UBIQUITINATION OF EPOR AND P85 IN LIGAND INDUCED

EPOR DOWN-REGULATION

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Erythropoietin (Epo) is the primary cytokine that drives red blood cell production and signals through its receptor, the EpoR, on erythroid progenitor cells. Epo binding to EpoR activates Janus kinase 2 (JAK2), which phosphorylates cytoplasmic tyrosines of the EpoR. Signaling proteins bind these phosphotyrosines through SH2 domains, leading to the survival and proliferation of erythroid progenitor cells and the differentiation of these progenitors into mature erythrocytes. Therefore, EpoR signaling is essential for red blood cell production.

To maintain physiologic numbers of circulating red blood cells EpoR signaling is also subject to negative regulation. Mutations in EpoR or JAK2 that abrogate negative regulation cause erythrocytosis in hematological malignancies. Primary familial and congenital polycythemia (PFCP) is a proliferative disorder characterized by erythrocytosis and

hypersensitivity of erythroid progenitors to Epo. Defects in negative regulation of EpoR signaling contribute to the etiology of PFCP. However, the underlying molecular mechanisms are poorly understood.

Here we show that ubiquitination of EpoR controls internalization, lysosomal sorting, degradation and signaling of the EpoR. Ubiquitination of EpoR at K256 is necessary and sufficient for efficient Epo-induced receptor internalization, while ubiquitination at K428 promotes trafficking of activated receptors to the lysosomes for degradation. Interestingly, EpoR that cannot be ubiquitinated has reduced mitogenic activities and ability to stimulate the downstream signaling pathways. We propose that ubiquitination of the EpoR critically controls both receptor down-regulation and signaling.

Secondly, we identified a novel mechanism mediating Epo-dependent EpoR internalization. Epo induces Cbl-dependent ubiquitination of the p85, which binds to phosphotyrosines on EpoR. Ubiquitination allows p85 to interact with epsin-1, thereby driving EpoR endocytosis. Knockdown of Cbl, expression of its dominant negative forms, or expression of an epsin-1 mutant all compromise Epo-induced EpoR internalization. Mutated EpoRs mimicking those from PFCP patients cannot bind p85, co-localize with epsin-1, nor internalize upon Epo stimulation and exhibit Epo hypersensitivity. Restoring p85 binding to PFCP receptors rescues Epo-induced epsin-1 co-localization, EpoR internalization, and normalizes Epo hypersensitivity. Our results uncover the role of EpoR ubiquitination and a novel Cbl/p85/epsin-1 pathway in EpoR endocytosis and show that defects in this pathway contribute to excessive Epo signaling and erythroid hyperproliferation in PFCP.

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PRIOR PUBLICATIONS

1) **G. B. Bulut**, R.Sulahian, H. Yao and L.J.Huang, "Cbl ubiquitination of p85 is essential for EPO-induced EPOR endocytosis" Blood, 2013
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4) L.J.Huang, Y.M.Shen, **G. B. Bulut**, "Advances in understanding the pathogenesis of primary familial and congenital polycythaemia" (2010). Br J Haematol 148, 844-852

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LIST OF DEFINITIONS

μg: microgram

μl: microliter

μM: micro molar

μm: micron

Cbl: Casitas B lineage lymphoma

EEA1: Early Endosomal Antigen 1

Epo: erythropoietin

Lamp2: Lysosomal associated membrane protein 2

MTT: 3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2,5-diphenyl**t**etrazolium bromide

PFCP: Primary familial and congenital polycythemia

PV: Polycythemia Vera

shRNA: small hairpin RNA

siRNA: small interfering RNA

STAT5: The signal transducer and activator of transcription 5

Ub: ubiquitin

CHAPTER 1: INTRODUCTION

1.1 ERYTHROPOIESIS

Erythropoiesis is the process through which pluripotent hematopoietic stem cells differentiate into mature red blood cells (RBC). Main function of RBC is to carry oxygen to bodily tissues by reaching tiny capillaries. RBCs circulate for about 120 days before they senesce and need to be cleared from the circulation by splenic macrophages. Therefore our bodies require continuous replenishment of RBC with an incredible production rate of 2 million cells per every second. In normal adults, about 200 billion erythrocytes are produced daily to ensure adequate tissue oxygenation. (Richmond, Chohan and Barber 2005)

Developmentally, there are two types of erythropoiesis in mammals: primitive and definitive. Primitive erythropoiesis takes place in the yolk sac to produce larger erythroid cells, which are observed during early stages of fetal life. Definitive erythropoiesis happens in the fetal liver during late stages of fetal life and in the adult bone marrow forming small, enucleated, biconcave flexible erythrocytes. Human erythropoiesis only takes place in the bone marrow, whereas murine erythroid precursors exist both in bone marrow (90%) and the spleen (10%). These precursors in the spleen can expand in response in erythropoietic stress or in disease conditions resulting in splenomegaly. (Palis 2014, Richmond et al. 2005)

1.2 ERYTHROPOIETIN AND ITS RECEPTOR

The principle growth factor that regulates erythropoiesis is erythropoietin (Epo), which is made mainly by the peritubular cells of the renal cortex in kidney. Epo signals through its receptor, the EpoR, on erythroid progenitor cells. Epo binding to EpoR activates

the cytosolic tyrosine kinase JAK2, which phosphorylates cytoplasmic tyrosines of the EpoR. Signaling proteins bind these phosphotyrosines through SH2 domains, leading to the survival and proliferation of erythroid progenitor cells and the differentiation of these progenitors into mature erythrocytes. (Constantinescu, Ghaffari and Lodish 1999, Richmond et al. 2005) Epo production is increased under conditions of anemia (reduced RBC mass), hemorrhage or decreased cellular oxygen tension (hypoxia) causing erythropoiesis rate to increase by up to 10-fold.

1.3 SIGNALING FROM THE ERYTHROPOIETIN RECEPTOR

EpoR belongs to the cytokine receptor super-family, and consists of an extracellular domain, a single transmembrane domain, and a cytoplasmic domain. EpoR lacks intrinsic enzymatic activities and relies on the cytosolic tyrosine kinase JAK2 for signal transduction. JAK2 binding to the EpoR in the endoplasmic reticulum promotes its maturation and appearance on the plasma membrane (Huang, Constantinescu and Lodish 2001). At the cell surface, binding of Epo to the EpoR extracellular domain activates JAK2 kinase activity. Subsequently, activated JAK2 phosphorylates many of the tyrosine residues in the cytoplasmic domain of EpoR, thereby providing a platform for the recruitment and activation of signaling mediators through SH2 domain-mediated interactions. Consistent with their essential roles in erythropoiesis, Epo, EpoR, and JAK2 deficient mice die embryonically due to severe anemia (Wu et al. 1995, Neubauer et al. 1998, Parganas et al. 1998). On the other hand, constitutive activation of Epo signaling caused by mutations in EpoR or JAK2 results in excessive red cell production in myeloproliferative neoplasms.

Much is known of the signaling pathways activated by Epo through signaling mediators recruited to the EpoR cytoplasmic phosphorylated tyrosines (Fig 1). Because the majority of these studies have been performed on murine EpoR, we utilize the murine numbering system. The signal transducer and activator of transcription 5 (STAT5) is recruited to Tyr343 and Tyr401 and is subsequently phosphorylated by JAK2 (Gobert et al. 1996, Klingmuller et al. 1996). Upon phosphorylation, STAT5 translocates into the nucleus to initiate gene transcription of proteins such as the anti-apoptotic proteins Bcl-x (Socolovsky et al. 1999), Pim-1, and Oncostatin M (Menon, Fang and Wojchowski 2006a). The adaptor molecule growth factor receptor protein 2 (GRB2) binds to the EpoR upon stimulation, presumably via a predicted GRB2 binding site at Tyr464, and through the guanine nucleotide exchange factor for Ras, SOS, activates the Ras/MAPK pathway to promote cell proliferation (Barber et al. 1997a). The GRB2/SOS complex can also be recruited to Tyr401 via SH2-containing inositol-5-phosphatase (SHIP-1) (Mason et al. 2000) or protein tyrosine phosphatase SHP2 (Tauchi et al. 1996) to activate the Ras/MAPK pathway. The p85 regulatory subunit of Phosphoinositide 3-Kinase (PI3K) binds to phosphorylated Tyr429/Tyr431 or Tyr479 and recruits and activates the catalytic subunit of PI3K (Klingmuller et al. 1997, Sulahian, Cleaver and Huang 2009). PI3K stimulates AKT-associated survival pathways (Bouscary et al. 2003), inhibits pro-apoptotic activities of the Forkhead transcription factor Foxo3 (Kashii et al. 2000), and also promotes erythroid proliferation and maturation (Klingmuller et al. 1997, Zhao et al. 2006). In addition, phosphorylated Tyr464 and/or Tyr479 binds to the SH2 domain of the Src tyrosine kinase Lyn (Chin et al. 1998), which regulates the expansion and differentiation of erythroid

progenitors (Harder et al. 2004, Ingley et al. 2005, Karur et al. 2006, Tilbrook et al. 2001). The CRKL adapter protein, recruited to phosphorylated Tyr460 upon stimulation, is phosphorylated by Lyn to augment MAPK activation (Arai et al. 2001). Moreover, Epo stimulates a rise in intracellular calcium, possibly through an interaction with Tyr460 in the EpoR and the transient receptor potential channels, which contributes to differentiation (Hensold, Dubyak and Housman 1991, Gillo, Ma and Marks 1993, Chu et al. 2002, Hirschler-Laszkiewicz et al. 2009). In human EpoR, an additional juxtamembrane tyrosine, Tyr309, exists that modulates STAT1 activation (Arcasoy and Karayal 2005).

Interestingly, mice expressing a minimal tyrosine-null EpoR allele are able to support steady-state erythropoiesis but fail to efficiently support stress erythropoiesis (Zang et al. 2001, Menon et al. 2006b). Therefore, signaling independent of EpoR cytoplasmic tyrosines plays essential roles for steady-state erythropoiesis (Wojchowski et al. 2006). Although not entirely understood, the MAPK signaling axis seems to play an important role in these pathways (Menon et al. 2006a). Much remains to be uncovered of the protein components and pathways underlying Epo-induced tyrosine-independent signaling. Together, EpoR tyrosine dependent and independent signaling cascades ultimately result in the survival, proliferation, and differentiation of erythroid progenitor cells.

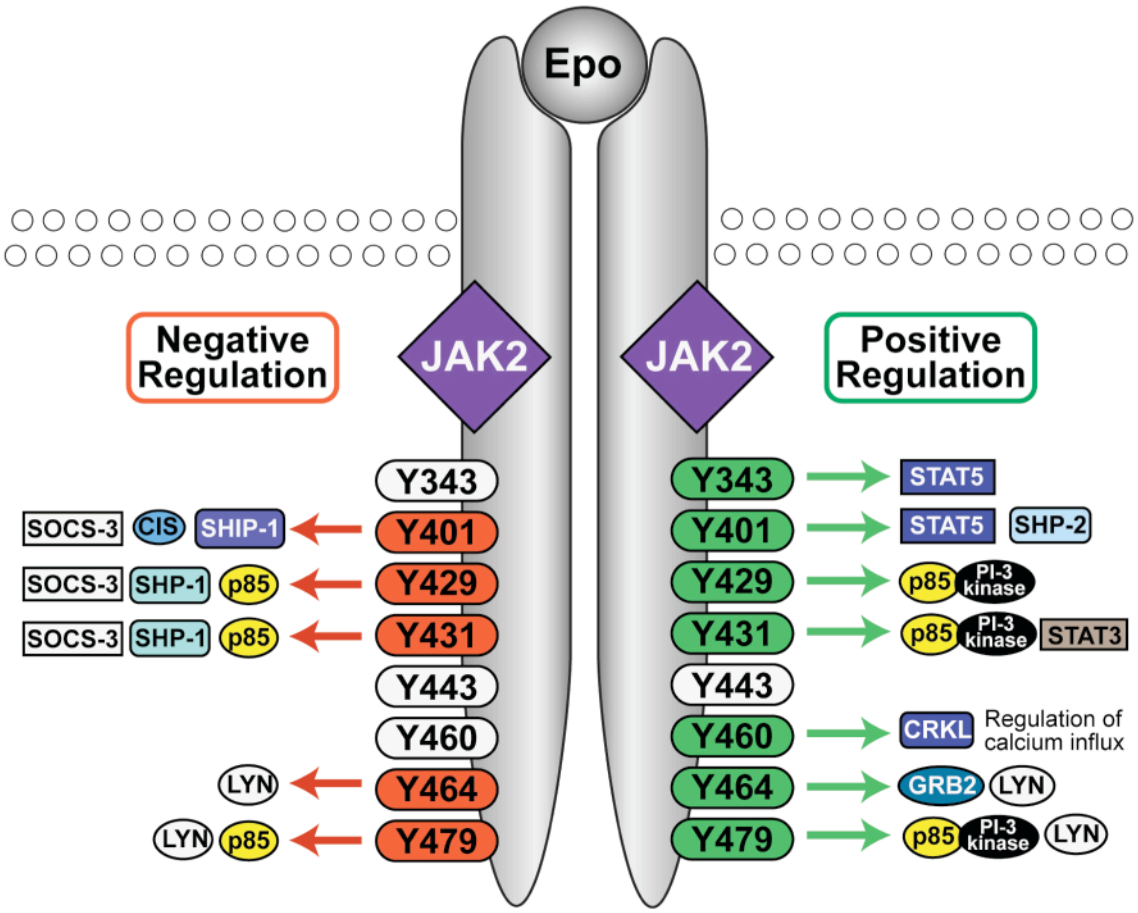


Figure 1: Signaling from the EpoR.

Proteins recruited to EpoR cytoplasmic tyrosine residues that positively or negatively regulate EpoR signaling are depicted

1.4 DOWN-REGULATION OF EPO SIGNALING BY PHOSPHATASES AND SOCS PROTEINS

In order to tightly regulate and to prevent uncontrolled erythropoiesis, negative regulators are also elicited upon activation to attenuate EpoR signal transduction (Fig. 1) (Wormald and Hilton 2004). For example, the tyrosine phosphatase SHP-1 (also known as SHPTP-1 or HCP) is recruited to phosphorylated Tyr429 and de-phosphorylates JAK2 (Yi et al. 1995, Klingmuller et al. 1995). The numbers of erythroid progenitors CFU-Es from SHP1-deficient (viable motheaten) mice are dramatically increased in the spleen and are hypersensitive to Epo (Van Zant and Shultz 1989). The SH2-containing inositol-5-phosphatase (SHIP-1) is recruited to Tyr401 of the EpoR (Mason et al. 2000) and SHIP-1 null mice show elevated formation of BFU-Es and CFU-Es in the bone marrow (Helgason et al. 1998). In addition, members of the suppressor of cytokine signaling (SOCS) family proteins and the cytokine-inducible SH2-domain-containing protein (CIS) can bind to cytokine receptors or the associated JAK kinases and attenuate signal transduction both by direct interference with signaling and by targeting the receptor complex for ubiquitin-mediated proteasomal degradation (Wormald and Hilton 2004). Upon Epo stimulation, SOCS-3 binds to Tyr401 or Tyr429 and Tyr431 (Sasaki et al. 1999, Hortner et al. 2002) and CIS binds to Tyr401 (Verdier et al. 1998), blocking access of STAT5 to the EpoR. SOCS1 interacts with the critical phospho-tyrosine residue Tyr1007 within the JAK2 catalytic loop and reduces its tyrosine-kinase activity and suppresses the tyrosine-phosphorylation and activation of STAT5 (Endo et al. 1997, Yasukawa et al. 1999). Transcription of SOCS-1, SOCS-3, and CIS is induced by Epo, which forms a negative feedback circuit (Starr et al.

1997). Moreover, over-expression of lymphocyte linker protein (Lnk), an adaptor protein that becomes tyrosine-phosphorylated following Epo induction, inhibits survival and differentiation of erythroid progenitors. Lnk-deficient mice display enhanced Epo-mediated signaling and erythropoiesis (Tong, Zhang and Lodish 2005).

1.5 ERYTHROCYTOSIS

Erythrocytosis, often used interchangeably with polycythemia ("many cells"), comprises a heterogeneous group of disorders characterized by the expansion of the erythrocyte compartment in the peripheral blood, reflected by an increase in hematocrit, hemoglobin content, or number of red blood cells (Cario 2005). Erythrocytosis is classified as congenital or acquired and may be primary or secondary. Primary erythrocytosis indicates that the erythrocytosis results from a molecular defect in the hematopoietic progenitor cells, whereas secondary erythrocytosis, although also a hematologic disorder, is due to aberrant regulation of erythropoiesis-promoting substances, mainly Epo, that act on these progenitors (Gordeuk, Stockton and Prchal 2005). Once the stimulus for increased Epo production is removed, the erythroid proliferation should return to the physiologic norm.

Most patients with primary erythrocytosis have polycythemia vera (PV), an acquired myeloproliferative neoplasm. In 2005, several groups identified somatic gain-of-function mutations in the JAK2 tyrosine kinase (V617F and exon 12), which functions downstream of Epo, in the vast majority of polycythemia vera patients (reviewed in (Levine and Gilliland 2008, Vainchenker, Dusa and Constantinescu 2008)). This prompted the World Health Organization to include JAK2 mutations in the diagnostic criteria for this disease (Patnaik and Tefferi 2009).

A small minority of patients with primary erythrocytosis has a congenital proliferative disorder of erythroid progenitor cells referred to as primary familial and congenital polycythemia (PFCP). PFCP is an autosomal-dominant disorder, although sporadic cases are also reported. PFCP is diagnosed when kindreds have isolated erythrocytosis without splenomegaly, low serum Epo levels, normal hemoglobin oxygen affinities, and bone marrow erythroid progenitors that exhibit Epo hypersensitivity (Prchal 2001). The natural history of PFCP shows no propensity to leukemic transformation nor is development of other myeloproliferative neoplasms observed (Prchal 2005). Clinically, PFCP patients may present with symptoms ranging from headaches, dizziness, epistaxis, exertional dyspnea to pruritis after bathing (Bourantas et al. 2006). Thrombotic and hemorrhagic events with premature morbidity and mortality have been reported (Prchal et al. 1995, Queisser et al. 1988), but many appear to have a benign clinical course (Prchal et al. 1985, Juvonen et al. 1991, Emanuel et al. 1992). Clinical symptoms are effectively relieved by phlebotomy; however, the increased risk of cardiovascular morbidity is not ameliorated by maintaining a normal hematocrit (Van Maerken et al. 2004, Cario 2005). Recent discoveries of the molecular defects involving the receptor for Epo that underlie this rare disorder will be discussed below.

1.6 PFCP MUTATIONS

The molecular basis of PFCP was not known until the study of a large family from Finland who had autosomal dominant erythrocytosis caused by excessive sensitivity of erythroid progenitors to Epo (Juvonen et al. 1991). Linkage analysis and later sequencing identified a mutation in the *EpoR* gene that was likely the cause of the erythrocytosis (de la

Chapelle et al. 1993a, de la Chapelle, Traskelin and Juvonen 1993b). This mutation (G6002A) leads to a premature stop codon at Trp439, resulting in a truncated EpoR. To date, 16 different EpoR alleles have been found in PFCP patients (Table 1).

