals, cultures were passed volumetrically to fresh medium with EPO at 0.3 U/mL. At day-21, numbers of viable cells and frequencies of non-viable cells were determined (mean values +/− SE, n=3) (left panel). For comparison, data for 3-d assays for cells cultured in EPO at 0.6 U/mL are provided (mean values +/− SE, n=3). **(E)** *PTPN18 effects on UT7epo cell survival following EPO withdrawal*: Exponentially growing UT7epo- EE-PTPN18, EE-PTPN18Y389F, and EE-EVEC cells were washed thrice and plated in IMDM, 10% FBS in the absence of EPO. Viability then was assayed at 20h and 24h post plating. At 20h, viability for each cell population was ≥95% (data not shown). At 24h, and as assayed via Annexin-V staining, significant cytoprotective effects were exerted by ectopically expressed PTPN18 (EE-PTPN18), and to a lesser extent EE-PTPN18-Y389F. Graphed values are means +/− SE (n=3). Representative primary flow cytometry data are also illustrated (right sub-panel).

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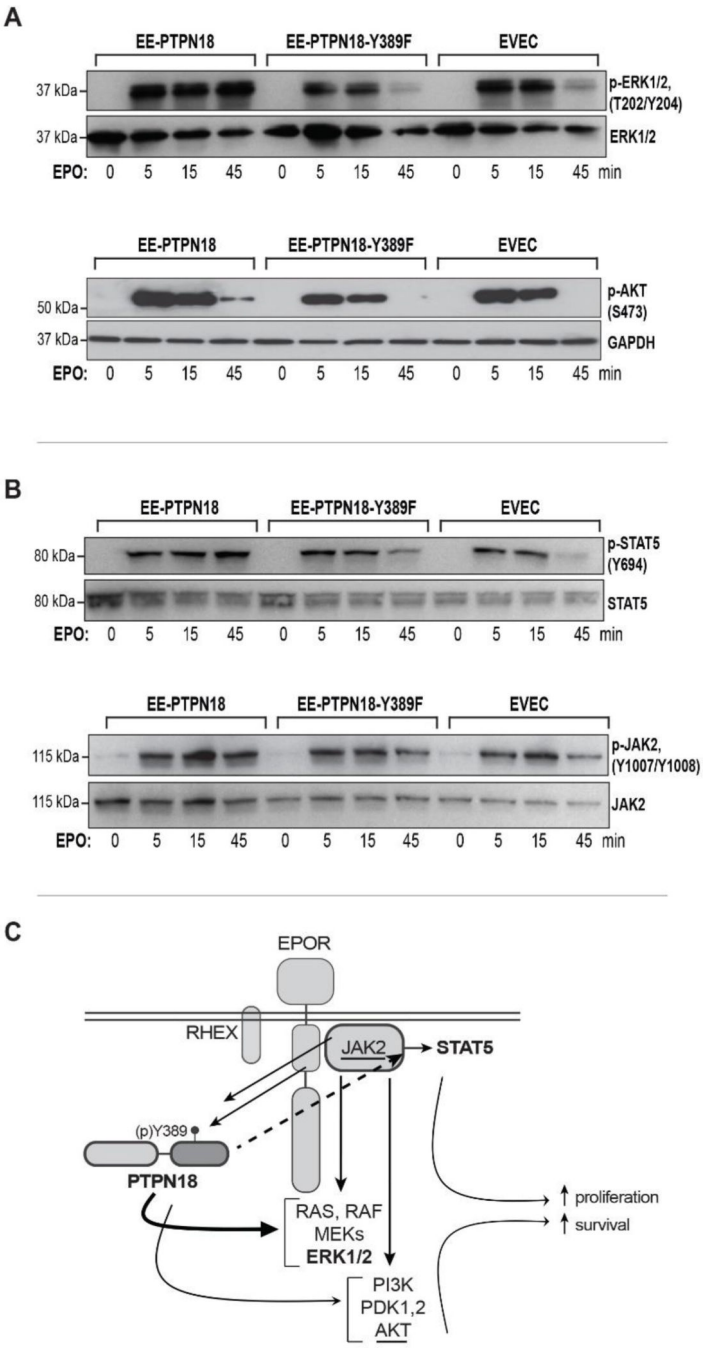

FIGURE 6. PTPN18 sustains the EPO- induced activation of ERK1/2, AKT and JAK2/STAT5.

UT7epo cells stably transduced with wt-PTPN18 ("EE-PTPN18"), PTPN18-Y389F ("EE-PTPN18-Y389F") or empty vector (EVEC) lentiviruses were plated at 1×10^5 cells/mL, and expanded to exponential growth phase (5 to 7×10^5 cells/mL). Cells were washed and plated at 7×10^5 cells per mL in IMDM 10% FBS without EPO. At 20 hours post EPO withdrawal, cells were challenged with EPO (1.5 U/mL), and at the indicated time points (0, 5, 15, 45 minutes) cells were brought to 2˚C, washed and processed for total protein extracts. Via western blot analyses, levels of activation site phosphorylated, and total ERK1/2, AKT,

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STAT5 and JAK2 were then determined. **(A)** *PTPN18 sustains the EPO- induced activation of ERK1/2, and AKT with Y389- dependency:* Cell lysates from UT7epo- EE-PTPN18, EE-PTPN18Y389F and EE-EVEC cells were analyzed by western blotting. Results shown are representative of two independent experiments. **(B)** *PTPN18 sustains the EPO- induced activation of STAT5 and JAK2 with Y389- dependency:* Cells, lysates and samples assayed for phospho- and total STAT5, and phospho- and total JAK2 are those analyzed in panel A. Results shown are representative of two independent experiments. **(C)** *Summarized effects of PTPN18 on EPO- induced ERK1/2, AKT and JAK2/STAT5 activation, and the heightened proliferation and survival of erythroid progenitor UT7epo cells:* The model outlined illustrates the EPOR, JAK2 and the EPOR associated adaptor protein RHEX together with PTPN18's observed positive effects on ERK1/2, AKT and JAK2/STAT5 signaling modules.

