Cytosolic lysine residues enhance anterograde transport and activation of the erythropoietin receptor

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Lysine residues are key residues in many cellular processes, in part due to their ability to accept a wide variety of post-translational modifications. In the present study, we identify the EPO-R [EPO (erythropoietin) receptor] cytosolic lysine residues as enhancers of receptor function. EPO-R drives survival, proliferation and differentiation of erythroid progenitor cells via binding of its ligand EPO. We mutated the five EPO-R cytosolic lysine residues to arginine residues (5KR EPO-R), eliminating putative lysine-dependent modifications. Overexpressed 5KR EPO-R displayed impaired ubiquitination and improved stability compared with wt (wild-type) EPO-R. Unexpectedly, fusion proteins consisting of VSVGtsO45 (vesicular stomatitis virus glycoprotein temperature-sensitive folding mutant) with wt or 5KR EPO-R cytosolic domains demonstrated delayed glycan maturation kinetics upon substitution of the lysine residues. Moreover, VSVG-wt EPO-R, but not VSVG-5KR EPO-R, displayed endoplasmic reticulum-associated ubiquitination. Despite similar cell-surface EPO-binding levels of both receptors and the lack of EPO-induced ubiquitination by 5KR EPO-R, the lysine-less mutant produced weaker receptor activation and signalling than the wt receptor. We thus propose that EPO-R cytosolic lysine residues enhance receptor function, most probably through ubiquitination and/or other post-translational modifications.

Key words: endoplasmic reticulum, post-translational modification, signalling, trafficking, ubiquitin, vesicular stomatitis virus glycoprotein.

INTRODUCTION

Lysine residues are key residues in many cellular processes. Owing to their positive charge, they play a major role in protein structure and function, often stabilizing protein–protein interactions or catalytic domains through non-covalent bonds [1,2]. In addition, lysine residues have a more specific role, directed by their ability to undergo different post-translational modifications (e.g. ubiquitination, acetylation, etc.) [3–5]. Lysine-dependent post-translational modifications have been implicated in protein metabolism and function; however, the nature and extent of their role in these processes remain largely unresolved. The EPO-R [EPO (erythropoietin) receptor] is unique among cytokine receptors owing to its strong intracellular retention and short half life, the underlying molecular mechanisms of which have not yet been fully resolved. We therefore questioned the involvement of the cytosolic lysine residues of EPO-R in receptor metabolism and activation.

EPO-R is a type I transmembrane protein ranging between 59 and 70 kDa [6,7] which belongs to the cytokine receptor superfamily [8]. Its ligand, EPO, is required for the survival, proliferation and differentiation of committed erythroid progenitor cells [9]. Members of the cytokine receptor family are devoid of intrinsic kinase activity and therefore rely on an associated tyrosine kinase(s) to initiate downstream signalling. EPO binding to cell-surface EPO-R results in activation of JAK2 (Janus kinase 2), the main tyrosine kinase associated with EPO-R, which undergoes autophosphorylation and then proceeds to phosphorylate the EPO-R homodimer at multiple cytoplasmic tyrosine residues [10]. Subsequently, different downstream effectors are recruited to the phosphorylated receptor, such as the transcription factor STAT5 (signal transducer and activator of transcription 5), MAPK (mitogen-activated protein kinase), and the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase) [11]. Concurrently, down-regulation pathways are activated, resulting in EPO-R dephosphorylation and degradation, mainly in the lysosome, as part of a classic regulatory loop [12–16].

Unlike other cytokine receptors (e.g. [17]), a striking property of the EPO-R is its enigmatically low expression at the cell surface, whereas most of the newly synthesized EPO-R remains sequestered intracellularly [18]. These metabolic features are similar in both transfected [18] and fetal liver cells that express EPO-R endogenously [19], supporting the idea that intrinsic features of the receptor molecule regulate its surface expression. Sequences in both the extracellular and intracellular domains of EPO-R have been documented to affect anterograde transport of the receptor from the ER (endoplasmic reticulum) [20–23].

