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Role of receptor ubiquitination in erythropoietin receptor signaling

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ROLE OF RECEPTOR UBIQUITINATION IN ERYTHROPOIETIN RECEPTOR SIGNALING

by

Daisuke Mayuzumi

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
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Thesis Supervisor: Associate Professor Frederick W. Quelle

ABSTRACT

Erythropietin (Epo), acting through its receptor (EpoR), is an essential hemotopietic growth factor that regulates the proliferation, differentiation, and survival of erythroid progenitor cells. Perturbations of Epo/EpoR function cause myeloproliferative disease, such as erythrocytosis, or myelodeficient disease, such as anemia. Therefore, defining the mechanisms by which Epo/EpoR control proliferation and differentiation of erythroid cell lineages attracts interest. Ubiquitin-dependent internalization and degradation is a common regulatory mechanisms affecting signaling from a variety of receptors. Although EpoR has been found to be ubiquitinated, the function of EpoR ubiquitination in the regulation of Epo signaling remains unclear. Therefore, the primary goal of this study was to define the role of EpoR ubiquitination in regulating Epo signaling activities and erythroid cell growth. We showed that EpoR was ubiquitinated in response to ligand stimulation, and that loss of EpoR ubiquitination reduced signaling activity and biological responses to low dosages of Epo. We also identified two EpoR lysines that were the primary targets for ubiquitination, and showed that either ubiquitination site supported the enhanced activities of wild-type-EpoR. Ubiquitination of EpoR was also associated with a change in the endocytic pathway mediating internalization of EpoR. Specifically, constitutive internalization of non-ubiquitinated EpoR was found to depend on dynamin activity, while internalization of ubiquitinated EpoR was dynamin-independent but could be inhibited by disrupting lipid raft microdomains in the plasma membrane. Interestingly, inhibiting internalization of ubiquitinated EpoR (by disrupting lipid

rafts) specifically reduced signaling from ubiquitinated receptors without affecting signaling from non-ubiquitinated receptors. Conversely, reducing internalization of non-ubiquitinated EpoR (by inhibiting dynamin) reduced its signaling activity without affecting signaling from ubiquitinated receptors. This strong correlation between EpoR internalization and signaling activity suggests a novel regulatory mechanism in which internalization of EpoR facilitates its signaling activity. In this regard, Epo-induced ubiquitination of EpoR promotes more efficient internalization of ligand-activated receptor and may contribute to enhanced responsiveness to low concentrations of Epo.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To my family

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ABSTRACT

Erythropietin (Epo), acting through its receptor (EpoR), is an essential hemotopoietic growth factor that regulates the proliferation, differentiation, and survival of erythroid progenitor cells. Perturbations of Epo/EpoR function cause myeloproliferative disease, such as erythrocytosis, or myelodeficient disease, such as anemia. Therefore, defining the mechanisms by which Epo/EpoR control proliferation and differentiation of erythroid cell lineages attracts interest. Ubiquitin-dependent internalization and degradation is a common regulatory mechanisms affecting signaling from a variety of receptors. Although EpoR has been found to be ubiquitinated, the function of EpoR ubiquitination in the regulation of Epo signaling remains unclear. Therefore, the primary goal of this study was to define the role of EpoR ubiquitination in regulating Epo signaling activities and erythroid cell growth. We showed that EpoR was ubiquitinated in response to ligand stimulation, and that loss of EpoR ubiquitination reduced signaling activity and biological responses to low dosages of Epo. We also identified two EpoR lysines that were the primary targets for ubiquitination, and showed that either ubiquitination site supported the enhanced activities of wild-type-EpoR. Ubiquitination of EpoR was also associated with a change in the endocytic pathway mediating internalization of EpoR. Specifically, constitutive internalization of non-ubiquitinated EpoR was found to depend on dynamin activity, while internalization of ubiquitinated EpoR was dynamin-independent but could be inhibited by disrupting lipid raft microdomains in the plasma membrane. Interestingly, inhibiting internalization of ubiquitinated EpoR (by disrupting lipid

rafts) specifically reduced signaling from ubiquitinated receptors without affecting signaling from non-ubiquitinated receptors. Conversely, reducing internalization of non-ubiquitinated EpoR (by inhibiting dynamin) reduced its signaling activity without affecting signaling from ubiquitinated receptors. This strong correlation between EpoR internalization and signaling activity suggests a novel regulatory mechanism in which internalization of EpoR facilitates its signaling activity. In this regard, Epo-induced ubiquitination of EpoR promotes more efficient internalization of ligand-activated receptor and may contribute to enhanced responsiveness to low concentrations of Epo.

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LIST OF ABBREVIATIONS

Arg	arginine
BFU-E	burst forming unit erythroid
CD45	cluster designation 45
Cdk2	cyclin-dependent kinase 2
CFU-E	colony forming unit erythroid
CIS	cytokine-inducible SH2
Cys	cysteine
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EDTA	ethylenediaminetetraacetic acid
EGFR	epitherial growth factor receptor
Epo	erythropoietin
EpoR	erythropoietin receptor
Erk	extracellular signal-regulated kinase
GH	growth hormone
GHR	growth hormone receptor
GPCR	G protein-coupled receptor
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HA	hemagglutinin

Hsp90	heat shock protein 90
IFNAR1	interferon-alpha/beta receptor alpha chain
IgG	immunoglobulin G
IL-3	interluekin-3
IL-2R	interluekin-2 receptor
IRS-2	insulin receptor substrate-2
Jak2	Janus kinase 2
MAPK	mitogen-activated protein kinase
MCD	methyl-β-cyclodextrin
MHC	major histocompatibility complex
NEM	N-ethylmaleimide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OβG	octhyl β-glucopyranoside
PAGE	polyacrylmide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PTP-1B	protein tyrosine phosphatase 1B
Ras	Rat sarcoma oncogene
SCF β-Trcp	Skp1-Cullin1-F-box β-transducin repeat-containing protein
SDS	sodium dodecyl sulfate
SHP-1	SH2 domain containing phosphatase 1
SOCS	suppressor of cytokine signaling

SOS	son of sevenless
Stat5	signal transducer and activator of transcription 5
TCL	total cell lysate
TGF-β	transforming growth factor beta
Tyr	tyrosine
Ub	ubiquitin

CHAPTER I

INTRODUCTION

Function of erythropoietin and erythropoietin signaling

Physiological role of erythropoietin

Erythropietin (Epo), a glycoprotein hormone, was identified as a hematopoietic growth factor in 1970, and the human Epo gene was successfully cloned in 1985 (Lin et al., 1985). The physiological role of Epo in erythropoiesis is to regulate production of red blood cells through the promotion of survival, proliferation, and differentiation of erythroid progenitor cells (Lacombe and Mayeux, 1998). Epo controls tissue oxygenation by regulating the production of red blood cells, which transport oxygen to peripheral tissues. Synthesis of Epo is under the control of oxygen-sensing mechanisms in the kidney, where low oxygen concentration in blood stimulates increased expression of Epo (Hardee et al., 2006). Significantly, patients with chronic renal failure cannot produce Epo in response to hypoxia and consequently suffer from serious anemia. Recombinant Epo is used to treat anemia associated with chronic renal failure (Lacombe and Mayeux, 1999). Recombinant Epo has also been applied to cancer patients in order to treat chemotherapy- or radiotherapy-induced anemia resulting from myelosuppression (Abdelrazik and Fouda, 2007; Ots et al., 2005).

Epo receptor

The Epo receptor (EpoR) is a member of the class I cytokine receptor family and was first identified as an Epo-binding protein expressed by normal and transformed erythroid cells (D'Andrea and Zon, 1990). Expression of EpoR is detected at the burst forming unit erythroid (BFU-E), colony forming unit erythroid (CFU-E), and erythroblast stages during erythroid maturation and decreases as erythroid cells mature (Yoshimura and Arai, 1996). Despite its importance to erythroid cell survival, the number of EpoR on the erythroid cell surface is very low, approximately one thousand receptors per cell (Lacombe and Mayeux, 1998). Functional domains of EpoR include a ligand-binding extracellular domain, transmembrane segment, and cytoplasmic domain. Binding of Epo induces activation of EpoR through conformational changes of homodimerized EpoR leading to activation of signaling and biological response (Livnah et al., 1999). However, EpoR can be activated independent of Epo binding. For example, mutation of the extracellular domain converting Arg-129 to Cys produces disulfide bonds between EpoR and stabilizes EpoR dimers in a constitutively active conformation (Longmore and Lodish, 1991). Similarly, Friend murine leukemia virus induces constitutive activation of EpoR mediated by disulfide bond formation between EpoR and the gp55 envelope protein (Hoatlin et al., 1990). These and other mechanisms that promote aberrant activation of EpoR lead to development of myeloproliferative diseases in mice and humans.

Epo-induced signal transduction

Epo-induced signal transduction is indispensable for proliferation and survival of erythroid cells. Loss of Epo-induced signal transduction in Epo-/- or EpoR-/- mice caused apoptosis and failure of erythropoiesis after the CFU stage (Wu et al., 1995). Epo signaling originates from the binding of Epo to EpoR, which induces a change in orientation of the cytoplasmic domains of homodimerized EpoR. The increased proximity of receptor-associated Janus kinase 2 (Jak2) is thought to allow transphosphorylation and activation of Jak2, and subsequent phosphorylation of tyrosines within the cytoplasmic domain of EpoR (Livnah et al., 1999). Jak2 has a pivotal role in Epo signal transduction. Similar to loss of Epo or EpoR, Jak2-/- embryos die due to anemia and failure of erythropoiesis (Neubauer et al., 1998). EpoR signaling activity is mediated through phosphorylation of tyrosines within the cytoplasmic domain of EpoR, which then become docking sites for downstream signaling effectors.

Role of the Stat5 pathway in Epo signaling and erythropoiesis

Once the EpoR is phosphorylated by activated Jak2 after Epo binding, signal transducer and activator of transcription 5 (Stat5) is recruited to two phosphorylated tyrosines of the cytoplasmic domain (Tyr-343 and Tyr-401) (Tilbrook and Klinken, 1999) and is phosphorylated by Jak2 (Fig. 1). Phosphorylation of Stat5 causes its dimerization and translocation to the nucleus, where it functions as a transcription factor for target genes. Stat5a-/-; Stat5b-/-

mice showed ineffective erythropoiesis due to decreased survival of erythroblast (Socolovsky et al., 2001), although the exact requirement for the Stat5 signaling pathway in erythropoiesis has been a subject of controversy. It is reported that Epo can induce expression of Bcl-xL and inhibit erythroid cell apoptosis through Stat5 activation (Silva et al., 1999). Furthermore, a correlation between Stat5 activation and erythroid cell differentiation was observed (Iwatsuki et al., 1997). On the other hand, one experiment using a mutant EpoR in which phosphorylation at Tyr-343 is deficient showed that Stat5 activation was correlated with erythroid cell proliferation (Damen et al., 1995) (Fig. 2). In addition, expression of a constitutively active form of Stat5 in Jak2-/- and EpoR-/- cells mitigated their proliferation defect and rescued erythropoiesis (Grebien et al., 2008). According to these findings, the function of the Stat5 signaling pathway significantly contributes to differentiation, proliferation, and survival of erythroid cells, although the exact role of Stat5 signaling pathway in promoting erythropoiesis remains unclear.

Role of the PI3-kinase pathway in Epo signaling and erythropoiesis

The phosphoinositide 3-kinase (PI3-kinase) pathway is activated by Epo through multiple mechanisms. Epo-induced phosphorylation of Tyr-479 of the EpoR cytoplasmic domain promotes its interaction with the p85 regulatory subunits of PI3-kinase (Damen et al., 1993) (Fig. 1). On the other hand, PI3-kinase can also be activated without direct association with phosphorylated EpoR, but rather through its association with phosphorylated insulin receptor

substrate-2 (IRS-2) (Verdier et al., 1997). The activated PI3-kinase pathway also contributes to proliferation of and inhibition of apoptosis in erythroid cells. Inhibition of apoptosis depends on activation of the serine/threonine kinase Akt, a downstream effector of PI3-kinase, and is associated with increased expression of the antiapoptotic protein Bcl-xL (Sui et al., 2000) (Fig. 2). Increased proliferation is also correlated with increased phosphorylation of Akt (Dai et al., 2005). In addition, it is reported that the PI3-kinase/Akt pathway contributes to cell cycle progression through enhancing phosphorylation of Cdk2 (Henry et al., 2004). According to these reports, it is suggested that the PI3-kinase pathway mediates antiapoptotic and proliferative effects promoting erythroid growth.

Role of the Ras/MAPK pathway in Epo signaling and erythropoiesis

The Ras/mitogen-activated protein kinase (MAPK) pathway is also activated by Epo binding (Fig. 1). Grb2 directly or indirectly interacts with phosphorylated EpoR and activates Ras through a guanine nucleotide exchange factor, SOS. Active GTP bound Ras activates Raf-1, resulting in the phosphorylation and activation of MEK. Finally, activated MEK activates the MAPK, Erk1, and Erk2 (Gobert et al., 1995) (Fig. 2). Activation of the Ras/MAPK pathway is associated with proliferation of erythroid cells, and rapid phosphorylation of Raf-1 and Erk1/2 in response to Epo is observed. Treatment with Raf-1 antisense oligonucleotide inhibits proliferation of erythroid cells (Carroll et al., 1991). In addition, it is reported that Epo synergistically activates Erk1/2 with stem cell factor and promotes erythropoiesis (Sui et al., 1998). These

findings indicate that the Ras/MAPK pathway contributes to erythroid proliferation in erythropoiesis.

Effect of other molecules on erythropoiesis

In normal erythroid progenitor cells, the expression of proto-oncogenes such as c-myc, c-fos, c-jun and pim-1 are induced by Epo (Pircher et al., 2000; Shimizu et al., 1996; Umemura et al., 1988). c-myc and c-fos are reported to contribute to the proliferation of erythroid progenitor cells (Umemura et al., 1988). Treatment of erythroid progenitor cells with antisense oligonucleotides to c-myc inhibits cell proliferation, but not differentiation or apoptosis (Bondurant et al., 1996). On the other hand, c-jun and pim-1 are reported to protect cells from apoptosis (Pircher et al., 2000; Shimizu et al., 1996). Thus, these proto-oncogenes also support erythroid growth by promoting proliferation and inhibiting apoptosis.

Function of Epo in nonhematopoietic cells

Although Epo is known to regulate the proliferation and differentiation of erythroid progenitor cells, recent studies also suggest a function for Epo in nonhematopoietic cells. For example, high expression of Epo and EpoR is observed during fetal brain development (Juul et al., 1999), and knockout of the EpoR gene showed abnormal brain development with decreased neuronal progenitor cells due to increased neuronal apoptosis (X. Yu et al., 2002). Epo and EpoR may also contribute to heart development, since $Epo^{-/-}$ and $EpoR^{-/-}$

mouse embryo died with defects of cardiac morphogenesis (Wu et al., 1999). In addition to brain and heart, the expression and function of Epo and EpoR is found in other organs such as retina or intestine (Chung et al., 2009; Guneli et al., 2007). Therefore, Epo and EpoR may support the normal development of a variety of organs by protecting tissues from apoptosis.

Expression of Epo and EpoR is also found during abnormal tissue development, such as tumor progression (Hardee et al., 2006). High expression of Epo and EpoR is correlated with increased proliferation in breast cancer cells (Acs et al., 2001). Proliferation of renal carcinoma cells is also promoted by Epo (Westenfelder and Baranowski, 2000). Alternatively, blockade of Epo signaling using Epo or EpoR antibodies causes increased apoptosis of ovarian and uterine cancer cells (Yasuda et al., 2001). According to all these reports, Epo and Epo induced signaling regulates cellular development by promoting proliferation and inhibiting apoptosis not only in erythroid progenitor cells, but also in nonhematopoietic cells or tumor cells.

On the other hand, recent studies report the absence of functional EpoR expression in cardiac, neuronal, renal, and tumor cells (Sinclair et al., 2010; Swift et al., 2010). Even though some studies have suggested proliferative function for EpoR signaling in nonhematopoietic cells, other recent studies have found that proliferative effects of EpoR signaling in nonhematopoietic cells are much less significant than in hematopoietic cells. Therefore, the importance of Epo signaling in nonhematopoietic cells is still controversial.

Regulation of Epo signaling

Transient activation of Epo signaling

Epo signaling activities are very transient, reaching maximum levels by 15-20 min and returning to near basal levels by 30-60 min. This tight regulation is important for controlling development of erythroid progenitor cells. Conversely, lack of proper regulatory activity promotes aberrant Epo signaling and Epo-dependent development. For example, C-terminal truncations of EpoR produce unrestrained EpoR signaling activities and are associated with benign erythroid polycythemia in humans (Watowich et al., 1999). Extended signaling activity of EpoR truncation mutants is thought to result from loss of a negative regulatory domain from EpoR. Unstrained signaling activity is also a cause of polycythemia vera, which results from a Jak2 mutation (V617F) that increases Jak2 activity in the absence of receptor ligands (Kralovics et al., 2005). Continuous activation of EpoR produced by virus infection also causes myeloproliferative diseases, such as erythroleukemia (Ruscetti, 1995). Conversely, disrupted or reduced production of Epo causes myelodeficient diseases, such as anemia (Elliott et al., 2008). Therefore, maintaining an appropriate balance of EpoR activation and inactivation is a critical factor in balancing the size of the erythroid cell population.

Downregulation of Epo signaling activities by SOCS family proteins

The ability of suppressor of cytokine signaling (SOCS) family proteins to suppress cytokine signaling activities was discovered in 1997. Overexpression of

SOCS-1, SOCS-3, or cytokine inducible SH2 (CIS) in transgenic mice showed that each protein can inhibit cytokine-induced signaling activities of cytokine family receptors, which suggests that these proteins can also function as a negative regulator of EpoR signaling (Fujimoto et al., 2000; Marine et al., 1999; Matsumoto et al., 1999). The expression of SOCS family proteins such as SOCS-1, SOCS-3, or CIS is strictly regulated at transcriptional levels, and is upregulated by Epo-induced activation of Stat family proteins (Starr et al., 1997).

Although SOCS-1 was discovered as a negative regulator of Epo signaling and SOCS-1-deficient CFU-erythroids showed hypersensitivity to Epo, studies using SOCS-1 knockout mice showed that SOCS-1 is not a critical regulator for erythropoiesis (Metcalf et al., 1999; Sarna et al., 2003). There are several different molecular mechanisms through which SOCS-1 can suppress Epo signaling. First, SOCS-1 binds to phosphorylated tyrosines of the Jak2 activation loop and inhibits its kinase activity toward downstream effectors (Yasukawa et al., 1999) (Fig. 1). Second, SOCS-1 associates with Jak2 and recruits ubiquitination machinery that targets Jak2 for ubiquitin-dependent degradation (Ungureanu et al., 2002).

CIS can also act as a feedback inhibitor of Epo signaling where its expression is induced by activated Stat5 (Matsumoto et al., 1997). The inhibitory action of CIS toward Epo signaling is mediated through its binding to Tyr-401 of EpoR where it competes with Stat5, consequently interrupting Stat5 binding to EpoR and negatively regulating Epo signaling activity (Fig. 1) (Sasaki et al.,

2000). Although CIS suppresses Epo signaling, CIS knockout mice do not have any apparent detectable deficiency of erythropoiesis (Yasukawa et al., 2000).

SOCS-3 is also reported to act as a negative regulator of Epo signaling. Overexpression of SOCS-3 in transgenic mice causes lack of erythropoiesis, whereas deletion of SOCS-3 in knockout mice enhanced erythropoiesis in embryos (Marine et al., 1999). A biologically relevant role for SOCS-3 in suppressing Epo signaling is indicated by observations that SOCS-3 attenuates Epo signaling activities. Similar to CIS, SOCS-3 binds to Tyr-401 of EpoR and inhibits Stat5 binding to EpoR (Sasaki et al., 2000) (Fig. 1). Similar to SOCS-1, SOCS-3 binds to the activation loop of Jak2 and blocks catalytic activity (Hansen et al., 1999).

Downregulation of Epo signaling activities by tyrosine phosphatases

Three different tyrosine phosphatases are reported as modulators of Jak2 kinase activities, SH2 domain containing phosphatase SHP-1, cluster designation 45 (CD45), and protein tyrosine phosphatase 1B (PTP-1B).

SHP-1 binds to Tyr-429 of the EpoR cytoplasmic domain and negatively regulates Jak2 activation (Fig. 1). Studies using a mutant EpoR lacking Tyr-429 showed a deficiency of SHP-1 binding to the receptor, hypersensitivities to Epo, and prolonged Jak2 phosphorylation after Epo treatment (Klingmuller et al., 1995). Motheaten mice in which the SHP-1 gene is naturally knocked-out also showed increased erythropoiesis (Green and Shultz, 1975). These results

indicate that SHP-1 phosphatase activity can attenuate Epo signaling activities, apparently by downregulation of Jak2 activities.

CD45 directly binds to Jak2 in the course of Jak2 dephosphorylation. Disruption of the CD45 gene results in enhanced activation of Jak2 and Stat5 in response to Epo stimulation, which consequently promotes increased erythropoiesis (Irie-Sasaki et al., 2001). This result suggests that CD45 is also a Jak phosphatase that downregulates Epo signaling activities.

PTP-1B also directly binds to Jak2 and inactivates Jak kinase activity. Mouse embryos deficient in PTP-1B showed hyperphosphorylation of Jak2, which indicates that PTP-1B is a negative regulator of Epo signaling (Myers et al., 2001).

Regulation of receptor signaling by internalization

Activation of cell surface-localized receptors by various stimuli transmits information to the inside of cells through various signal transduction pathways. Termination of information flow is controlled by various mechanisms, including desensitization or internalization of receptor. Internalization of receptors from the cell surface is one of these termination mechanisms. For example, G protein-coupled receptors are targeted for endocytosis after ligand stimulation, thus sequestering the receptors from the cell surface and terminating signaling activities (von Zastrow, 2001).

Internalization of the TGFβ receptor also controls TGFβ signaling. Endocytosis of TGFβ receptor mediated by the caveolar/lipid-raft pathway

contributes to the down regulation of its signaling activities (Finger et al., 2008). However, TGFβ receptors are also internalized through clathrin-mediated endocytosis. Unlike the caveolar/lipid-raft mediated endocytosis that down-regulates signaling activities, clathrin-mediated endocytosis of TGFβ receptor may function to promote its signaling activities (Di Guglielmo et al., 2003). Therefore, receptor internalization can lead to either increases or decreases in signaling activity. Moreover, changing the pathway of internalization can produce changes in signaling activity from ligand-activated receptors.

With regard to the cytokine receptor family, the β subunit of the interleukin-2 receptor (IL-2Rβ) is also reported to be internalized through a non-clathrin, caveolar/lipid-raft mediated pathway (Lamaze et al., 2001). However, whether this internalization of IL-2Rβ regulates its signaling activities positively or negatively remains unclear.

Regulation of receptor signaling by ubiquitination

Ubiquitin-mediated degradation of proteins

Ubiquitin (Ub), a 76-amino acid protein, is highly conserved among species. Ub can be attached to lysine residues of a variety of proteins, which targets them for proteosomal or lysosomal degradation. Defects in Ub-dependent regulation result in many diseases, ranging from developmental abnormalities to neurodegenerative diseases and cancer.