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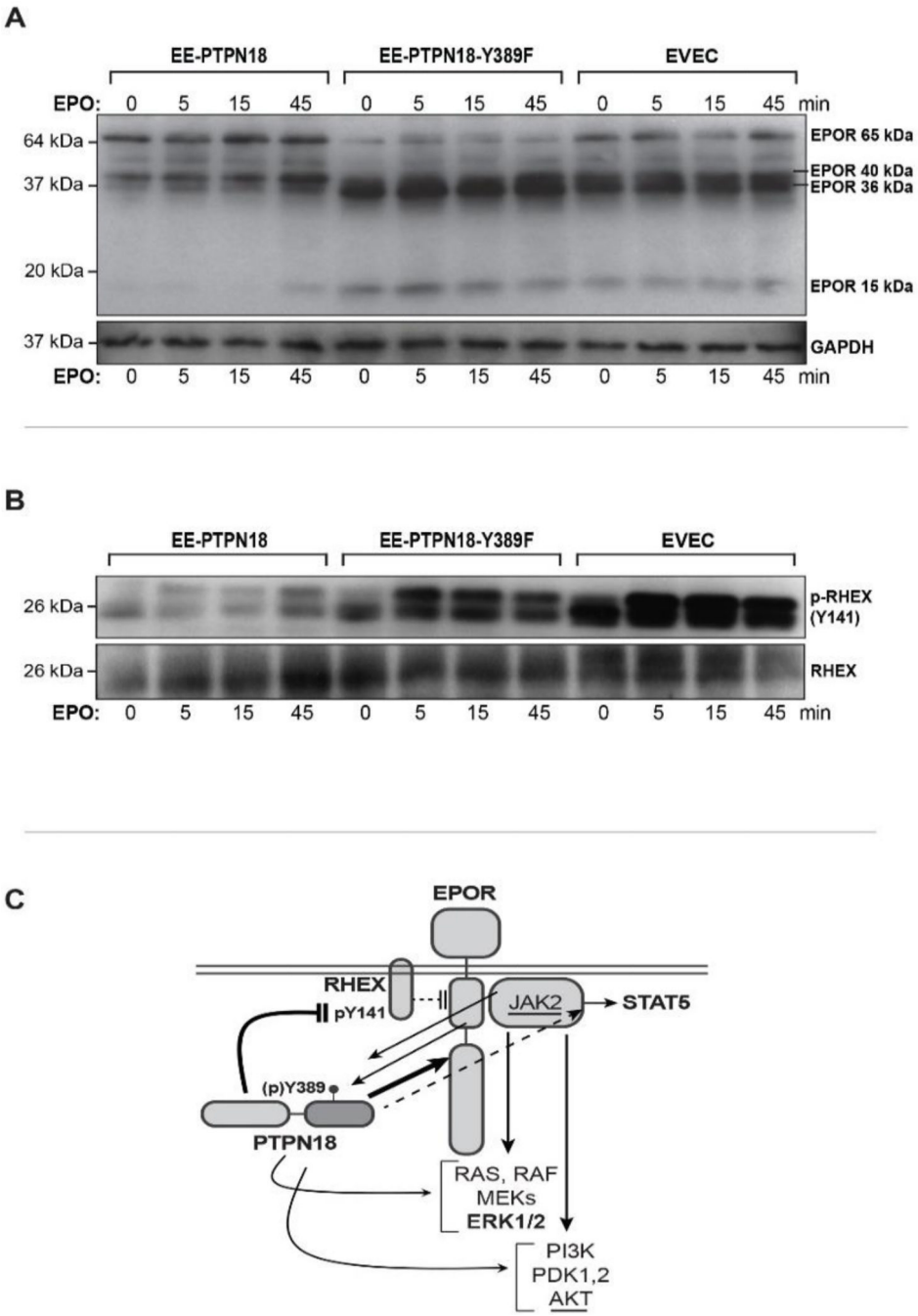

FIGURE 7. PTPN18 increases levels of high molecular weight EPOR forms, and inhibits the EPO- induced phosphorylation of RHEX-pY141.

(A) *PTPN18 increases the formation of high MW EPOR forms*: In UT7epo cells ectopically expressing ("EE") PTPN18, PTPN18-Y389F or empty vector (EVEC), possible effects on apparent EPOR processing were assessed via western blotting. EPO was withdrawn for 20 hours, followed by EPO challenge (1.5 U/mL). At indicated intervals, cell lysates and protein extracts were prepared. Western blotting was then performed using hEPOR specific antibody c1.1. EPOR forms identified include 65K, 40K, 38K and 15K Mr species (see

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reference #77). **(B)** *PTPN18 limits the EPO- induced phosphorylation of RHEX-pY141*: In UT7epo EE-PTPN18, EE-PTPN18-Y389F and EVEC cells, possible effects of ectopically expressed PTPN18 or PTPN18-Y389F on the p-Y phosphorylation of the EPOR associated adaptor protein RHEX were assessed via western blotting (for p-Y phosphorylated RHEX, it's migration in SDS PAGE is shifted to ~28K MW. **(C)** *Model for PTPN18 regulation of RHEX-pY141 phosphorylation and EPOR processing*: In a working model, PTPN18 is suggested to limit EPOR turnover via a pathway potentially associated with PTPN18 inhibition of RHEX-pY141 phosphorylation.