Ubiquitination is a widely studied lysine-dependent post-translational modification. First discovered as a signal for proteosomal degradation of ubiquitin-tagged proteins, it has since been found to participate in modulation of a wide range of cellular processes, including transcription, DNA repair, endocytosis, endosomal sorting and viral infection [24–26]. Ubiquitination of EPO-R has been demonstrated in different contexts [15,16,27–30]. Most of the studies link EPO-R ubiquitination to down-regulation of receptor signalling; upon activation of the receptor with EPO, the E3 ubiquitin ligase β-TrCP (β-transducin repeat-containing protein) ubiquitiates the
EPO-R, whereas the receptor is still located at the cell surface, resulting in partial degradation of the tyrosine-phosphorylated EPO-R cytosolic domain [15,27]. Eventually, cleaved and uncleaved EPO-Rs reach the lysosome where they are fully degraded. The E3 ubiquitin ligase FLRF (fetal liver ring finger; RNF41) ubiquitinates and down-regulates EPO-R, but in an EPO-independent manner. FLRF overexpression reduces the steady-state levels of the receptor and attenuates differentiation of haemopoietic progenitors [30].

A third ubiquitin ligase, p33RUL, also ubiquitinates EPO-R independent of EPO. However, in contrast with the former two ligases, this ubiquitination transduces EPO-dependent survival and proliferation signals, and does not affect EPO-R stability [29]. These findings have broadened our perspective of the role of EPO-R ubiquitination and call for a more direct approach to examine the effects of EPO-R ubiquitination on receptor metabolism and function.

In the present study we show that the EPO-R is ubiquitinated with and without EPO, depending on its cytosolic lysine residues. In line with this, an EPO-R mutant in which all five cytosolic lysine residues were substituted with arginine residues acquired prolonged stability. Moreover, we present evidence for ER-associated EPO-R ubiquitination. Unexpectedly, we found that mutation of the EPO-R cytosolic lysine residues resulted in impairment of receptor functions, namely delayed ER-to-Golgi transport and reduced EPO-mediated signalling.

EXPERIMENTAL

Antibodies

Rabbit polyclonal antibodies against the first 15 N-terminal residues of EPO-R are described in [18]. Anti-EPO H-162 (sc-7956) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-ubiquitin P4D1 (sc-8017, Santa Cruz Biotechnology), anti-HA (haemagglutinin; MMS-101R, Covance), anti-GFP (green fluorescent protein; 1814460, Roche), anti-actin C4 (69100, ImmunO) mouse monoclonal antibodies, and anti-ubiquitin P4D1 (sc-8017, Santa Cruz Biotechnology) were used for immunoprecipitation and/or Western blot analysis. Antibodies were diluted in 5% milk in TBS-T [TBS (Tris-buffered saline, 25 mM Tris/HCl, pH 7.4) with 0.05% Tween 20], except for anti-ubiquitin antibody, which was diluted 1:500 in TBS-T containing 1% BSA.

Plasmids

Mouse EPO-R and HA–EPO-R cDNA (GenBank® accession number AM193090) were cloned into pXM or pcDNA3 respectively, as described previously [20,31]. 5KR EPO-R was constructed by sequential mutations of the wt (wild-type) EPO-R cDNA according to the QuikChange® protocol (Stratagene). The lysine codons were mutated to arginine by point mutation of the second A nucleotide to G (AAG to AGG), except for Lys348, which was replaced by a double mutation (AAG to AGA). Fusion proteins of the thermoreversible folding mutant of the VSVG (vesicular stomatitis virus glycoprotein) (VSVGtsO45), EPO-R cytosolic domain and YFP (yellow fluorescent protein) were constructed as described previously [21]. All constructs were verified by DNA sequencing.

Cell culture and transfection

HEK (human embryonic kidney)-293 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FCS (fetal calf serum) and transiently transfected using the calcium-phosphate method [32]. BaF3 cells stably expressing EPO-R or HA–EPO-R were cultured in RPMI 1640 medium supplemented with 10% FCS containing rHuEPO (recombinant human EPO) (0.5 unit/ml; Eprex) [18]. Experiments conducted in BaF3 cells were repeated in at least two independent stably transfected clones. Non-transfected BaF3 cells were maintained in RPMI 1640 medium with 10% FCS supplemented with 10% conditioned medium from WEHI-3B cells as a source for IL-3.