Ubiquitination of proteins is a multistep process. First, the carboxy terminus of ubiquitin forms a thiol-ester bond with a ubiquitin-activating enzyme

(E1) in a reaction coupled to ATP hydrolysis. Then, the ubiquitin polypeptide is transferred to a ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin protein ligase (E3) transfers the ubiquitin from the E2 enzyme to a lysine residue of a target protein. E3 enzymes recognize the targeted protein, therefore directing the specificity of ubiquitination.

Targeted proteins can also be poly-ubiquitinated, in which ubiquitin can form a multiple ubiquitin chain via the successive conjugation of ubiquitin moieties to lysine residues of previously ligated ubiquitin units. Polyubiquitin chains connected through different ubiquitin lysines can mediate different activities. For example, Lys-48 linked polyubiquitination is often associated with proteasome-dependent-degradation, while Lys-63 linked polyubiquitination can influence protein interactions or localization. Altogether, different modes of mono- or polyubiquitination can have diverse effects on targeted proteins. For cell surface receptors, these effects are most commonly associated with changes in receptor internalization and targeting for proteasomal or lysosomal degradation (Weissman, 2001).

Regulation of receptor signaling by ubiquitin-mediated internalization

One of the primary regulatory mechanisms of receptor signaling is their ubiquitin-mediated internalization and degradation. These mechanisms are reported for a variety of receptors, including the epidermal growth factor receptor (EGFR), growth hormone receptor (GHR), or interleukin-2 receptor (IL-2R) (Hicke, 1997).

Endocytosis and degradation of ligand-induced EGFR is a primary mechanism for downregulation of EGFR signaling. The desensitization process of EGFR signaling is mediated by the RING finger E3 ligase, c-Cbl. c-Cbl ubiquitinates EGFR, which results in its endocytosis and degradation (Waterman et al., 1999). This ubiquitin-mediated endocytosis of EGFR facilitated by c-Cbl is characterized by monoubiquitination or multiple monoubiquitination rather than polyubiquitination (Mosesson et al., 2003).

IL-2R is also a target for ubiquitination, and its signaling activities are negatively regulated by its ubiquitin-mediated degradation (C. L. Yu and Burakoff, 1997). Similarly, GHR signaling is negatively regulated by ubiquitin-mediated degradation of GHR. There are two different degradation pathways for GHR, ligand-independent and ligand-dependent degradation pathway. Ligand-independent degradation is mediated by a protein chaperon complex including Hsp90. Ligand-dependent degradation is mediated by c-Cbl recruited to activated GHR, which ubiquitinates GHR and targets it for endocytosis and degradation (Lipkowitz, 2003).

Multiple types of ubiquitination affect receptor internalization

The ubiquitination of proteins has been widely associated with protein degradation, particularly where lysine-48-linked polyubiquitin chain formation targets proteins for degradation by proteosomal machinery (Chau et al., 1989). However, other types of ubiquitination promote receptor internalization and degradation through different mechanisms. For example, MHC class I molecules

are polyubiquitinated through lysine 63 linkage and their polyubiquitination is necessary for its endocytosis and lysosomal degradation (Duncan et al., 2006). On the other hand, monoubiquitination or multiple monoubiquitination are sufficient to drive endocytosis and subsequent lysosomal degradation of receptor tyrosine kinases (Haglund et al., 2003; Mosesson et al., 2003). Therefore, different types of receptor ubiquitination may function as signals for endocytosis and degradation of different receptors.

Although in many cases receptor ubiquitination is an important negative regulator of receptor signaling, some observations suggest that an intact ubiquitination system can be necessary for proper signaling activities. In particular, defects in GHR ubiquitination have been associated with inhibition of Stat5 activation after GH stimulation, suggesting that ubiquitination of GHR potentiates GHR signaling activities (Strous et al., 1997). Therefore, receptor ubiquitination may work for not only the negative regulation but also the positive regulation of receptor signaling activities.

Regulation of Epo signal transduction by ubiquitination

Regulation of EpoR activation by proteosomal and lysosomal degradation

Similar to the regulation of other receptors and their signaling activities, EpoR can be targeted for internalization and degradation by ubiquitin-mediated mechanisms. Signaling activities of the EpoR are very transient, with maximum

levels reached by 15-20 min and returning to basal levels by 30-60 min. How much EpoR internalization and degradation contribute to the transient nature of its signaling activity is unclear. However, EpoR has five lysines in its cytoplasmic domain, which could be targets for ubiquitination. Several reports indicate that EpoR is a target for ubiquitination and that Epo signaling activities are regulated by ubiquitin-mediated receptor internalization and degradation. Epo-induced activation of Stat5 in UT-7 human EpoR-expressing cells is prolonged by treatment with proteosomal inhibitors (Verdier et al., 1998). This suggests that proteosomal degradation down-regulates Epo signaling. Moreover, proteosomal inhibitors have been shown to block degradation of internalized Epo-EpoR complexes and allow the replenishment of cell surface EpoR pools (Verdier et al., 2000). After Epo-EpoR complexes are internalized, cell surface EpoR pools are supplemented and maintained with newly synthesized EpoR. According to this report, proteosomal machinery was not only involved in the degradation of EpoR, but also regulated the surface EpoR replenishment process.

Evidence also suggests that lysosomal degradation can regulate of Epo signaling activities. Similar to the inhibition of proteosomal degradation, inhibition of lysosomal degradation slows EpoR degradation kinetics after Epo stimulation. Also, treatment with both proteosomal and lysosomal degradation inhibitors have been reported to further reduced the degradation of EpoR after Epo stimulation (Walrafen et al., 2005).

All these reports are consistent with a role for EpoR ubiquitination in regulating Epo signaling activities by promoting degradation of EpoR through proteosomal and lysosomal machinery.

Ubiquitin ligases specific for EpoR

SCFβ-Trcp (Skp1-Cullin1-F-box β-transducin repeat-containing protein) was originally identified as an E3 ubiquitin ligase for the NF-κB inhibitor, IκB (Yaron et al., 1998). It is also known that this E3 ubiquitin ligase mediates ubiquitination and degradation of the prolactin receptor, the IFNαR1 subunit of the type I interferon receptor, and the GH receptor (Kumar et al., 2004; Li et al., 2004; van Kerkhof et al., 2007). SCFβ-Trcp binds to $DSGX_{2+n}S$ consensus sequence motifs in substrates, targeting them for ubiquitination. EpoR also has this DSG SCFβ-Trcp recognition motif within its cytoplasmic domain, and associates with SCFβ-Trcp. Serine to alanine mutation of the EpoR DSG motif disrupted interaction with SCFβ-Trcp and reduced ubiquitination of EpoR after Epo stimulation (Meyer et al., 2007). Also, EpoR DSG mutations were found to increase the duration of EpoR phosphorylation and increased the sensitivity to low concentration of Epo (Meyer et al., 2007). These observations suggest that EpoR ubiquitination by SCFβ-Trcp contributes to the down-regulation of Epo signaling activities.

A different EpoR ubiquitin ligase, $p33^{RUL}$, was identified by yeast-2-hybrid-screen. Ligase activity-deficient mutants of $p33^{RUL}$ and knockdown of $p33^{RUL}$ using siRNA caused a deficiency of EpoR ubiquitination. Also, $p33^{RUL}$ mutants

downregulated Epo-induced expression of several downstream effectors, such as c-myc, bcl-2, bcl-xl, and inhibited Epo-dependent survival and proliferation of factor-dependent cells (Friedman et al., 2003). This suggested that EpoR ubiquitination by $p33^{RUL}$ may promote Epo-induced proliferation.

Genetic aberrations of EpoR that cause hypererythrocytosis

A point mutation that causes erythroleukemia

Constitutively activating mutations in EpoR were isolated using an *in vitro* retroviral transduction system (Longmore and Lodish, 1991). It was found that a single point mutation at codon 129 resulting in an arginine to cysteine substitution in the extracellular domain of EpoR caused erythroleukemia. Introduction of this EpoR mutant cDNA into mice using virus-mediated gene delivery induced continuous signal transducing activities and cell proliferation in the absence of ligand stimulation, resulting in development of erythroleukemia. Therefore, a single point mutation at codon 129 is an oncogenic mutation that induces factor-independent erythroid proliferation. This activating mutation was found to alter glycosylation of the extracellular domain and reduce receptor turnover (Yoshimura et al., 1990), however, no structural alternations of receptor intracellular domains have been associated with this mutation.

A truncation mutation that causes familial erythrocytosis

Familial erythrocytosis is a myeloproliferative disorder that is autosomal dominantly inherited. Resent studies have determined that familial erythrocytosis is caused by genetic mutations of EpoR. It was demonstrated that part of the EpoR cytoplasmic domain is truncated in affected individuals and those truncations made the erythroid cells hypersensitive to Epo compared with wild-type EpoR-expressing erythroid cells (Arcasoy et al., 1997; Arcasoy et al., 2002; de la Chapelle et al., 1993). Although it has been proposed that this enhanced EpoR activity is due to loss of a negative regulatory domain (de la Chapelle et al., 1993), the complete molecular mechanisms responsible for this phenotype remain unclear.

Purpose of study

Epo, acting through EpoR, is an essential hematopoietic growth factor that regulates the proliferation, differentiation, and survival of erythroid progenitors. While Epo/EpoR function controls the production of red blood cells by regulating erythroid progenitors, defects in Epo/EpoR regulation cause myeloproliferative diseases, such as erythrocytosis, or myelodeficient disease, such as anemia. Therefore, the elucidation of the mechanisms controlling Epo/EpoR signaling and proliferation/differentiation of erythroid progenitors attracts interest.

EpoR expressed on the erythroid cell surface mediates Epo-induced activities, such as Jak2-dependent activation of Stat5/PI-3 kinase/MAPK

signaling pathways. Epo-EpoR signaling activities are transient even in the continued presence of ligand, indicating that mechanisms of regulation exist beyond simple engagement of receptors by Epo ligands. Disruption of these regulatory mechanisms may contribute to myeloproliferative/myelodeficient disease. Indeed, C-terminal truncated forms of EpoR produce unrestrained Epo-EpoR signaling activities in humans, indicating that C-terminal domains are important for regulation of the EpoR signaling. However, the mechanism through which this regulation is effected remains incompletely understood.

One of the regulatory mechanisms of receptor signaling after ligand stimulation is receptor internalization and degradation by ubiquitin-dependent pathways. These regulatory mechanisms are well characterized in the case of several receptors including GHR and EGFR. Interestingly, EpoR has also been found to be ubiquitinated, which indicates that EpoR internalization and degradation by ubiquitin-dependent pathways are potential mechanisms regulating EpoR signaling activities. However, the function of EpoR ubiquitination in the regulation of Epo signaling is still unclear.

Several studies using proteosome/lysosome degradation inhibitors suggested that both proteosomes and lysosomes degrade activated EpoR. Moreover, it was found that inhibition of EpoR degradation with proteosomal inhibitors prolonged Epo signaling activities. These results suggest that EpoR ubiquitination and its degradation are important for turning off Epo signaling. However, not only EpoR but also other downstream effectors such as Jak2, Stat5, PI3 kinase and Akt are targets for ubiquitin-mediated degradation (Chen et

al., 2006; Fang et al., 2001; Kamizono et al., 2001; Suizu et al., 2009). Therefore, the approach using proteosomal/lysosomal inhibitors is not appropriate to specifically evaluate EpoR ubiquitination effects on Epo signaling activities.

The major goal of this study is to clarify the role of EpoR ubiquitination in regulating EpoR signaling activities and erythroid cell growth. In order to accomplish this goal, three specific aims are to be investigated.

1. To examine the effect of EpoR ubiquitination on regulation of Epo signaling activities and cell growth
2. To determine the EpoR domains required for its ubiquitination and Ub-mediated effects on signaling activities and cell growth
3. To elucidate the mechanism through which EpoR ubiquitination affects Epo signaling activities and cell growth

Addressing these aims will enhance an understanding of the role of EpoR ubiquitination in regulation of Epo signaling activities and erythroid cell growth.

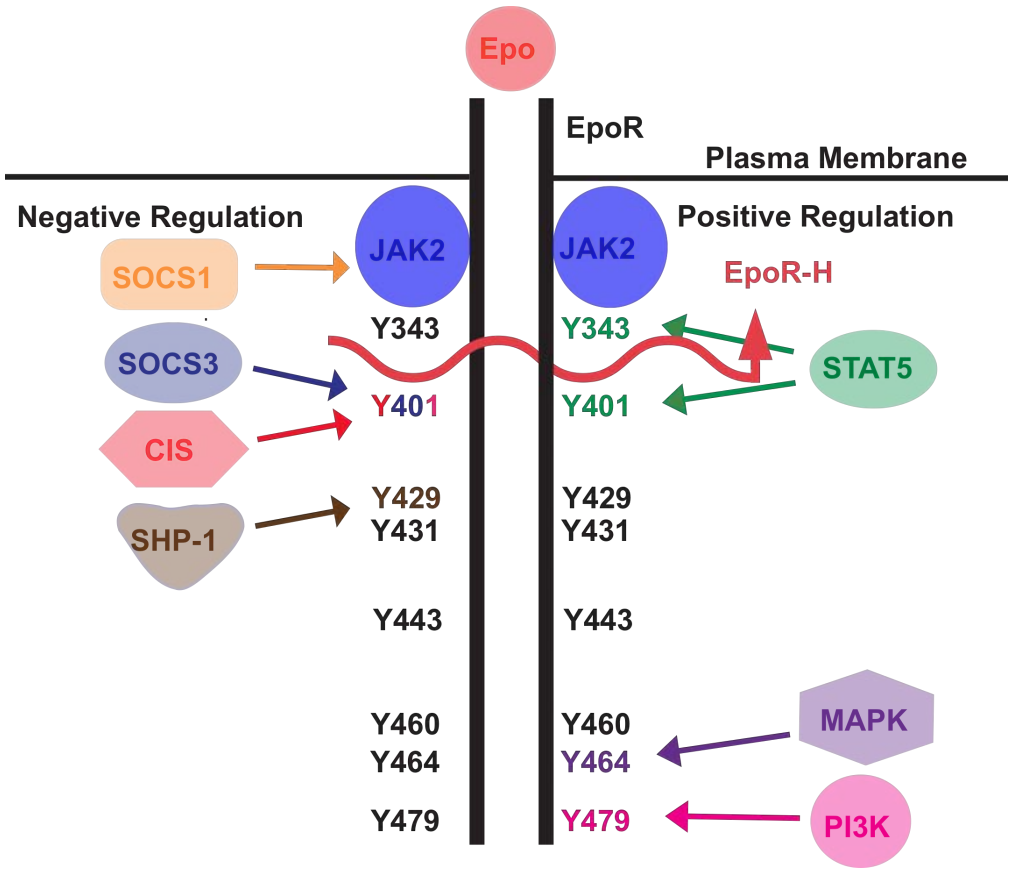


Figure 1. Schematic diagram of cytoplasmic domain of EpoR and identified binding sites for downstream effectors.

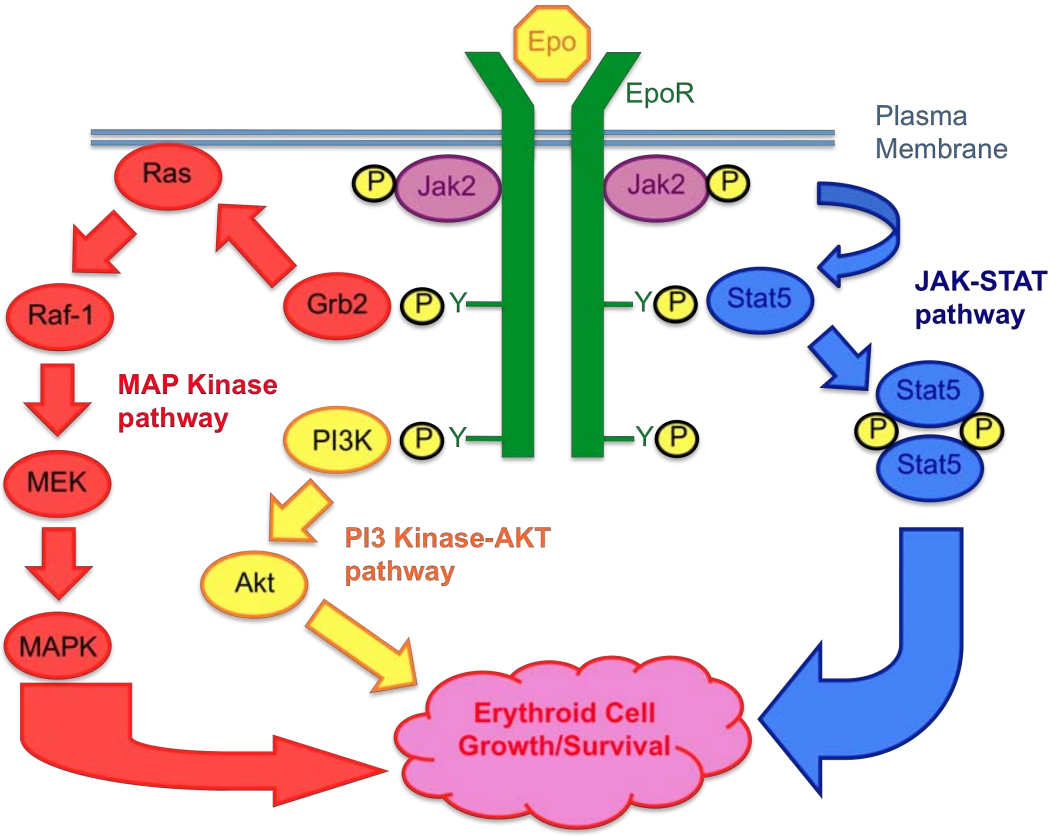


Figure 2. Model of proposed signaling pathways for the growth and survival of erythroid progenitor cells.

CHAPTER II
EFFECT OF EPOR UBIQUITINATION ON REGULATION OF EPO SIGNALING ACTIVITIES AND CELL GROWTH

Rationale

Ubiquitin-mediated internalization and degradation of receptors is a common regulatory mechanism in receptor signaling. Although EpoR has five lysine residues in its cytoplasmic domain that could be targets for ubiquitination, it is still unclear if Epo signaling activities and subsequent physiological response of erythroid progenitors are regulated by EpoR ubiquitination. Analyses using proteosomal or lysosomal degradation inhibitors have assessed a general role for ubiquitination in Epo signaling. However, several downstream effectors, such as Jak2, Stat5, PI3 kinase and Akt are also targets for ubiquitin-mediated degradation. Therefore, previous approaches using pharmacological inhibitors of ubiquitin-mediated degradation do not specifically address the effects of direct EpoR ubiquitination on Epo signaling activities. In this chapter, we constructed a mutated EpoR lacking all five cytoplasmic lysines (5KR-EpoR) in order to define the effects of EpoR ubiquitination on Epo signaling and Epo-dependent cell growth.

Materials and methods

Antibodies, reagents, and chemicals

Rat monoclonal anti-HA antibodies conjugated to horse-radish peroxidase (3F10) and mouse monoclonal anti-ubiquitin antibodies were purchased from Roche and Santa Cruz Biotechnology, respectively. Mouse monoclonal antibodies specific to phosphorylated Stat5A/B (Tyr-694/699) were obtained from Upstate Biotechnology. Mouse monoclonal anti-phosphotyrosine antibodies were purchased from Millipore. Mouse monoclonal antibodies specific to phosphorylated Erk (Tyr-204) were purchased from Santa Cruz Biotechnologies. Rabbit anti-Jak2 antibodies and rabbit anti-Stat5A antibodies were produced in our laboratory. Rabbit anti-rat IgG antibodies were obtained from Sigma Aldrich. Donkey anti-mouse IgG antibodies conjugated to IRDye and Donkey anti-rabbit IgG antibodies conjugated to IRDye were purchased from LI-COR. Recombinant erythropoietin (Epogen) was purchased from Amgen. Protein A-Sepharose and High Capacity Neutravidin-Agarose were obtained from Amersham Bioscience and Thermo Scientific, respectively. Cell-titer 96-well Aqueous MTS cell proliferation solution was purchased from Promega. EZ-link Sulfo-NHS-Biotin was purchased from Thermo Scientific. MG132 was from Calbiochem. Protease inhibitor and Phosphatase inhibitor cocktails for mammalian cells were obtained from Sigma Aldrich. N-ethylmaleimide (NEM) was from Fisher Scientific. Octhyl β-D-glucopyranoside (OβG) was purchased from Sigma Aldrich.

Cell lines and culture conditions

Cell lines used for experiments were derived from the BaF3 mouse pre-B cell line. Cells were maintained in RPMI1640 medium containing 10% fetal bovine serum, 2 mM glutamine, and recombinant murine IL-3 (70 pg/ml). Cells were cultured at 37°C in 5% CO_2.

DNA constructs

DNA constructs (pXM-HA-WT, pXM-HA-5KR) expressing WT or 5KR-EpoR in which all lysines on cytoplasmic domain were mutated to arginine were previously made by our lab (Fig. 3).

Establishment of stable cell lines that express WT or 5KR-EpoR

BaF3 cells were grown and transiently transfected by electroporation with pXM-HA-WT or pXM-HA-5KR, vectors for expressing HA epitope-tagged wild-type EpoR (WT-EpoR) of the EpoR with five-cytoplasmic lysine to arginine substitution (5KR-EpoR), respectively. After transfection, the BaF3 cells were grown with RPMI media containing 5 U/ml Epo to select for functional expression of the transfected cDNAs, and clonal cell lines were obtained by limiting dilution. Finally, Western blotting analysis using anti-HA antibodies was performed to identify subclones that express equivalent levels of EpoR-HA protein (Fig. 4).

EpoR ubiquitination assay

In order to assess ubiquitination of EpoR, cell surface receptors were isolated by a sequential precipitation technique from BaF3 cells expressing WT- or 5KR-EpoR constructs. Cells were starved in ligand-free RPMI media for 3 hrs. Starved cells were stimulated with 10 U/ml Epo for 15 min at 37°C, and were resuspended in ice-cold PBS to terminate signaling and movement of proteins within membranes. Cell surface proteins were then labeled with sulfo-NHS-biotin (0.25 mg/ml) for 30 min at 4°C. Glycine (0.1 M) was added to inactivate free NHS-biotin, and the labeled cells were collected by centrifugation (5 min at 500 x *g*). Cells were suspended in lysis buffer (10 mM tris-base, 1% triton X-100, 5 mM EDTA, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovandate, 0.1% SDS, 0.5% deoxycholic acid, 60 mM octhyl β-D-glucopyranoside (OβG), 10 mM N-ethylmaleimide (NEM), 20 μM MG-132, 1x protease inhibitor cocktail, and 1x phosphatase inhibitor cocktail, pH=7.6) for 30 min. at 4°C. Lysates were cleared of insoluble material by centrifugation for 10 min at 10,000 x *g*. For the first precipitation, total EpoR was immunoprecipitated with anti-HA antibodies and eluted with SDS sample buffer (125 mM tris-base, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.008% bromophenol blue). Eluted samples were diluted 1/10 with lysis buffer, and biotinylated cell surface proteins were precipitated with neutravidin-agarose. Finally, sequentially precipitated proteins were eluted with SDS sample buffer and analyzed by Western blotting.