EpoR mutation	Consequences of the Mutation	Reference
del5828-5829	Frameshift: P381Q-*	(Al-Sheikh et al. 2008)
G5881T	E399*	(Arcasoy et al. 2002)
del5938-5941	Frameshift: G418P-LLPALSTLSWTPAPSSCVHGHCALSCPLPHPT-*	(Petersen et al. 2004)
del5957-5958	F424*	(Al-Sheikh et al. 2008)
G5959T	E425*	(Kralovics and Prchal 2001)
C5964G	Y426*	(Kralovics, Sokol and Prchal 1998)
5967insT	Frameshift: I428Y-PGPQLPALASMDTVP-*	(Kralovics et al. 1997a)
del5971C	Frameshift: L429W-TPAPSSCVHGHCALSCPLPHPT-*	(Al-Sheikh et al. 2008)
dup5968-5975	Frameshift: D430E-SWTPAPSSCVHGHCALSCPLPHPT-*	(Watowich et al. 1999)
5974insG	Frameshift: D430G-PQLPALASMDTVP-*	(Sokol et al. 1995)
del5985-5991	Frameshift: Q434C-VHGHCALSCPLPHPT-*	(Arcasoy et al. 1997, Kralovics et al. 1997a)
C5986T	Q434*	(Furukawa et al. 1997)
G6002A	W439*	(Percy et al. 1998, de la Chapelle et al. 1993b)
G6003A	W439*	(Rives et al. 2007)
A6146G	N487S (Missense Mutation)	(Le Couedic et al. 1996)
C6148T	P488S (Missense Mutation)	(Kralovics et al. 1997b, Sokol et al. 1994)

Table 1: EpoR mutations in Primary Familial and Congenital Polycythemia.

Abbreviations: Ins, insertion; del, deletion; dup, duplication; *, termination codon. In the shortest deletion, P381 corresponds to C356 in the murine EpoR, and in the longest deletion, W439 corresponds to R414 in the murine EpoR.

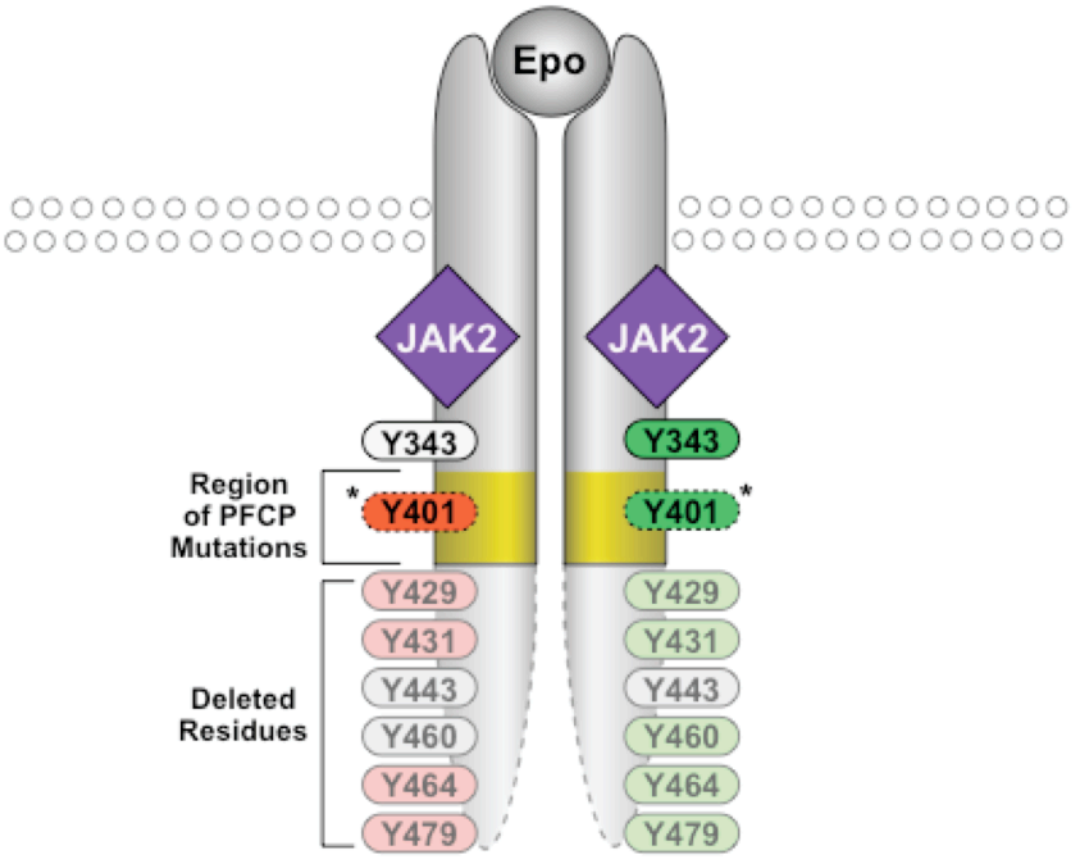


Figure 2: Truncated EpoR from PFCP patients.

EpoR from PFCP patients lack parts of the EpoR cytoplasmic domain. Y401 is retained in some but not all of the PFCP receptors.

All these mutations reside in exon VIII, which encodes the C-terminal portion of the EpoR cytoplasmic domain. Except for two missense mutations that may not be linked to the polycythemic phenotype (Sokol et al. 1994, Le Couedic et al. 1996), all other mutations result in premature termination and, consequently, these mutant receptors lack portions of the C-terminal EpoR cytoplasmic domain (Fig 2). Because these truncated EpoRs lack Tyr429 and/or Tyr401, loss of negative regulation by SHP-1, SOCS-3, CIS, or SHIP1 was assumed to be the underlying cause for PFCP. Consistent with this notion, prolonged JAK2 and

STAT5 activation and STAT5 DNA binding activity were observed in cells expressing PFCP EpoR mutants (Arcasoy, Harris and Forget 1999, Arcasoy and Karayal 2005).

A mouse model of PFCP was generated by replacing the murine EpoR with a truncated human EpoR identified in PFCP patients. To mimic the heterozygous state of truncated EpoR in patients, the murine *EpoR* gene was replaced with a wild-type and a mutant *EpoR*. These mice develop polycythemia within 3-6 weeks of age and have increased hematocrit and elevated hemoglobin concentrations, mimicking the human disorder (Divoky et al. 2001). Homozygous mice of the mutant allele are also polycythemic (Divoky et al. 2001).

1.7 DOWN-REGULATION OF EPOR SIGNALING

Recent advances in the knowledge of mechanisms underlying EpoR down-regulation lead to the hypothesis that deregulation of EpoR down-regulation may also contribute to PFCP (Fig 3). Upon Epo stimulation, cell-surface EpoR is internalized and degraded; few, if any, EpoR molecules recycle back to the cell surface (Beckman et al. 1999, Levin et al. 1998, Walrafen et al. 2005). Because cell-surface levels of EpoR control cellular Epo sensitivity (Suzuki et al. 2002), Epo-induced internalization is an efficient way to rapidly decrease Epo responsiveness. It also may bring about destruction of activated protein complexes and terminate signaling. Upon Epo stimulation, cell-surface EpoR is internalized via clathrin-mediated endocytosis, and this process requires both JAK2 kinase activity and EpoR cytoplasmic tyrosines (Sulahian et al. 2009). Phosphorylated Tyr429, Tyr431, and Tyr479 in the EpoR cytoplasmic domain share redundant functions in mediating Epo-dependent EpoR internalization via binding to the p85α or p85β subunit of PI3K (Sulahian et al. 2009).

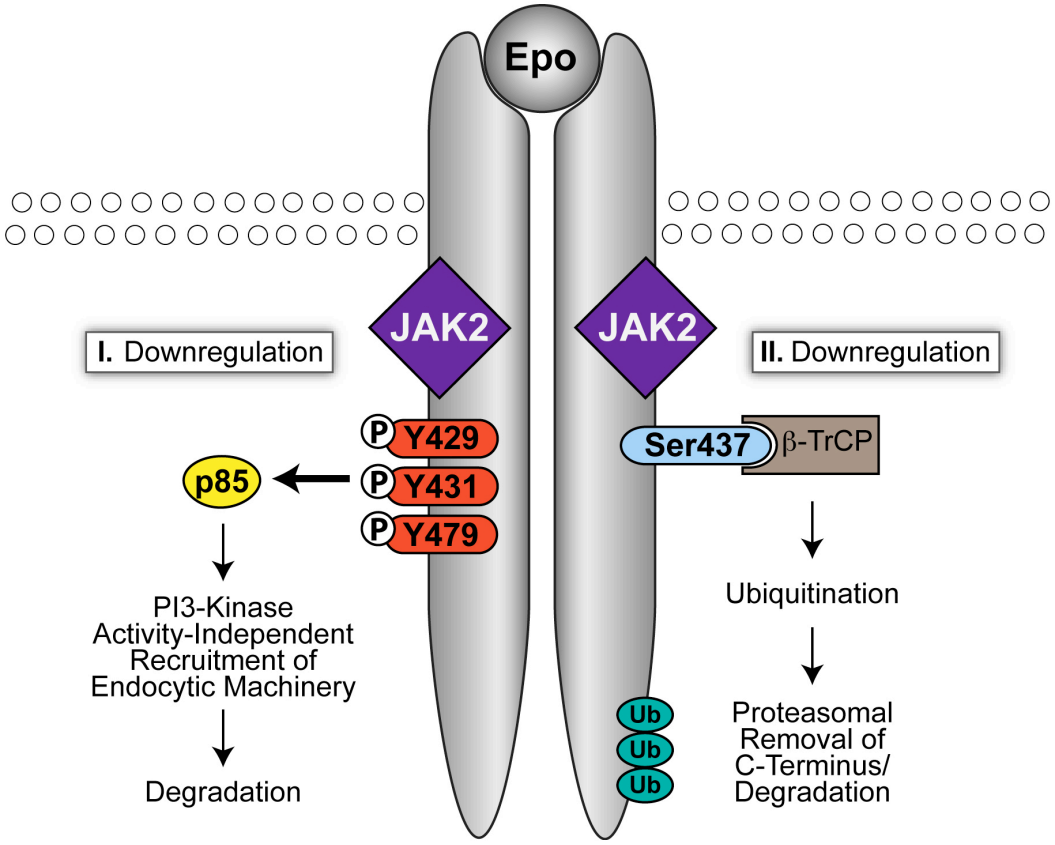


Figure 3: Epo-induced EpoR down-regulation.

Upon Epo stimulation, EpoR is down-regulated through two mechanisms, one dependent on p85 and the other on β-TrCP-mediated proteasomal degradation.

All truncated EpoR alleles from PFCP patients lack Tyr429, Tyr431, and Tyr479 (Fig 2). None of the mutants tested bound p85β and none were internalized or degraded upon Epo stimulation (Sulahian et al. 2009). Consistent with these results, erythroid progenitors expressing PFCP receptors are hypersensitive to Epo (Sulahian et al. 2009). Importantly, fusion of $KY^{429}LY^{431}L$ but not KFLFL (where Y429 and Y431 were replaced with Phe), residues encompassing Tyr429 and Tyr431, to a PFCP receptor restores both p85β binding and ligand-induced internalization and reverses Epo hypersensitivity in primary erythroid

progenitors (Sulahian et al. 2009). Therefore, failure to recruit p85 and internalize may contribute to the prolonged signaling of the truncated EpoRs in PFCP.

Besides phospho-tyrosine-based down-regulation, several lines of evidence indicate that ubiquitination also plays an important role in EpoR down-regulation. Epo binding rapidly stimulates EpoR ubiquitination at the plasma membrane and JAK2 kinase activity is required for EpoR ubiquitination (Walrafen et al. 2005). Epo-induced degradation of the EpoR is sensitive to inhibitors of both proteasomal and lysosomal function (Walrafen et al. 2005). Because an EpoR fragment containing the EpoR C-terminus was detected upon stimulation in the presence of both proteasomal and lysosomal inhibitors but not with lysosomal inhibitors alone (Walrafen et al. 2005), part of the EpoR cytoplasmic domain may be removed by the proteasome upon Epo stimulation and the remaining part of the receptor may be sorted to the lysosome for degradation (Walrafen et al. 2005). Although the exact boundary is not clear, it was proposed that this would remove the tyrosine residues and terminate tyrosine-based signaling. Later work demonstrated that β-Transducin repeat-containing protein (β-TrCP), the substrate-binding subunit of a cullin–RING ubiquitin ligase complex (Petroski and Deshaies 2005), is responsible for Epo-induced EpoR ubiquitination (Meyer et al. 2007). Mutation of Ser437 in the β-TrCP binding motif in the EpoR cytoplasmic domain abolishes inducible binding of β-TrCP to the EpoR and also blocks EpoR ubiquitination and degradation (Meyer et al. 2007). In addition, cells expressing EpoR with a Ser437 mutation are hypersensitive to Epo and show prolonged presence of phosphorylated EpoR (Meyer et al. 2007). Because PFCP EpoRs lack the β-TrCP binding

motif, these truncated receptors are not expected to recruit β-TrCP upon stimulation nor be degraded by the proteasome and later lysosome upon stimulation.

1.8 SUMMARY AND SPECIFIC AIMS OF THIS STUDY

Two pathways of Epo-induced EpoR down-regulation are known so far. One pathway depends on internalization mediated through p85 and the other utilizes ubiquitin-based proteasomal degradation. We observed an internalization mechanism at high Epo concentration that depends on JAK2 but not cytoplasmic EpoR tyrosines. We thus surmised that at low Epo concentrations, EpoR is internalized via the p85-dependent pathway and subsequently degraded in the lysosome, whereas at high Epo concentrations, EpoR is down-regulated through β-TrCP-mediated ubiquitination and degradation. This proposal is consistent with findings that endocytosis pathway of EGF receptors varies with EGF concentrations (Sigismund et al. 2005). Truncated PFCP receptors lack essential determinants for both pathways. Therefore, PFCP EpoRs not only cannot recruit negative regulators such as phosphatases to inactivate JAK2, these mutant receptors also are defective in Epo-induced receptor down-regulation. Consequently, these defects result in hypersensitivity to Epo and prolonged signaling.

Importantly, although EpoR ubiquitination has been implicated in EpoR down-regulation, the exact mechanism of how ubiquitination of the EpoR itself contributes to this regulation is missing. In addition, there could be other E3 ligases playing essential roles in the EpoR down-regulation, which have not been identified yet.

Secondly, the molecular mechanisms of p85 mediated EpoR down-regulation are not fully understood. Interestingly, although binding of p85 to the EpoR recruits and activates the

catalytic subunit of PI3K, inhibition of the PI3K activity with Wortmannin does not impair EpoR internalization (Sulahian et al. 2009). Therefore, we speculate that p85 promotes EpoR internalization via a novel PI3K kinase activity-independent mechanism, to recruit the endocytic machinery.

The **specific aims** of this study are as follows:

1) Characterize the role of ubiquitination on the EpoR itself in EpoR down-regulation.
    a. Determine the function of individual EpoR cytoplasmic lysines in EpoR internalization, degradation and signaling properties.
    b. Find the consequences of expressing an EpoR that cannot be ubiquitinated.
2) Identify the molecular mechanism of p85 mediated EpoR down-regulation.
    a. Find how p85 links EpoR to endocytic machinery, identify molecular partners and their role in EpoR signaling.

CHAPTER 2: UBIQUITINATION REGULATES THE INTERNALIZATION, ENDO-LYSOSOMAL SORTING, AND SIGNALING OF THE ERYTHROPOIETIN RECEPTOR

2.1 ABSTRACT

Ubiquitination is a common mechanism of down-regulation of mitogenic receptors. Here we show that ubiquitination of the erythropoietin receptor (EpoR) at K256 is necessary and sufficient for efficient Epo-induced receptor internalization, while ubiquitination at K428 promotes trafficking of activated receptors to the lysosomes for degradation. Interestingly, EpoR that cannot be ubiquitinated has reduced mitogenic activities and ability to stimulate the STAT5, Ras/MAPK and PI3K/AKT signaling pathways. We therefore propose that ubiquitination of the EpoR critically controls both receptor down-regulation and downstream signaling.

2.2 INTRODUCTION

Erythropoietin (Epo) is the primary cytokine controlling red blood cell production and its function is mediated through the Epo receptor (EpoR). EpoR lacks intrinsic enzymatic activities and relies on the cytosolic tyrosine kinase JAK2 for signal transduction. Epo binding to the EpoR activates JAK2, which in turn phosphorylates tyrosine residues in the EpoR cytoplasmic domain and initiates signaling cascades including the STAT5, the Ras/Mitogen Activated Protein Kinase (MAPK), and the Phosphoinositide 3-Kinase

(PI3K)/AKT pathways that ultimately result in the survival, proliferation, and differentiation of erythroid progenitor cells (Constantinescu et al. 1999, Richmond et al. 2005).

In addition, to elicit downstream signaling, Epo binding results in rapid internalization of activated EpoR and its targeting to the lysosomes for degradation (Sulahian et al. 2009, Neumann et al. 1993, Walrafen et al. 2005). Endocytosis of the EpoR critically controls the amplitude and duration of Epo signaling, as the cell-surface level of EpoR controls cellular Epo sensitivity (Suzuki et al. 2002), and endocytosis may lead to destruction of activated protein complexes to terminate signaling (Meyer et al. 2007). We previously showed that Epo-induced EpoR internalization requires both JAK2 kinase activity and EpoR cytoplasmic tyrosine residues (Sulahian et al. 2009). Specifically, Y429, Y431, and Y479 in the EpoR cytoplasmic domain, by binding to the p85 regulatory subunit of PI3K, mediate Epo-dependent EpoR internalization through a PI3K kinase activity-independent mechanism (Sulahian et al. 2009). JAK2 also stimulates ubiquitination of the EpoR, most likely through the E3 ubiquitin ligase β-TrCP (β-transducin repeat-containing protein), which contributes to receptor endocytosis and down-regulation (Walrafen et al. 2005, Meyer et al. 2007). However, the exact processes controlled by ubiquitination of the EpoR are not entirely understood.

Here we used cellular reconstitution with EpoR variants to show that ubiquitination of the EpoR itself promotes ligand-induced receptor internalization and lysosomal degradation. Of the five EpoR cytoplasmic lysine residues, ubiquitination of K256 mediates Epo-induced EpoR internalization while ubiquitination of K428 promotes Epo-induced EpoR degradation. Surprisingly, a lysineless EpoR in which all cytoplasmic lysine residues were replaced with

arginines showed reduced mitogenic activity and attenuated activation of downstream signaling pathways. Therefore, ubiquitination of the EpoR regulates both its endocytosis and signal transduction.

2.3 MATERIALS AND METHODS

Plasmid constructs, cell lines, and reagents - All EpoR mutants were generated in the pMX-IRES-GFP vector using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. JAK2 and JAK2KD (kinase deficient) were generated in the pMX-IRES-CD4 vector. γ2A cells stably expressing wild-type or mutant HA-EpoRs with JAK2 or JAK2KD were generated by infecting a γ2A cell line that stably expresses the ecotropic murine leukemia virus receptor with retroviruses encoding wild-type or mutant EpoR and JAK2 or JAK2KD. Populations of cells expressing GFP and CD4 above a predetermined level were isolated by FACS sorting (Huang et al. 2001, Tong et al. 2006). BaF3 cells stably expressing wild-type or mutant HA-EpoRs were generated similarly with retroviruses encoding wild-type or mutant EpoR followed by FACS sorting. Antibodies were from the following sources: HA, Covance; JAK2 and phospho-JAK2, Millipore; EEA1, LAMP2 and STAT5, Santa Cruz; actin, Sigma; phospho-AKT, phospho-ERK, Cell Signaling, phospho-STAT5, BD Biosciences; ubiquitin, Enzo Life Sciences. HA affinity resin was from Roche Applied Science and Flag affinity resin was from Sigma. Horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence system were from Amersham Biosciences. Lactacystin was from Boston Biochem.