Ubiquitination assay

BaF3 ([4–5] × 10^6) or HEK-293 (7 × 10^6) cells were harvested and lysed on ice for 45 min in 50 mM Tris/HCl (pH 7.4)-based lysis buffer, containing 1% Triton X-100, 0.5% DOC (deoxycholate), 150 mM NaCl and 5 mM EDTA, supplemented with freshly added 10 mM NEM (N-ethylmaleimide) and Complete™ PIC (protease inhibitor cocktail; Roche), and subsequently centrifuged at 20800 g for 15 min at 4°C. A 10% aliquot of each cleared lysate was kept as TCL (total cell lysate), whereas the rest was transferred to new tubes containing 0.1% SDS (final concentration), and 1 μl of anti-HA or anti-GFP antibody was added before shaking the tubes overnight at 4°C. The next day, 20 μl of Protein G beads (Santa Cruz Biotechnology) was added prior to a further 1 h of shaking at 4°C. Immunoprecipitates were washed four times in ice-cold lysis buffer containing 0.1% SDS, 10 mM NEM and PIC, followed by two washes in ice-cold TBS. Protein was eluted from the beads with sample buffer and boiled for 5 min before resolving by SDS/PAGE (6 or 7.5% gels).

CHX (cycloheximide) chase and Endo H (endoglycosidase H) treatment

Equal amounts of BaF3 cells were incubated in growth medium containing 35 μM CHX for the times indicated. Cells were lysed in PBS-based lysis buffer, containing 1% Triton X-100, 0.5% DOC, 5 mM EDTA and freshly added PIC. Endo H treatment was performed as described previously [33].

VSVG–EPO-R transport assay

HEK-293 cells were transiently transfected with VSVG-wt (VSVGtsO45-cytosolic-wt-EPO-R–YFP) or VSVG-5KR (VSVGtsO45-cytosolic-5KR-EPO-R–YFP) cDNA for 5 h at 37°C, washed twice with DMEM and incubated at 40°C overnight. The next day, cells were transferred to 32°C to allow VSVG transport along the secretory pathway and stopped by washing with ice-cold PBS on ice at the times indicated. Cell lysates were subjected to cleavage by Endo H, followed by Western blot analysis with anti-HA antibodies.
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Figure 1  Schematic representation of wt and 5KR EPO-R

Schematic representation of wt EPO-R and of 5KR EPO-R, harbouring lysine-to-arginine mutations in the cytosolic domain. TM, transmembrane. 1 and 2 refer to the conserved box 1 and 2 regions. EPO-Rs with an inserted HA tag in the extracellular domain were employed in some experiments.

Cell-surface EPO-R levels

Cells were starved of EPO and FCS for 1 h at 37°C, and then incubated with saturating levels of rHuEPO (20 units/ml) in RPMI 1640 medium containing 10% FCS for 1 h at 4°C [21]. Subsequently, cells were washed three times in ice-cold PBS, lysed and subjected to Western blot analysis with anti-EPO antibodies.

JAK2–EPO-R co-immunoprecipitation

Cell pellets were lysed in 25 mM Tris/HCl (pH 7.4), 1% digitonin, 150 mM NaCl, 1 mM EDTA and freshly added PIC, for 45 min on ice, and debris was removed by 15 min centrifugation at 20800 g at 4°C. Lysates (2%) were kept for analysis of TCL, whereas the rest was rotated overnight at 4°C with 4 μl of anti-HA antibody. Protein G–Sepharose beads (20 μl) were added for 1 h at 4°C, followed by three washes in 25 mM Tris/HCl (pH 7.4), 0.1% digitonin, 150 mM NaCl and 1 mM EDTA and two washes in ice-cold TBS. Precipitated protein was eluted from the beads by boiling in sample buffer for 5 min. Samples were subjected to Western blot analysis with anti-JAK2 antibodies.

Cell-cycle assay

BaF3 cells stably expressing wt or 5KR EPO-Rs were incubated overnight in RPMI 1640 medium containing 10% FCS and the indicated EPO concentrations or IL-3 (control). The next day, cells were washed three times in ice-cold PBS and stained with 50 μg/ml PI (propidium iodide; Sigma), along with 0.1% Triton X-100 and 0.1% citric acid. Samples were then incubated on ice for 1 h and analysed on a FACSort flow cytometer (BD Biosciences) with WINMDI software.