For Western blotting, precipitated EpoR was resolved on 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Then, membranes were probed with anti-HA antibodies to determine levels of precipitated, biotinylated, HA-tagged receptors, or probed with anti-ubiquitin antibodies to detect EpoR ubiquitination.

Cell viability and cell proliferation assays

MTS assay

MTS assay was performed using BaF3 cells expressing WT or 5KR-EpoR that had been starved by incubation in cytokine-free media for 3hrs. Cells were plated in 96-well plates (2 x 10^5 cells/ml) with specified concentrations of Epo or IL-3. Plates were incubated at 37°C, 5% CO_2 for 48 hrs. Then, cells were treated with CellTiter 96 AQueous MTS solution and incubated for 60 min. Metabolized MTS products were measured by absorbance at 490 nm on a microplate reader.

Cell proliferation and viability analysis

Trypan blue cell counts were performed to assess proliferation and viability of cells expressing WT- or mutant EpoR. Cells were starved for 3 hrs in cytokine-free media and plated in 12 well dishes (1 x 10^5 cells/ml) in media containing the indicated concentrations of Epo or IL-3. Cell numbers were determined at 24, 48, and 72 hrs time points by mixing culture samples with trypan blue solution and counting live (trypan blue-excluded) and dead (trypan

blue-stained) cells on a hemocytometer. For each sample, at least 100 total cells were counted.

Signaling activity assay

Activation of downstream effectors, such as phospho-Jak2, phospho-Stat5, or phospho-Erk was used as indicators of EpoR signaling activity. BaF3 cells expressing the WT- or mutant EpoR were starved for 3 hrs in cytokine-free media and stimulated with the indicated concentrations of Epo at 37°C. Cells were lysed by suspension in SDS sample buffer after the specified stimulation time period. Cell lysates were resolved on 7.5% SDS-PAGE gels, and gels were subjected to Western blotting using anti-phospho Stat5 or anti-phospho Erk antibodies. For the analysis of phospho-Jak2, cells were lysed using lysis buffer and immunoprecipitated with anti-Jak2 antisera and Protein A-Sepharose. Immunoprecipitates were eluted with SDS sample buffer and analyzed on 7.5% SDS-PAGE gels, with subsequent Western blotting with anti-phosphotyrosine antibodies. Total Jak2 and Stat5 levels were also analyzed by Western blotting using anti-JAK2 and anti-STAT5 antibodies. Blots were visualized by staining with fluorescently tagged secondary antibodies and were quantified on an Odyssey fluorescent imaging system. Levels of phosphorylated Jak2, Stat5, and Erk was normalized to levels of total Jak2, Stat5, and Erk protein, respectively.

Stability of cell surface EpoR

BaF3 cells expressing WT- or 5KR-EpoR were starved for 3 hrs in cytokine-free media and were stimulated at 37°C with 10 U/ml Epo for the indicated periods of time. At each time point, Epo stimulation was terminated by resuspension of the cells in ice-cold PBS, and cell surface EpoR was biotinylated (0.25 mg/ml, 30 min, 4°C) and isolated by precipitation with neutravidin-agarose. Cell surface EpoR was measured by Western blotting using anti-HA antibodies. The stability of cell surface receptors after Epo stimulation was calculated by comparing the intensity of cell surface receptor at each time point with its intensity at time zero.

Results

EpoR is ubiquitinated after Epo stimulation

Ubiquitin can be attached to lysine residues of a variety of proteins, often targeting them for proteosomal or lysosomal degradation. EpoR has five lysine residues within its cytoplasmic domain that could be targets for ubiquitination. In order to define a role for EpoR ubiquitination, we constructed an EpoR mutant lacking all five cytoplasmic lysines (5KR-EpoR) and compared it to the wild type EpoR (WT-EpoR) for its ability to be ubiquitinated in response to Epo stimulation. Since most EpoR expressed in hematopoietic cells is retained within the endoplasmic reticulum (Hilton et al., 1995), we used surface biotinylation to specifically identify ubiquitination of EpoR at the plasma membrane. BaF3 cells

expressing HA-tagged WT- or 5KR-EpoR were stimulated with 10 U/ml Epo for 15 min and the surface EpoR was isolated by sequential precipitation with anti-HA antibodies and neutravidin. Western blotting analysis using anti-ubiquitin antibody showed a difference between the precipitated receptors, where WT-EpoR but not 5KR-EpoR contained high molecular weight ubiquitinated species (Fig. 5, left panel). Although these ubiquitinated species could include proteins co-precipitating with EpoR, a dark exposure of the anti-HA immunoblot detected high molecular weight species of WT-EpoR consistent with direct ubiquitination of this receptor (Fig. 5, right panel). By contrast, no high molecular weight species of 5KR-EpoR were detected. Although receptor ubiquitination was detectable in unstimulated cells, Epo-treatment dramatically increased WT-EpoR ubiquitination. This enhancement of receptor ubiquitination after ligand stimulation appears similar to mechanisms of TGF-β receptor regulation in which TGF-β stimulation enhances receptor ubiquitination and promotes its internalization (Di Guglielmo et al., 2003; Ebisawa et al., 2001). Altogether, these results demonstrated that WT-EpoR is inducibly ubiquitinated after Epo stimulation, and that this activity is disrupted by the lysine to arginine substitution in 5KR-EpoR.

WT-EpoR is more sensitive than 5KR-EpoR to low doses of Epo

In order to investigate the physiological effect of EpoR ubiquitination, we used the MTS assay to assess the ability of Epo to support viability and

proliferation for clonal BaF3 cell lines expressing WT- or 5KR-EpoR. To examine biological response caused by the EpoR-specific effects, results were normalized with values obtained from IL-3-stimulation, since IL-3 receptors are endogenously expressed in BaF3 cells and regulate the same signaling pathways (e.g. Jak2 and Stat5) that are activated by EpoR. In these analyses, the number of viable cells supported by higher dosages of Epo did not differ between the cell lines expressing WT- or 5KR-EpoR (Fig. 6). This suggests that the ability to support survival and proliferation at saturating concentrations of Epo does not differ between WT and 5KR-EpoR. However, at low dosages of Epo, greater numbers of viable cells were found in WT-EpoR as compared to 5KR-EpoR cultures. This suggests that WT-EpoR is more capable of mediating effective signaling when fewer receptors are occupied by ligand.

In order to separate effects on viability versus proliferation, cell counts were obtained and viability was assessed by trypan blue exclusion. Proliferation rates at high dosages of Epo or IL-3 did not differ between cells expressing WT-EpoR versus 5KR-EpoR (Fig. 7). In contrast, at low dosages of Epo (0.1 U/ml), cells expressing WT-EpoR continued to proliferate, whereas cell numbers did not increase in 5KR-EpoR cultures. In addition, the viability of 5KR-EpoR cultures was significantly reduced compared to WT-EpoR cultures at a low dosage of Epo (Fig. 8). Altogether, these results suggest that EpoR ubiquitination may be an important factor affecting responsiveness of erythroid cells to lower concentrations of Epo.

Signaling activity induced by low-dose Epo is reduced for 5KR-EpoR

Reduced proliferation and viability supported by non-ubiquitinatable 5KR-EpoR suggest that signaling activities affecting these responses may also be reduced. Therefore, we examined Epo signaling activities in cells expressing WT- or 5KR-EpoR at different dosages of Epo. Epo signaling activities were analyzed by measuring phosphorylation of Jak2, Stat5, or Erk, with activities at each dosage of Epo normalized to the maximum signal induced by saturating concentrations of Epo (30 U/ml). In these analyses, induced activation of Jak2 (Fig. 9), Stat5 (Fig. 10), and Erk (Fig. 11) were each reduced in 5KR-EpoR cells treated with intermediate concentrations of Epo (0.12 - 3.3 U/ml). These results were consistent with the reduced ability of 5KR-EpoR to support viability and proliferation at sub-maximal doses of Epo, and suggest that enhanced physiological responses of cells expressing WT-EpoR may result from enhanced signaling activities of WT-EpoR at lower dosages of Epo.

We also tested whether enhanced physiological responses of cells expressing WT-EpoR could be related to changes in the duration of Epo signaling activities by WT- versus 5KR-EpoR. Stat5 phosphorylation was measured at several time points after Epo or IL-3 stimulation and normalized to its maximal phosphorylation at 15 min. Signal duration in WT-EpoR was similar to that of 5KR-EpoR, suggesting that the enhanced biological response of WT-EpoR expressing cells was not due to increased signal duration of WT-EpoR signaling (Fig. 12).

Together, these results suggest that EpoR ubiquitination may be an important factor promoting increased signaling activity by EpoR when activated by lower concentrations of Epo. Ubiquitination does not appear to affect the duration or maximal level of signaling activity mediated by EpoR, suggesting that the inherent ability of these receptors to interact with signaling proteins may not be significantly altered by ubiquitination. Instead, EpoR ubiquitination may directly or indirectly facilitate these signaling interactions at sub-saturating concentrations of ligand.

Surface WT-EpoR is less stable than 5KR-EpoR after Epo stimulation

If EpoR ubiquitination facilitates Epo signaling, the number of plasma membrane localized EpoR might also be regulated by ubiquitination. In order to test this possibility, we measured the relative level of surface EpoR as a function of time after ligand stimulation. Compared with WT-EpoR, 5KR-EpoR was more slowly depleted from the cell surface after Epo stimulation (Fig. 13). Nonetheless, lack of ubiquitination did not prevent Epo-induced depletion of surface of EpoR, and WT- and 5KR-EpoR levels where reduced to similar levels by 60 minutes after Epo stimulation. Notably, this enhanced stability of surface 5KR-EpoR did not produce enhanced signaling activities or biological responses. By contrast, enhanced signaling activity and biological responses were associated with WT-EpoR that is also more rapidly depleted from the cell surface. These results suggest that ubiquitin-mediated internalization of EpoR might facilitate more efficient signaling from the EpoR.

Conclusions

Previous studies using proteosomal and lysosomal degradation inhibitors have shown that the EpoR is a target for ubiquitination, and that the EpoR is subject to proteosomal and lysosomal degradation (Verdier et al., 1998; Verdier et al., 2000; Walrafen et al., 2005). However, several downstream effectors of Epo signaling are also targets for ubiquitin-mediated degradation. Therefore, the simple use of proteosomal and/or lysosomal degradation inhibitors does not appropriately assess the specific role of direct EpoR ubiquitination in the signaling activities and biological responses induced by Epo. Here, we generated a ubiquitination-deficient mutant of the EpoR (5KR-EpoR), and assessed the consequences resulting from loss of EpoR ubiquitination on Epo signaling activities and cellular response.

Ubiquitination assays demonstrated that Epo stimulation dramatically increased WT-EpoR ubiquitination, but not ubiquitination of 5KR-EpoR. This result indicates that one or more of the five cytoplasmic lysines of EpoR is a target for ubiquitination. Also, the simultaneous enhancement of EpoR ubiquitination and activation of Epo-induced signaling suggests that EpoR ubiquitination may be involved in the regulation of Epo signaling activities. The majority of ubiquitinated EpoR from Epo-treated cells migrated near 150 kDa in size, suggesting that multiple ubiquitinations of the 80 kDa mature EpoR must be induced. With only five potential sites of ubiquitination, at least some ubiquitinations must be in the form of polyubiquitin chains. It is also possible that mono-ubiquitination occurs at some sites of EpoR. However, we have not

observed significant ubiquitinated EpoR species at or below 100 kDa that would be consistent with simple mono-ubiquitination at one or two sites.

Our studies show that the biological consequences of EpoR ubiquitination are associated with increased viability and proliferation of cells at low concentrations of Epo. These results indicate that ubiquitination of the EpoR has positive rather than negative effects on responses to Epo. This was consistent with the analysis of signaling activity, where Jak2, Stat5, and Erk were more effectively activated by WT-EpoR at low dosages of Epo. Because both Erk and Stat5 activation are downstream of Jak2 in the Epo signaling pathway, the reduced Stat5 and Erk phosphorylation induced by low-dose Epo activation of 5KR-EpoR may simply be a consequence of reduced Jak2 activation under these conditions.

How lack of EpoR ubiquitination might reduce Jak2 activation is uncertain. But, this effect does not appear to involve differences in maximal signaling activity or the duration of signaling activity, since these properties did not significantly differ between WT and 5KR-EpoR. We did find that 5KR-EpoR was depleted less efficiently from the cell surface, suggesting the possibility that EpoR lacking ubiquitination may be internalized differently than ubiquitinated WT-EpoR. However, it is also possible that this difference reflects changes in recycling of receptors. For example, if 5KR-EpoR were recycled faster than WT-EpoR, the net effect would appear to be a reduced rate of depletion from the cell surface for 5KR-EpoR, compared to WT-EpoR. Still, the correlation between reduced overall internalization of receptor and reduced signaling activity by 5KR-

EpoR suggests the possibility that receptor internalization may facilitate signaling by EpoR.

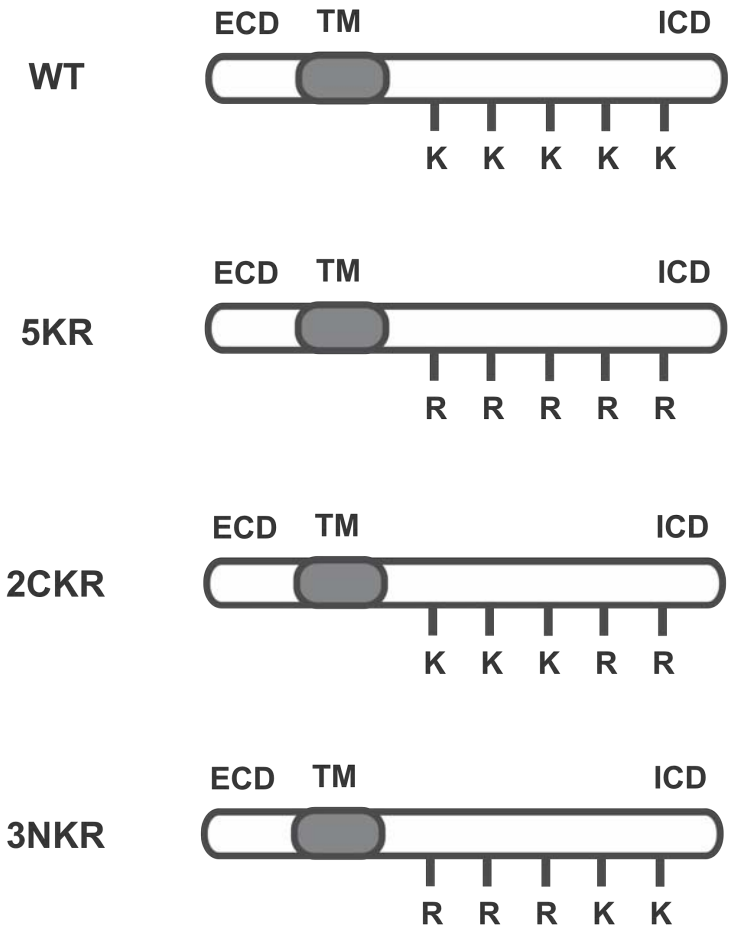


Figure 3. Schematic diagram of indicating K to R substitutions in mutant EpoR.
ECD=Extracellular domain, TM=Transmembrane domain, ICD=Intracellular domain. 5KR=all cytoplasmic lysines are mutated to arginines. 2CKR=2 C-terminal cytoplasmic lysines are mutated to arginines. 3NKR=3 N-terminal cytoplasmic lysines are mutated arginines.

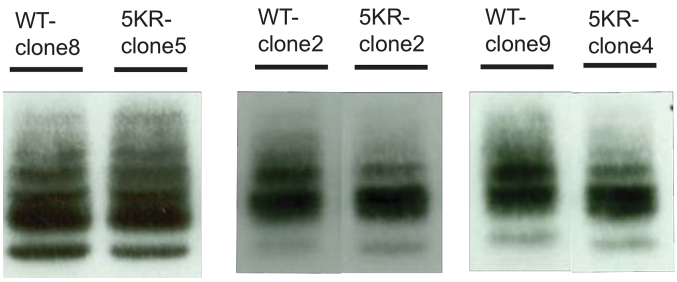


Figure 4. Screen for equivalent expression of EpoR constructs.
Total cell lysates were prepared from clonal cell lines expressing WT- or 5KR-EpoR. Equal amounts of protein from each sample were assayed for EpoR expression by SDS-PAGE and Western Blotting with anti-HA antibodies.

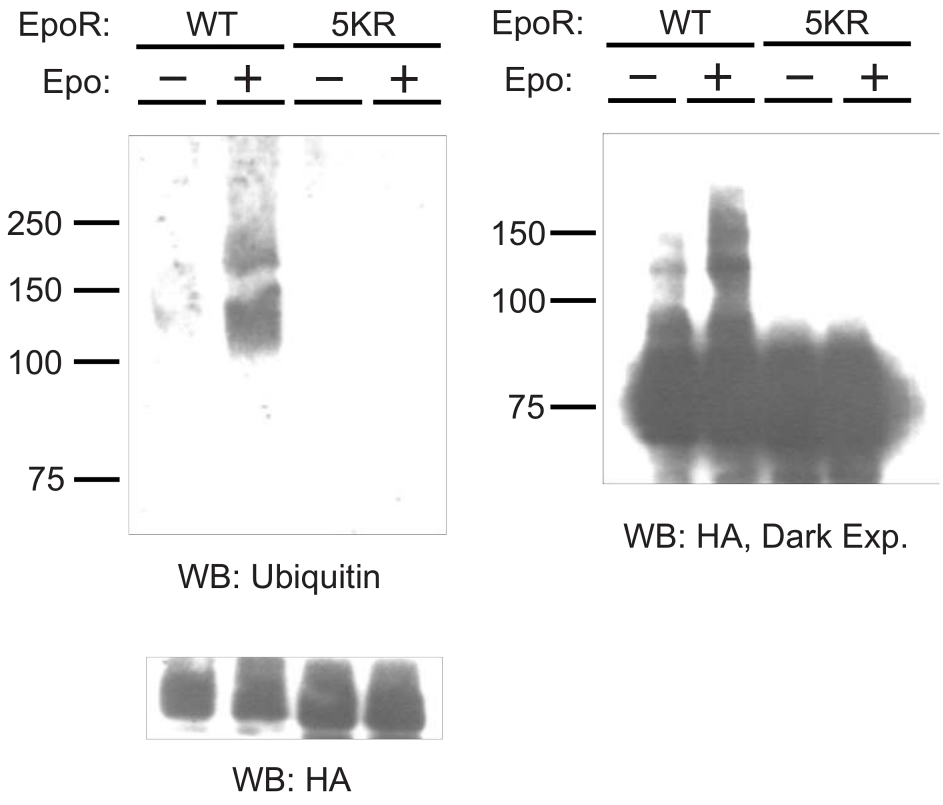


Figure 5. Ubiquitination of WT- and 5KR-EpoR in BaF3 cells.
BaF3 cells expressing WT or 5KR-EpoR were unstimulated or stimulated with 10 U/ml Epo for 15 min at 37°C. Then, surface EpoR was labeled with a membrane-impermeable biotin reagent. After the cells were lysed, cell surface EpoR was sequentially precipitated with agarose conjugated anti-HA antibodies and neutravidin. Precipitates were western blotted (WB) with anti-HA or anti-ubiquitin antibodies.

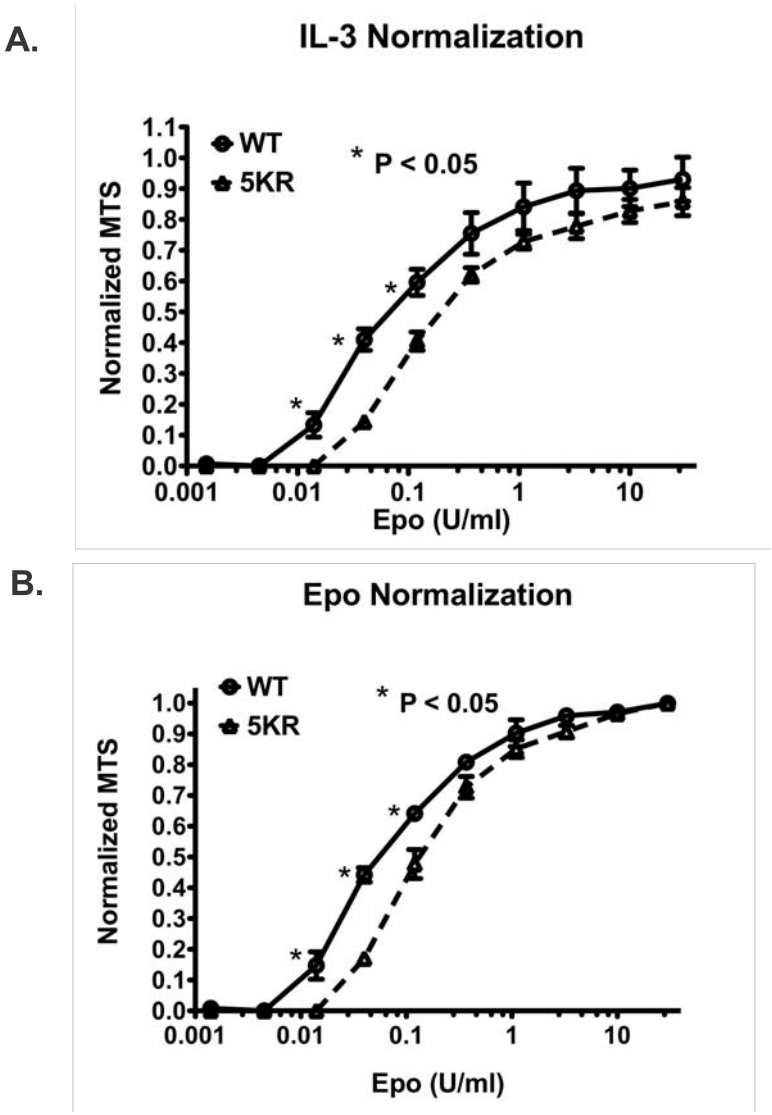
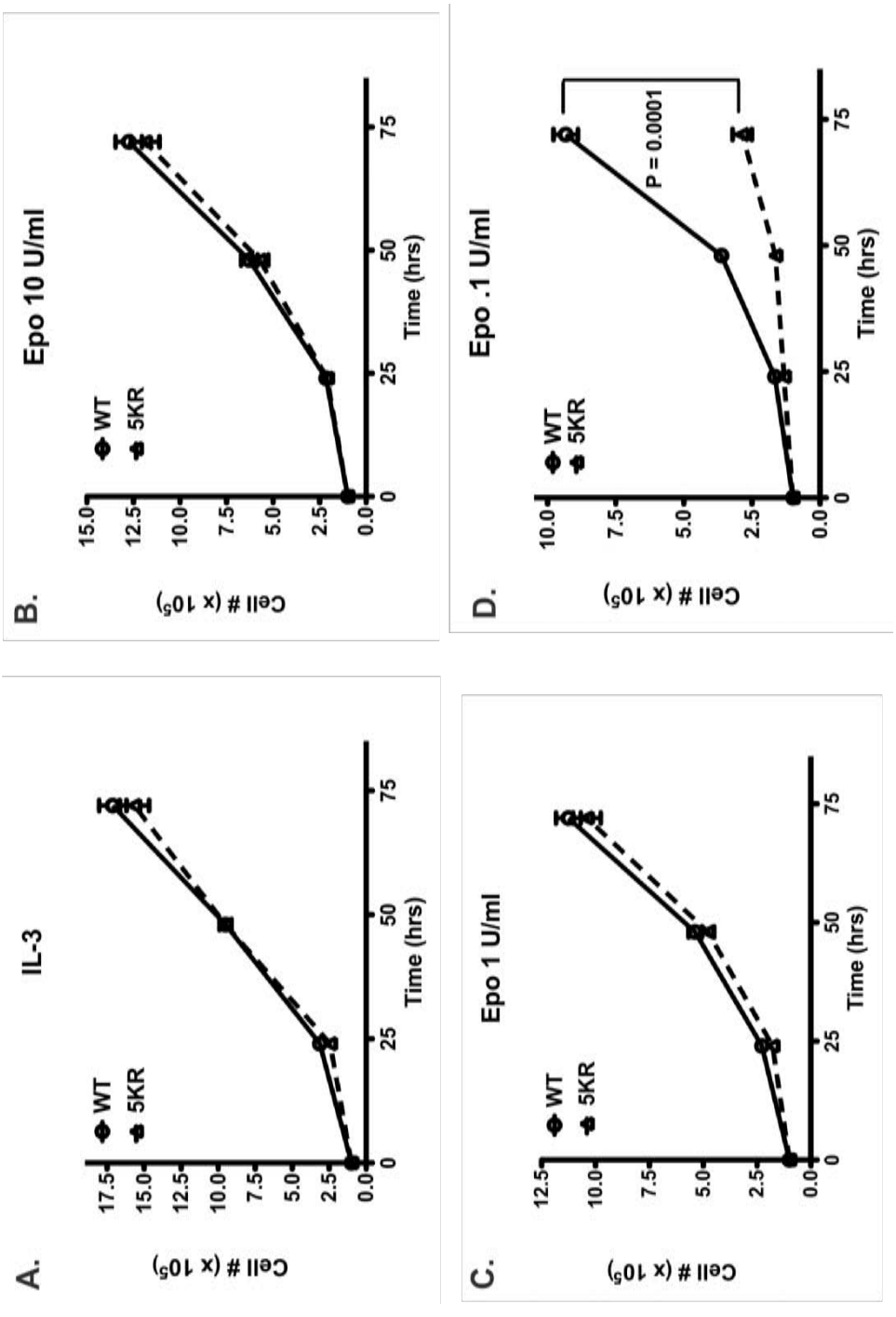


Figure 6. Epo-responsiveness of BaF3 cells expressing WT- or 5KR-EpoR.
BaF3 cells expressing WT- or 5KR-EpoR were incubated with different concentrations of Epo for 48 hrs and viable cell numbers were measured by the MTS assay. Data represent the average of four independent cell lines for each EpoR construct. **(A)** Normalized to cells cultured in 1.5 ng/ml IL-3, **(B)** Normalized to maximum dosages of Epo.