Flow cytometry and data analysis - Internalization of surface EpoR was measured by flow cytometry and analyzed as described previously (Sulahian et al. 2009). For signaling analysis, BaF3 cells stably expressing various EpoRs were starved for 4 hrs in RPMI media with 1% BSA, pre-treated with 100mM cycloheximide for 60 minutes to reduce the maturing pool of receptors that could replenish the cell-surface pool of EpoR during Epo stimulation, then stimulated with different concentrations of Epo for the indicated times. For kinetics experiments, 30U/mL of Epo was used for induction. Subsequently, cells were fixed with 1.6% paraformaldehyde, permeabilized with methanol at 4°C, and stained with allophycocyanin (APC)-conjugated phospho-STAT5 (1:30), phospho-ERK (1:100), or phospho-AKT (1:100) antibodies. Fluorescence was analyzed by flow cytometry on a FACS Calibur (BD Biosciences), and median fluorescence from 20,000 cells was used for data analysis.

Immuno-precipitation and immuno-blotting - γ2A cells stably expressing wild-type or mutant EpoRs were transfected with a plasmid expressing Flag-tagged ubiquitin. 48 hours later cells were treated with 100mM cycloheximide for 1 hr to reduce the intracellular maturing pool of the EpoR. Subsequently, 20mM lactacystin or 40mM MG132 was added for 1 hr followed by Epo induction. Cells were lysed with 1% NP-40 lysis buffer with protease inhibitors and 10mM N-ethylmaleimide. Lysates were immuno-precipitated with HA or Flag affinity resins. The precipitates were eluted with SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies to HA, Flag, or ubiquitin. Bound antibodies were detected by the ECL chemiluminescence system

after incubation with horseradish peroxidase-coupled secondary antibodies. Cell lysates were also immuno-blotted with antibodies to HA, phosphorylated JAK2, JAK2, or actin.

Immunofluorescence - γ2A cells stably expressing HA-EpoR or HA-5KR were seeded on glass coverslips. Coverslips were blocked and stained with anti-HA antibodies, fixed, permeabilized, and incubated with antibodies for EEA1 as described in (Sulahian et al. 2009) except that rabbit anti-HA antibodies were used (1:125) when co-staining with LAMP2. Coverslips were incubated with LAMP2 antibodies (1:50) and the appropriate fluorescence conjugated secondary antibodies. Coverslips were mounted onto slides with a semi-permanent Mowiol mounting medium (Calbiochem). Fluorescent images were taken on a Leica TCS SP5 confocal microscope with 63X oil objective lenses with numeric aperture of 1.25N. Confocal section images were acquired by Leica acquisition software and analyzed with Image J and Adobe Photoshop.

Glycosidase digestion of EpoR and MTT cell proliferation assay - Glycosidase treatment of the various EpoR constructs with EndoH (Endoglycosidase H, New England Biolabs), or with PNGaseF (Peptide N-Glycosidase F, New England Biolabs) and neuraminidase (New England Biolabs), and MTT cell proliferation assay were performed as described (Tong et al. 2006).

2.4 RESULTS

2.4.1 Ubiquitination of the EpoR promotes ligand-induced internalization and endocytosis –

To characterize the role of ubiquitinated EpoR in ligand-induced EpoR endocytosis and down-regulation, we engineered a mutant EpoR unable to be ubiquitinated, 5KR, in

which all five cytoplasmic lysines of the receptor are replaced with arginines. We examined EpoR ubiquitination in γ2A cells stably co-expressing HA-EpoR and JAK2, HA-EpoR and kinase deficient JAK2 (JAK2KD), or HA-5KR and JAK2. These cells also transiently express Flag-tagged ubiquitin. After pre-treatment with 20μM lactacystin to inhibit proteasomal activities, cells were induced with Epo, and cell lysates were immunoblotted with anti-HA antibodies to detect HA-EpoR (Fig. 4A) or immunoprecipitated with anti-HA antibodies prior to anti-ubiquitin and anti-Flag immunoblotting (Fig. 4B). EpoR was ubiquitinated in the presence of JAK2 but not in JAK2KD. Based on the apparent molecular weight that exceeds mono-ubiquitinated and multi-ubiquitinated (mono-ubiquitinated at multiple sites) EpoR, the EpoR is most likely poly-ubiquitinated. These results do not preclude the presence of low-level mono- or multi-ubiquitinated EpoRs at abundance below our detection limit. Our observation is consistent with previous reports showing that EpoR is poly-ubiquitinated upon Epo stimulation in a JAK2 kinase activity-dependent manner (Walrafen et al. 2005, Yen et al. 2000). In contrast, 5KR promptly activated JAK2 in response to Epo but did not become ubiquitinated (Fig. 4A,B). Similar results were obtained in the presence of another proteasomal inhibitor MG132 (Data not shown).

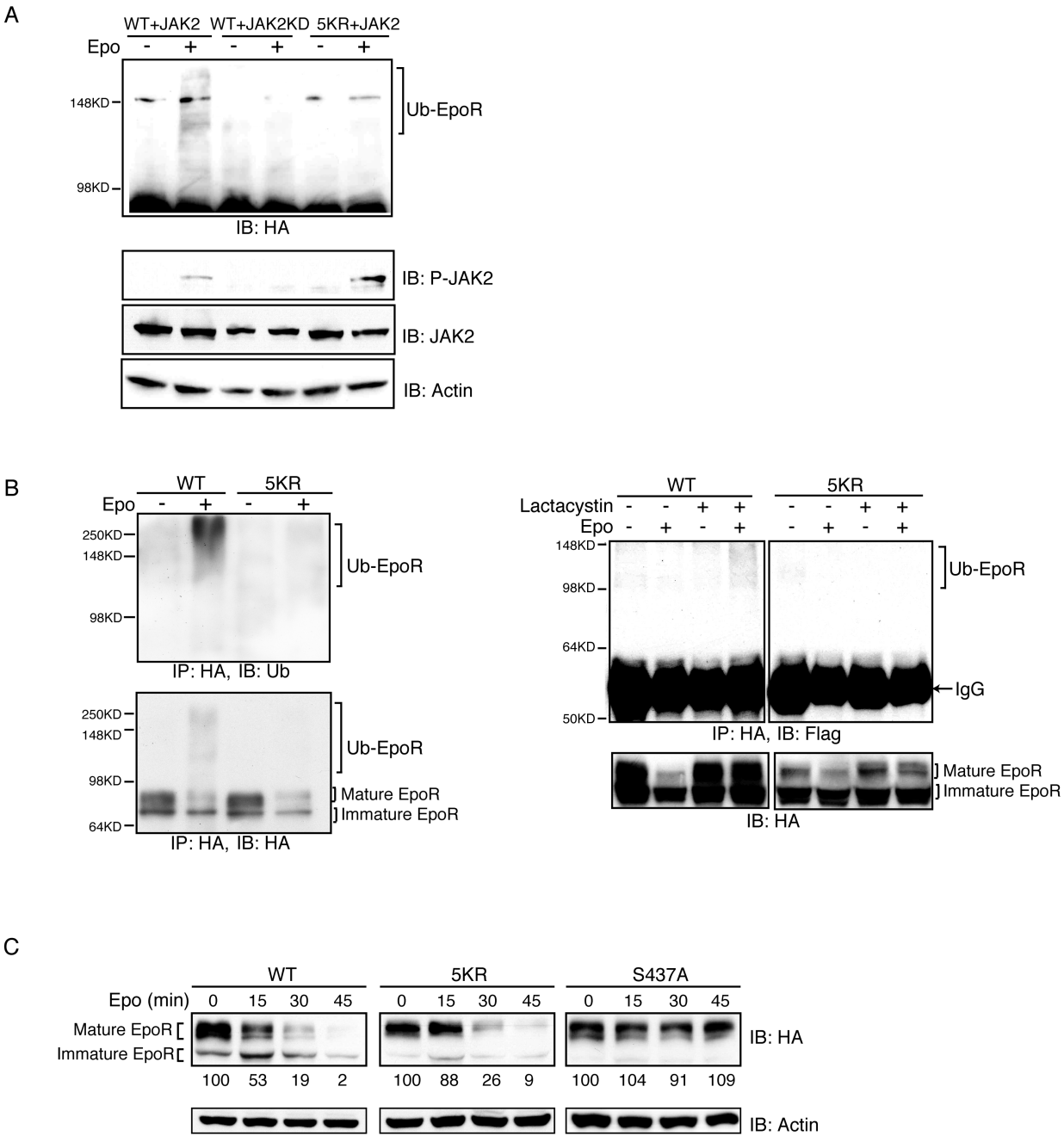


Figure 4: Ubiquitination of the EpoR requires JAK2 kinase activity and promotes ligand-induced EpoR down-regulation.

(A, B) Upon Epo stimulation, ubiquitinated EpoR species were detected in cells stably co-expressing HA-EpoR with wild-type JAK2 but not with kinase-deficient JAK2 (JAK2KD) (A). In cells stably

co-expressing HA-5KR with JAK2, Epo induced JAK2 activation but did not induce ubiquitination of the EpoR, as determined by anti-HA immunoblotting (A) and by anti-ubiquitin and anti-Flag immunoblotting of anti-HA immunoprecipitates (B). (C) Upon Epo induction, mature HA-5KR and HA-S437A receptors exhibited defective degradation compared to wild-type HA-EpoR. Numbers below the HA immunoblots indicate quantified intensity of the mature EpoR bands normalized to actin by Image J. WT: wild-type EpoR. IB: immuno-blot. IP: immuno-precipitation. P-JAK2: phosphorylated active JAK2.

To determine the effect of EpoR ubiquitination on receptor internalization, we used flow cytometry to determine the internalization kinetics. In these assays, cell surface HA-tagged receptors were detected by staining non-permeabilized cells with anti-HA antibodies followed by allophycocyanin (APC)-conjugated secondary antibodies. Because HA-EpoR and HA-5KR were expressed in a bicistronic vector that also expressed GFP, GFP fluorescence in each cell is proportional to the total amount of the receptors present whereas APC fluorescence indicated cell-surface expression. To quantify the amounts of receptor at the cell surface, the median APC fluorescence intensity was used. HA-EpoR and HA-5KR were expressed at similar levels (as judged by median GFP fluorescence, data not shown) and their cell surface EpoR expression levels were similar (judged by median APC fluorescence pre-stimulation, Fig. 5A). Epo promoted rapid internalization of HA-EpoR but not HA-5KR. (Fig. 5A) Attenuated internalization of HA-5KR compared to HA-EpoR was also observed in the hematopoietic cell line BaF3 (Fig. 5B). Pretreatment with monensin, a well-known inhibitor of receptor recycling (Wang et al. 2002, Varghese et al. 2008), had no effect on the internalization of HA-5KR (data not shown), indicating that its higher levels of surface expression was not due to increased recycling. Therefore, EpoR cytoplasmic lysines are important for ligand-induced EpoR internalization.

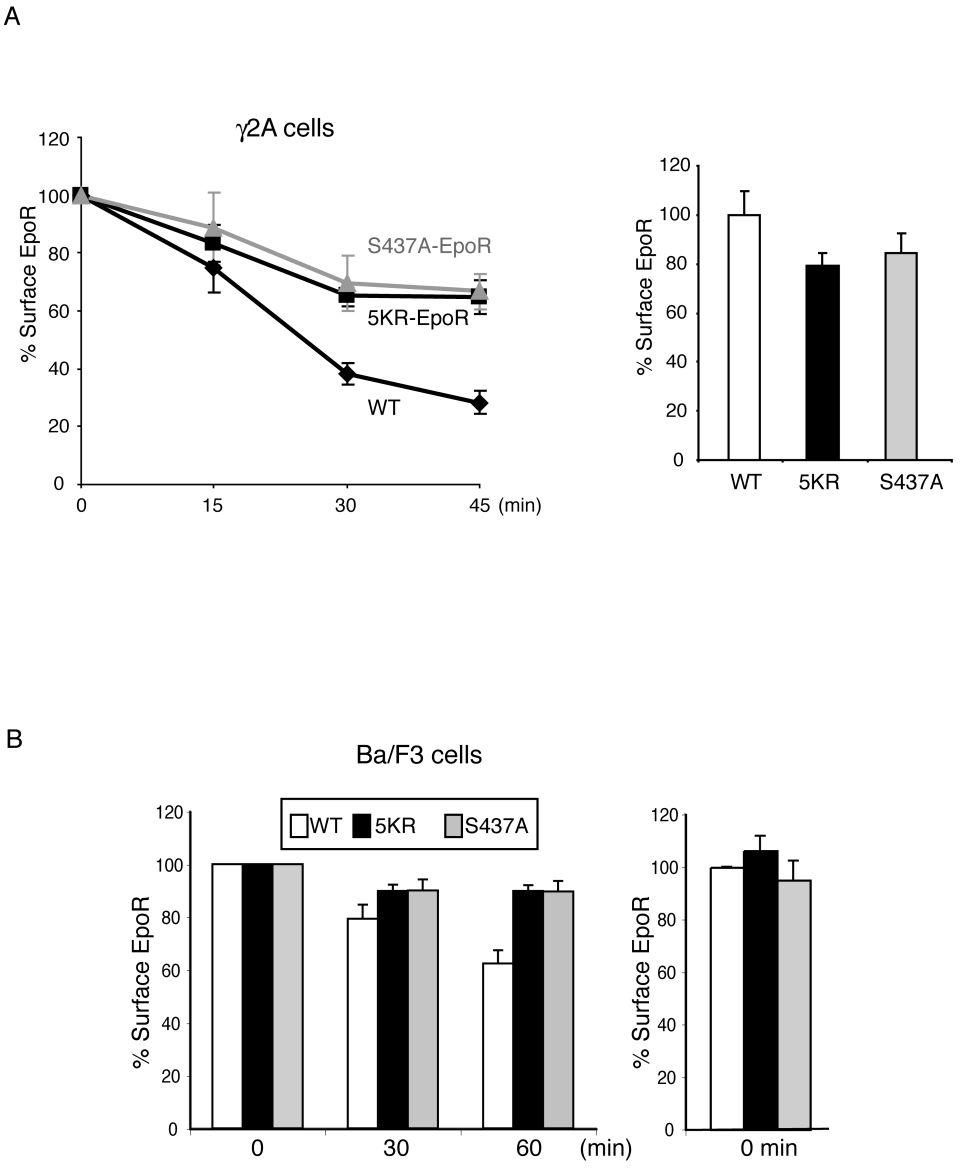


Figure 5: EpoR cytoplasmic lysines are important for Epo-induced receptor internalization.

Levels of cell-surface receptors at indicated time points after Epo induction were quantified by flow cytometry using APC-conjugated anti-HA antibodies in non-permeabilized γ2A. (A) and BaF3 (B) cells. Median APC fluorescence was used to quantify cell surface expression of receptors, and results are normalized to that of the un-induced sample for each receptor. Relative levels of cell-surface 5KR and S437A in un-induced cells normalized to wild-type EpoR are shown on the right.

The fate of cell-surface EpoR and 5KR upon Epo induction was investigated by confocal immunofluorescence microscopy. Non-permeabilized cells were labeled with anti-HA antibodies prior to Epo induction. Cells were stimulated with Epo, fixed, permeabilized and visualized with fluorescence-conjugated secondary antibodies. This approach thus followed the fate of only surface-tagged receptors prior to Epo treatment. HA-EpoR moved from the plasma membrane to an internal compartment that co-localized with the early endosomal marker EEA1 (early endosome antigen 1) within 25 min after Epo treatment (Fig. 6). At 33 min post Epo stimulation, HA-EpoR staining partially co-localized with the lysosomal-associated membrane protein 2 (LAMP2), a lysosomal marker, indicating that the EpoR had reached the lysosomes (Fig. 7). Consistent with previous results that Epo-induced internalization of HA-5KR receptors was impaired, a substantial portion of HA-5KR receptors were not internalized and remained on the surface (Fig. 6,7). The fraction of HA-5KR that was internalized co-localized with EEA1 and LAMP2 (Fig. 6,7). Consistent with the defect in internalization, ligand-induced degradation of HA-5KR was impaired compared to HA-EpoR (Fig. 4C).

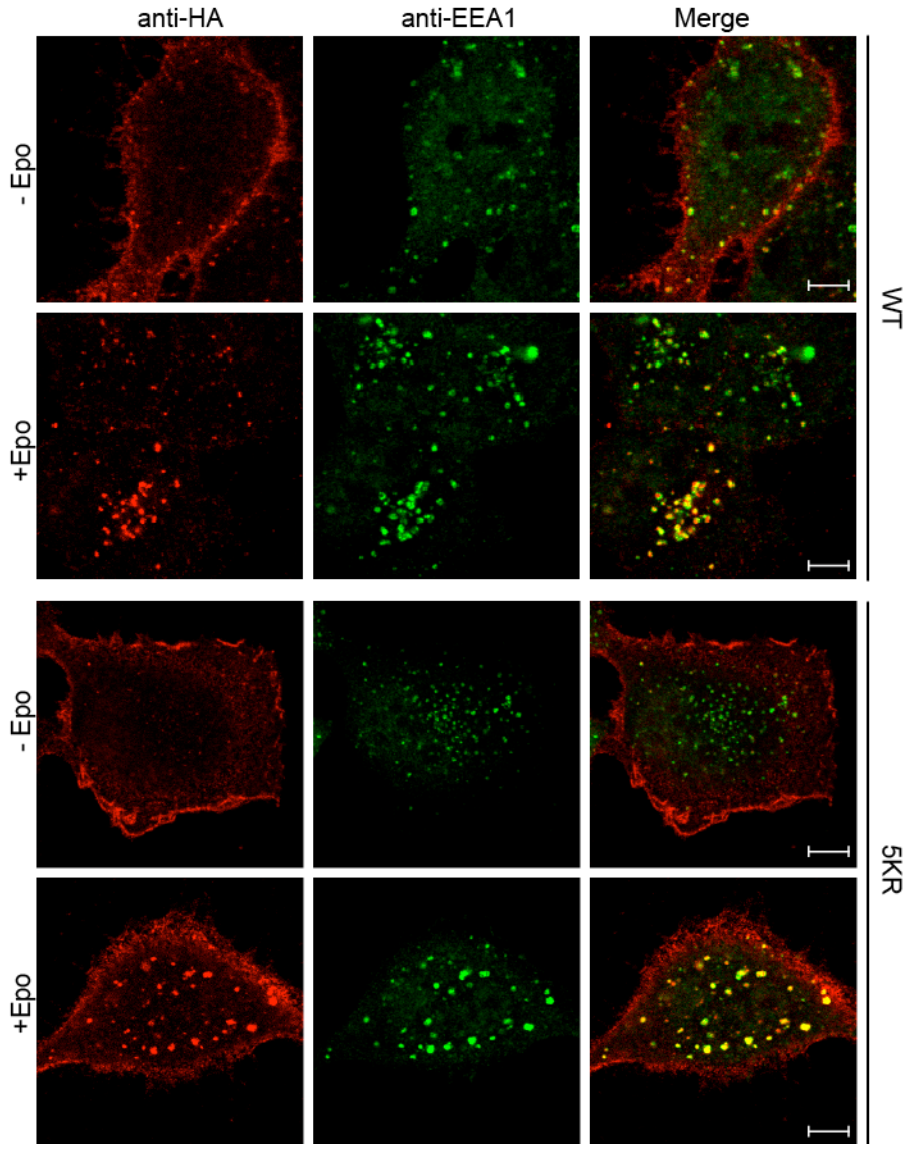


Figure 6: 5KR is defective in sorting to early endosomes.