Statistical analysis

Each experiment was performed at least three times, and representative data are shown. Data presented along with the line graph in Figure 3(B) depict the calculated mean ± S.E.M. values, as obtained by a two-tailed, paired Student’s t test. Data in Figures 5, 8 and 9 depict the calculated mean ± S.E.M. values, as obtained by a two-tailed, equal variance Student’s t test. P values < 0.05 were considered significant.

RESULTS

EPO-R cytosolic lysine residues mediate receptor ubiquitination and degradation

To address the role of EPO-R cytosolic lysine residues, we constructed an EPO-R mutant in which all five cytosolic lysine residues were replaced by arginine residues (K256,276,348,388,428R, termed 5KR EPO-R) (Figure 1). Arginine is considered a conservative replacement for lysine as both amino acids are positively charged and relatively large [35], and it is frequently used to abolish ubiquitination or as a general substitution for the residue. First, we determined whether the arginine substitutions indeed prevented ubiquitination of EPO-R. As shown in Figure 2, HA-tagged wt EPO-R expressed in HEK-293 cells was ubiquitinated, whereas ubiquitination of 5KR EPO-R was barely detected.

EPO-R is degraded mainly by the lysosomal pathway [15,36]. However, proteasomal degradation of immature EPO-Rs [37] and proteasomal cleavage of ubiquitinated EPO-Rs following activation at the cell surface [15,27] have been implicated. We questioned whether the lack of cytosolic lysine residues, and consequently impaired EPO-R ubiquitination, affects receptor stability.

Wt and 5KR EPO-Rs were stably transfected into pro-B IL-3-dependent BaF3 cells. This cell line was chosen owing to the wealth of information on it from studies of EPO-R signal transduction and structure–function relationships. It thus allows...
Figure 3  Cytosolic lysine residues confer EPO-R degradation

BaF3 cells \(10^6\) stably expressing wt or 5KR EPO-R were treated with the translation inhibitor CHX in culture medium (RPMI 1640 medium containing 0.5 unit/ml rHuEPO and 10 % FCS) for the time periods indicated. Lysates were incubated for 1 h at 37°C with or without Endo H prior to Western blot analysis with an anti-EPO-R antibody, and re-probed with an anti-JAK2 antibody as a loading control. Black and grey arrows indicate mature and immature EPO-R forms respectively. A representative of seven independent experiments is depicted. (B) The graph represents quantification of total wt and 5KR EPO-R of the blot shown in (A), normalized to JAK2 levels. The numerical values depict the fold increase of 5KR EPO-R in comparison with wt EPO-R for 60 and 120 min of CHX chase, calculated from the quantification of six independent experiments, \(^*P = 0.02, ^{**}P = 0.001\).

A.

![Western blot analysis of wt and 5KR EPO-R with Endo H treatment](image)

B.

![Graph showing quantification of EPO-R degradation](image)

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EPO-R cytosolic lysine residues facilitate receptor maturation

To further assess the differences in wt and 5KR EPO-R metabolism, we analysed the stability of two forms of the EPO-R, immature (ER-associated) and mature (post-ER). Following CHX chase, cell lysates were incubated with Endo H, which cleaves N-linked high-mannose-containing oligosaccharides from immature glycoproteins residing in early compartments of the secretory pathway (i.e. ER and cis-Golgi) [20]. As demonstrated in Figure 3(A), both mature and immature forms of 5KR EPO-R were apparent for a longer time compared with wt EPO-R. The persistence of immature 5KR EPO-R directed our focus to ER-asssociated EPO-R metabolism.

Transport processes along the secretory pathway have been extensively examined via the thermoreversible folding mutant of the VSVG (VSVGtsO45), as it enables synchronizing the release of newly synthesized molecules from the ER and tracking their journey to the plasma membrane (e.g. [39,40]). When incubated at 40°C, VSVGtsO45 is misfolded and accumulates within the ER, whereas upon temperature-shift to 32°C, it acquires the correct conformation and assembly, and is released from the ER to be efficiently expressed at the plasma membrane. Using this experimental design, one can determine the potency of selected motifs in modifying the trafficking kinetics of VSVGtsO45 when fused to the parent molecule [21].