Figure 7. Proliferation of BaF3 cells expressing WT- or 5KR-EpoR. BaF3 cells expressing WT- or 5KR-EpoR were seeded at 1 x 10^5/ml and allowed to grow at the indicated dose of Epo or 1.5 ng/ml IL-3 for 72 hrs. Viable cells were counted using trypan blue staining at 24, 48, and 72 hrs. Data represents the average of three independent cell lines for each EpoR construct. **(A)** 1.5 ng/ml IL-3, **(B)** 10 U/ml Epo, **(C)** 1 U/ml Epo, **(D)** 0.1 U/ml Epo.


A.
IL-3
B.
Epo 10 U/ml
C.
Epo 1 U/ml
D.
Epo .1 U/ml
WT
5KR
Cell # (x 10⁵)
Time (hrs)
P = 0.0001

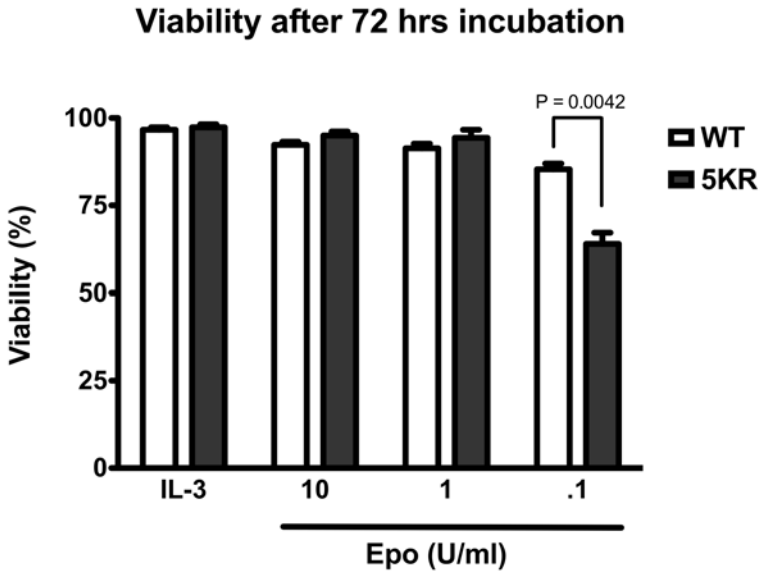


Figure 8. Viability of BaF3 cells expressing WT- or 5KR-EpoR.
After a 72 hrs incubation with 1.5 ng/ml IL-3, 10 U/ml Epo, 1 U/ml Epo, or 0.1 U/ml Epo, viable and dead cells were counted using trypan blue exclusion, and the percent viability was calculated. Data represents the average of three independent cell lines for each EpoR construct.

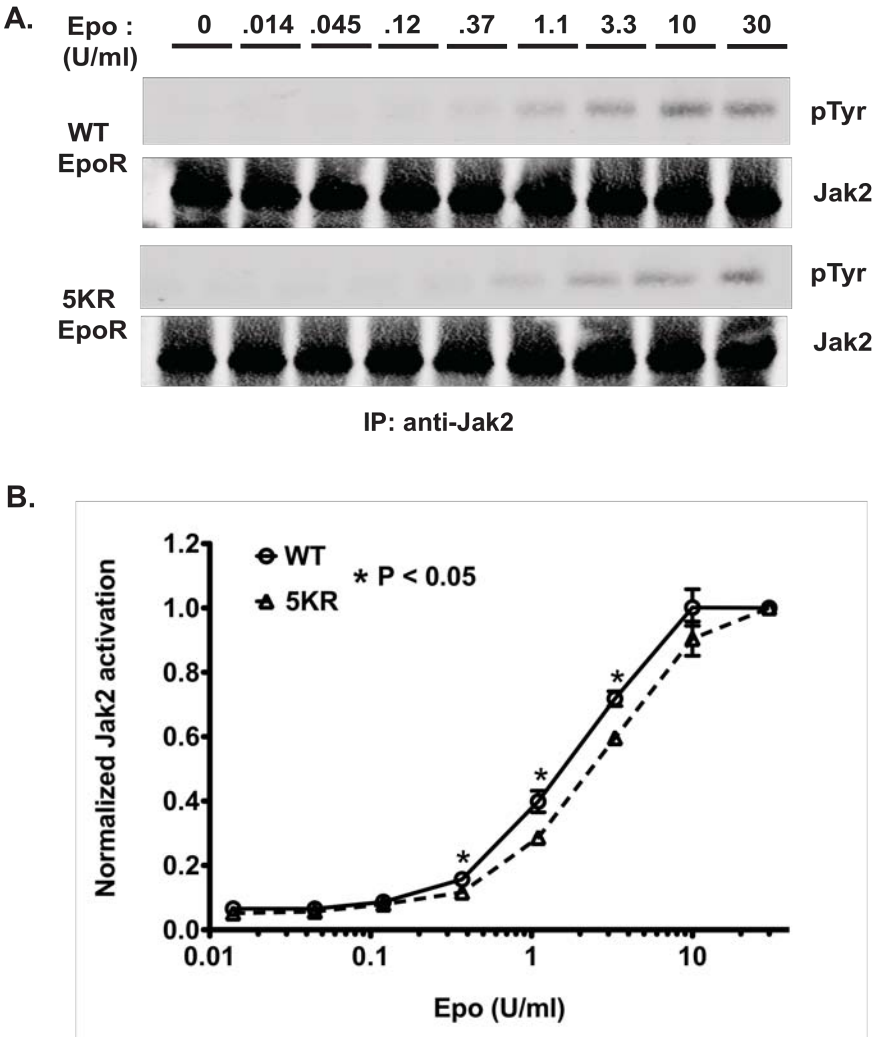


Figure 9. Jak2 activation in BaF3 cells expressing WT- or 5KR-EpoR. BaF3 cells expressing WT- or 5KR-EpoR were incubated with the indicated concentrations of Epo for 15 min. Jak2 was immunoprecipitaed using anti-Jak2 antibody, followed by Western blotting using anti-phosphotyrosine (pTyr) or anti-Jak2 (Jak2) antibodies **(A)**. Jak2 phosphorylation in each lane was normalized to total Jak2. To allow comparison between experiments, each Jak2 activity at each dose was normalized to the Jak2 activity at the maximum dosage of Epo **(B)**. Data represent the average of three independent cell lines for each EpoR construct.

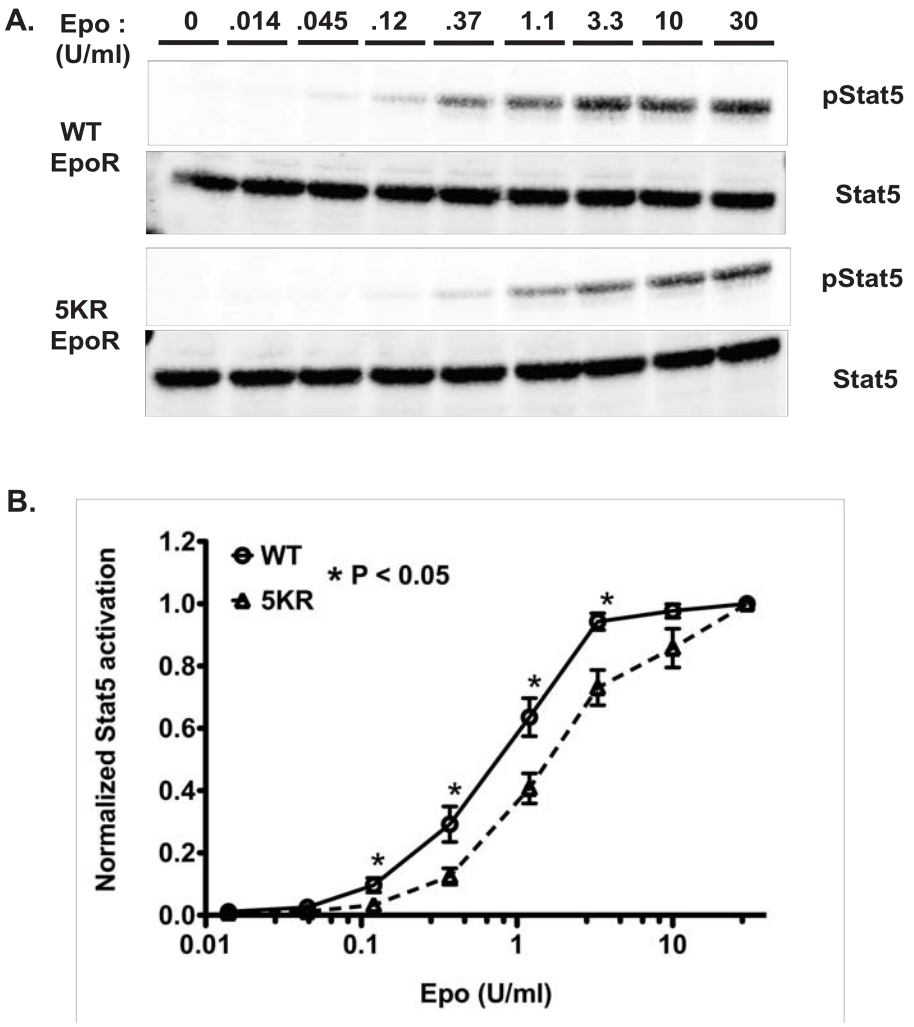


Figure 10. Stat5 activation in BaF3 cells expressing WT- or 5KR-EpoR. BaF3 cells expressing WT- or 5KR-EpoR were incubated with the indicated concentrations of Epo for 15 min. TCL was analyzed by Western blotting using anti-phosphoStat5 (pStat5) or anti-Stat5 antibodies (Stat5) **(A)**. The level of phosphorylated Stat5 for each lane was normalized to total Stat5 level. To allow comparison between experiments, the level of phosphorylated Stat5 at each dose was normalized to the level of phosphorylated Stat5 at the maximum dosage of Epo **(B)**. Data represent the average of three independent cell lines for each EpoR construct.

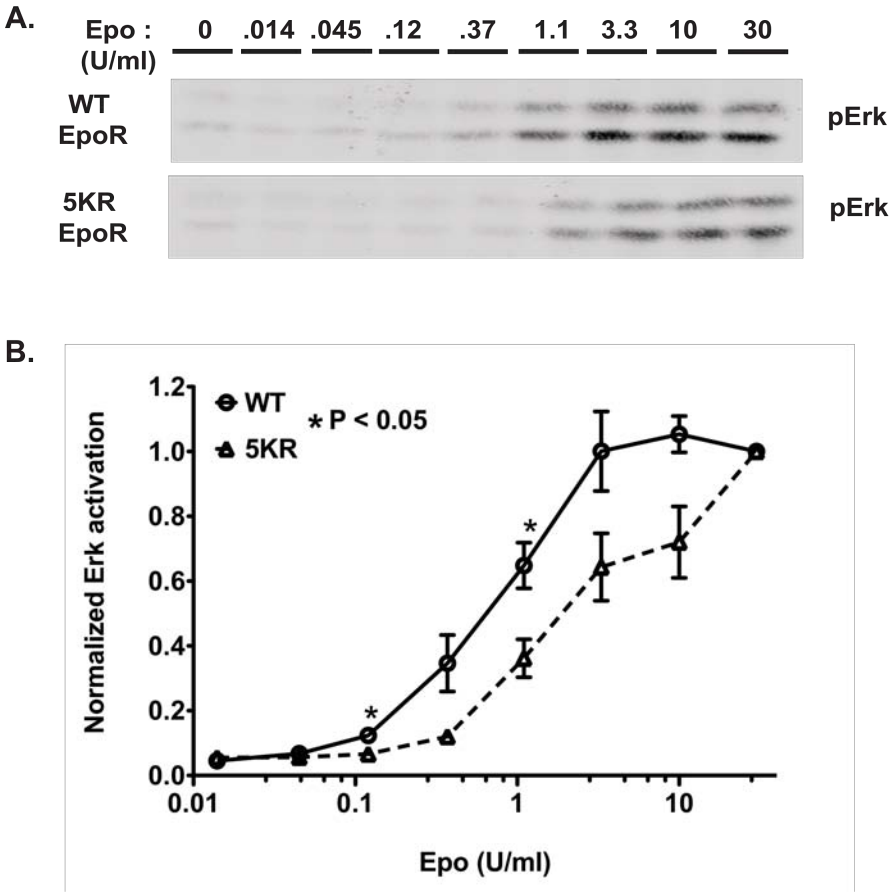


Figure 11. Erk activation in BaF3 cells expressing WT- or 5KR-EpoR. BaF3 cells expressing WT- or 5KR-EpoR were incubated with the indicated concentrations of Epo for 15 min. TCL was analyzed by Western blotting using anti-phosphoErk antibody (pErk) **(A)**. To allow comparison between experiments, Erk activity for each lane was normalized to the Erk activity at the maximum dosage of Epo **(B)**. Data represent the average of three independent cell lines for each EpoR construct.

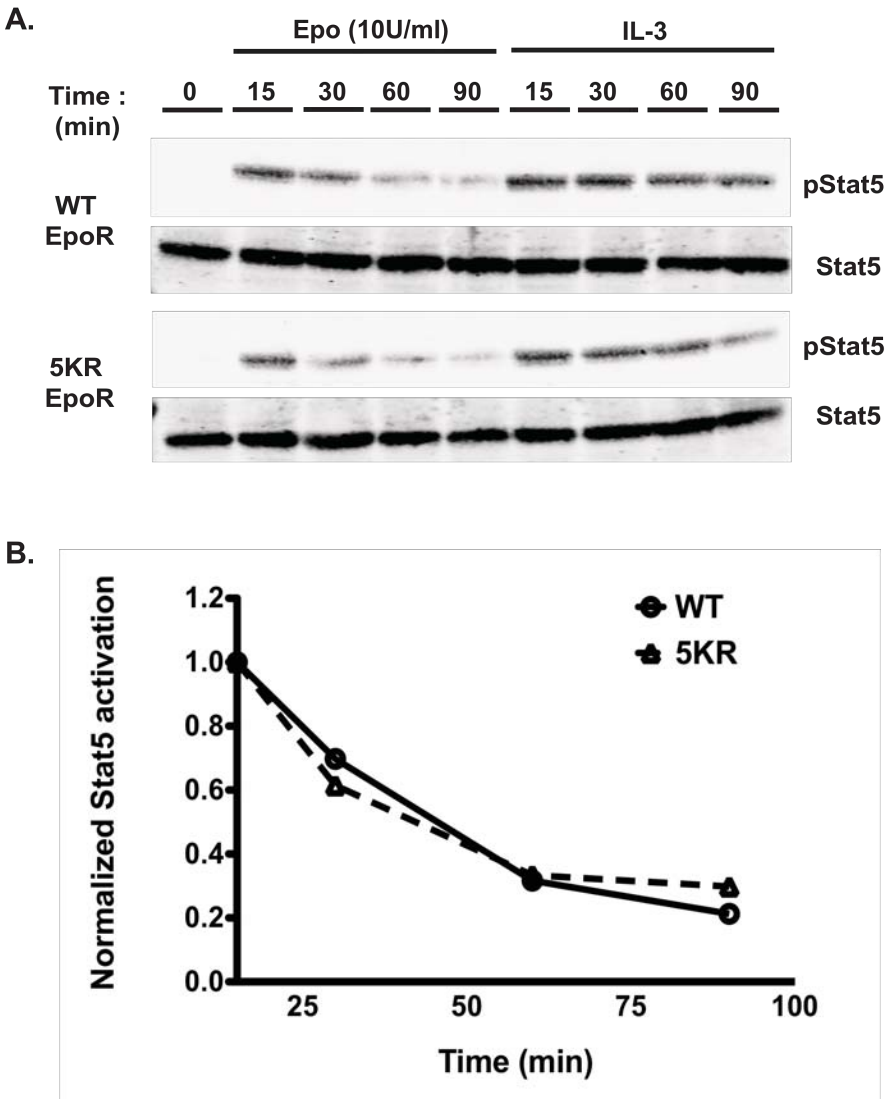


Figure 12. Duration of Epo signaling in BaF3 cells expressing WT- or 5KR-EpoR.
BaF3 cells expressing WT- or 5KR-EpoR were incubated with 10 U/ml Epo or 1.5 ng/ml IL-3 for the indicated time. TCL was analyzed by Western blotting using anti-phosphoStat5 (pStat5) or anti-Stat5 (Stat5) antibodies **(A)**. To assess the duration of Epo signaling, each level of pStat5 was normalized to the maximum level of pStat5 at 15 min **(B)**.

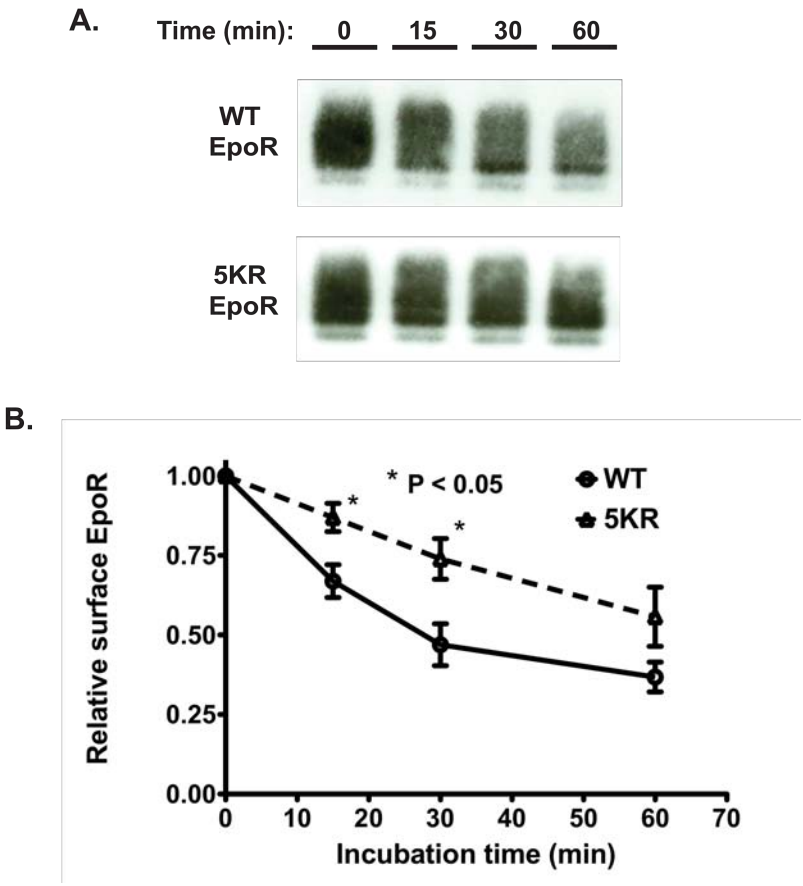


Figure 13. Ligand-induced depletion of surface EpoR.
BaF3 cells expressing WT- or 5KR-EpoR were stimulated with 10U/ml Epo for the indicated time period, and surface EpoR was then labeled with biotin. Biotin-labeled EpoR was precipitated from cell lysates with neutravidin, and precipitated proteins were immunoblotted with anti-HA antibody **(A)**. The amount of biotin-labeled EpoR at each time point was normalized to the amount of surface EpoR present before addition of ligand (time=0) **(B)**. Data represents the average of three independent cell lines for each EpoR construct.

CHAPTER III
EPOR SITES OF UBIQUITINATION AND THEIR ROLE IN EPO SIGNALING AND BIOLOGICAL RESPONSE

Rationale

The previous chapter showed that EpoR lacking sites of ubiquitination (5KR-EpoR) supports reduced signaling activity and biological responses to low dosages of Epo. These results suggest the possibility that ubiquitination of EpoR facilitates increased responsiveness to low dosages of Epo. However, there is still a possibility that attenuated effects mediated by 5KR-EpoR are due to structural changes caused by mutation of one or more receptor lysines, and are not directly related to the loss of receptor ubiquitination. To address this possibility, it is necessary to know which EpoR lysines can support receptor ubiquitination, and whether enhanced receptor function is associated with ubiquitination at one or more EpoR lysines. In this chapter, we used EpoR mutants retaining single cytoplasmic lysines to assess the contribution of each potential site to receptor ubiquitination and functional responses to Epo.