HA-EpoR or HA-5KR receptors were labeled with anti-HA antibodies prior to Epo stimulation for 25 min. Cells were fixed and immuno-stained with anti-EEA1 antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocal images (single section) are presented. Original magnification 63X (Leica TCS SP5). Scale bars: 5µm.

The E3 ubiquitin ligase β-TrCP has been implicated in EpoR ubiquitination, and mutation of the predicted β-TrCP recognition motif around S462 in the human EpoR abolishes β-TrCP binding and blocks EpoR ubiquitination (Meyer et al. 2007). We therefore

examined the internalization and down-regulation of the corresponding mutant murine EpoR, S437A. Epo-induced internalization of HA-S437A was less efficient, and its degradation was also defective (Fig. 5 and 4C). Together, these results suggest that EpoR ubiquitination plays an important role in ligand-induced EpoR internalization and down-regulation.

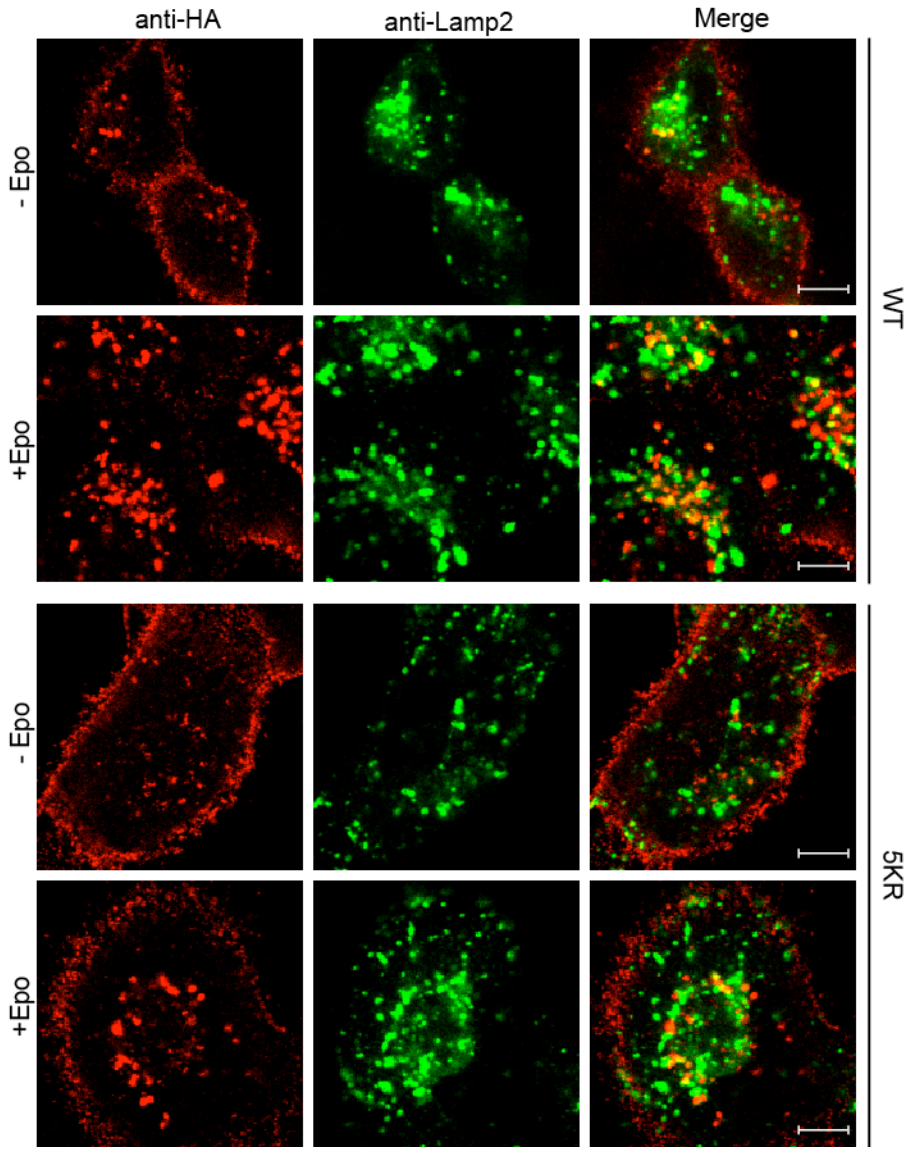


Figure 7: 5KR is defective in sorting to lysosomes.

HA-EpoR or HA-5KR receptors were labeled with anti-HA antibodies prior to Epo stimulation for 33 min. Cells were fixed and immuno-stained with anti-LAMP2 antibodies followed by appropriate

fluorescence-conjugated secondary antibodies. Representative confocal images (single section) are presented. Original magnification 63X (Leica TCS SP5). Scale bars: 5μm.

To examine if ubiquitination may be sufficient to target the EpoR to the endosomes, we fused to the C-terminus of the HA-EpoR either ubiquitin or monoUb, the K48R/K63R ubiquitin variant which cannot support poly-ubiquitination, and examined the localization. We also examined EpoR fused to ubiquitin with K48R or K63R mutations. EpoR fused with ubiquitin (EpoR-Ub), ubiquitin with K48R mutation (EpoR-UbK48R), ubiquitin with K63R mutation (EpoR-UbK63R) or ubiquitin with K48R/K63R mutations (EpoR-monoUb) all had few mature receptors expressed on the cell surface even without stimulation, in contrast to wild-type EpoR (Fig. 8), indicating improper processing. Therefore, whether ubiquitination of the EpoR is sufficient to trigger internalization is not clear.

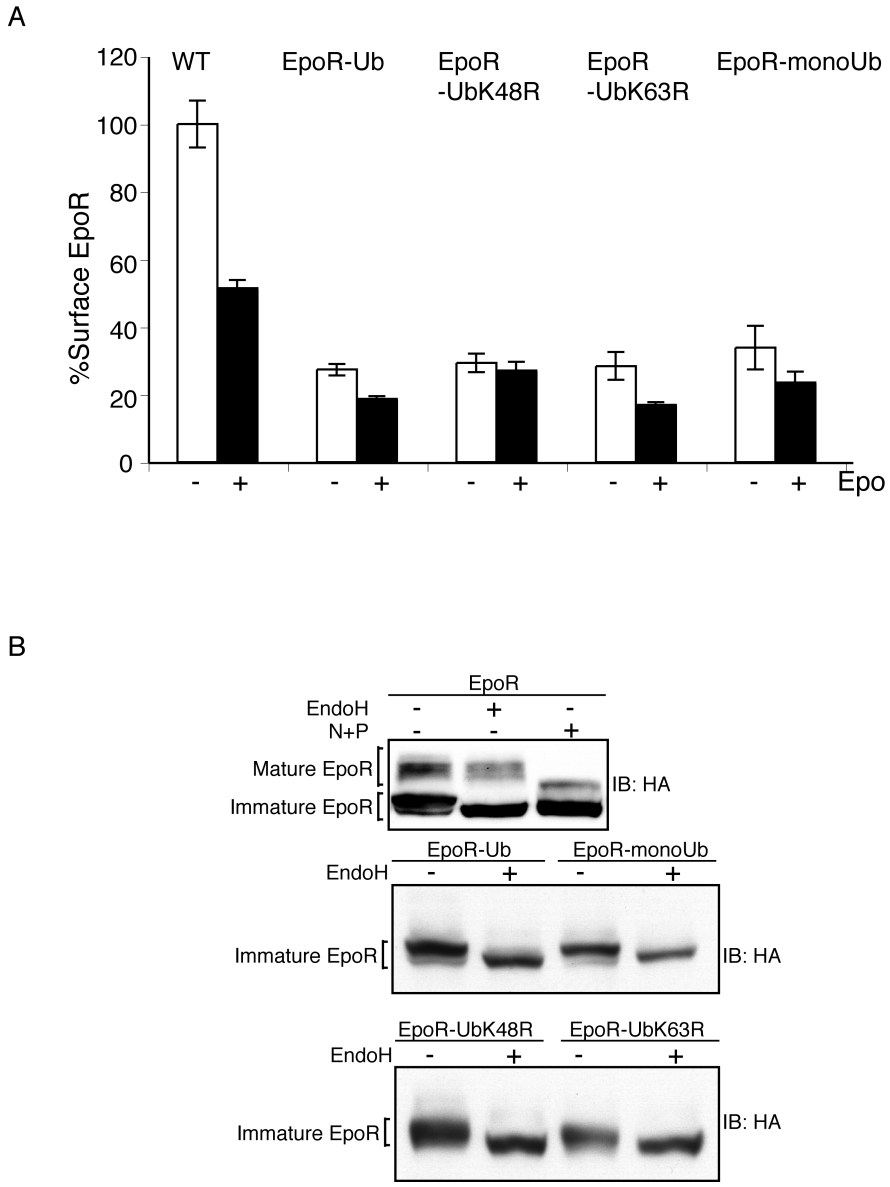


Figure 8: Fusion of Ub, K48R-Ub, K63R-Ub or monoUb to the EpoR results in receptors that are not processed properly.

(A) In contrast to wild-type EpoR, EpoR-Ub, EpoR-UbK48R, EpoR-UbK63R, and EpoR-monoUb are not properly expressed on the cell surface with or without stimulation in γ2A cells. (B) Mature receptor species (defined by resistance to Endo H treatment) were not detected for EpoR-Ub,

EpoR-UbK48R, EpoR-UbK63R, or EpoR-monoUb. EndoH: endoglycosidase H. N+P: neuraminidase and PNGaseF.

2.4.2 Ubiquitination of K256 mediates Epo-induced EpoR internalization whereas ubiquitination of K428 regulates EpoR degradation –

In order to determine which of the five lysine residues are responsible for EpoR internalization, ubiquitination, and down-regulation, we generated cells that express EpoR variants in which individual lysine was replaced with arginine. These mutated receptors were examined for Epo-induced internalization and degradation. As shown in Figure 9A, K256R resulted in defective internalization similar to 5KR, whereas K276R, K348R, K388R, or K428R had little effect. Similar results were also observed in BaF3 cells (data not shown). In addition, restoring K256 on the lysineless background (R4K256) rescued receptor internalization (Fig. 9B). Moreover, R4K256 restored EpoR poly-ubiquitination upon stimulation (Fig. 9C). Therefore, ubiquitination of K256 is both necessary and sufficient for ubiquitin-based Epo-induced EpoR internalization. We also examined mutant EpoRs with single lysine replacement in Epo-induced down-regulation.

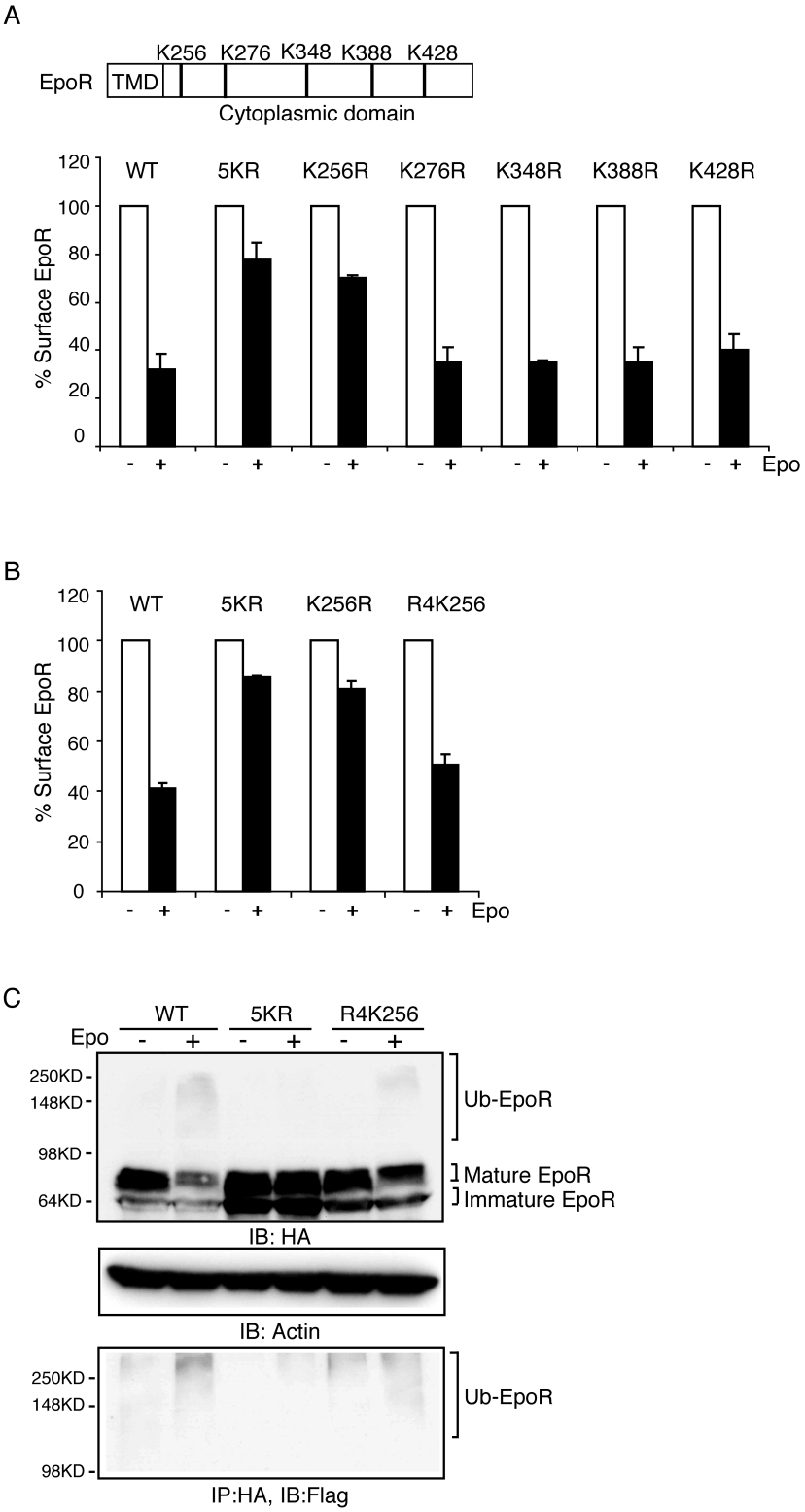
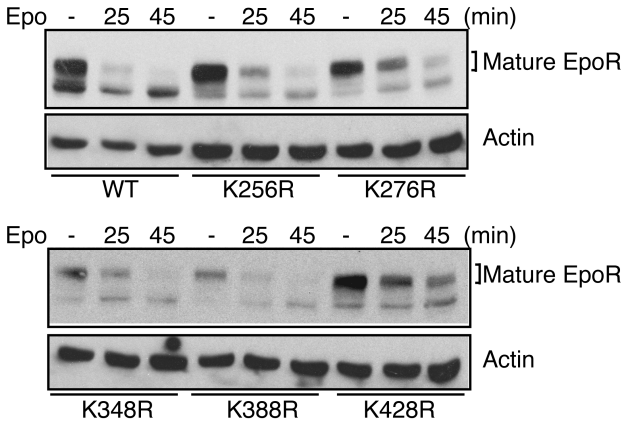

A
K256 K276 K348 K388 K428
EpoR
TMD
Cytoplasmic domain
WT
5KR
K256R
K276R
K348R
K388R
K428R
% Surface EpoR
120
100
80
60
40
20
0
- + - + - + - + - + - + - +
Epo
B
WT
5KR
K256R
R4K256
% Surface EpoR
120
100
80
60
40
20
0
- + - + - + - +
Epo
C
WT
5KR
R4K256
Epo - + - + - +
250KD
148KD
98KD
64KD
Ub-EpoR
Mature EpoR
Immature EpoR
IB: HA
IB: Actin
250KD
148KD
98KD
Ub-EpoR
IP:HA, IB:Flag

Figure 9: Ubiquitination of K256 promotes Epo-induced EpoR internalization.

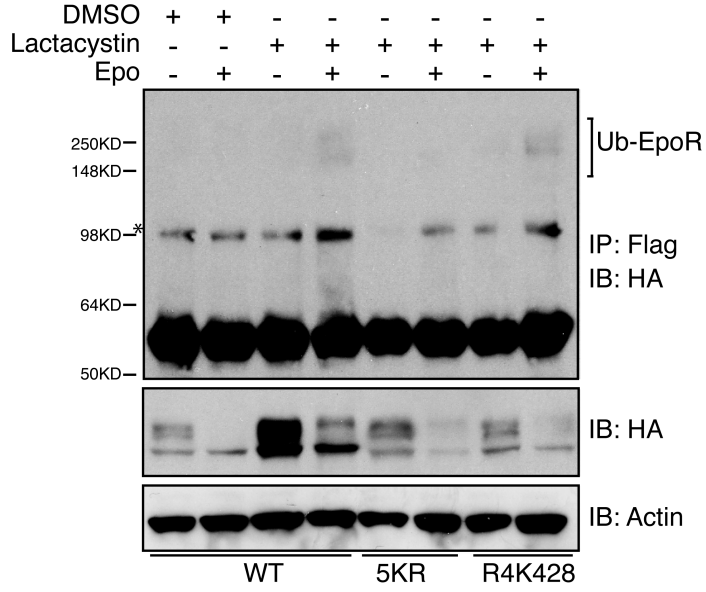
(A) K256R exhibited impaired internalization upon Epo stimulation. Receptor internalization was detected by flow cytometry in γ2A cells stably expressing JAK2 and either wild-type or mutant EpoRs. Schematic diagram of the EpoR cytoplasmic lysines are shown on top. TMD: transmembrane domain. (B) Add-back of K256 on lysineless EpoR (R4K256) rescues efficient receptor internalization in γ2A cells. (C) R4K256 but not 5KR becomes ubiquitinated upon Epo stimulation.

As shown in Figure 10A, only K428R, but not mutations of other lysine residues, resulted in a dramatic decrease in degradation upon stimulation. Consistent with this observation, the portion of internalized K428R receptors that co-localized with LAMP2 was reduced compared to wild-type EpoR (44% vs. 65%). Therefore, although K428 internalizes normally, internalized receptors are not efficiently sorted to the lysosome for degradation. Furthermore, restoration of K428 on 5KR (R4K428) rescued receptor poly-ubiquitination (Fig. 10B) and restored normal Epo-dependent receptor degradation (Fig. 10C). In summary, these results suggested that upon Epo stimulation, ubiquitination at K256 promotes receptor internalization, while ubiquitination at K428 promotes endo-lysosomal sorting and degradation.

A


Epo - 25 45 - 25 45 - 25 45 (min)
Mature EpoR
Actin
WT
K256R
K276R
Epo - 25 45 - 25 45 - 25 45 (min)
Mature EpoR
Actin
K348R
K388R
K428R

B


DMSO + + - - - - - -
Lactacystin - - + + + + + +
Epo - + - + - + - +
250KD
148KD
98KD
64KD
50KD
Ub-EpoR
IP: Flag
IB: HA
IB: HA
IB: Actin
WT
5KR
R4K428

C

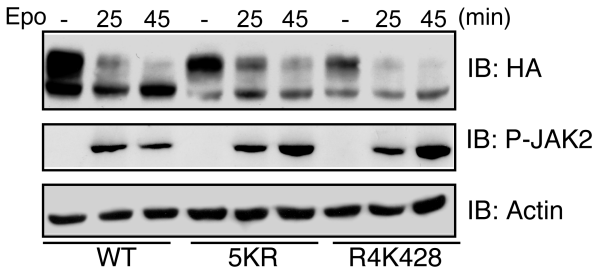

Epo - 25 45 - 25 45 - 25 45 (min)
IB: HA
IB: P-JAK2
IB: Actin
WT
5KR
R4K428

Figure 10: Ubiquitination of K428 promotes Epo-induced EpoR degradation.