We generated a fusion protein of VSVGtsO45 fused to the cytosolic domain of 5KR EPO-R (Figure 4), corresponding to the previously reported VSVG-wt EPO-R fusion protein [21]. To analyse the contribution of EPO-R cytosolic lysine residues in ER-to-Golgi trafficking, we captured VSVG–EPO-R fusion proteins in the ER by incubating the cells at 40°C overnight. Upon shift to 32°C, the permissive temperature for VSVG anterograde transport along the secretory pathway, VSVG-5KR acquired glycan maturation at a slower rate than VSVG-wt (Figure 5). Notably, after 3 h at the permissive temperature, the observed differences between the glycan-mature fractions of VSVG-wt and VSVG-5KR were diminished.

EPO-R cytosolic domain undergoes ubiquitination while associated with the ER

The VSVG–EPO-R fusion proteins, a useful tool for the isolation of ER-associated processes, enabled addressing the question of whether the fusion proteins are already ubiquitinated at the ER. Figure 6(A) demonstrates that at 40°C, when VSVG and VSVG–EPO-Rs are retained in the ER, VSVG-wt was extensively polyubiquitinated (expressed by the smear between 150 kDa and 250 kDa marks), whereas ubiquitination of VSVG-5KR was similar to the background levels of VSVG alone (Figure 6A). Treatment with Endo H was used to ensure ER localization of the fusion proteins (Figure 6B). VSVG-5KR migrates slightly faster...
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Figure 4  Schematic representation of the VSVG–EPO-R fusion proteins

Figure 5  Delayed maturation of the VSVG-5KR EPO-R fusion protein
(A) HEK-293 cells (10^6) transiently transfected with VSVG-wt or VSVG-5KR fusion proteins were incubated overnight at 40 °C to trap newly synthesized VSVG fusion proteins in the ER. Cells were then shifted to 32 °C, the permissive temperature for ER exit, for the times indicated. Cell lysates were treated with Endo H and subjected to Western blot analysis with an anti-GFP antibody. Black and white arrowheads indicate mature and immature VSVG–EPO-R fusion proteins respectively. (B) The histogram represents quantification of four independent experiments, *P = 0.04, **P = 0.002.

than VSVG-wt; however, following incubation with Endo H, there was a clear downward shift of the whole population, confirming that both samples consist solely of immature proteins.

EPO-R cytosolic lysine residues mediate EPO-induced ubiquitination and enhance receptor activation
Ubiquitination has been demonstrated as a major pathway leading to EPO-R down-regulation upon receptor activation with EPO [15,27]. We therefore examined wt and 5KR EPO-R ubiquitination upon EPO-induced stimulation. As opposed to ligand-independent ubiquitination of EPO-R (Figure 2), this experiment was conducted in BaF3 cells stably expressing HA-tagged wt or 5KR EPO-Rs, which transduce EPO-mediated growth and survival signals. Cells were subjected to stimulation with EPO, and subsequently the EPO-Rs were immunoprecipitated from the lysates with anti-HA antibodies and analysed by Western blotting for ubiquitin moieties. Consistent with the previous assays (Figures 2 and 6), ubiquitination of wt EPO-R was induced in response to EPO, whereas ubiquitination of 5KR EPO-R was impaired (Figure 7).

On the basis of the premise that ubiquitination down-regulates EPO-R signalling [15,27], we thus expected 5KR EPO-R to present an increased and/or prolonged EPO-driven phosphorylation pattern. However, 5KR EPO-R displayed weaker tyrosine phosphorylation of the receptor in response to EPO, with the most striking difference detected after 5 min of incubation with EPO (Figure 8A). Both receptors also displayed similar phosphorylation–dephosphorylation kinetics (Figure 8A). The higher levels of total 5KR EPO-R were attributed to the increased stability of the receptor. Thus, all quantifications were calculated with respect to actin, and not to total EPO-R.