Materials and methods

DNA constructs

DNA constructs that express 3NKR- (two C-terminal lysines are retained within the intracellular domain) or 2CKR- (three N-terminal lysines are retained within the intracellular domain) EpoR were previously made by our lab (Fig. 3).

DNA constructs that express single-lysine EpoR (one lysine exists within the intracellular domain (256K, 276K, 348K, 388K, or 428K)) were generated by PCR. Primers were prepared to replicate the sequence of WT-EpoR and were centered on each of the five lysine codons of the cytoplasmic domain. WT-EpoR primers were then used to amplify the region of the EpoR cDNA coding for the cytoplasmic domain, using 5KR-EpoR as the template. Amplified cDNA fragments were then cloned into existing pXM-HA-WT-EpoR constructs, replacing the region coding for the cytoplasmic domain. The resulting full length EpoR cDNAs retained four lysine-to-arginine mutations from the 5KR-EpoR template while restoring one lysine codon to the original WT-EpoR sequence (Fig. 14). Each construct was fully sequenced over the amplified region.

Establishment of stable cell lines expressing 3NKR-, 2CKR-, or single-lysine EpoR constructs

Establishment of stable cell lines for expressing 3NKR-, 2CKR- or single-lysine EpoR cDNAs was performed as described in Chapter II. Western Blotting with anti-HA antibodies was used to identify clones with equivalent expression of EpoR constructs (Fig. 15).

EpoR ubiquitination assay, cell proliferation assay, signaling activity assay

Assays for ubiquitination, proliferation, and signaling were performed as described in Chapter II.

Results

Two C-terminal lysines, 388K and 428K, are primary targets for EpoR ubiquitination

In order to identify the target lysines for EpoR ubiquitination, we generated single-lysine mutants of EpoR, and assessed ubiquitination of the EpoR mutants expressed in BaF3 cells, as previously described in Chapter I. As shown in Fig. 16, EpoR mutants retaining a single lysine at 388K or 428K were ubiquitinated to a level similar to WT-EpoR. Conversely, ubiquitination of receptors retaining only 256K, 276K, or 348K was only weakly detected. These results suggest that the two C-terminal lysines, 388K and 428K, are primary targets for EpoR ubiquitination. We also assessed ubiquitination of EpoR mutants retaining multiple lysines (Fig. 17), and found that 3NKR-EpoR (retaining lysines at 388K and 428K) was able to support efficient ubiquitination of EpoR. Ubiquitination of 2CKR-EpoR (retaining 256K, 276K, and 348K) was more readily detected than the respective single-lysine EpoR mutants, but appeared less dependent on Epo-induced activation. This result also suggests that the two C-terminal lysines, 388K and 428K, are primary targets for Epo-induced ubiquitination of EpoR.

388K or 428K support ubiquitination of EpoR and enhanced functional responses to Epo

In order to characterize the effect of 388K or 428K ubiquitination on functional responses to Epo, we assessed proliferation and viability for BaF3 cell lines expressing these single-lysine EpoRs. When treated with high dose Epo (≥

1 U/ml) or IL-3, cells expressing the single-lysine-EpoR mutants proliferated at rates similar to WT-EpoR cells (Fig. 18). Cells expressing 388K-EpoR also proliferated at rates comparable to WT-EpoR in response to low dosages of Epo (0.1 U/ml), and cells expressing 428K-EpoR exhibited enhanced proliferation compared to 5KR-EpoR. Conversely, cells expressing 256K-, 276K-, or 348K-EpoR mutants were not significantly different from 5KR-EpoR. Similarly, 388K or 428K restored wild-type maintenance of viability, while 256K-, 276K-, or 348K-EpoRs did not support any higher viability than 5KR-EpoR in the presence of low dose Epo (Fig. 19). These results indicate that 388K or 428K appear to be the primary functional targets of EpoR ubiquitination promoting Epo-induced responses. Furthermore, ubiquitination of either site is sufficient to support nearly wild-type responses to Epo. Conversely, weak ubiquitination at any of the N-terminal lysines (256K, 276K, and 348K) does not significantly recover function compared to 5KR-EpoR, and even more robust ubiquitination of receptors retaining all these sites only partly restored these functional responses (Fig. 20, Fig. 21). Therefore, one or both of the C-terminal lysines (388K and 428K) appears to be required and sufficient to restore near wild type responses that are depend on ubiquitination of EpoR.

C-terminal lysines (388K or 428K) enhance EpoR signaling activity

The previous experiments showed that attenuated cell viability and proliferation induced through 5KR-EpoR was rescued by restoration of the C-terminal lysines of EpoR. These functional responses are tightly associated with

EpoR signaling activities. Therefore, if the attenuated biological response of 5KR-EpoR was restored by the addition of C-terminal lysines, attenuated signaling activities of 5KR-EpoR may also be recovered. Indeed, at low dosages of Epo (≤ 1 U/ml), 388K and 428K-EpoR exhibited similar levels of Stat5 activation compared with WT EpoR, while 256K, 276K, or 348K did not enhance signaling compared to 5KR-EpoR (Fig. 22 and 23). Similarly, 3NKR-EpoR, but not 2CKR-EpoR, recovered Stat5 activation at low dosages of Epo (0.33 U/ml or 0.1 U/ml), compared to 5KR-EpoR (Fig. 24 and 25). These results are consistent with the increase in viability and proliferation associated with the restoration of C-terminal lysines (388K and 428K), and suggest that these sites of ubiquitination are important to maintain EpoR signaling activities, and promote physiological responses to low concentrations of Epo.

Conclusions

In the previous chapter, we showed that the mutations disrupting EpoR ubiquitination are associated with reduced signaling and physiological responses to Epo. However, this correlation does not rule out the possibility that structural changes, rather than loss of ubiquitination, are responsible for the reduced responsiveness of 5KR-EpoR. In order to address this possibility, we identified the target lysines for EpoR ubiquitination and tested if restoring individual sites of EpoR ubiquitination also restored signaling activities and functional responses to Epo. In this analysis, two C-terminal lysines (388K and 428K) were significant

sites of ubiquitination, while the three N-terminal lysines (256K, 276K, and 348K) were only weakly ubiquitinated.

Consistent with a role for ubiquitination in promoting EpoR signaling, receptors that contained either ubiquitinatable lysine (388K or 428K) displayed near wild-type signaling activity and biological responses to low dosages of Epo. Thus, no single lysine appears to be required for full EpoR activity, suggesting that disrupted structure resulting from lysine-to-arginine mutations is not a likely explanation for the reduced activity of 5KR-EpoR. Similarly, retention of any one of the N-terminal lysines (256K, 276K, or 348K) did not support significant ubiquitination or increased responsiveness to low dose Epo, while retention of all these N-terminal lysines (2CKR) supported increased ubiquitination and increased responsiveness compared to the non-ubiquitinatable receptor (5KR). Thus, there appears to be a strong correlation between the ability of EpoR mutants to support ubiquitination and their ability to mediate efficient signaling activity in the presence of low concentrations of ligand, suggesting that receptor ubiquitination is a likely mediator of this increased responsiveness.

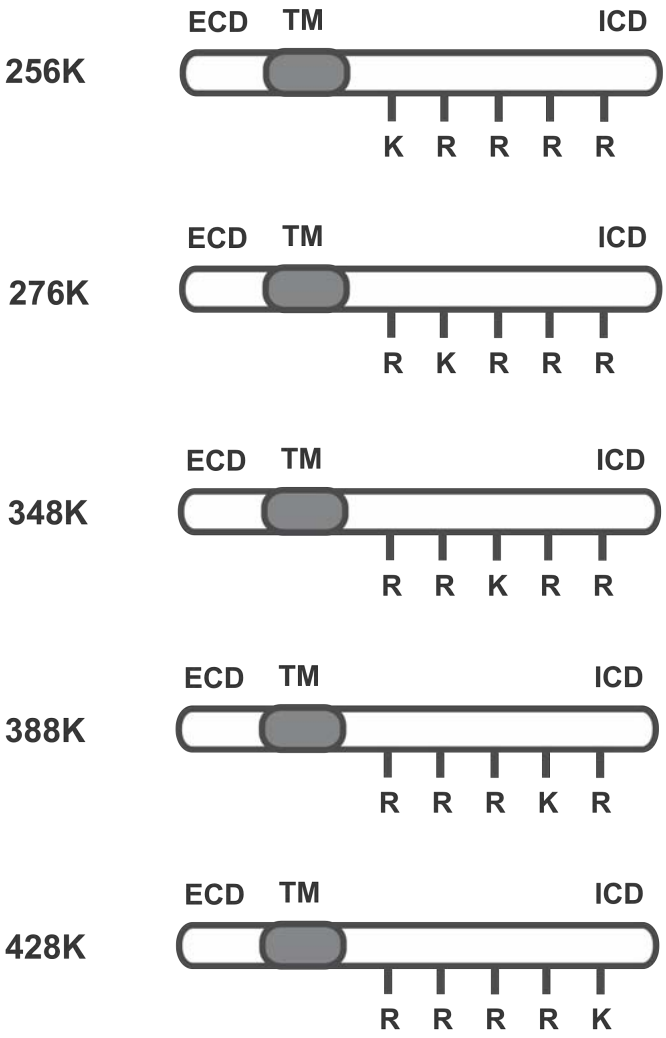


Figure 14. Schematic diagram of single-lysine EpoR mutants.
ECD=Extracellular domain, TM=Transmembrane domain, ICD=Intracellular domain. Single K mutants=Only one lysine is retained at the indicated position of the cytoplasm domain.

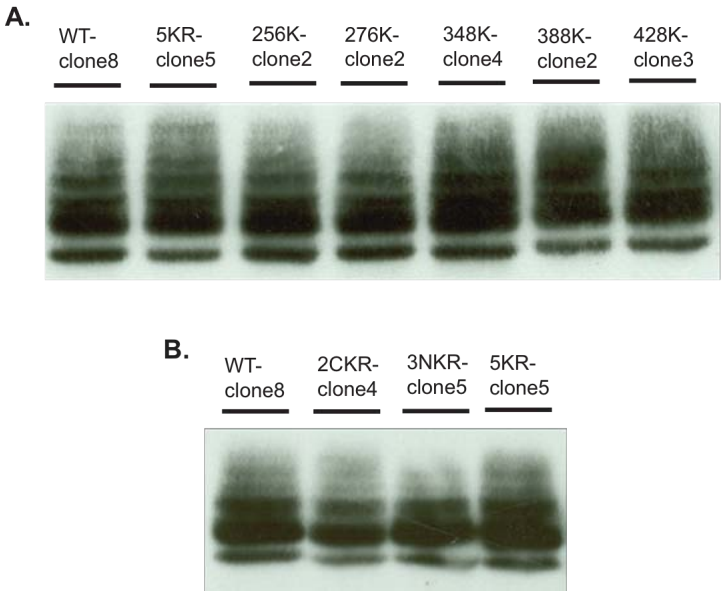


Figure 15. Screen for equivalent expression of EpoR constructs. Total cell lysates were prepared from clonal cell lines expressing single-lysine or block-lysine EpoR mutants. Equal amounts of protein from each sample were assayed for EpoR expression by SDS-PAGE and Western Blotting with anti-HA antibodies. Example of one clone for each construct are shown. **(A)** Single-lysine EpoR mutants, **(B)** Block-lysine EpoR mutants

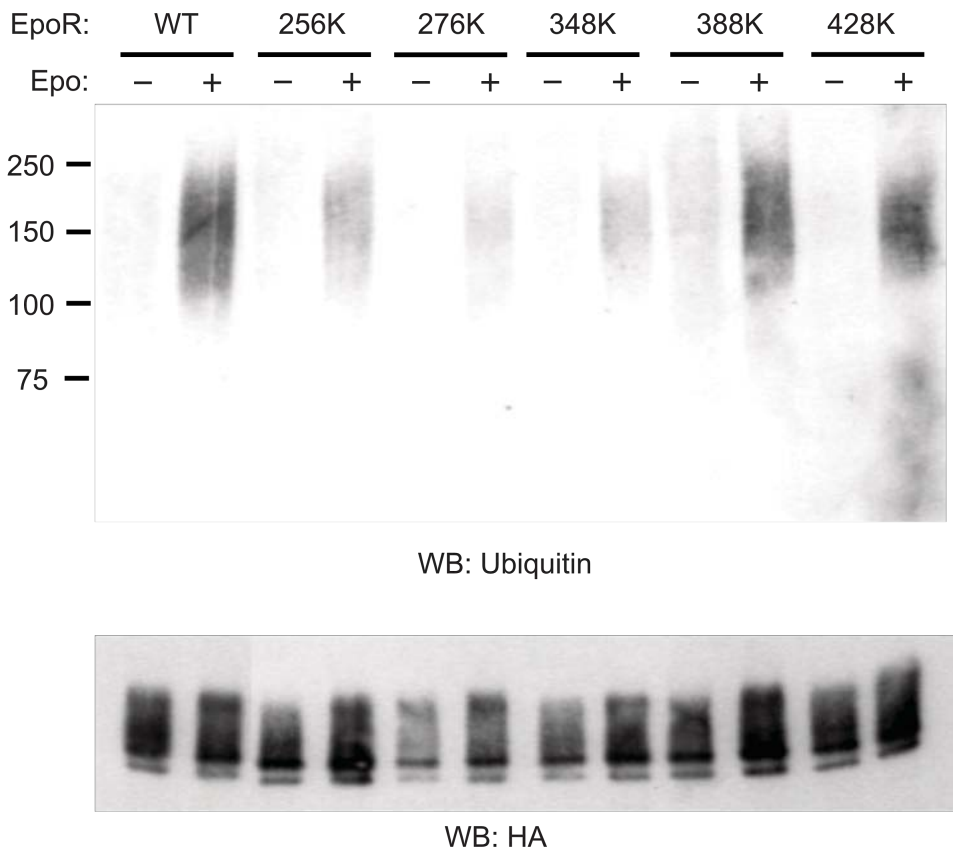


Figure 16. Ubiquitination of single-lysine EpoR mutants in BaF3 cells. BaF3 cells expressing WT or single-lysine EpoR were unstimulated or stimulated with 10 U/ml Epo for 15 min at 37°C. Then, surface EpoR was labeled with a membrane-impermeable biotinylation reagent. After the cells were lysed, EpoR was sequentially precipitated with agarose conjugated anti-HA antibodies and neutravidin resins. Precipitates were western blotted (WB) with anti-HA or anti-ubiquitin antibodies.

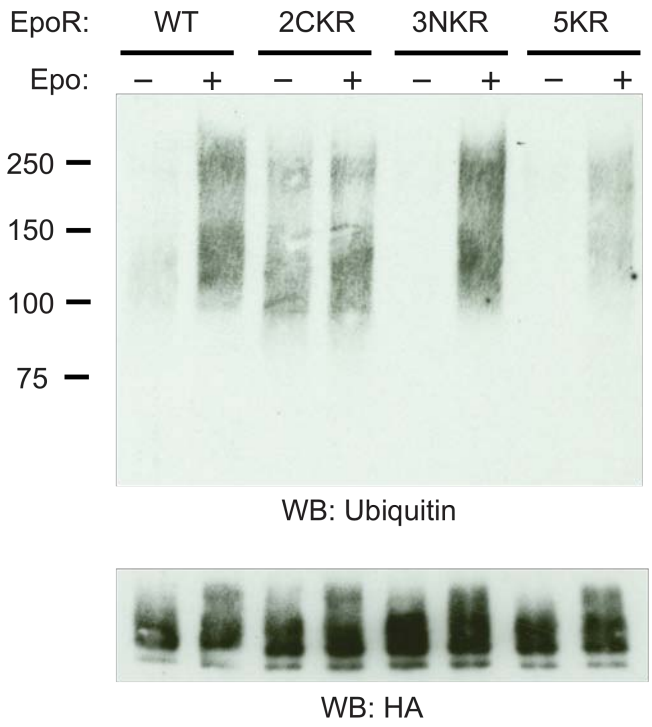
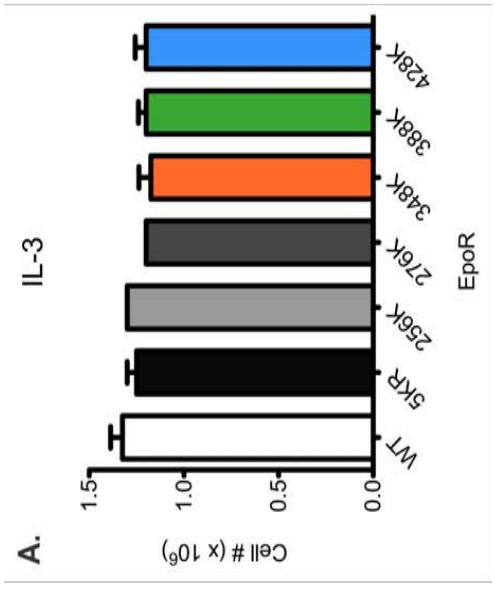
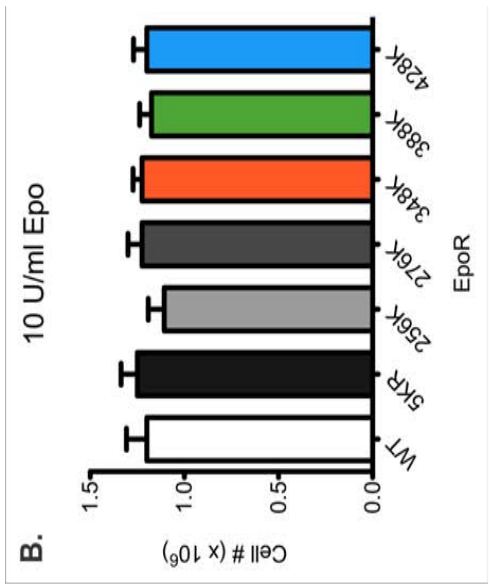
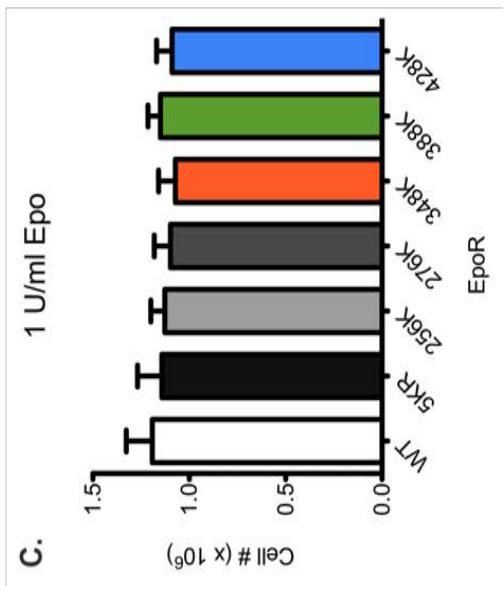


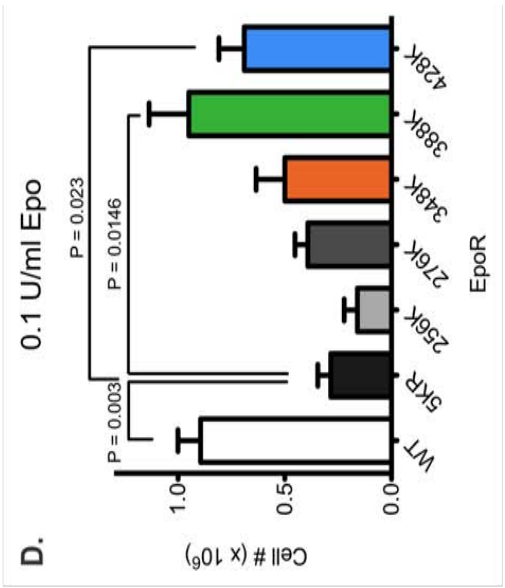
Figure 17. Ubiquitination of block-lysine EpoR mutants in BaF3 cells. BaF3 cells expressing WT or block-lysine-EpoR were unstimulated or stimulated with 10 U/ml Epo for 15 min at 37°C. Then, surface EpoR was labeled with a membrane-impermeable biotinylation reagent. After the cells were lysed, EpoR was sequentially precipitated with agarose conjugated anti-HA antibodies and neutravidin resins. Precipitates were western blotted (WB) with anti-HA or anti-ubiquitin antibodies.