(A) K428R is defective in receptor degradation upon stimulation in γ2A cells. (B) Add-back of K428 on lysineless EpoR (R4K428) but not 5KR becomes ubiquitinated upon Epo stimulation. * indicates a non-specific band. (C) Add-back of K428 on lysineless EpoR (R4K428) rescues Epo-induced receptor degradation.

2.4.3 EpoR mutant that cannot be ubiquitinated has dysregulated signaling –

To assess the consequences of defective EpoR ubiquitination on signaling, we determined the mitogenic activities of wild-type EpoR or 5KR in the IL-3 dependent hematopoietic BaF3 cells. BaF3 cells stably expressing the different EpoRs at similar levels were grown in the absence of IL-3 but with various concentrations of Epo, and their mitogenic activity was measured. As shown in Figure 11A, cells expressing 5KR grew normally in Epo above 1 U/mL. However, at lower Epo concentrations, the mitogenic activity of cells expressing 5KR was impaired. As controls, cells expressing all constructs grew similarly in IL-3 conditioned media (WEHI, data not shown). Therefore, defective ubiquitination of the EpoR is associated with reduced mitogenic activities, despite normal JAK2 activation.

The three main signaling pathways stimulated by Epo are the STAT5, Ras/MAPK and PI3K/AKT pathways. To determine the mechanism underlying the mitogenic activity defect of 5KR, we examined the activation kinetics of these pathways using flow cytometry and fluorescence-conjugated antibodies specific to the active forms of STAT5, ERK, and AKT. Activation of all three proteins was maximal at 20 min after induction with saturating Epo concentrations (30U/mL) (Fig. 11B). We thus examined activation of STAT5, ERK, and AKT by wild-type EpoR or 5KR at 20 min post induction with various Epo concentrations.

Consistent with the reduced mitogenic activity, 5KR showed Epo hyposensitivity in activating all three pathways compared to wild-type EpoR (Fig. 11C). Because endosomes have been implicated in amplifying signaling, we hypothesized that impaired sorting of activated 5KR receptors to endosomes may be responsible for the reduced signaling through the STAT5, ERK, or AKT pathways. In line with this hypothesis, K256R, defective in internalization, also exhibited Epo hyposensitivity in activating ERK and AKT as well as reduced mitogenic activities as compared to wild-type EpoR (Fig. 11D,E).

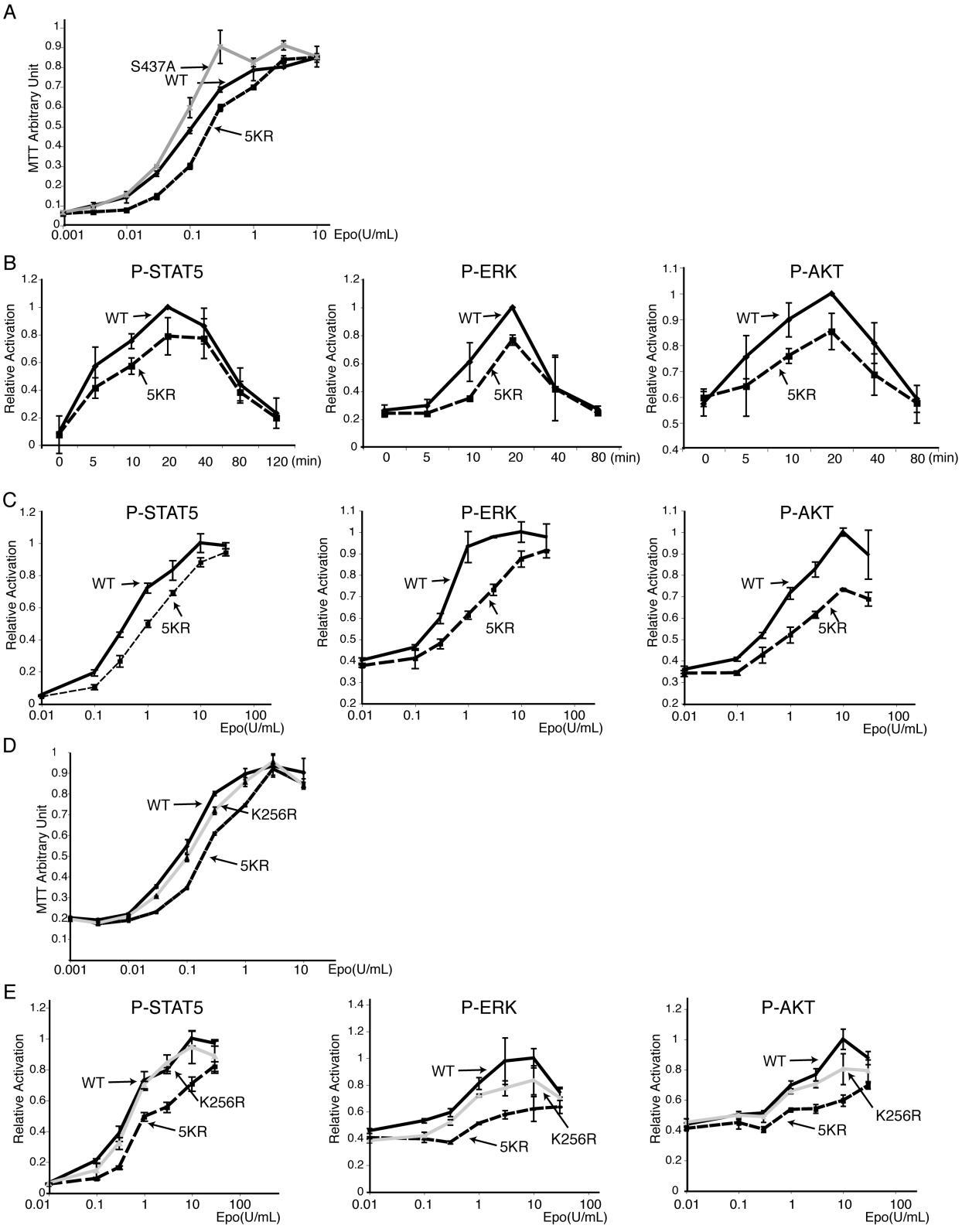

A
MTT Arbitrary Unit
S437A
WT
5KR
Epo(U/mL)
B
P-STAT5
P-ERK
P-AKT
Relative Activation
WT
5KR
0 5 10 20 40 80 120 (min)
0 5 10 20 40 80 (min)
C
P-STAT5
P-ERK
P-AKT
Relative Activation
WT
5KR
Epo(U/mL)
D
MTT Arbitrary Unit
WT
K256R
5KR
Epo(U/mL)
E
P-STAT5
P-ERK
P-AKT
Relative Activation
WT
K256R
5KR
Epo(U/mL)

Figure 11: 5KR-expressing cells are hyposensitive to Epo and defective in activating three major downstream pathways.

(A) BaF3 cells stably expressing HA-5KR are less sensitive to Epo than those expressing wild-type EpoR. In contrast, cells expressing HA-S437A are hypersensitive to Epo. Mitogenic activities were measured by MTT assays after 48 hrs. Data for each construct are normalized to growth in IL-3 conditioned media. (B) BaF3 cells stably expressing 5KR activated STAT5, MAPK, and AKT with similar kinetics compared to wild-type EpoR. Activation was assessed with 30U/mL Epo over time using flow cytometry with APC-conjugated antibodies to phospho-STAT5, phospho-ERK, and phospho-AKT, respectively. Median APC fluorescence was used for analysis. Results were normalized to the maximal activation of wild-type EpoR. Data represent three independent experiments. (C) BaF3 cells stably expressing 5KR are defective in activating STAT5, MAPK, and AKT compared to wild-type EpoR. Activation of STAT5, MAPK, and AKT was assessed at indicated Epo concentrations at 20 min post induction by flow cytometry as described in (B). (D) BaF3 cells stably expressing K256R have reduced mitogenic activities compared to cells expressing wild-type EpoR. (E) Activation of MAPK and AKT is reduced in cells expressing K256R compared to wild-type EpoR.

Meyer et al. showed that a mutant human EpoR lacking the β-TrCP binding site was hypersensitive to Epo (Meyer et al. 2007). Consistent with their results, we also observed a small but reproducible degree of Epo hypersensitivity for the corresponding mutant murine EpoR, S437A, which contrasts to the hyposensitivity of the 5KR receptor (Fig. 11A). One possibility is that one or more signaling proteins in the activated EpoR complex other than the EpoR itself may be substrates of β-TrCP and their ubiquitination negatively regulates EpoR signaling. In summary, in addition to receptor internalization and trafficking, ubiquitination of the EpoR plays an important role in controlling downstream signaling.

2.5 CONCLUSIONS

Here we show that ubiquitination of the EpoR is required for efficient internalization and lysosomal degradation. Two lysine residues, K256 and K428, in the EpoR cytoplasmic domain, both poly-ubiquitinated upon Epo stimulation, contribute to distinct steps in the endocytosis process. Specifically, K256 mediates Epo-induced receptor internalization, while

K428 is not required for receptor internalization but regulates endo-lysosomal sorting and receptor degradation. Surprisingly, an EpoR lacking cytoplasmic lysines (5KR) exhibits attenuated activation of the STAT5, Ras/MAPK and PI3K/AKT pathways and has reduced mitogenic activity. Therefore, ubiquitination of the EpoR critically controls both receptor endocytosis and downstream signaling.

CHAPTER 3: CBL UBIQUITINATION OF P85 IS ESSENTIAL FOR EPO-INDUCED EPOR ENDOCYTOSIS

3.1 ABSTRACT

Epo binding to the EpoR elicits downstream signaling that is essential for red blood cell production. One important negative regulatory mechanism to terminate Epo signaling is Epo-induced EpoR endocytosis and degradation. Defects in this mechanism play a key role in the overproduction of erythrocytes in primary familial and congenital polycythemia (PFCP). Here we have identified a novel mechanism mediating Epo-dependent EpoR internalization. Epo induces Cbl-dependent ubiquitination of the p85 regulatory subunit of PI3K, which binds to phosphotyrosines on EpoR. Ubiquitination allows p85 to interact with the endocytic protein epsin-1, thereby driving EpoR endocytosis. Knockdown of Cbl, expression of its dominant negative forms, or expression of an epsin-1 mutant devoid of ubiquitin-interacting motifs all compromise Epo-induced EpoR internalization. Mutated EpoRs mimicking those from PFCP patients cannot bind p85, co-localize with epsin-1, nor internalize upon Epo stimulation and exhibit Epo hypersensitivity. Similarly, knockdown of Cbl also causes Epo hypersensitivity in primary erythroid progenitors. Restoring p85 binding to PFCP receptors rescues Epo-induced epsin-1 co-localization, EpoR internalization, and normalizes Epo hypersensitivity. Our results uncover a novel Cbl/p85/epsin-1 pathway in EpoR endocytosis and show that defects in this pathway contribute to excessive Epo signaling and erythroid hyperproliferation in PFCP.

3.2 INTRODUCTION

In order to maintain physiologic numbers of circulating red blood cells EpoR signaling is also subject to negative regulation, and the balance between positive and negative regulation allows the system to maintain homeostasis. Mutations in EpoR or JAK2 that abrogate negative regulation cause erythrocytosis in hematological malignancies. (Huang, Shen and Bulut 2010, Levine 2012)

A key element in negative regulation of Epo signaling is Epo-dependent endocytosis and degradation of the EpoR. (Sulahian et al. 2009, Walrafen et al. 2005) This process also controls cellular Epo sensitivity and clears Epo from the circulation. (Suzuki et al. 2002, Gross and Lodish 2006) We previously showed that the p85 regulatory subunit of PI3K, by binding to three EpoR cytoplasmic tyrosine residues (Y^{429}, Y^{431} and Y^{479}) phosphorylated by JAK2, is an essential mediator of Epo-induced EpoR internalization via a PI3K kinase activity-independent mechanism. (Sulahian et al. 2009) This mechanism is physiologically relevant because truncated EpoRs from patients with primary familial and congenital polycythemia (PFCP) lack the three key tyrosines, do not bind p85, and cannot be internalized upon Epo stimulation. (Sulahian et al. 2009, Huang et al. 2010) PFCP erythroid progenitors are hypersensitive to Epo. (Skoda and Prchal 2005) Appending the pentapeptide $KY^{429}LY^{431}L$ that includes p85-binding sites to a PFCP EpoR rescues receptor internalization, down-regulation and normalizes signaling in erythroid progenitor cells. (Sulahian et al. 2009) Importantly, the molecular mechanism of how p85 mediates EpoR internalization is not understood.

Ubiquitination plays an important role in the endocytosis of many surface receptors. (Haglund and Dikic 2005, Pickart 2001, Thien and Langdon 2005) Ubiquitination involves

the covalent attachment of the protein ubiquitin (Ub) to lysine residues of target proteins. In general, ubiquitination is controlled by a three-step reaction initialized by E1-mediated Ub activation, followed by conjugation to an E2 enzyme and finally substrate targeting by an E3 ligase. The attachment of one Ub molecule to target proteins, mono-ubiquitination, mediates protein trafficking and intracellular signaling. (Sigismund, Polo and Di Fiore 2004) Additional Ub molecules can also be added to lysine residues in the previously attached Ub, forming Ub chains. Poly-ubiquitination can signal proteins for degradation. (Welchman, Gordon and Mayer 2005) Ubiquitinated proteins interact with other proteins containing ubiquitin-binding domains such as ubiquitin interacting motif (UIM) to form complex signaling networks. (Husnjak and Dikic 2012)

Cbl (Casitas B-lineage lymphoma, also known as c-Cbl) is an E3 ligase that plays a crucial role in endocytic down-regulation of receptor tyrosine kinases. (Kales et al. 2010, Swaminathan and Tsygankov 2006) Cbl contains an N-terminal tyrosine kinase-binding (TKB) domain, followed by a linker and a RING finger domain. The linker critically regulates Cbl ligase activity, (Kales, Ryan and Lipkowitz 2012) and mutations in the linker such as deletion of Y^{368} (ΔY368) or Y^{371} (ΔY371) abolish Cbl ligase activity and result in cell transformation. (Thien, Walker and Langdon 2001) Cbl was originally identified as the cellular counterpart of v-Cbl that causes myeloid leukemia and lymphomas in mice, (Langdon et al. 1989) and Cbl mutations are recently implicated in human myeloid leukemia and neoplasms. (Kales et al. 2010) It was also noted that Cbl-/- mice exhibit erythroid hyperplasia and extramedullary hematopoiesis. (Naramura et al. 1998, Murphy et al. 1998) A functional linkage between EpoR and Cbl is suggested by the ability of Epo to induce

phosphorylation of Cbl. (Barber et al. 1997b, Odai et al. 1995) The contribution of Cbl to erythropoiesis is not known.

Epsin-1 is an endocytic adaptor protein that promotes clathrin-mediated endocytosis. Through its N-terminal ENTH (Epsin N-terminal homology) domain, it binds to PtdIns(4,5)P2 and induces membrane curvature. (Horvath et al. 2007) Importantly, epsin-1 contains binding sites for clathrin and AP-2, as well as three UIMs to bind ubiquitinated cargos. (Sen et al. 2012, Wendland 2002) Therefore, epsin-1 links ubiquitinated cargos to the endocytic machinery.

In this chapter, we describe the molecular mechanism underlying p85-mediated EpoR internalization upon Epo induction. We show that Epo stimulates ubiquitination of p85 via Cbl, and that ubiquitinated p85 recruits epsin-1, thereby inducing EpoR internalization and down-regulation. We also show that mutated EpoRs mimicking those from PFCP patients fail to activate this pathway. These studies identify a novel molecular mechanism that drives Epo-induced EpoR endocytosis.

3.3 MATERIALS AND METHODS

Plasmid constructs, cell lines, and reagents - γ2A and BaF3 cells stably expressing wild-type or mutant HA-EpoRs were generated as previously described. (Tong et al. 2006) p85α and p85β function redundantly to mediate Epo-induced EpoR internalization, (Sulahian et al. 2009) so we focused on characterizing p85β in this study and will refer to p85β as p85 henceforth. C-terminal biotinylation tagged p85 constructs were engineered in the pEBB-TB vector. (Maine et al. 2010) p85 fragments were generated by site directed mutagenesis or conventional PCR and subcloning. Cbl and its mutants in the pSRα vector were kind gifts of

Dr. Wallace Langdon. Cbl-/- mouse embryonic fibroblasts (MEFs) were kind gifts of Dr. Brian Druker. Epsin-1 and epsin-1(ΔUIM) tagged with enhanced cyan fluorescent protein (ECFP) were from Addgene. Recombinant Flag-Ub and Ub(KR) were from Boston Biochem. Antibodies were from the following sources: mouse anti-HA, Covance; JAK2, phospho-JAK2, phospho-tyrosine antibody 4G10, Millipore; actin and Flag, Sigma; streptavidin agarose, Thermo Scientific; streptavidin-HRP, Biolegend; Cbl, BD Biosciences; rabbit anti-HA, pY^{731} Cbl, Alexa Fluor 488 conjugated anti-myc and GAPDH; Cell Signaling; p85β and epsin-1, Santa Cruz; anti-ubiquitinated proteins antibody FK2-conjugated beads, MBL; T7 tag, Novagen. Anti-GFP antibody that also recognizes CFP was a gift from Dr. Joachim Seemann. Horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence system were from Amersham Biosciences.

Flow cytometry and data analysis - Internalization of surface EpoR was measured by flow cytometry and analyzed as described previously. (Sulahian et al. 2009) The data were acquired on BD FACS Calibur and analyzed by Flow Jo 8.8.4.

Immunoprecipitation and immunoblotting - γ2A/HA-EpoR/JAK2 cells were transfected with different p85 constructs and induced with Epo. Cell lysates were immunoprecipitated with antibodies as indicated. Under non-denaturing conditions, cells were lysed with 1% NP-40 lysis buffer with phosphatase and protease inhibitors. Under denaturing conditions, cells were lysed with buffer containing 6M urea or 1% SDS at 65°C or 100°C for 10 minutes, which was subsequently diluted with NP-40 lysis buffer prior to immunoprecipitation with FK2 beads. Immunoprecipitation and immunoblotting were

performed as described. (Sulahian et al. 2009) Cell lysates were also immuno-blotted with antibodies to HA, phosphorylated JAK2, JAK2, p85, phosphorylated Cbl, Cbl, GAPDH, or actin as indicated.

Cbl knockdown - siRNAs for Cbl (SMART pool ON-TARGET plus L-003003-00-0005) or control siRNA (ON-Target plus D-0011810-01-05) were from Dharmacon. 48 hours post transfection with DharmaFect (Thermo Scientific); cells were induced with Epo and assayed for EpoR internalization. Retroviral shRNA vectors were generated in pWH99 that also expresses GFP (kindly provided by Dr. Harvey Lodish). The hairpin sequence targeting Cbl is: ACCCAGGAACAATATGAATTA, and control shRNA is: CAACAAGATGAAGAGCACCAA. BaF3 cells were co-transduced with two retroviral vectors: one expresses Cbl shRNA or a control shRNA with GFP and the other expresses HA-EpoR with CD4. Cells positive for both GFP and CD4 were isolated via flow cytometry sorting, and Epo-induced EpoR internalization and MTT assays were performed. To determine Epo sensitivity in Ter119- erythroid progenitors, cells were transduced with retroviruses expressing shRNA against Cbl or control shRNA and cultured in expansion conditions (described below). At indicated days, cell viability was determined by MTT assays.