Two parameters are important to verify when addressing mutant EPO-R signalling: cell-surface expression and JAK2 binding. Since 5KR EPO-R traffics along the secretory pathway at a slower rate than wt EPO-R, cell-surface receptor levels may also vary. As a means of estimating surface EPO-R levels, we thus measured the ability of wt and 5KR EPO-R-expressing BaF3 cells to bind EPO at 4 °C. As shown in Figure 8(B), EPO bound to each cell line at similar levels, excluding this factor. Binding of JAK2 to wt and 5KR EPO-R was estimated via immunoprecipitation of HA–EPO-Rs from BaF3 cells and blotting for associated endogenous JAK2 (Figure 8C). Wt and 5KR EPO-Rs were immunoprecipitated with the same amount of JAK2.

To test the biological outcome of wt and 5KR EPO-R signalling, we evaluated the sensitivity of BaF3 cells harbouring wt or 5KR EPO-R to decreasing levels of EPO. Since EPO drives proliferation and survival signals, cells depleted of the hormone stop dividing and undergo apoptosis. To test the overall
Figure 6 EPO-R cytosolic domain undergoes ubiquitination on lysine residues while retained in the ER

(A) HEK-293 cells (7 × 10^6) transiently transfected with VSVG, VSVG-wt or VSVG-5KR fusion proteins, and non-transfected (NT) cells, were incubated overnight at 40°C to trap newly synthesized VSVG protein in the ER. VSVG fusion proteins were immunoprecipitated from the cell lysates with an anti-GFP antibody. Immunoprecipitated (IP) proteins were resolved by SDS/PAGE and subjected to Western blot (WB) analysis. The blot was probed with an anti-ubiquitin (Ub) antibody (P4D1) followed by an anti-GFP antibody. The molecular mass in kDa is indicated on the right-hand side.

(B) TCLs were treated with Endo H for 1 h at 37°C, resolved by SDS/PAGE, and subjected to Western blot analysis with an anti-GFP antibody. Please note, 5KR EPO-R-based mutants run faster on SDS/PAGE, thus Endo H sensitivity should be compared with the (−) Endo H lane of each receptor separately. A representative of four independent experiments is depicted.

Figure 7 Lack of EPO-induced ubiquitination of 5KR EPO-R

BaF3 cells (4 × 10^5) stably expressing HA-tagged wt or 5KR EPO-R were starved of serum and EPO for 1 h at 37°C, and subsequently activated with 10 units/ml rHuEPO for the times indicated. EPO-Rs were immunoprecipitated (IP) with an anti-HA antibody, resolved by SDS/PAGE, and subjected to Western blot (WB) analysis. The blot was probed with an anti-ubiquitin (Ub) antibody (P4D1) followed by an anti-HA antibody. A representative of four independent experiments is depicted. The molecular mass in kDa is indicated on the right-hand side. NT, non-transfected.

We provide novel evidence for lysine-dependent ubiquitination of the ER-associated EPO-R cytosolic domain. The cytosolic lysine residues also contribute to EPO-R function, by enhancing receptor transport along the secretory pathway and enhancing EPO-driven signals. These results shed further light on the role of lysine residues in the regulation of complex protein function.

DISCUSSION

In the present study we demonstrate that the lysine residues in the EPO-R cytosolic domain modulate EPO-R metabolism and function. These conclusions were drawn from experiments with an EPO-R mutant in which the five cytosolic lysine residues were conservatively substituted with arginine residues.

EPO-R ubiquitination

In the present study we show that EPO-R ubiquitination, both in the presence and in the absence of EPO, is dependent on its cytosolic lysine residues (Figures 2 and 7). Although others have shown ubiquitination of EPO-R that is independent of EPO [28–30], the cellular compartments in which this ubiquitination takes place were not demonstrated. Our data clearly show that the EPO-R cytosolic domain can undergo ubiquitination while localized in the ER (Figure 6). This is the first evidence of ER-associated ubiquitination of EPO-R, or of any other cytokine receptor. Further studies are required to identify the ubiquitin ligase responsible for this ER-associated ubiquitination of the EPO-R.