Figure 18. Proliferation of BaF3 cells expressing single-lysine EpoR mutants.
BaF3 cells expressing single-lysine EpoR mutants were seeded at 1 x 10^5/ml and allowed to grow at the indicated dose of Epo or 1.5 ng/ml IL-3 for 72 hrs, after which viable cells were counted using trypan blue staining. Data represents the average of three independent cell lines for each EpoR construct. **(A)** 1.5 ng/ml IL-3, **(B)** 10 U/ml Epo, **(C)** 1 U/ml Epo, **(D)** 0.1 U/ml Epo


A.
IL-3
Cell # (x 10^6)
1.5
1.0
0.5
0.0
WT
5kR
256k
276k
348k
388k
428k
EpoR


B.
10 U/ml Epo
Cell # (x 10^6)
1.5
1.0
0.5
0.0
WT
5kR
256k
276k
348k
388k
428k
EpoR


C.
1 U/ml Epo
Cell # (x 10^6)
1.5
1.0
0.5
0.0
WT
5kR
256k
276k
348k
388k
428k
EpoR


D.
0.1 U/ml Epo
P = 0.003
P = 0.0146
P = 0.023
Cell # (x 10^6)
1.0
0.5
0.0
WT
5kR
256k
276k
348k
388k
428k
EpoR

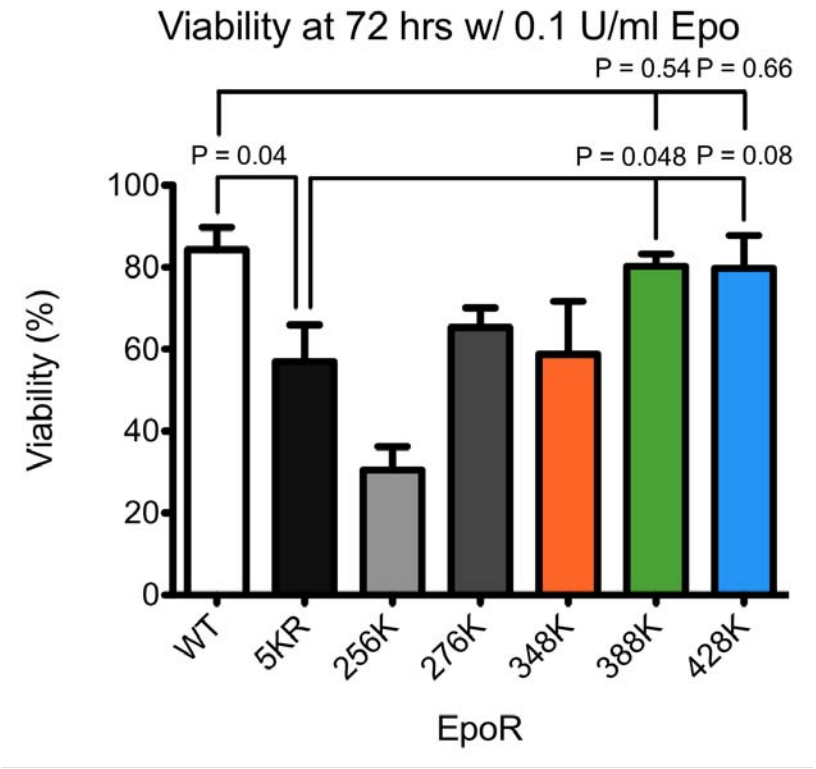
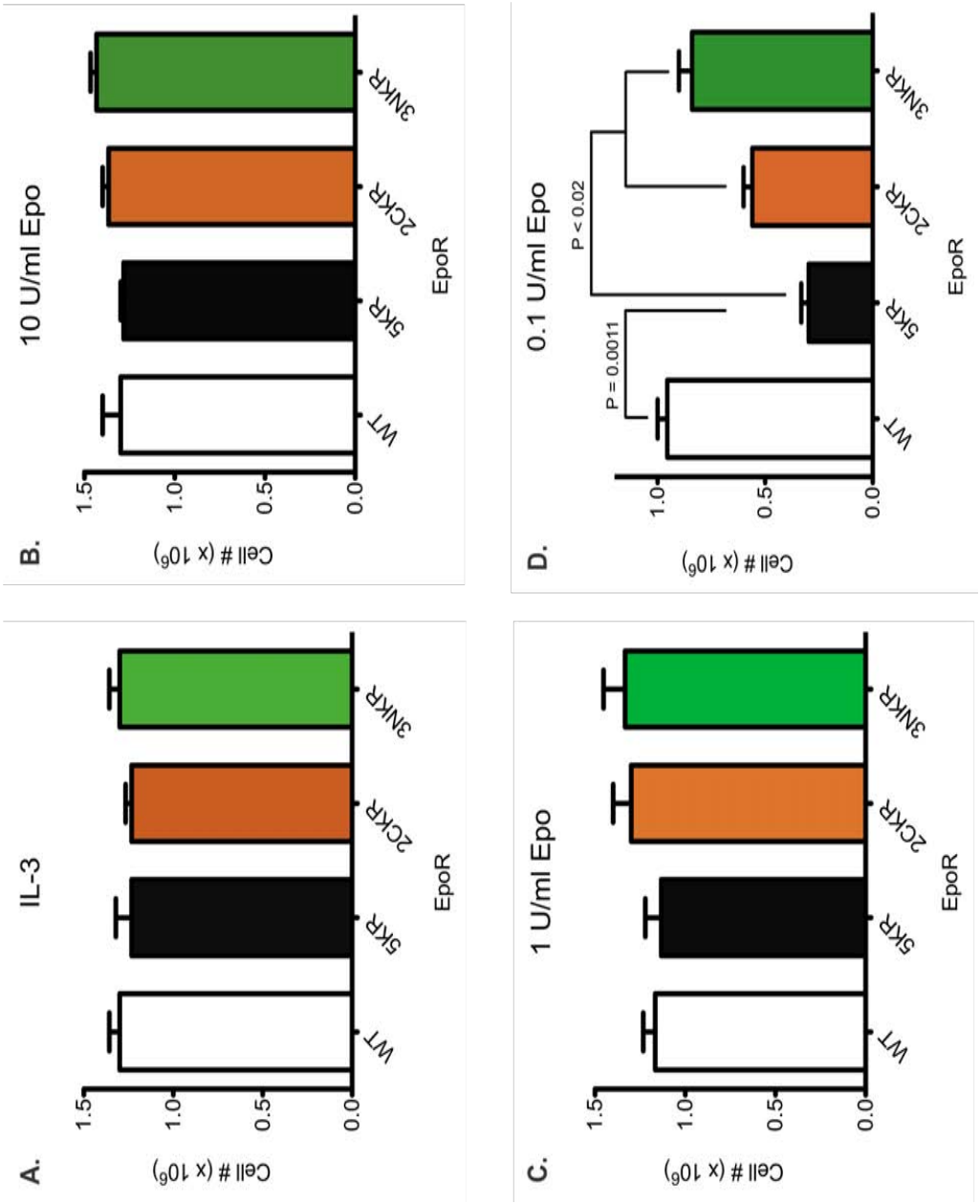


Figure 19. Viability of BaF3 cells expressing Single-lysine EpoR mutants. After 72 hrs incubation with 0.1 U/ml Epo, viable and dead cells were counted using trypan blue and percent viability was computed. Data represents the average of three independent cell lines for each EpoR construct.

Figure 20. Proliferation of BaF3 cells expressing block-lysine EpoR mutants.
BaF3 cells expressing Block-lysine-EpoR mutants were seeded at 1 x 10^5/ml and allowed to grow at the indicated dose of Epo or 1.5 ng/ml IL-3 for 72 hrs, after which viable cells were counted using trypan blue staining. Data represents the average of three independent cell lines for each EpoR construct. **(A)** 1.5 ng/ml IL-3, **(B)** 10 U/ml Epo, **(C)** 1 U/ml Epo, **(D)** 0.1 U/ml Epo


A.
IL-3
B.
10 U/ml Epo
C.
1 U/ml Epo
D.
0.1 U/ml Epo
Cell # (x 10^6)
1.5
1.0
0.5
0.0
WT
5KR
2CKR
3NKR
EpoR
P = 0.0011
P < 0.02

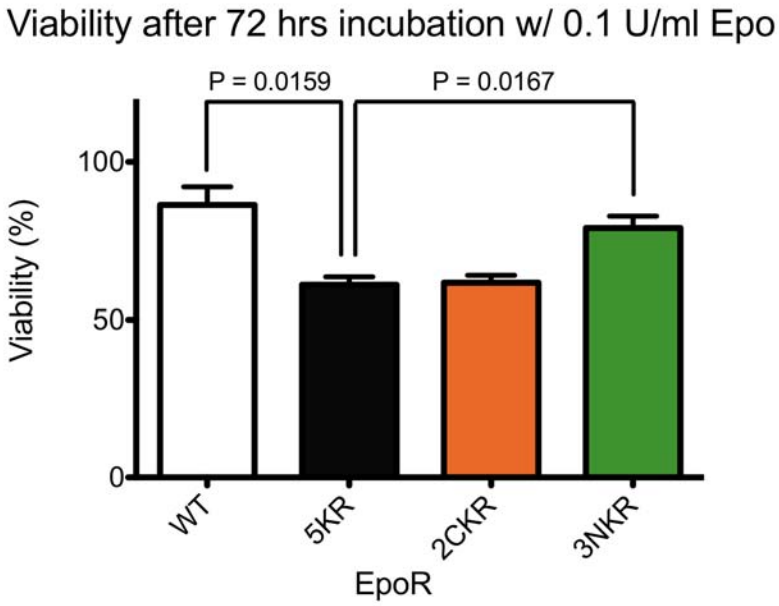


Figure 21. Viability of BaF3 cells expressing block-lysine EpoR mutants. After 72 hrs incubation with 0.1 U/ml Epo, viable and dead cells were counted using trypan blue staining and the percent viability was computed. Data represents the average of three independent cell lines for each EpoR construct.

Figure 22. Stat5 activation in BaF3 cells expressing single-lysine EpoR mutants.
BaF3 cells expressing single-lysine EpoR were incubated with indicated concentrations of Epo for 15 min. TCL was analyzed by western blotting using anti-phosphoStat5 or anti-Stat5 antibody.

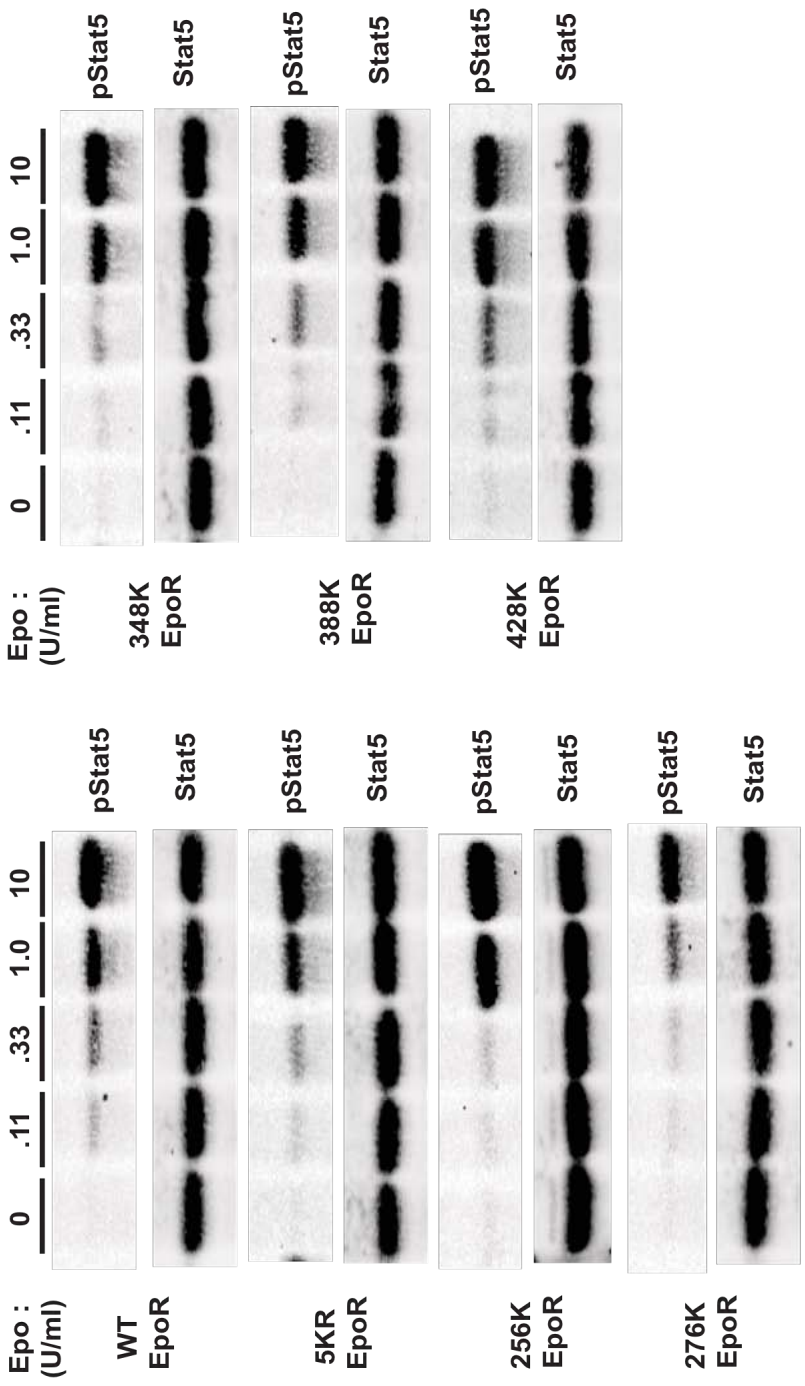
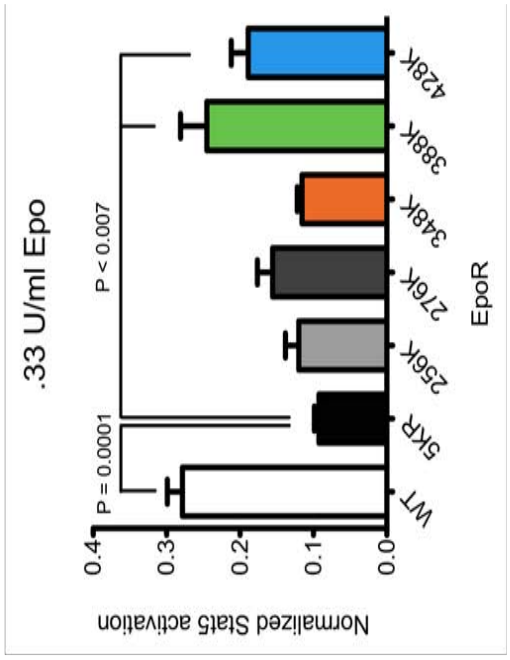
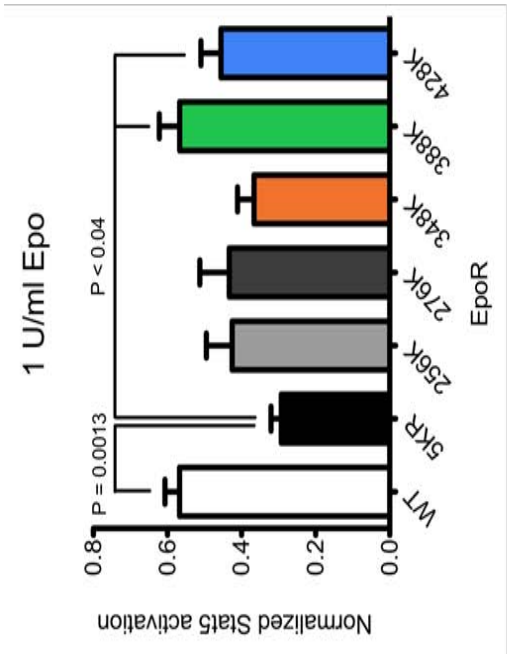
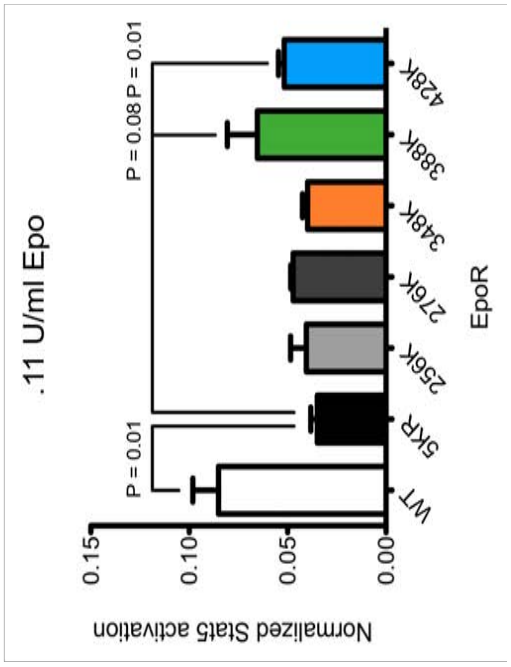

Epo :
(U/ml)
0
.11
.33
1.0
10
WT
EpoR
5KR
EpoR
256K
EpoR
276K
EpoR
348K
EpoR
388K
EpoR
428K
EpoR
pStat5
Stat5

Figure 23. Stat5 activation in BaF3 cells expressing single-lysine EpoR mutants.
pStat5 activities were normalized to total Stat5 to control for loading. To allow comparison between experiments, each Stat5 activity was normalized to the Stat5 activity at the maximum dosage of Epo. Data represent the average of three independent cell lines for each EpoR construct over multiple experiments.


.33 U/ml Epo
Normalized Stat5 activation
0.0
0.1
0.2
0.3
0.4
P = 0.0001
P < 0.007
WT
5KR
256K
276K
348K
388K
428K
EpoR


1 U/ml Epo
Normalized Stat5 activation
0.0
0.2
0.4
0.6
0.8
P = 0.0013
P < 0.04
WT
5KR
256K
276K
348K
388K
428K
EpoR


.11 U/ml Epo
Normalized Stat5 activation
0.00
0.05
0.10
0.15
P = 0.01
P = 0.08 P = 0.01
WT
5KR
256K
276K
348K
388K
428K
EpoR

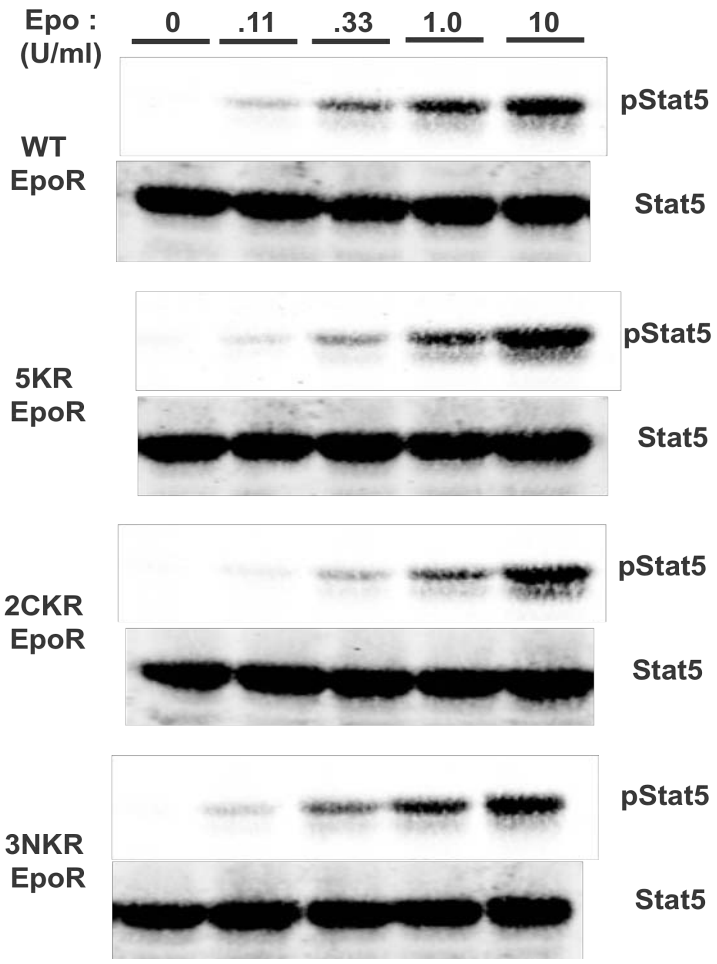


Figure 24. Stat5 activation in BaF3 cells expressing block-lysine EpoR mutants.
BaF3 cells expressing block-lysine EpoR were incubated with indicated concentrations of Epo for 15 min. TCL was analyzed by western blotting using anti-phosphoStat5 or anti-Stat5 antibodies.

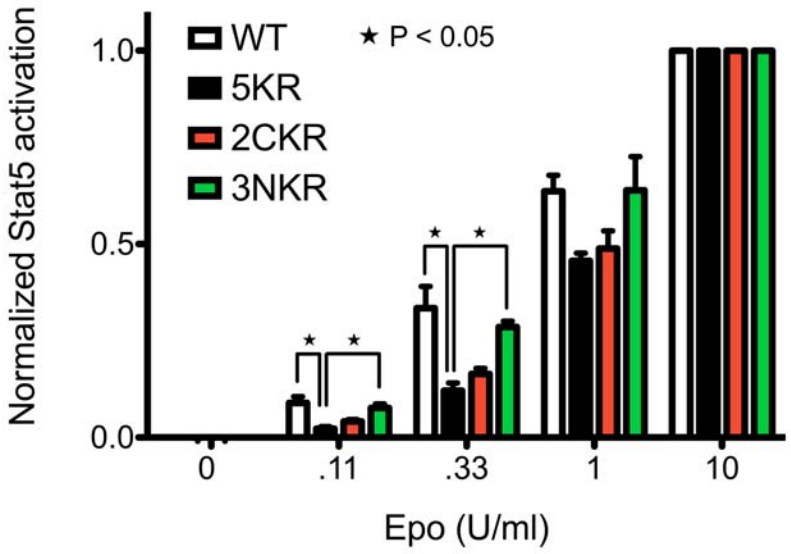


Figure 25. Stat5 activation in BaF3 cells expressing block-lysine EpoR mutants.
pStat5 activities were normalized to total Stat5 to control for loading. To allow comparison between experiments, each Stat5 activity was normalized to the Stat5 activity at the maximum dosage of Epo. Data represents the average of three independent cell lines for each EpoR construct.

CHAPTER IV
EPOR SIGNALING DEPENDS ON RECEPTOR INTERNALIZATION

Rationale

Wild-type responsiveness of EpoR strongly correlates with receptor ubiquitination, suggesting that ubiquitination may facilitate efficient signaling from EpoR. How ubiquitination may contribute to EpoR regulation remains unclear. However, ubiquitination of receptors is associated with regulated internalization (Govers et al., 1999; Haglund et al., 2003; Hicke and Riezman, 1996; Kumar et al., 2007). Internalization itself can have different effects on receptor signaling activity. For example, internalization of G-protein coupled receptors is an important mechanism reducing responsiveness (desensitization) to receptor ligands (Hicke et al., 1998; Luttrell and Lefkowitz, 2002). On the other hand, internalization of TGF-β receptors has been suggested as an important step in formation of a fully active receptor signaling complex (Di Guglielmo et al., 2003). A similar ubiquitination-dependent internalization mechanism could potentially facilitate EpoR signaling activities. In this regard, we have observed that WT-EpoR is more rapidly depleted from the cell surface after Epo stimulation than is 5KR-EpoR, suggesting that internalization of WT-EpoR is mediated differently than 5KR-EpoR. In this chapter, we have characterized the mechanisms mediating internalization of WT- and 5KR-EpoR and tested the requirement for internalization in promoting signaling from these receptors.

Materials and methods

Antibodies, reagents, and chemicals

EZ-link Sulfo-NHS-SS-Biotin was purchased from Thermo Scientific. Dynasore monohydrate and Methyl-β-cyclodextrin (MCD) were obtained from Sigma Aldrich.

Signaling activity assay

Activation of Stat5 was assessed as an indicator of Epo signaling activities. BaF3 cells expressing WT- or mutant EpoR were starved for 2.5 hrs in cytokine-free media and treated with or without 80 μM Dynasore or 5 mM MCD for 30 min. Then, cells were stimulated with 10 U/ml of Epo for 15 min. Stimulated cells were lysed using SDS sample buffer, followed by Western blotting with anti-Stat5 or anti-phospho-Stat5 antibodies.

EpoR internalization assay

BaF3 cells expressing WT- or 5KR-EpoR were starved for 2 hrs in cytokine-free media and treated with or without 20 mM Ammonium Chloride (NH_4Cl) or 20 μM MG-132 for 60 min. Then, cell surface EpoR was labeled with sulfo-NHS-ss-biotin (0.25 mg/ml, 30 min, 4°C). A sample of cells was immediately resuspended in ice-cold 50 mM DTT-PBS to remove the cell surface biotin-label and identify any remaining labeled receptor populations that were not specifically internalized. The remaining sample was treated with or without 10

U/ml Epo at 37 °C for 20 min. Then, Epo stimulation was terminated by resuspension of cells in ice-cold PBS, containing 50 mM DTT to remove biotin-labels remaining at the cell surface. Finally, cells were lysed and internalized EpoR was isolated by Neutravidin pulldown. Internalized EpoR was measured by Western blotting using anti-HA antibodies.