***In vitro* ubiquitination assay** - Recombinant polyhistidine-tagged Cbl protein was purified from BL21 bacteria via Ni-NTA agarose (Invitrogen). T7-tagged p85 proteins immunoprecipitated from HEK293T cells on Protein A beads were incubated with recombinant Cbl in ubiquitination reaction mixture containing E1, E2 and Flag-tagged wild-

type Ub or lysineless Ub(KR) (E2 is UbcH5B, ubiquitination buffer is 25mM Tris-HCl pH 7.6, 5mM MgCl2, 100mM NaCl, 1mM DTT, 2mM ATP and 10mM N-ethylmaleimide. Auto-ubiquitination kit, Enzo Life Sciences) at 37°C for 1 hr. After extensive washing, p85 on Protein A resin was spun down and eluted by SDS sample buffer, ran on SDS-PAGE, and probed with anti-Ub, anti-Flag, or anti-p85 antibodies.

***In vitro* kinase assay** - Cbl immunoprecipitated from HEK293T cells was incubated with glutathion agarose of GST-tagged wild-type or kinase-deficient JAK2 kinase domain in kinase assay as described. (Zhao et al. 2009)

Immunofluorescence - γ2A cells stably expressing HA-EpoR, HA-EpoR(5KR), HA-EpoR(PFCP) or HA-EpoR(Rescue) with JAK2 were seeded on glass coverslips. Coverslips were blocked and stained with rabbit anti-HA antibodies prior to Epo induction. Subsequently, cells were fixed, permeabilized, and incubated with the indicated antibodies. Coverslips were incubated with the appropriate fluorophore conjugated secondary antibodies and were mounted onto slides with Mowiol mounting medium (Calbiochem). Fluorescence images were taken on a Leica TCS SP5 or Zeiss LSM510 confocal microscope with a 63X oil objective with numeric apertures of 1.25 or 1.4. Confocal section images were analyzed with Image J and Adobe Photoshop. Mander's coefficients were calculated using WCIF Intensity Correlation Analysis plug-in in Image J.

Isolation of erythroid progenitor cells - Ter119- erythroid progenitors were isolated from E13.5 – E14.5 Balb/c murine fetal livers as described. (Sulahian et al. 2009) For endogenous p85 ubiquitination experiment, Ter119- cells were expanded in StemPro34

medium with Epo, stem cell factor, insulin-like growth factor-1, and dexamethasone for 3 days. (von Lindern et al. 1999) The cells were starved for 4 hours and induced with Epo at indicated time points. For immunofluorescence, Ter119- cells expressing HA-EpoR were seeded on poly-L-lysine coated coverslips and starved prior to immunostaining as described above.

MTT assay - 10,000 BaF3 cells and 30,000 primary erythroid progenitors were seeded per well of a 96 well plate and MTT assay was performed (Promega).

3.4 RESULTS (BULUT ET AL. 2013)

3.4.1 p85 becomes ubiquitinated upon Epo stimulation –

We showed previously that p85 mediates Epo-induced EpoR internalization in a PI3K kinase activity-independent manner, but the mechanism is not clear. We discovered that p85 becomes ubiquitinated upon Epo stimulation in γ2A/HA-EpoR/JAK2 cells that stably express HA-tagged EpoR and JAK2. Ubiquitinated p85 was detected in these cells transiently expressing p85 and Flag-tagged ubiquitin (Flag-Ub) by immunoprecipitation with anti-p85 antibodies and immunoblotting with anti-Flag antibodies (Fig. 12A, left), and also by immunoprecipitation with anti-ubiquitinated proteins antibody (FK2) and immunoblotting with anti-p85 antibodies (Fig. 12A, right). To eliminate the possibility that a p85-associated protein of similar molecular weight becomes ubiquitinated upon Epo stimulation, we performed immunoprecipitation with FK2 antibody under denaturing conditions followed by immunoblotting with anti-p85 antibodies. Similar to previous results, Epo-induced ubiquitination of p85 was observed (Fig. 12B). Based on the apparent molecular weight on

SDS-PAGE, p85 likely is mono-ubiquitinated upon Epo stimulation. However, some higher molecular weight ubiquitinated p85 species (seen as high molecular weight smear) were also detected under denaturing conditions. Importantly, we verified our results on endogenous p85 protein in the physiologically relevant primary erythroid progenitors. Erythroid progenitors can be isolated from E13.5-14.5 murine fetal livers by depletion of Ter119+ cells. (Sulahian et al. 2009, Socolovsky et al. 1999) These Ter119- erythroid progenitors are highly Epo-responsive and can undergo terminal differentiation *in vitro* into reticulocytes. We expanded these cells in media containing Epo, stem cell factor, insulin-like growth factor-1, and dexamethasone to keep them in an undifferentiated state as described.(von Lindern et al. 1999)

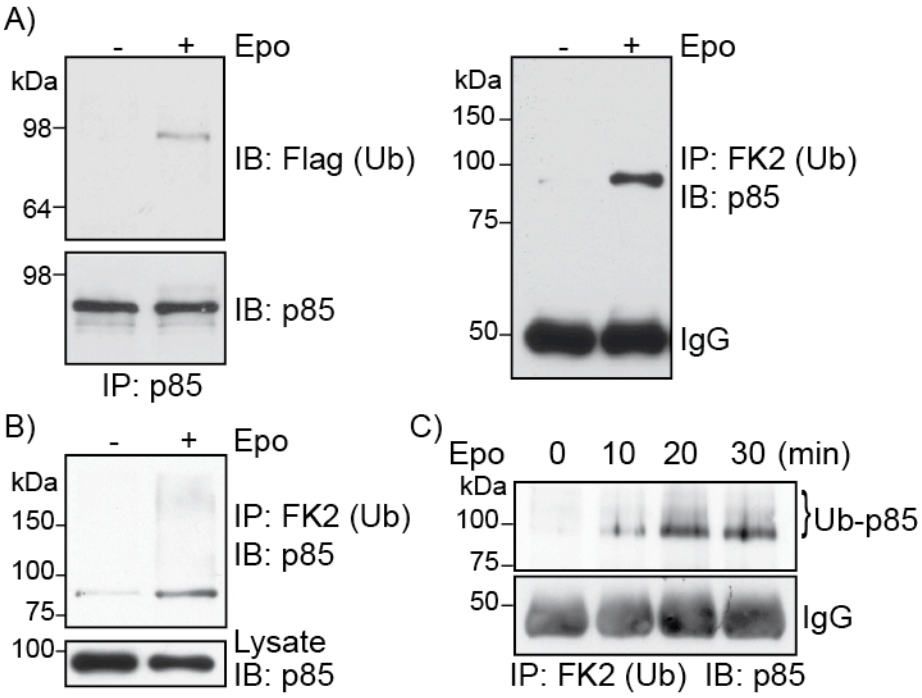


Figure 12: p85 becomes ubiquitinated upon Epo stimulation.

A) γ2A/HA-EpoR/JAK2 cells transiently expressing p85 and Flag-tagged Ub were stimulated with Epo for 15 minutes. Immunoprecipitated p85 under non-denaturing conditions was blotted with the indicated antibodies. B) Ubiquitinated p85 was also detected by immunoprecipitation using FK2 antibody-conjugated beads under denaturing conditions and immunoblotting for p85. C) Epo induces endogenous p85 ubiquitination in primary Ter119- erythroid progenitor cells. FK2: anti-ubiquitinated proteins antibody. IP: immunoprecipitation. IB: immunoblot.

These cells were washed, starved for 4 hrs, stimulated with Epo, and ubiquitinated proteins were immunoprecipitated by FK2 antibody under denaturing conditions and immunoblotted with anti-p85 antibodies. Ubiquitination of endogenous p85 was readily detectable as early as 10 minutes post Epo stimulation, and the amount of ubiquitinated p85 reached a stable level 20 minutes post Epo stimulation (Fig. 12C). Therefore, consistent with our results in cell lines, Epo-induced p85 ubiquitination was observed in primary erythroid progenitors. We surmised that ubiquitinated p85 may serve as an endocytic signal to internalize EpoR upon Epo stimulation.

3.4.2 Epo induces p85 binding to Cbl –

The E3 ligase Cbl, which mediates ubiquitination and endocytosis of multiple receptor tyrosine kinases, was previously shown to bind p85 in stimulated B-lymphocytes and in Jurkat T cells.(Kim, Kim and Pillai 1995, Hartley, Meisner and Corvera 1995) In addition, erythroid progenitors appeared to have expanded in Cbl-/- mice. (Naramura et al. 1998) We thus examined the possibility that Cbl is the E3 ligase that ubiquitinates p85 upon Epo stimulation.

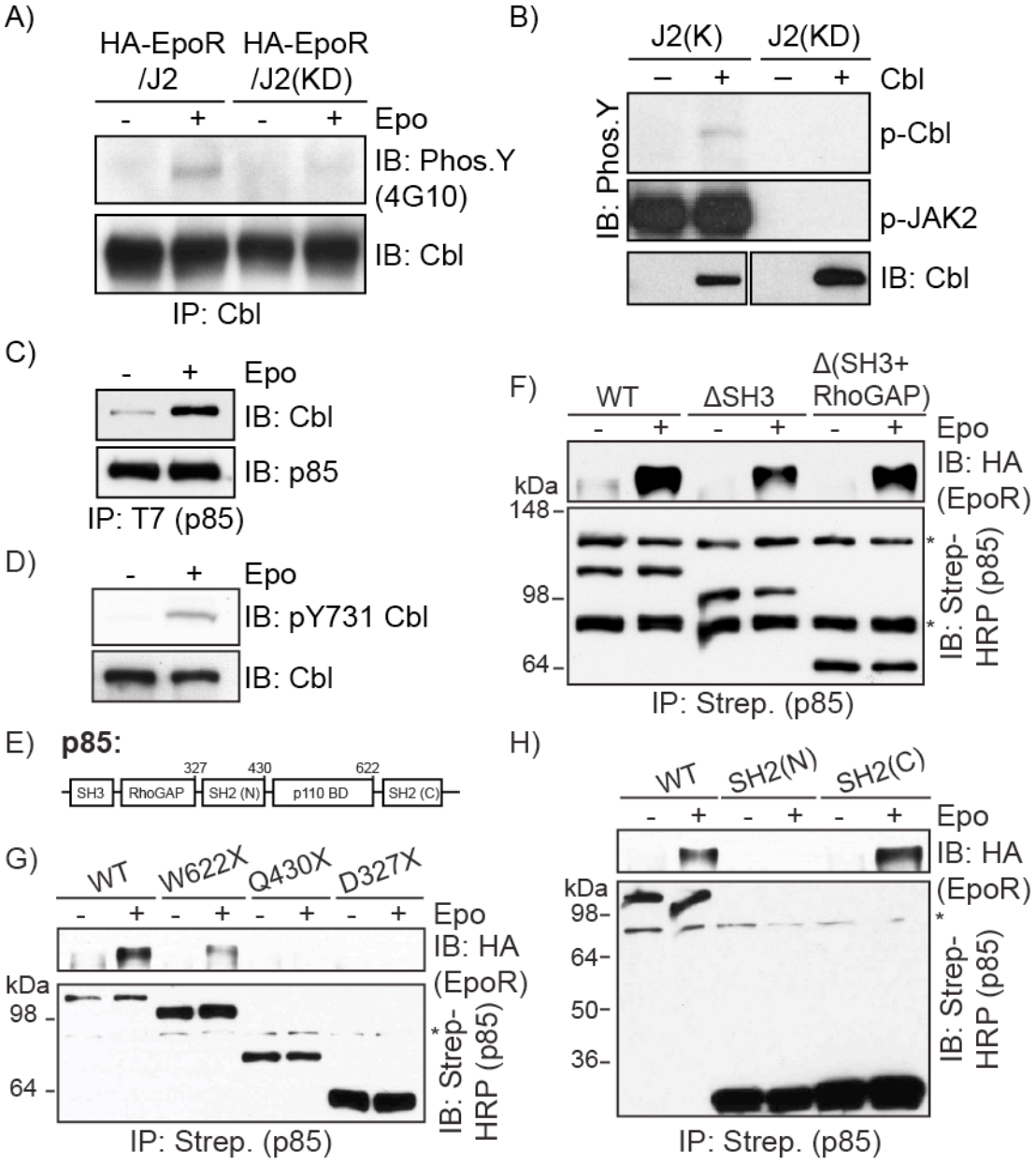


Figure 13: Epo induces p85 interaction with Cbl.

A) Cbl becomes tyrosine phosphorylated upon Epo stimulation in γ2A/HA-EpoR cells expressing wild-type JAK2 but not kinase-deficient (KD) JAK2. Cell lysates were subjected to immunoprecipitation by Cbl antibodies and immunoblotted with 4G10 to detect phospho-tyrosine. B) JAK2 phosphorylates Cbl *in vitro*. Cbl proteins immunoprecipitated from HEK293T cells were incubated with GST-tagged wild-type or kinase-deficient JAK2 kinase domain in *in vitro* kinase assay. J2(K): JAK2 kinase domain, J2(KD): kinase-deficient JAK2 kinase domain. C) Epo induces p85 interaction with Cbl. γ2A/HA-EpoR/JAK2 cells transiently expressing Cbl and T7-tagged p85 were induced with Epo. p85 was immunoprecipitated with T7 antibodies and the immunoprecipitates

were probed with antibodies against Cbl. D) Epo induces phosphorylation of Y^{731} in the p85-binding motif of Cbl. E) p85 domain structure. SH3: src-homology 3 domain. RhoGAP: RhoGAP homology domain. SH2: src-homology 2 domain. p110BD: p110-binding domain. F-H) The C-terminal SH2 domain of p85 mediates inducible binding to EpoR upon stimulation. Biotin-tagged p85 fragments were transiently expressed in γ2A/HA-EpoR/JAK2 cells, isolated with streptavidin agarose, and probed with anti-HA to detect bound EpoR. In G), W622X, Q430X, and D327X are truncations where residues W^{622}, Q^{430} or D^{327} were mutated to a stop codon. IP: Immunoprecipitation, IB: Immunoblot. * indicates non-specific bands.

Epo induced tyrosine phosphorylation of Cbl in γ2A/HA-EpoR/JAK2 cells, similar to previous reports, but not γ2A/HA-EpoR/JAK2KD cells, which express kinase-deficient JAK2 (Fig. 13A). (Barber et al. 1997b, Odai et al. 1995) Cbl phosphorylation is likely mediated by JAK2 because we observed that JAK2 could directly phosphorylate Cbl in *in vitro* kinase assays (Fig. 13B). Importantly, we found that Epo induced p85 binding to Cbl (Fig. 13C). Consistent with prior findings that the N-terminal SH2 domain of p85 binds to Cbl Y^{731} in the predicted p85-binding motif (Y^{731}EAM), (Songyang and Cantley , Hunter et al. 1999, Hakak, Hsu and Martin 2000, Fournier et al. 2000, Alcazar et al. 2009) Epo induced phosphorylation of Cbl Y^{731} (Fig. 13D).

We also determined p85 domain(s) required for binding to EpoR upon Epo stimulation using constructs of individual p85 domains fused to a biotinylation sequence, which is biotinylated by the endogenous mammalian machinery, to allow isolation via streptavidin agarose (Fig. 13E-H). The SH3 and RhoGAP homology domains were dispensable for EpoR binding (Fig. 13F). Consistent with the fact that p85 binds to phosphotyrosines on the EpoR; W622X lacking the C-terminal SH2 domain had greatly reduced binding to EpoR (Fig. 13G). Conversely, the C-terminal SH2 domain alone, but not the N-terminal SH2 domain, was sufficient to bind to EpoR (Fig. 13H). Therefore, the C-terminal SH2 domain of p85 likely mediates EpoR binding. The p110-binding domain also

somehow contributed to the p85-EpoR interaction, because the residual binding of W622X to EpoR was abolished in Q430X (Fig. 13G). Together, these results suggested that upon Epo stimulation, the C-terminal SH2 domain of p85 binds to phosphorylated Y^{429}, Y^{431}, or Y^{479} of EpoR. In addition, Cbl Y^{731} becomes phosphorylated upon Epo stimulation, facilitating Cbl binding to p85.

3.4.3 Cbl and its E3 ligase activity are essential for Epo-induced EpoR internalization –

We examined if Cbl contributes to Epo-induced EpoR internalization. We used siRNAs to knockdown Cbl in γ2A/HA-EpoR/JAK2 cells. As shown in Figure 14A, Epo-induced EpoR internalization was blocked in Cbl knockdown cells compared to cells transfected with control siRNAs, measured using anti-HA antibodies to label the exofacial HA-tag of EpoR. We also determined if Cbl contributes to Epo-induced EpoR internalization in hematopoietic BaF3 cells. BaF3 cells were transduced with two retroviral vectors, one expressing Cbl shRNA or a control shRNA and GFP, and the other expressing HA-EpoR and human CD4 after an internal ribosomal entry site. Cells transduced with both vectors were isolated via flow cytometry sorting and examined for Epo-induced EpoR internalization. As shown in Figure 14B, Epo-induced EpoR internalization was mildly but significantly impaired in cells expressing Cbl shRNA compared to control cells, possibly due to modest knockdown efficiency.