Of the different types of ubiquitin chains that may be attached to a substrate protein, Lys48-linked ubiquitin chains are the most studied [25]. Tagging a protein with these chains leads to its rapid degradation in the proteasome [41]. Therefore proteasome inhibitors are commonly used to assist in detecting ubiquitinated proteins. Notably, ubiquitination of ER-associated VSVG-wt was easily observed, even in the absence of proteasome inhibitors (Figure 6). Moreover, proteasome inhibitors did not enhance ubiquitination of the fusion protein (results not shown), suggesting that this ubiquitination does not necessarily lead to proteasomal degradation.

Loss of the cytosolic lysine residues in 5KR EPO-R resulted in increased receptor stability (Figure 3), observed also by the increased steady-state 5KR EPO-R levels in comparison to wt EPO-R in many experiments (including different cell lines and different stable transfection clones). Moreover, persistence of the immature 5KR EPO-R form was observed, indicating impaired degradation in the early compartments of the secretory pathway or impaired anterograde transport (Figure 3A). The increased stability upon substitution...
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Figure 8 Reduced EPO-mediated 5KR EPO-R activation

(A) BaF3 cells (2 × 10⁶) stably expressing wt or 5KR EPO-R were starved of serum and EPO for 1 h at 37°C, and subsequently activated with 10 units/ml rHuEPO for the times indicated. Cell lysates were subjected to Western blot analysis and probed with an anti-phosphotyrosine antibody (4G10) and an antibody directed against the EPO-R N-terminal domain. The histogram represents quantification of 5KR EPO-R activation after 5 min with EPO in comparison with wt EPO-R. Phosphorylated EPO-R values were divided by actin, as a loading control, and normalized to phosphorylated wt EPO-R. Quantification is of six independent experiments, *P = 0.001. (B) BaF3 cells (10⁶) stably expressing HA-tagged wt or 5KR EPO-Rs were starved of serum and EPO for 1 h at 37°C, and subsequently incubated with 20 units/ml rHuEPO for 1 h at 4°C to measure cell-surface EPO-R. The cells were then washed three times in ice-cold PBS, lysed and subjected to Western blot analysis with the antibodies indicated. The histogram represents 5KR EPO-R cell-surface expression in comparison with wt EPO-R, in which the recovered EPO was normalized to actin. Quantification is of five independent experiments, P = 0.47. (C) BaF3 cells stably expressing HA-tagged wt or 5KR EPO-Rs were lysed and subjected to immunoprecipitation (IP) with an anti-HA antibody. Immunoprecipitated proteins were separated on SDS/PAGE and subjected to Western blot analysis. Endogenous co-immunoprecipitated JAK2 was recovered by anti-JAK2 antibody, after which the blot was probed with anti-HA and anti-actin antibodies as controls. NT, non-transfected.

of the lysine residues in the EPO-R is likely to be related to impaired degradation through the ubiquitin–proteasome pathway. Importantly, the unexpected effects on 5KR EPO-R transport and signalling efficiency constitute the main novelty of the present work.

EPO-R transport

Considerable research has focused on the enigmatically low cell-surface expression of EPO-R. Sequence determinants that facilitate or impair EPO-R maturation have been characterized. Improper folding of the extracellular domain is thought to be the main reason for ER retention of a large population of EPO-Rs. This notion is based on the findings that different mutations in the luminal WSXWS motif alter the efficiency of receptor processing in the ER and expression at the cell surface [22,42], and that the ER chaperone BiP (immunoglobulin heavy-chain-binding protein) has a prolonged association with newly synthesized EPO-R molecules [43]. In addition, the cytosolic domain carries sequence determinants that retard EPO-R vesicular transport from the ER, thereby affecting receptor levels at the cell surface [21,23,44].

The results of the present study suggest lysine-dependent recruitment of proteins to the EPO-R cytosolic domain that assist in EPO-R maturation, processing and/or transport. 5KR EPO-R displayed slower ER-to-Golgi maturation kinetics in the context of the VSVG-5KR fusion protein (Figure 5), also suggested in the CHX chase of the full-length 5KR EPO-R (Figure 3). VSVG-wt and VSVG-5KR fusion proteins eventually acquired a similar population of mature receptors. Furthermore, full-length wt and 5KR EPO-Rs were expressed at similar levels at the plasma membrane (Figure 8B). This may indicate strict regulation of surface EPO-R levels, supporting the notion that EPO-R low surface levels are of biological significance. Alternatively, another pathway(s) contributing to cell-surface levels may also have been affected, and thus the overall surface-expression level of the EPO-Rs eventually became similar (e.g. mature 5KR EPO-R may be more stable than mature wt EPO-R).