Results

Internalized EpoR is targeted for lysosomal degradation

According to previous analysis, both ubiquitinated and non-ubiquitinated EpoR are depleted from the cell surface after Epo stimulation (Fig. 13). In order to determine if this depletion is due to the internalization of EpoR, we assayed actual internalization of EpoR. This assay showed that EpoR was internalized in either the presence or absence of Epo (Fig. 26). Since internalized EpoR might be targeted for degradation, we also tested whether they could be protected by inhibitors of the proteasome (MG-132) or lysosome (NH_4Cl). Only NH_4Cl significantly protected internalized EpoR, suggesting that these receptors are largely targeted to lysosomes. Although we consistently observed a very modest increase in internalized EpoR from Epo-treated cells, substantial internalization was also found in the absence of Epo, indicative of constitutive internalization. We also tested the internalization of 5KR-EpoR. Similar to WT-EpoR, 5KR-EpoR was internalized in either the presence or absence of Epo (Fig. 27).

EpoR is internalized by dynamin-independent and by dynamin-dependent Mechanisms

Analysis of EpoR internalization showed that Both WT- and 5KR-EpoR were internalized constitutively. However, the reduced rate of 5KR-EpoR depletion from the cell surface after Epo stimulation (Fig. 13) suggests the possibility that internalization of activated WT and 5KR receptors might be differently regulated. In order to assess mechanisms regulating EpoR internalization, we tested the requirement for dynamin-dependent mechanisms (Fig. 28). Treatment of cells with a dynamin-inhibitor (Dynasore) greatly reduced all internalization of 5KR-EpoR. Dynasore also reduced internalization of WT-EpoR in the absence of Epo stimulation, suggesting that constitutive EpoR internalization is dynamin-dependent. However, Dynasore did not significantly affect Epo-induced internalization of WT-EpoR. This observation suggests that activated, ubiquitinated EpoR is internalized through a different mechanism (dynamin-independent) than that which mediates constitutive internalization of EpoR in the absence of ligand or the absence of receptor ubiquitination (dynamin-dependent). Moreover, inhibition of this dynamin-dependent internalization appeared to uncover an Epo-induced increase in internalization (Fig. 28), suggesting that ligand-induced internalization of EpoR is largely mediated through a dynamin-independent mechanism.

Inhibition of dynamin-dependent internalization reduces 5KR-EpoR signaling activities

Since internalization of Epo-induced 5KR-EpoR but not WT-EpoR was dependent on dynamin activity, we assessed the effect of dynamin-inhibition on Epo signaling. Indeed, Stat5 activation by 5KR-EpoR was significantly reduced by Dynasore treatment (Fig. 29, panel A and B). However, IL-3 induced Stat5 activation was not significantly affected by Dynasore-treatment, indicating that reduced Stat5 phosphorylation was specific to 5KR-EpoR and did not result from a non-specific effect of dynamin inhibition in these cells (Fig. 29, panel C). Interestingly, Stat5 activation by WT-EpoR was not significantly affected by Dynasore-treatment. Thus, inhibition of dynamin-dependent internalization correlated with a corresponding reduction in signaling activity, suggesting that internalization may be required for efficient signaling by EpoR.

EpoR internalization correlates temporally with signaling activity

The apparent correlation between reduced internalization and reduced signaling activity suggests that EpoR internalization may be necessary for efficient signaling. If this is the case, then these activities would be expected to happen at similar times after receptor activation. In order to test this correlation, EpoR internalization and signaling activities were assessed as a function of time after Epo-treatment. Indeed, both Epo-induced EpoR internalization and Stat5 phosphorylation reached maximal levels approximately 15 min. after ligand

stimulation (Fig. 30). This correlation is consistent with a role for EpoR internalization in promoting signaling activities leading to Stat5 phosphorylation.

WT-EpoR internalization and signaling are lipid raft-dependent

Lipid rafts act as organizational centers for signaling and internalization. In order to test their involvement in EpoR internalization and signaling, we disrupted rafts with a cholesterol depleting agent, Methyl-cyclodextrin (MCD). Internalization of WT-EpoR was significantly reduced by MCD (Fig. 31), suggesting that its internalization is substantially dependent on lipid-rafts. However, MCD had little effect on internalization of 5KR-EpoR in either the absence and presence of Epo. Together, these observations appear to define distinct routes of internalization for WT- and 5KR-EpoR; where non-ubiquitinated 5KR-EpoR is internalized through a dynamin-dependent, raft-independent mechanism. By contrast, ubiquitinated WT-EpoR is internalized through raft-dependent, dynamin-independent pathways. Even though the internalization of ubiquitinated EpoR was blocked by the disruption of lipid rafts, it is still not clear if the reduction in EpoR signaling results from the lack of EpoR localization within lipid rafts or the lack of its internalization from lipid rafts. If ubiquitinated EpoR, but not non-ubiquitinated EpoR, is localized within lipid rafts, another question is if the ubiquitination of EpoR direct its localization to lipid raft microdomains. These questions will need to be answered in future studies.

To test the requirement for lipid rafts in EpoR signaling, Epo-induced Stat5 activation was assessed in cells treated with MCD (Fig. 32). Similar to effects on

internalization, MCD-treatment significantly reduced Epo-induced Stat5 activation by WT-EpoR, while MCD had little effect on Stat5 activation mediated by 5KR-EpoR. Thus, reduced internalization of WT-EpoR resulting from disruption of lipid rafts is correlated with reduced signaling from these receptors.

Conclusions

In Chapters II and III, it was shown that ubiquitinated EpoR produces more efficient Epo-induced signaling activities and biological responses than non-ubiquitinated EpoR, suggesting that ubiquitination of EpoR is necessary for efficient Epo signaling activities. However, the mechanism of how ubiquitination enhances EpoR signaling activities was unclear. Therefore, we tried to elucidate the mechanisms in this Chapter IV. Signaling from some receptors has been linked to their internalization, and we had found that reduced signaling by non-ubiquitinated 5KR-EpoR was associated with a reduced rate of depletion from the cell surface. Therefore, we assessed potential differences in internalization between WT- and 5KR-EpoR.

Under normal conditions, both WT- and 5KR-EpoR were internalized in the presence or absence of Epo stimulation and were substantially protected by a lysosomal degradation inhibitor, indicating that constitutive internalization and lysosomal degradation are common fates of these receptors. Despite this gross similarity, evidence here suggests that the dominant mechanism of internalization differs between WT-EpoR and the non-ubiquitinated 5KR-EpoR. Specifically, Dynasore-treatment significantly reduced all internalization of 5KR-EpoR,

suggesting that the non-ubiquitinated EpoR is largely internalized through dynamin-dependent mechanisms. Internalization of unstimulated WT-EpoR was moderately reduced, but dynamin-inhibition did not inhibit internalization of WT-EpoR after Epo-treatment. Thus, Epo-induced ubiquitination of WT-EpoR appears to alter trafficking of receptors toward a dynamin-independent internalization pathway. Although WT-EpoR ubiquitination is significantly induced by Epo-treatment, ubiquitination of unstimulated receptor is also detectable. Thus, some level of ubiquitination may direct dynamin-independent internalization of WT-EpoR and reduce effects of Dynasore even in the absence of Epo stimulation. Still, Epo-induced increases in WT-EpoR internalization were only significant in the presence of Dynasore, suggesting that constitutive dynamin-dependent mechanisms also contribute to internalization of WT-EpoR. By contrast, only constitutive internalization of non-ubiquitinated EpoR was observed, where dynamin-inhibition equivalently reduced internalization of 5KR-EpoR in the presence or absence of Epo stimulation.

Finally, the different forms of EpoR were shown to substantially differ in their dependence on lipid rafts for internalization, with internalization of WT-EpoR significantly reduced by cholesterol depletion which had little effect on internalization of non-ubiquitinatable 5KR-EpoR. Thus, the dynamin-independent mechanism mediating internalization of ubiquitinated EpoR appears to depend on intact lipid raft microdomains of the plasma membrane. How is the internalization of ubiquitinated EpoR affected by lipid raft microdomains? Studies of EGFR, T-cell antigen receptor, and IgE receptor FcεRI have shown that

ubiquitination of activated receptors is necessary for lipid raft-dependent endocytosis of receptors (Balbis et al., 2007; Barr et al., 2006; Molfetta et al., 2009; Sigismund et al., 2005). According to the reports, internalization of ubiquitinated receptors through lipid raft microdomains requires interaction with Ub-interacting motif-containing proteins eps15, eps15R, and epsin, suggesting that these proteins are involved in directing ubiquitinated cargo to lipid raft microdomains. However, functional interaction of these proteins with lipid raft-specific structural proteins, such as caveolin, has not been identified. Thus, the mechanism responsible for lipid raft localization of ubiquitinated receptors is incompletely understood. Still, the ubiquitination of EpoR and its interaction with Ub-interacting motif-containing proteins might direct a mechanism that shifts the receptor from clathrin-coated pits responsible for dynamin-dependent internalization to lipid raft microdomains where internalization is cholesterol-dependent.

Evidence here also suggests that internalization facilitates EpoR signaling. First, we show that there is a temporal correlation between detectable internalization and Stat5 activation, indicating that the onset of signaling activity at least does not precede detectable receptor internalization. A functional role for internalization in EpoR signaling is suggested by the ability of specific inhibitors of EpoR internalization to also inhibit signaling activity. Specifically, MCD disruption of lipid rafts reduced internalization and signaling from WT-EpoR, while dynamin-inhibition reduced internalization and signaling from 5KR-EpoR. Importantly, the lack of effect of MCD on internalization and signaling of 5KR-

EpoR, and the lack of effect of Dynasore on internalization and signaling from Epo-stimulated WT-EpoR indicate that the observed effects on internalization and signaling from WT-EpoR or 5KR-EpoR, respectively, did not result from non-specific disruption of cellular processes.

How does internalization of EpoR activate EpoR signaling? Receptor internalization mediated through dynamin- or lipid raft-dependent mechanisms and its delivery to early endosome has been reported to facilitate signal activation for TGF-βR or activation of several signaling molecules regulated by EGFR (Balbis et al., 2007; Di Guglielmo et al., 1994; Di Guglielmo et al., 2003; Vieira et al., 1996). TGF-βR is internalized through a dynamin-dependent mechanism and is delivered to endosomes where it interacts with SMAD anchor for receptor activation proteins (SARA), a step required for recruitment of SMAD2 and signal activation (Di Guglielmo et al., 2003). EGFR internalized through lipid raft microdomains is also delivered to endosomes where several signaling molecules, such as Grb2, Shc, or Erk are recruited, forming a signaling complex for signal activation (Balbis et al., 2007; Teis et al., 2002). If this would be the case, internalized EpoR directed through either dynamin- or lipid raft-dependent mechanisms might be delivered to endosomes where it recruits signaling effectors, such as Stat5, Grb2, or PI3K. This recruitment might be direct or could be effected indirectly through interaction with scaffolding or adaptor proteins present within endosomes. This would result in effective signal activation only after receptor internalization.

Altogether, these results indicate that the internalization of EpoR is necessary for efficient Epo signaling activities. Although internalization of ubiquitinated and non-ubiquitinated receptors are differently regulated, either route of internalization appears to be able to deliver receptors to sites capable of supporting signaling activities.

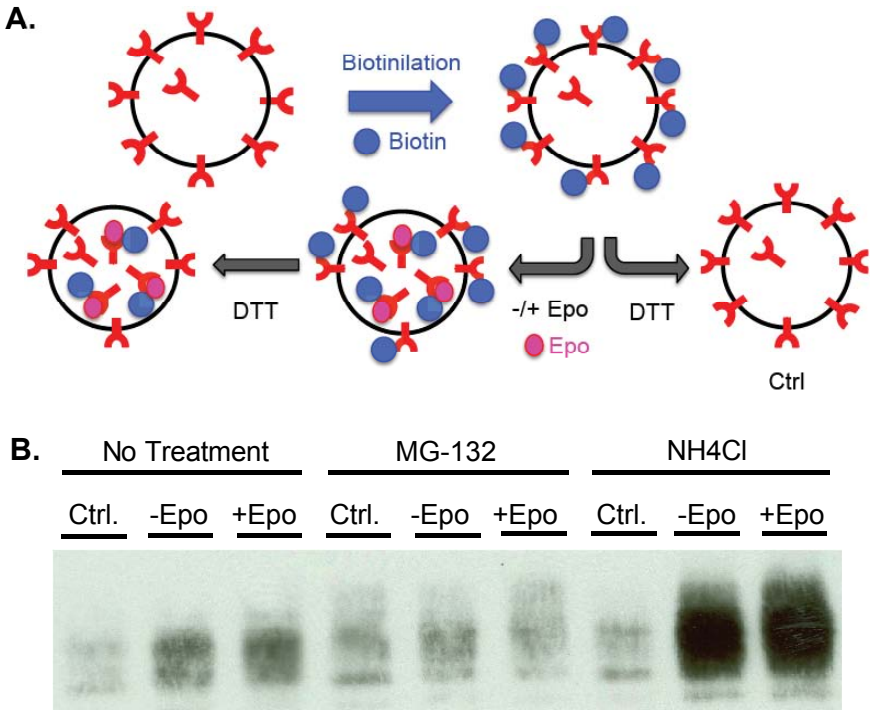


Figure 26. Internalization of WT-EpoR.
BaF3 cells expressing WT-EpoR were treated with 20 μM MG-132, 20 mM NH_4Cl, or left untreated for 60 min. Identification of internalized EpoR was performed as diagramed **(A)**. Before Epo stimulation, surface EpoR was labeled with a cleavable membrane-impermeable biotinylation reagent. A sample of biotinylated cells was immediately treated with DTT to remove the cell surface label and identify remaining labeled receptor populations that were not specifically internalized (Ctrl). The remaining cells were treated with (+Epo) or without (-Epo) 10 U/ml Epo for 20 min., after which cells were cooled to 4 °C and the biotin-label of surface EpoR was cleaved by DTT treatment. Cell were lysed and internalized biotin-labeled EpoR was precipitated with neutravidin. Precipitated EpoR was immunoblotted with anti-HA antibodies **(B).**

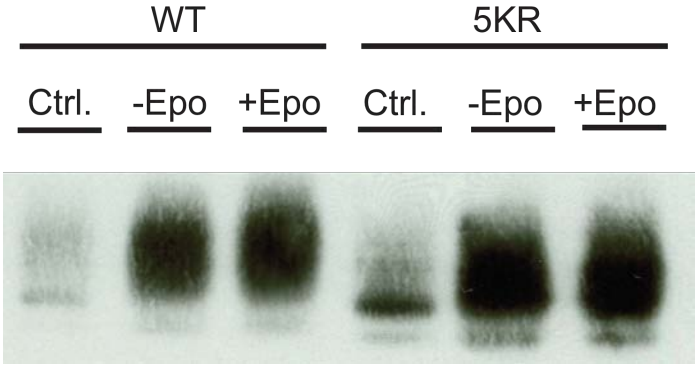
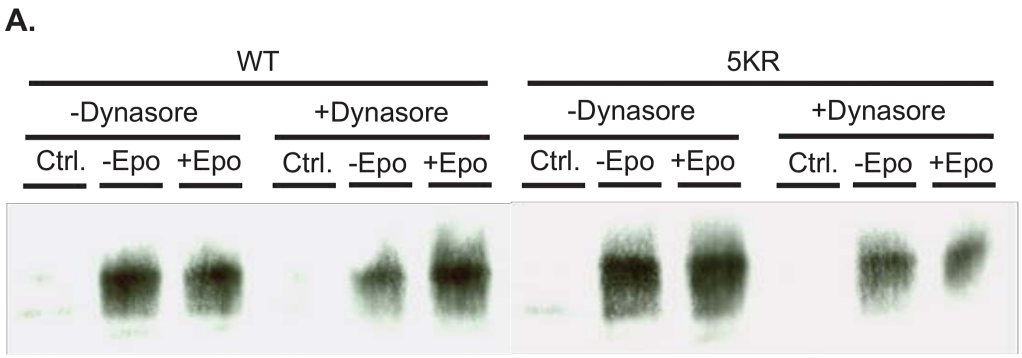
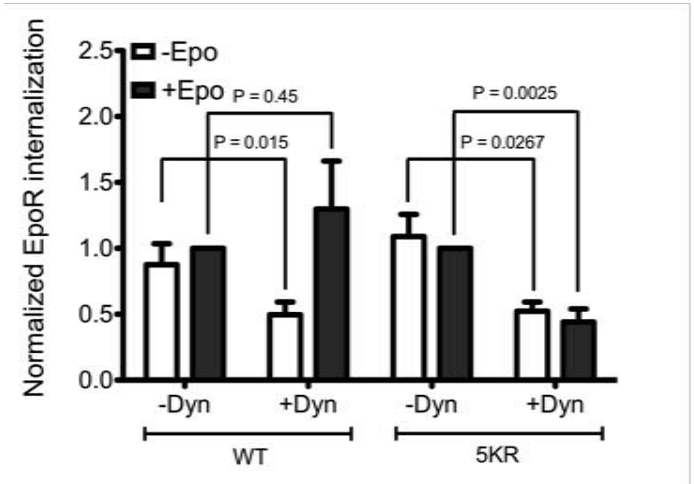


Figure 27. Internalization of WT- and 5KR-EpoR in the presence of NH_4Cl. BaF3 cells expressing WT or 5KR-EpoR was treated with 20 mM NH_4Cl for 60 min. Receptor internalization was then assessed as described for **Fig. 26**. Precipitated EpoR was immunoblotted with anti-HA antibodies.

Figure 28. Dynamin dependence of EpoR internalization.
BaF3 cells expressing WT- or 5KR-EpoR was treated with 20 mM NH_4Cl in the absence or presence of 80 μM Dynasore for 60 min. Receptor internalization was then assayed as described for **Fig. 26 (A)**. Internalized EpoR was quantified and normalized to the value from Epo-treated cells (+Epo, -Dynasore) so that results from multiple cell lines could be pooled **(B)**. Results shown represent the average of three independent clones and two repeated experiments for each EpoR construct. The percent increase in EpoR internalization induced by Epo-treatment was computed from data represented in Panel B **(C)**.


A.
WT
5KR
-Dynasore
+Dynasore
-Dynasore
+Dynasore
Ctrl.
-Epo
+Epo
Ctrl.
-Epo
+Epo
Ctrl.
-Epo
+Epo
Ctrl.
-Epo
+Epo


B.
Normalized EpoR internalization
2.5
2.0
1.5
1.0
0.5
0.0
-Epo
+Epo
P = 0.45
P = 0.015
P = 0.0025
P = 0.0267
-Dyn
+Dyn
-Dyn
+Dyn
WT
5KR

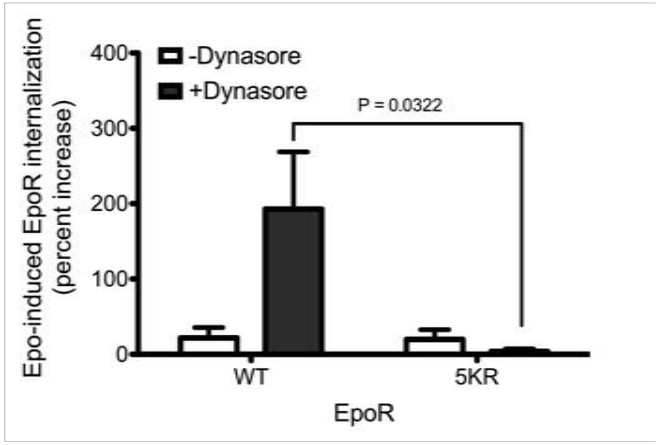
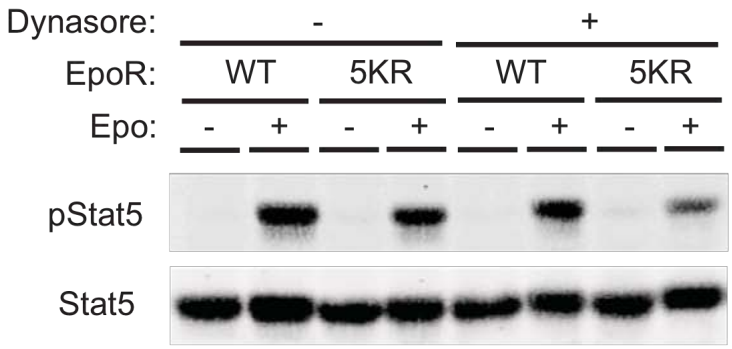
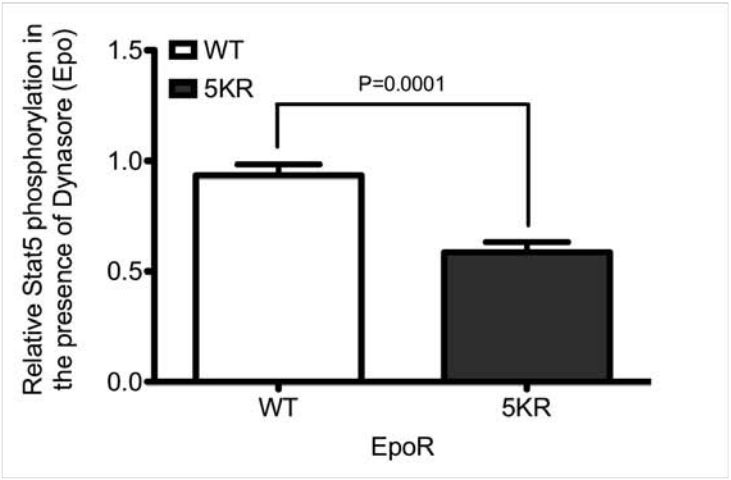

C.
Epo-induced EpoR internalization
(percent increase)
400
300
200
100
0
-Dynasore
+Dynasore
P = 0.0322
WT
5KR
EpoR

Figure 29. Dynamin-dependence of Epo signaling activity.
BaF3 cells expressing WT- or 5KR-EpoR were treated with 80 μM Dynasore for 30 min and then stimulated with 10 U/ml Epo for 15 min. Whole cell lysates were immunoblotted with antibodies to phospho-Stat5 (pStat5) or total Stat5 (Stat5) **(A)**. pStat5 was quantified and normalized to total Stat5 to control for loading, and the ratio of Epo-activated Stat5 in the presence and absence of Dynasore (+Dynasore/-Dynasore) was computed to assess inhibition of EpoR signaling resulting from Dynasore-treatment **(B)**. pStat5 stimulated by 1.5 ng/ml IL-3 (not shown) was quantified and normalized to total Stat5 to control for loading, and the ratio of Epo-activated Stat5 in the presence and absence of Dynasore (+Dynasore/-Dynasore) was computed to assess inhibition of EpoR signaling resulting from Dynasore-treatment **(C)**. Values shown represent the average of three independent cell lines for each EpoR construct.

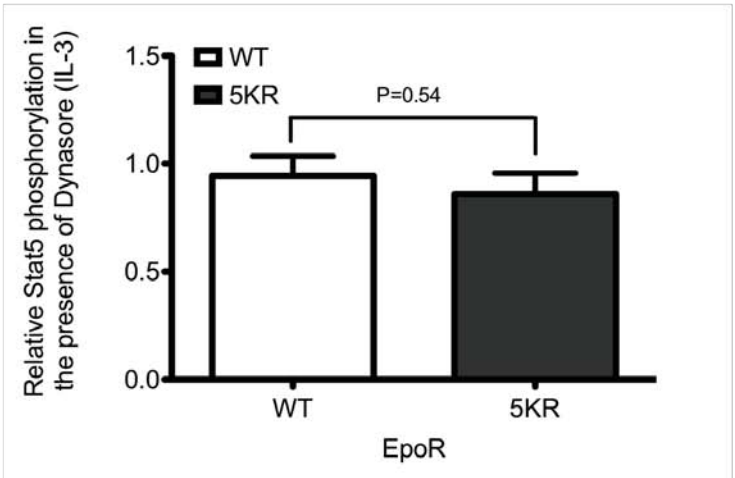
A.