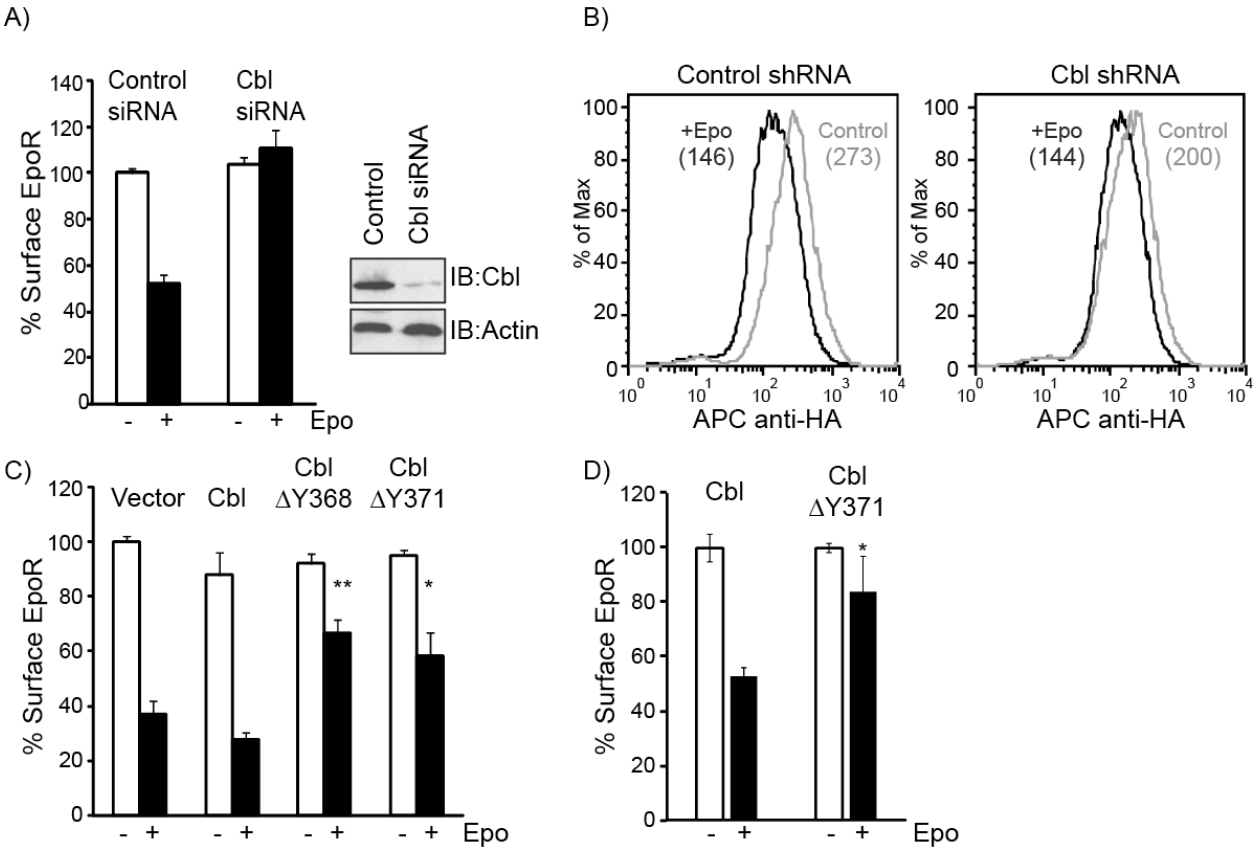


Figure 14: Cbl and its E3 ligase activity are essential for Epo-induced EpoR internalization.
A) siRNAs for Cbl or control siRNAs were transfected into γ2A/HA-EpoR/JAK2 cells and levels of surface EpoR pre and post 45 minutes of Epo stimulation were analyzed by flow cytometry. For each sample, EpoR surface expression was normalized to that from samples expressing control siRNA prior to Epo stimulation. Knockdown efficiency of Cbl is shown. Immunoblotting of actin was used as a loading control. B) Cbl knockdown in BaF3 cells impairs Epo-induced EpoR internalization. Cells expressing shRNA for Cbl or control shRNA, identified by GFP expression, were gated for analyses. Numbers in parenthesis are median fluorescence intensity, which represents EpoR surface expression. Representative histograms from 3 independent experiments are shown. C) Epo-induced EpoR internalization is impaired in γ2A/HA-EpoR/JAK2 cells expressing ligase-deficient Cbl mutants (ΔY368 and ΔY371). D) Epo-induced EpoR internalization is significantly impaired by expression of dominant negative Cbl in Ter119- erythroid progenitor cells. *$p<0.05$, **$p<0.005$ (Student's t test).

To determine if E3 ligase activity of Cbl is required for EpoR internalization, we examined the effect of two ligase-deficient Cbl mutants, ΔY368 and ΔY371 on Epo-induced EpoR internalization. (Kassenbrock and Anderson 2004, Thien et al. 2001) As shown in Figure 14C, Cbl(ΔY368) and Cbl(ΔY371) both had a dominant negative effect on the

internalization of EpoR in γ2A/HA-EpoR/JAK2 cells, while expression of wild-type Cbl had no effect, demonstrating that Cbl E3 ligase activity is critical for Epo-induced EpoR internalization. Importantly, these results were verified in Ter119- erythroid progenitors (Fig. 14D). Together, our results showed that Epo induces p85 ubiquitination and its interaction with Cbl, and that Cbl and its ligase activity play a crucial role in Epo-induced EpoR internalization.

3.4.4 Cbl ubiquitinates p85 in vitro –

To corroborate our results, we examined if Cbl can directly ubiquitinate p85 using an *in vitro* ubiquitination assay. Incubation of recombinant Cbl and Flag-Ub proteins with T7-tagged p85, immunoprecipitated from HEK293T cells, in *in vitro* ubiquitination assays resulted in the appearance of higher molecular weight ubiquitinated p85 species, detectable by immunoblotting with anti-Flag or anti-p85 antibodies. In contrast, these species were not detected in the absence of Cbl (Fig. 15A). To determine the nature of ubiquitination on p85, we also examined p85 ubiquitination with a lysineless Ub mutant that cannot form ubiquitin chains and only supports mono-ubiquitination (Ub(KR)). In reactions with recombinant Ub(KR), high molecular weight ubiquitinated p85 species were still detected, indicating that Cbl can ubiquitinate p85 on multiple sites *in vitro* (Fig. 15B). We also examined p85 ubiquitination in cells from Cbl-/- animals. Cbl-/- mouse embryonic fibroblasts (MEFs) were immortalized and transfected with vectors expressing HA-EpoR, JAK2, p85 and Flag-Ub. Epo-induced p85 ubiquitination was not observed in Cbl-/- MEFs, and re-expression of Cbl in these cells rescued Epo-induced p85 ubiquitination (Fig. 15C). These results demonstrate that p85 is ubiquitinated by Cbl upon Epo stimulation.

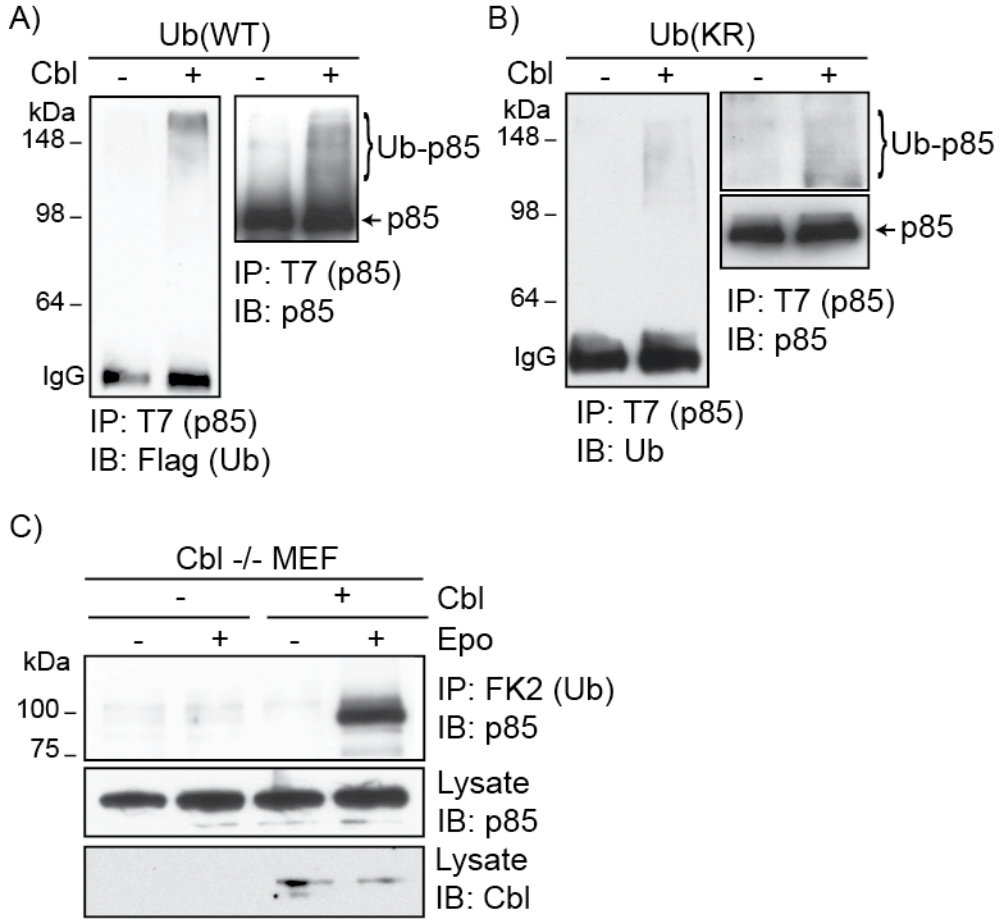


Figure 15: Cbl ubiquitinates p85 in vitro.

A) T7-tagged p85 immunoprecipitated from HEK293T cells using anti-T7 antibody and Protein A beads was incubated with 200ng of recombinant Cbl purified from BL21 in in vitro ubiquitination assay using recombinant E1, E2 (UbcH5B) and Flag-tagged wild-type Ub. After extensive washing, ubiquitinated p85 species were eluted from Protein A beads by SDS sample buffer and immuno-blotted with the indicated antibodies. B) p85 can be ubiquitinated at multiple sites. In vitro ubiquitination assay was performed with a lysineless ubiquitin mutant (KR) that cannot form ubiquitin chains. C) p85 ubiquitination is lost in Cbl-/- MEFs and is restored in Cbl-/- MEFs reconstituted with Cbl.

3.4.5 Epo induces co-localization of epsin-1 with the EpoR –

We determined the functional consequence of p85 ubiquitination by Cbl. Because Ub can trigger the internalization of cargos by binding to endocytic ubiquitin-binding proteins, (Reider and Wendland 2011) we examined candidate endocytic proteins known to bind

ubiquitinated cargos and tested if they become colocalized with EpoR upon Epo stimulation. We found that epsin-1, which couples ubiquitinated cargos through its ubiquitin-interacting motifs (UIMs) to clathrin and AP-2, (Horvath et al. 2007) co-localized with the EpoR upon Epo induction (Fig. 16A). Consistent with these results, Epo induced association between p85 and epsin-1 tagged with enhanced cyan fluorescent protein (ECFP) (Fig. 16B). To rule out that epsin-1 is recruited to EpoR itself, which is also ubiquitinated upon Epo stimulation, we examined a lysine-less EpoR mutant that cannot be ubiquitinated (HA-EpoR(5KR)). (Bulut et al. 2011) HA-EpoR(5KR), similar to wild-type HA-EpoR, co-localized with epsin-1 upon Epo stimulation (Fig. 17), suggesting that recruitment of epsin-1 to EpoR does not require a ubiquitin moiety on EpoR itself and may instead bind to ubiquitinated p85.

We also tested the effect of expressing a ligase-deficient Cbl mutant on co-localization of EpoR with epsin-1. As shown in Fig. 16C, cells expressing wild-type myc-tagged Cbl (identified by Alexa488-conjugated anti-myc) exhibited co-localization (blue arrows, upper merge panel), similar to non-expressing cells. However, Cbl(ΔY368) expressing cells showed greatly reduced EpoR colocalization with epsin-1 (blue arrows, lower merge panel). As controls, co-localization between EpoR and epsin-1 was normal in untransfected cells (white arrow, lower merge panel). We also tested if EpoR co-localizes with endosomal markers upon longer Epo treatment. Both γ2A/HA-EpoR/JAK2 (data not shown) and Ter119- progenitors exhibited co-localization of HA-EpoR with early endosome antigen EEA1 upon 25 minutes Epo induction (Fig. 18).

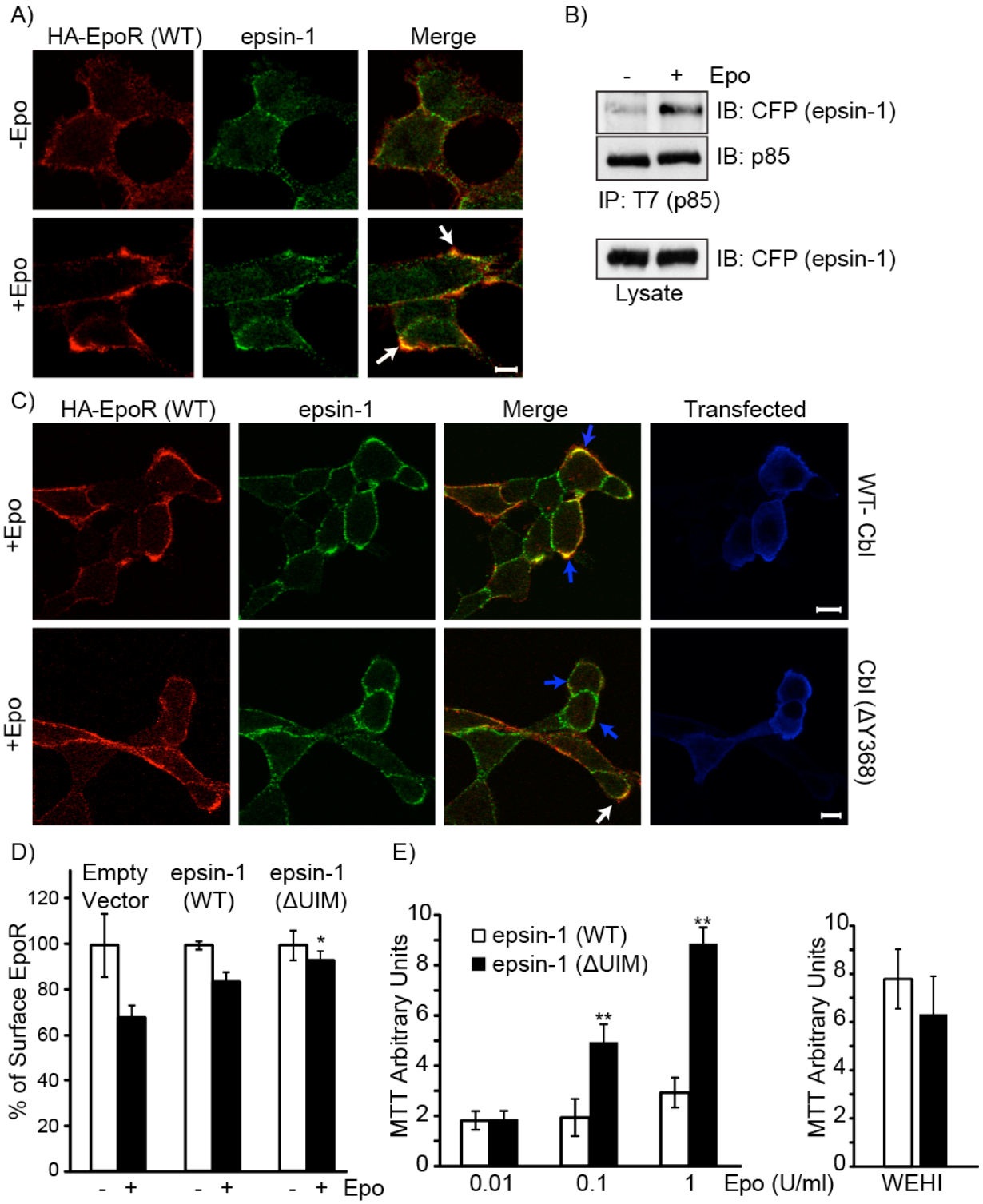


Figure 16: Epo induces EpoR co-localization with epsin-1.

A) Endogenous epsin-1 becomes co-localized with wild-type EpoR upon Epo stimulation. Non-permeabilized γ2A/HA-EpoR/JAK2 cells were stained with anti-HA antibodies to label the exofacial HA-tag of the EpoR as described previously.5 Cell were then treated with Epo for 12 minutes, fixed, permeabilized, immuno-stained for epsin-1 and visualized by confocal microscopy. Scale bar is 5 μm. Representative co-localizaion areas were marked with arrows. B) Epo induces p85 binding to epsin-1. ECFP-tagged epsin-1 and T7-tagged p85 were transiently expressed in γ2A/HA-EpoR/JAK2. Anti-T7 immunoprecipitates were immunoblotted with anti-GFP antibody to detect epsin-1. C) EpoR-epsin-1 co-localization is impaired in cells expressing a ligase-deficient Cbl mutant. γ2A/HA-EpoR/JAK2 cells were transfected with either wild-type myc-tagged Cbl or Cbl(ΔY368). 48hrs post transfection, cells were stained as described above, except Alexa 488 anti-myc antibody was used to mark transfected cells. Blue arrows mark transfected cells, whereas white arrow shows co-localization in an untransfected cell. D) Expression of epsin-1(ΔUIM) impaired EpoR internalization. Epo-induced EpoR internalization was determined by flow cytometry in γ2A cells cotransfected with vectors expressing HA-EpoR, JAK2, and ECFP-tagged epsin-1 (either wild-type or the ΔUIM mutant). Data was normalized to EpoR surface expression of unstimulated control cells. *$p<0.05$ between epsin-1(WT) and epsin-1(ΔUIM). E) BaF3 cells expressing HA-EpoR and epsin-1 (either wild-type or the ΔUIM mutant) were grown in RPMI media containing 2% FBS with different concentrations of Epo. Cell growth was measured using MTT assays. Cells grew similarly in WEHI media and 10% FBS. Epsin-1(ΔUIM) expressing cells exhibit Epo hypersensitity. **$p<0.005$.

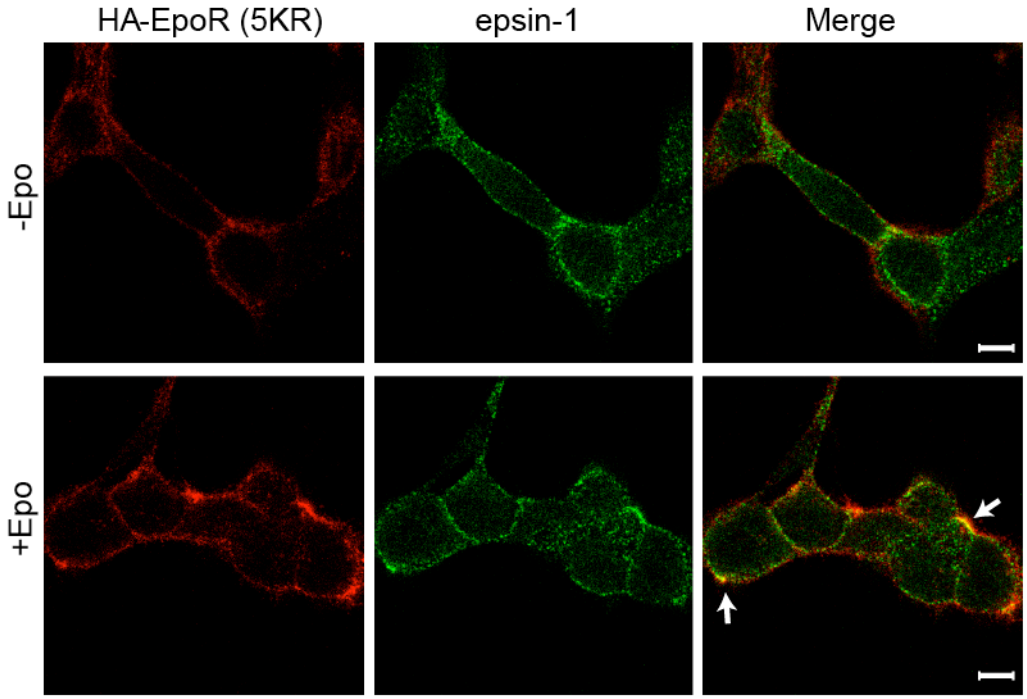


Figure 17: EpoR ubiquitination is not required for colocalization with epsin-1.

Epo induces colocalization of HA-EpoR(5KR) with endogenous epsin-1 similar to wild-type HA-EpoR. Scale bar is 5 μm. A representative co-localization area was marked with an arrow.