EPO-R activation

Since both wt and 5KR EPO-Rs are expressed at similar levels at the cell surface (Figure 8B), and the latter did not undergo ligand-induced ubiquitination (Figure 7), we expected this mutant EPO-R to confer augmented duration and amplitude of EPO-induced signalling. Surprisingly, 5KR EPO-R was tyrosine-phosphorylated to a lower level than the wt receptor upon stimulation with EPO (Figure 8A). In line with this, cells expressing 5KR EPO-R were less sensitive to EPO-driven proliferation and survival signals at low concentrations of EPO (Figure 9) corresponding to physiological EPO levels [27]. We
thus conclude that the lysine residues are true enhancers of the ability of the receptor to undergo EPO-induced activation.

These results do not contradict previous data regarding down-regulation of EPO-R signalling by the ubiquitin–proteasome system, since the experimental systems differ in their approach. The data on β-TrCP-mediated down-regulation were obtained using a S462A EPO-R mutant, thus abolishing binding of the ubiquitin ligase to the receptor [27], which may in itself affect EPO-R function. In the present study, we mutated all five lysine residues, directly abolishing both β-TrCP-mediated and any other ubiquitination, as well as all other lysine-dependent effects. Rather than being contradictory, these different outcomes emphasize the wealth of pathways and the complexity of the signalling process [26].

JAK2 has been documented to play a dual role in EPO-R metabolism; in addition to its crucial part in EPO-R activation, it also facilitates EPO-R trafficking along the secretory pathway [23]. JAK2 binds the ER-associated EPO-R in the box 1 and box 2 regions (Figure 1), which have been thoroughly explored [45]. Two of the five cytosolic lysine residues reside within this area, although their mutation to alanine does not alter JAK2 binding or EPO-R activity [23,46]. Our findings that wt and 5KR EPO-R bind JAK2 to a similar extent (Figure 8C) imply that impaired JAK2 binding is not the underlying factor for retarded trafficking and impaired activity of 5KR EPO-R. Moreover, JAK2 binding to 5KR EPO-R, as well as activation of the main EPO-R signalling pathways (i.e. JAK2/STAT5 and PI3K/Akt; results not shown), lead us to the conclusion that structural changes due to the mutations themselves are minor, if any.

Model for lysine-dependent modulation of EPO-R function

The major difference between lysine and arginine residues is their ability to undergo post-translational modifications. Post-translational modifications frequently regulate the function and fate of a protein or pathway (reviewed in [47]). Some modifications are highly stable (such as glycosylation) and some are highly transient (such as phosphorylation). The latter may be thought of as fine modulators of protein function, as they are extremely dynamic and respond to subtle changes within the cell.

The observations in the present study fit the profile of post-translational modifications. The lysine residues are necessary and sufficient to promote the functions of wt EPO-R that were abolished in 5KR EPO-R, thereby acting as a simple and common site that can appear in many different proteins. A slight migratory difference in SDS/PAGE between wt and 5KR EPO-R is evident (Figure 7). This difference is also maintained in the VSVG–EPO-R fusion proteins (Figure 6B). Since arginine is slightly larger than lysine, the 5KR mutations were predicted to reduce...
lysine residues other than Lys48. Thus the nature of the cargos is carried out by way of atypical ubiquitin chains, linked through a cargo protein from the ER to the Golgi (Figure 6). This may transport from the Golgi. The results of the present study indicate that ubiquitination such as endocytosis and anterograde transport from the Golgi[49]. The results of the present study demonstrate that ubiquitin-mediated regulation of EPO-R trafficking and signalling, in line with the results of the presented study.

AUTHOR CONTRIBUTION
Liron Yosha conceived the study, designed and performed experiments, evaluated data and wrote the paper. Orly Ravid performed experiments and evaluated data. Nathalie Benc-Califa performed experiments. Drorit Neumann conceived the study, designed experiments, evaluated data and wrote the paper.

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