Dynasore:
-
+
EpoR:
WT
5KR
WT
5KR
Epo:
- + - + - + - +
pStat5
Stat5

B.


Relative Stat5 phosphorylation in the presence of Dynasore (Epo)
1.5
1.0
0.5
0.0
WT
5KR
P=0.0001
WT
5KR
EpoR

C.


Relative Stat5 phosphorylation in the presence of Dynasore (IL-3)
1.5
1.0
0.5
0.0
WT
5KR
P=0.54
WT
5KR
EpoR

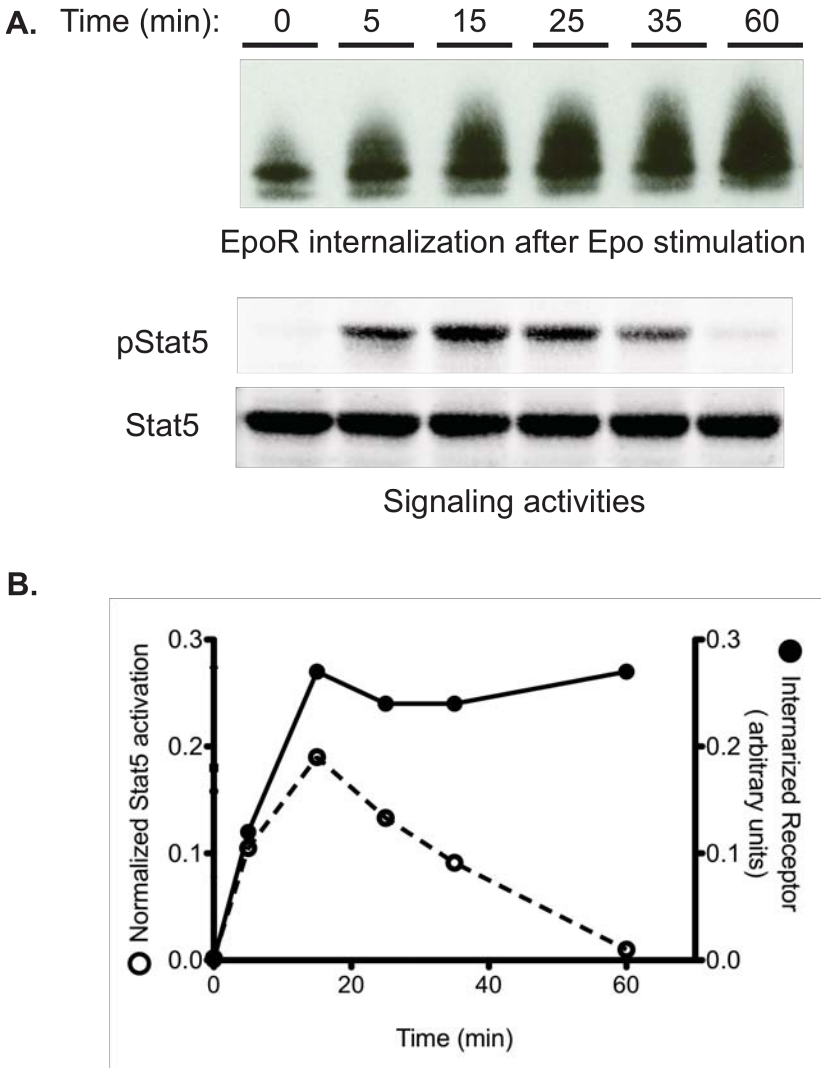


Figure 30. Correlation of signal activation with EpoR internalization. BaF3 cells expressing WT-EpoR were treated with 80 μM Dynasore and 20mM NH_4Cl for 60 min and then surface EpoR was labeled with a cleavable membrane impermeable biotinylation reagent. At indicated time points after Epo stimulation (10 U/ml), internalized EpoR was assessed as described for **Fig. 26**. Also, whole cell lysates were directly immunoblotted with antibodies to phospho-Stat5 (pStat5) or total Stat5 (Stat5) **(A)**. Internalized EpoR was quantified and normalized to the value from cells at time 0 (-Epo). pStat5 was quantified and normalized to total Stat5 to control for loading **(B)**.

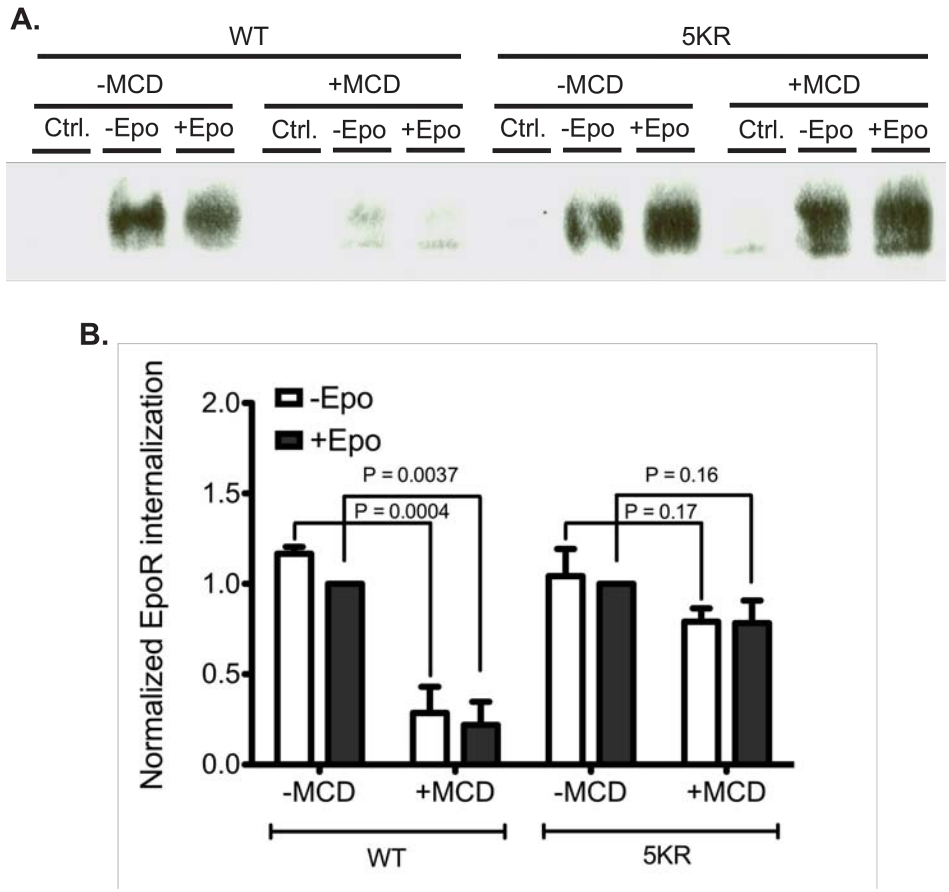


Figure 31. Raft-dependence of EpoR internalization.
BaF3 cells expressing WT- or 5KR-EpoR was treated with 20 mM NH_4Cl in the absence or presence of 5 mM MCD for 60 min. Receptor internalization was then assayed as described for **Fig. 26 (A)**. Internalized EpoR was quantified and normalized to the value from Epo-treated cells (+Epo, -MCD) so that results from multiple cell lines could be pooled **(B)**. Results shown represent the average of three independent clones and two repeated experiments for each EpoR construct.

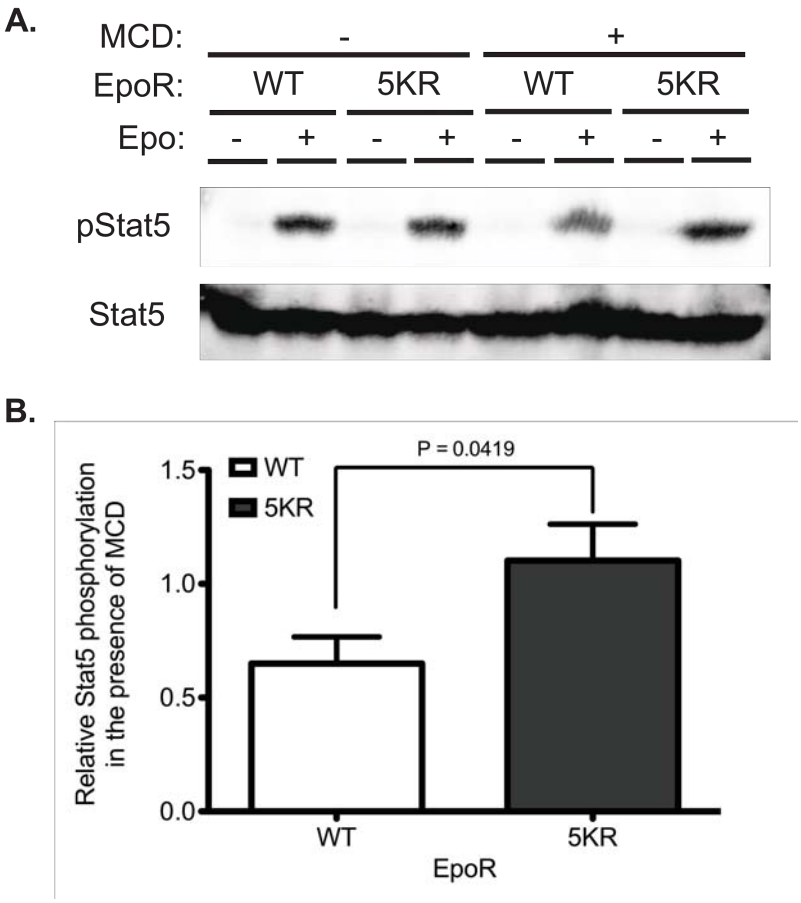


Figure 32. Raft-dependence of Epo signaling activity.
BaF3 cells expressing WT- or 5KR-EpoR were treated with 5 mM MCD for 30 min and then stimulated with 10 U/ml Epo for 15 min. Whole cell lysates were immunoblotted with antibodies to phospho-Stat5 (pStat5) or total Stat5 (Stat5) **(A)**. pStat5 was quantified and normalized to total Stat5 to control for loading, and the ratio of Epo-activated Stat5 in the presence and absence of MCD (+MCD/-MCD) was computed to assess inhibition of EpoR signaling resulting from MCD-treatment **(B)**. Values shown represent the average of three independent cell lines for each EpoR construct.

CHAPTER V
DISCUSSION

The proper regulation of cytokine growth factors and signaling from their receptors controls the normal function of hematopoietic cell lineages. Production of erythrocytes delivering oxygen to body tissues is strictly governed by Epo, acting through its receptor, EpoR, which regulates the proliferation, differentiation, and survival of erythroid progenitors (Lacombe and Mayeux, 1998, 1999). Conversely, defects in Epo/EpoR regulation can cause myeloproliferative diseases or anemia (Hoatlin et al., 1990; Jelkmann, 2004; Longmore and Lodish, 1991). Therefore, elucidating mechanisms that normally control biological responses to Epo attracts interest.

Ubiquitination of receptors has often been associated with negative regulation of receptor signaling as it can promote their internalization and degradation (Hicke and Riezman, 1996; Rocca et al., 2001; van Kerkhof et al., 2000). EpoR has previously been shown to be ubiquitinated, and inhibition of proteosome/lysosome degradation can stabilize receptors and reportedly prolong Epo signaling, suggesting that EpoR ubiquitination and its degradation might function as a negative regulator of Epo signaling activities (Verdier et al., 1998; Verdier et al., 2000). However, many downstream effectors of Epo signaling pathways are targeted for ubiquitin-dependent degradation, thus making the general inhibition of ubiquitin-dependent degradation a poor indicator of effects that are specifically mediated through direct ubiquitination of EpoR (Chen et al., 2006; Fang et al., 2001; Kamizono et al., 2001; Suizu et al., 2009). Therefore, the

purpose of this work was to clarify the role of EpoR ubiquitination in regulating EpoR signaling and Epo-dependent biological responses.

Role of EpoR ubiquitination in Epo signaling activities and functional responses

In Chapter II, we explored the effect of EpoR ubiquitination on the regulation of Epo signaling activities, as well as functional responses promoting proliferation and survival. We showed that ubiquitination of the EpoR was dramatically increased by Epo stimulation, suggesting a potential role for ubiquitination of EpoR in regulation of Epo signaling. Interestingly, a non-ubiquitinatable form of EpoR (5KR-EpoR) was found to mediate reduced signaling activity compared to WT-EpoR at low concentrations of Epo. This was also associated with a reduced ability to promote proliferation and survival of cells expressing non-ubiquitinatable EpoR. Based on these observations, we concluded that ubiquitination of EpoR is required for efficient signaling activity, especially in response to low dosages of Epo. Loss of EpoR ubiquitination reduced all Epo-induced signaling activities assessed in this work, suggesting that the requirement for receptor ubiquitination may be an early event in the EpoR signaling pathway. Jak2 activation is generally considered the earliest assayable activity resulting from EpoR activation, and is required for all downstream effects, including Stat5, PI3K, and MAPK pathway activation. Thus, the observation that Epo-induced Jak2 activation is less effectively mediated by non-ubiquitinatable EpoR is consistent with a uniform reduction in all assayable signaling activities and biological responses mediated by Epo. The reduction in

Jak2 phosphorylation associated with 5KR-EpoR was only moderate, compared to the larger effects observed on downstream effector pathways (Stat5 and Erk). However, This is consistent with the normal amplification of activities as signals progress downstream in signaling pathways. Thus, small changes in the level of Jak2 activity would be expected to produce relatively larger changes in the activities of its downstream effectors (Stat5 and Erk).

EpoR domains supporting ubiquitination and promoting signaling activities

In Chapter III, we defined EpoR domains that can support its ubiquitination, ubiquitin-dependent signaling activities, and physiological responses. Two C-terminal lysines (388K and 428K) were shown to be primary targets for ubiquitination. β-Trcp, an E3 ubiquitin ligase for IFNαR1 and IκBα, has been reported to act as an E3 ubiquitin ligase for EpoR (Meyer et al., 2007). In ubiquitination of IFNαR1 and IκBα, β-Trcp binds a consensus sequence, DSGXXS, and ubiquitinates lysines adjacent to this site (Kumar et al., 2003; Yaron et al., 1998). EpoR also possesses a partial consensus sequence for β-Trcp binding in the C-terminus of its intracellular domain. If β-Trcp ubiquitinates EpoR in the same manner as IFNαR1 or IκBα, targeting of the C-terminal lysines (388K and 428K) would be a reasonable outcome. In this regard, it would be interesting to test if mutation of the β-Trcp binding site results in the same changes in biological function and signaling activities associated with loss of two C-terminal ubiquitination sites of EpoR. The functional outcome of ubiquitination

at either C-terminal lysine appears to be enhanced signaling and biological response, since EpoR mutants retaining only a single lysine at position 388 or 428 support nearly wild-type activities in response to low concentrations of Epo. The observation that either of these two lysines support this enhanced responsiveness argues that ubiquitination itself is responsible for this effect. Conversely, EpoR mutants containing single lysines at positions 256, 276, or 348 were not significantly ubiquitinated and did not restore Epo-responsiveness compared to EpoR lacking all cytoplasmic lysines (5KR). Therefore, we conclude that ubiquitination of EpoR facilitates its signaling activities and subsequent effects on cell proliferation and survival.

How does ubiquitination facilitate EpoR signaling?

In Chapter IV, we addressed the possibility that ubiquitination effects on Epo signaling might be related to changes in EpoR internalization. We demonstrated that both ubiquitinated and non-ubiquitinated EpoR were constitutively internalized in the absence or presence of ligand. A significant fraction of internalized EpoR is protected by NH_4Cl treatment, and is apparently destined to be degradated by lysosomal machinery. Internalized EpoR has also been reported to recycle to the cell surface (Walrafen et al., 2005), but how this fraction of internalized EpoR is affected by ubiquitination was not addressed in our studies. Nonetheless, we did find a reduced rate of ligand-induced depletion from the cell surface for non-ubiquitinated EpoR, suggesting that receptor ubiquitination might affect EpoR internalization. Indeed, we found internalization

of ligand-activated WT-EpoR is dependent on different mediators compared to non-ubiquitinated EpoR mutants, which were specifically inhibited by disruption of lipid rafts or inhibition of dynamin, respectively. These observations suggest that ubiquitination of EpoR shifts its internalization from a mechanism dependent on dynamin toward a distinct pathway dependent on maintenance of lipid raft microdomains. Whether ubiquitination actually causes a relocalization of EpoR into lipid rafts, or simply effects internalization of lipid-raft-localized EpoR remains to be determined.

Our studies also find a strong correlation between internalization of EpoR and the ability of EpoR to activate signaling pathways. Specifically, we show that disruption of lipid rafts inhibits internalization and signaling from WT-EpoR, while inhibition of dynamin reduces internalization and signaling from non-ubiquitinated EpoR. Interestingly, we also show a correlation in the time course of Epo-induced EpoR internalization and Epo-induced signaling activity, suggesting the possibility that internalization is a requirement prior to effective signaling from ligand-activated EpoR. If this is the case, internalization mediated through constitutive dynamin-dependent mechanisms or ubiquitination-induced lipid raft-dependent mechanisms would both be capable of delivering activated EpoR to an appropriate signaling platform.

Potential mechanisms regulating EpoR signaling

We have shown that EpoR is rapidly ubiquitinated after Epo stimulation, and that ubiquitination of EpoR facilitates its internalization by a dynamin-

independent and lipid-raft-dependent endocytic pathway. Furthermore, this raft-dependent EpoR internalization facilitates its signaling activities. Thus, we suggest that Epo-induced ubiquitination promotes the internalization of EpoR through lipid-raft-microdomains which can deliver activated EpoR to an appropriate intracellular signaling platform supporting Jak2 activation and phosphorylation of Stat5 (Fig. 33). On the other hand, non-ubiquitinated EpoR is constitutively internalized by a dynamin-dependent and lipid-raft-independent endocytic pathway. But, when EpoR is activated without direct ubiquitination (e.g. 5KR-EpoR), constitutive internalization mechanisms can also capture active EpoR for delivery to an appropriate signaling platform. In this scenario, concentrations of Epo that can effectively saturate cell surface EpoR may be expected to produce essentially equivalent signaling activity through WT or non-ubiquitinatable receptors. But, at low concentrations of Epo, the random capture of ligand-bound EpoR by constitutive internalization mechanisms may be expected to deliver fewer active receptors to intracellular signaling domains compared to WT-EpoR which is selectively internalized through lipid rafts in response to Epo-induced ubiquitination. This prediction is consistent with our observation that non-ubiquitinated forms of EpoR mediate reduced signaling activity and biological responsiveness to low concentrations of Epo.

The mechanisms proposed in this thesis represent novel concepts affecting EpoR signaling. Notably, rather than acting as a negative regulator, we have identified a role for EpoR ubiquitination in facilitating Epo-induced responses.

Future directions

Although lipid rafts define organizational centers for membrane proteins, they are also well known to function as signaling platforms themselves (Brown and London, 1998; Lajoie et al., 2009; Lingwood and Simons, 2010). While our studies strongly correlate EpoR internalization with signaling activities, we cannot rule out the possibility that localization to lipid-raft domains directly supports EpoR signaling. It is also not known if Epo-induced ubiquitination promotes lipid raft localization, or if it simply promotes internalization of receptors already localized in lipid rafts. These concepts could be addressed by defining the degree of lipid raft localization in the presence or absence of Epo or as a function of the ubiquitinated state of EpoR. Similarly, it will be important to determine if lipid raft microdomains present on the plasma membrane contain active signaling complexes of EpoR, or whether signaling EpoR complexes are localized to signaling platforms in intracellular compartments. Therefore, analyses of EpoR signaling activities within subcellular fractions may provide important insights into how trafficking of activated EpoR affects its signaling activities.

Finally, the role of EpoR ubiquitination in primary erythroid cells should be investigated in the future. Such an analysis could be addressed using transgenic mice in which WT or 5KR-EpoR is used to rescue erythropoiesis in an EpoR-/- background. In particular, it would be interesting to determine if loss of EpoR ubiquitination alters the concentration-dependent response of primary erythroid cells to Epo, or if it has significant effects on the development of red blood cells in vivo. Such studies elucidating the role of EpoR ubiquitination in erythropoiesis of

primary erythroid cells could provide conclusive evidence of the physiological and clinical importance of EpoR ubiquitination.

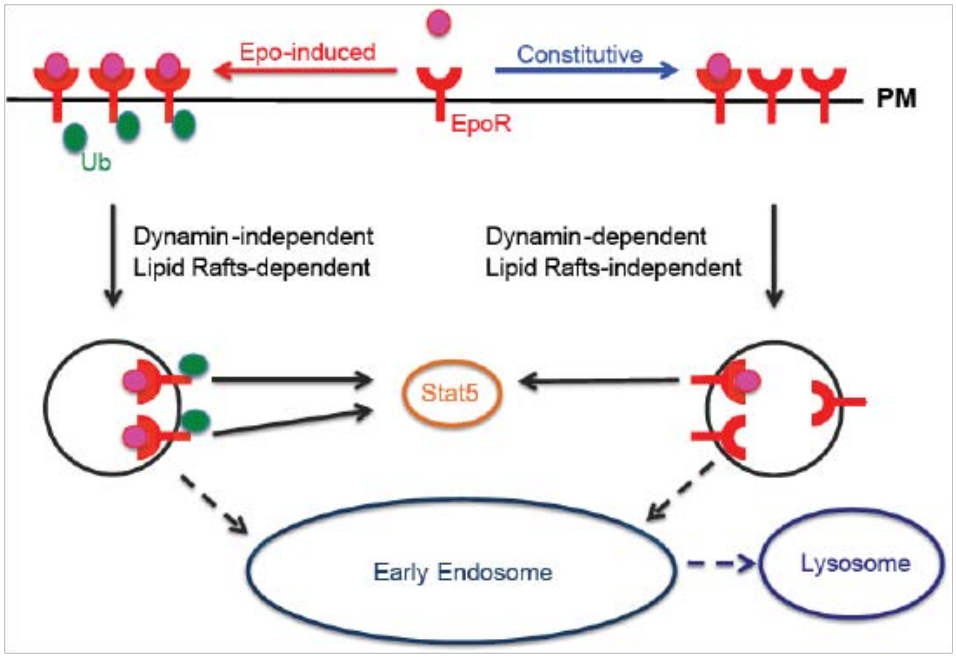


Figure 33. Potential mechanisms of EpoR internalization and Epo signaling.
After Epo stimulation, ubiquitinated EpoR is internalized by a dynamin-independent, lipid-raft dependent mechanism where all internalized receptor can signal to Stat5. Non-ubiquitinated EpoR is constitutively internalized by a dynamin-dependent, lipid-raft-independent mechanism. However, capture of ligand-activated EpoR by constitutive internalization can still deliver active EpoR to signaling platforms supporting activation of signaling pathways leading to Stat5 activation.

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