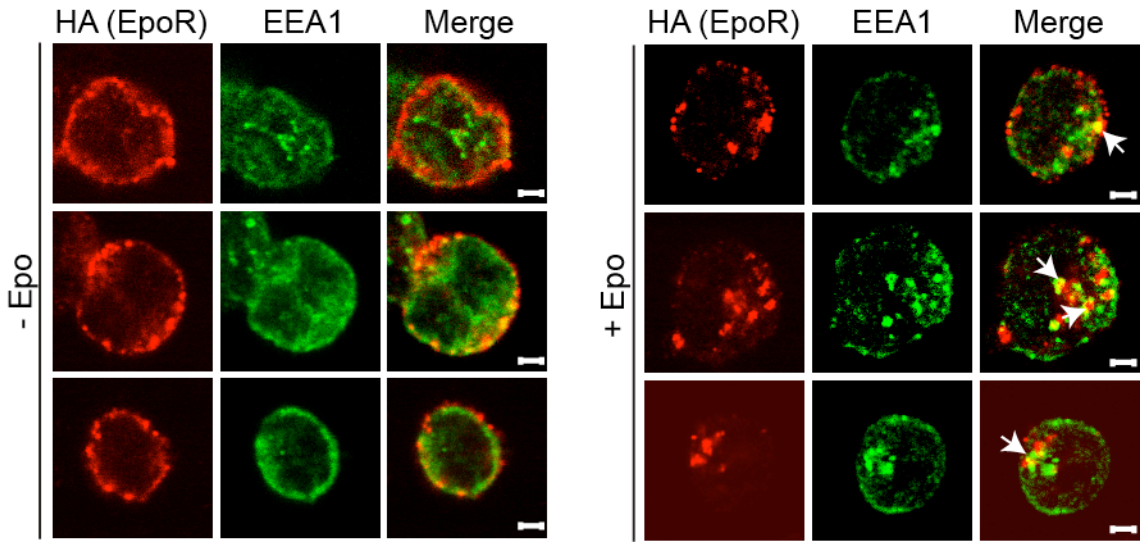


Figure 18: HA-EpoR is endocytosed upon Epo and co-localizes with EEA1 in primary erythroid progenitors.

Ter119- erythroid progenitors (expressing HA-EpoR) were immunostained with antibodies against HA and EEA1. Following 25 min Epo induction, surface labeled receptors co-localize with early endosomal marker EEA1. Scale bar is 2.5 μm.

To corroborate these results, we examined an epsin-1 mutant lacking its UIMs and thus cannot interact with ubiquitinated cargos (epsin-1(ΔUIM)). Contrary to wild-type epsin-1, epsin-1(ΔUIM) expression impaired Epo-induced EpoR internalization (Fig. 16D). Consistent with this defect in EpoR internalization, BaF3/HA-EpoR cells expressing epsin-1(ΔUIM) exhibited increased Epo sensitivity as compared to cells expressing wild-type epsin-1 (Fig 16E). Together, our results support a model in which Epo induces binding of EpoR and p85, which is ubiquitinated by Cbl. Ubiquitination allows p85 to recruit epsin-1 via its UIMs to the EpoR/p85 complex and engages the endocytic machinery for EpoR internalization and down-regulation (Fig. 20C).

3.4.6 Truncated PFCP EpoR is defective in epsin-1 co-localization upon Epo stimulation –

Erythroid progenitors from PFCP patients harbor truncated EpoRs and display Epo hypersensitivity. (Skoda and Prchal 2005) These PFCP receptors lack p85-binding sites. (Sulahian et al. 2009) We examined the ability of a truncated EpoR that was engineered to mimic a PFCP EpoR allele, HA-EpoR(PFCP), and its rescue counterpart with appended $KY^{429}LY^{431}L$ p85-binding sites, HA-EpoR(Rescue), to recruit epsin-1 upon Epo stimulation (Fig. 19A). Consistent with the inability to recruit p85, HA-EpoR(PFCP) failed to co-localize with epsin-1 upon Epo stimulation (Fig. 19B). Importantly, HA-EpoR(Rescue) co-localized with epsin-1 similar to wild-type HA-EpoR (Fig. 19C). Importantly, EpoR internalization was restored and Epo hypersensitivity was normalized in erythroid progenitors expressing HA-EpoR(Rescue) compared to HA-EpoR(PFCP). (Sulahian et al. 2009)

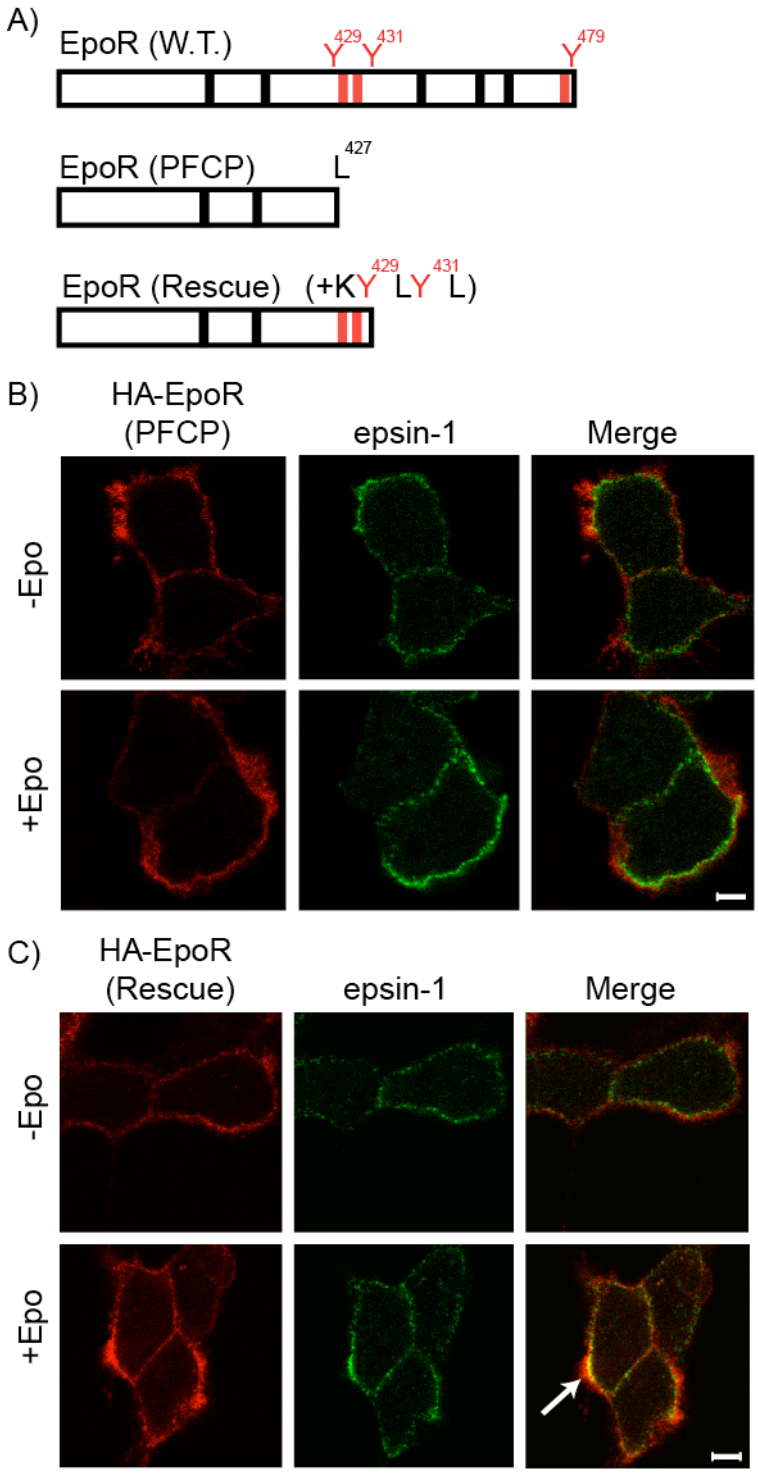


Figure 19: Appendage of p85-binding sites restores Epo-induced epsin-1 co-localization of a truncated EpoR that mimics those from PFCP patients in γ2A cells.

A) Schematic diagram of PFCP and rescue EpoR constructs. B) PFCP EpoR did not co-localize with epsin-1 upon Epo stimulation. C) Rescue EpoR appended with p85-binding sites rescued Epo-induced epsin-1 co-localization. A representative co-localization area was marked with an arrow.

3.4.7 Cbl knockdown increases Epo sensitivity in primary erythroid progenitors –

To implicate the physiological relevance of Cbl in EpoR endocytosis and signaling, we knocked down Cbl in hematopoietic BaF3 cells and in primary Ter119- erythroid progenitors and examined their Epo sensitivity. As shown in Figure 20A, IL-3 dependent BaF3 cells stably expressing HA-EpoR with Cbl shRNA or control shRNA grew similarly in control media containing IL-3 (WEHI conditioned media). In contrast, despite a modest knockdown efficiency and a slightly lower HA-EpoR surface expression level Cbl knockdown BaF3 cells had higher mitogenic activities in media containing 0.1U/ml and 0.01U/ml Epo compared to control knockdown cells. Similarly, at comparable transduction efficiency (based on GFP fluorescence, data not shown), Cbl knockdown in Ter119- cells caused Epo hypersensitivity at different Epo concentrations compared to control cells (Fig. 20B). Therefore, knockdown of Cbl phenocopied deletion of p85-binding sites from EpoR as in PFCP EpoRs and resulted in Epo hypersignaling in primary erythroid progenitors. These results elucidated the physiological relevance of our Cbl/p85/epsin-1 pathway to internalize and down-regulate EpoR upon Epo stimulation.

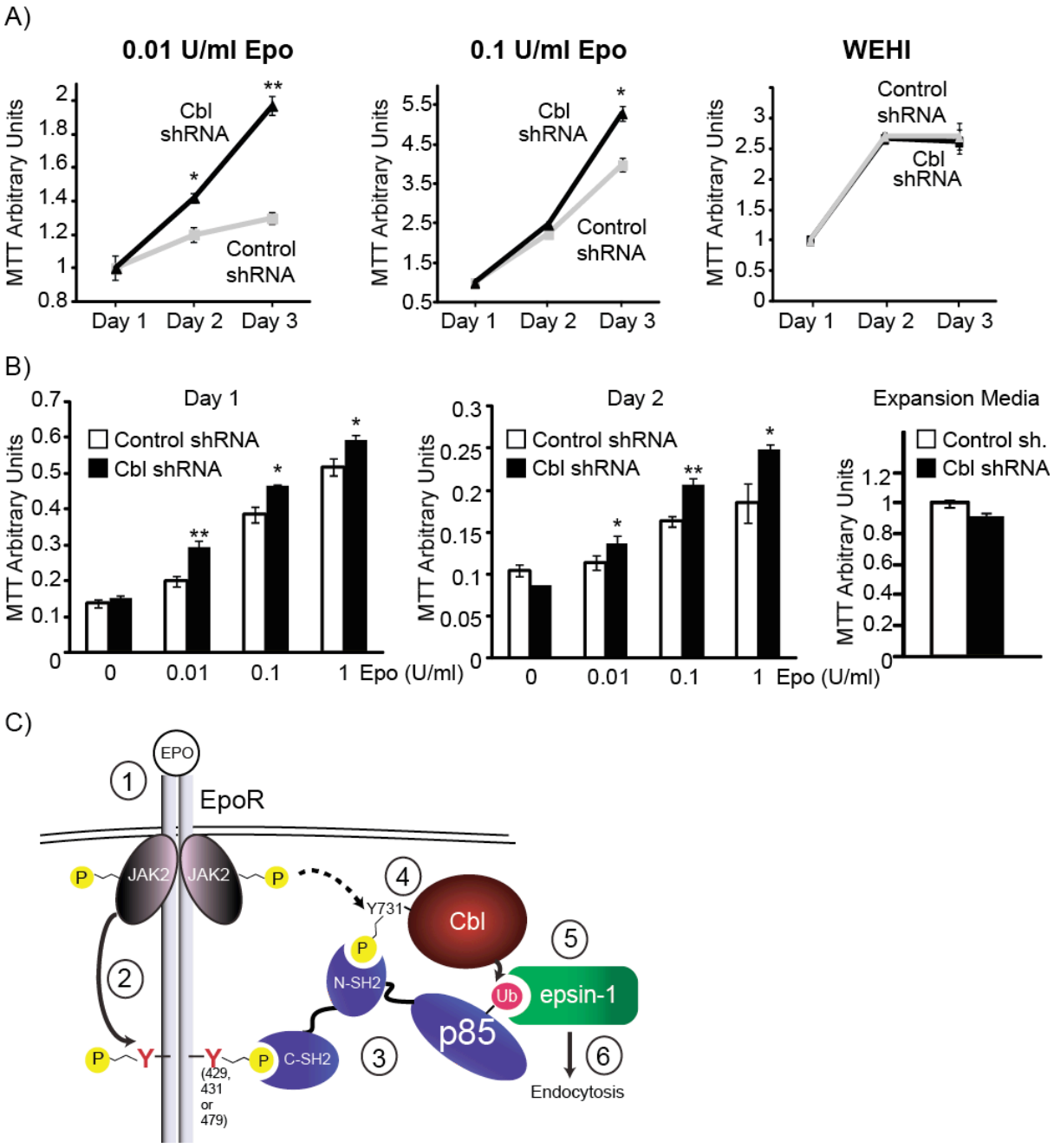


Figure 20: Cbl knockdown results in hypersensitivity to Epo.

A) BaF3 cells expressing HA-EpoR and either Cbl shRNA or control shRNA were grown in RPMI media containing 2% FBS with different concentrations of Epo. Cell growth was measured using MTT assays. Cells grew similarly in control media containing no Epo but IL-3 (WEHI media) and 10% FBS. *p<0.05, **p<0.005. B) Expression of Cbl shRNA but not control shRNA in primary Ter119- erythroid progenitors resulted in Epo hypersensitivity in IMDM media containing 2% FBS and different concentrations of Epo. Cells grew similarly in control expansion media (2U/ml Epo).

*p<0.05, **p<0.005. C) Working model of p85-mediated EpoR internalization. Epo stimulates binding of p85 to both Cbl and the EpoR through its SH2 domains, possibly forming a tripartite complex. Epo also induces Cbl ubiquitination of p85, which in turn recruits epsin-1 through its UIMs to further enlist the endocytic machinery for EpoR internalization.

3.5 CONCLUSIONS

In this study, we identified a novel Cbl/p85/epsin-1 pathway by which Epo induces EpoR endocytosis and down-regulation. In our model (Fig. 20C), upon Epo stimulation (1) JAK2 becomes activated and (2) phosphorylates EpoR cytoplasmic tyrosine residues, including Y^{429}, Y^{431} and Y^{479}, which (3) serve as docking sites for SH2 domain-mediated binding of p85 to EpoR. In addition, (4) Cbl Y^{731} becomes phosphorylated upon Epo stimulation, facilitating Cbl binding to p85. (5) Cbl ubiquitinates p85, enabling recruitment of epsin-1 to ubiquitinated p85 through its UIMs. (6) Binding of the EpoR/p85 complex to epsin-1 connects it to the clathrin-mediated endocytic machinery to induce EpoR internalization. We also showed that mutated EpoRs mimicking those found in PFCP patients cannot bind p85 nor recruit epsin-1 to engage the endocytic machinery, thus do not internalize upon Epo stimulation and exhibit Epo hypersensitivity. Similarly, knockdown of Cbl also causes Epo hypersensitivity in primary erythroid progenitors. Restoring p85 binding to PFCP receptors rescues Epo-induced epsin-1 co-localization and normalizes Epo hypersensitivity. Our results elucidate the molecular mechanism underlying Epo-induced p85-mediated EpoR internalization and demonstrate that defect in this pathway may contribute to the etiology of PFCP.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 EPOR UBIQUITINATION

Ubiquitination plays a crucial role in ligand-induced internalization and down-regulation of many cytokine receptors, despite differences in the molecular mechanisms used to couple ubiquitination to these trafficking processes. In our first study, we investigated the role of ubiquitination of the EpoR itself in EpoR down-regulation by generating single lysine mutants of the receptor in our cellular reconstitution system. Our first key finding is that ubiquitination of K256 promotes Epo-induced EpoR internalization. Ubiquitinated K256 may interact with adaptor proteins that bind to ubiquitin and facilitate receptor internalization (Barriere et al. 2007), or may aid in another "endocytic signal", such as the signal we previously identified that involves the recruitment of p85 (Sulahian et al. 2009). Ubiquitination of K428 is not important for internalization but regulates Epo-induced receptor sorting to the lysosome and degradation. These results are consistent with recent evidence suggesting that HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), a protein with a ubiquitin-interacting motif, plays a critical role in the transport of the ubiquitinated gp130 from the early endosome to the late endosome for degradation (Tanaka et al. 2008). The ubiquitin–proteasome machinery has also been implicated in transporting the prolactin receptor from endosome to lysosome (van Kerkhof et al. 2001). Like the EpoR, the prolactin and type I interferon (IFNAR1) receptors use ubiquitination to promote both receptor internalization and targeting to the lysosomes (Varghese et al. 2008, Kumar et al.

2003). gp130 requires ubiquitination for lysosomal degradation, but not for ligand-induced internalization (Tanaka et al. 2008).

In addition to the contribution from EpoR ubiquitination shown in this study, p85 recruitment to cytoplasmic tyrosines Y429, Y431, and Y479 promotes EpoR internalization (Sulahian et al. 2009). Therefore, the EpoR employs multiple, and possibly cooperative and/or partially redundant, mechanisms to regulate receptor endocytosis. The relative contribution of these mechanisms to the overall EpoR endocytosis and whether one mechanism is preferred under certain physiological conditions are unclear. Interestingly, both ubiquitination-based and tyrosine-based mechanisms for EpoR internalization require JAK2 kinase activity, underscoring the contribution of JAK2 to EpoR signal transduction. Prolactin-stimulated ubiquitination and internalization of the prolactin receptor also requires JAK2 kinase activity (Swaminathan et al. 2008); however, JAK2 activity is not a universal requirement for ubiquitination and internalization because internalization of the growth hormone receptor does not require JAK2 kinase activity (Alves dos Santos, ten Broeke and Strous 2001), and the thrombopoietin receptor c-Mpl, while requiring JAK2 kinase activity for internalization, does not have need of JAK2 for ubiquitination (Saur et al. , Hitchcock et al. 2008).

Our conclusions that ubiquitination of the EpoR is required for efficient ligand-induced receptor internalization and positively regulates downstream signaling by EpoR/JAK2 contrast published work of Meyer et al. (Meyer et al. 2007), which suggested that EpoR ubiquitination is not important for internalization and lysosomal sorting. Instead, it affects Epo-induced receptor degradation and negatively regulates mitogenic activity. We reason

that the different results regarding receptor internalization and sorting may stem from the differences in assay sensitivity, as degradation of internalized Epo was used to infer receptor internalization and sorting in that report. Alternatively, the human EpoR may behave differently from the murine EpoR. With regard to the role of EpoR ubiquitination in signaling, one possibility is that EpoR is ubiquitinated by multiple E3 ligases and that β-TrCP is only responsible for ubiquitination events that promote receptor internalization and degradation. Consistent with this possibility, K428, which is involved in lysosomal sorting/degradation of EpoR, lies proximally to the consensus β-TrCP binding site. A second possibility is that β-TrCP ubiquitinates another component of the EpoR/JAK2 signaling complex, whose ubiquitination negatively regulates EpoR signaling.

Upon Epo stimulation, 5KR was capable of activating JAK2 normally; however, the amplitude of STAT5, ERK, and AKT activation was reduced. Consistent with these results, the mitogenic response of 5KR cells to Epo was defective compared to cells expressing wild-type EpoR. Therefore, JAK2 activation at the cell surface is not sufficient for optimal down-stream signaling of the EpoR. One hypothesis is that EpoR endocytosis kinetics and intracellular compartments critically contribute to sustaining and/or amplifying the STAT5, Ras/MAPK and PI3K/AKT pathways. This hypothesis is based on recent findings regarding receptors such as the EGFR, TGFβ-receptor, and G-protein coupled receptors, that intracellular compartments possess "signaling" capability to sustain signals originated from the cell surface as well as generate new signals (Fehrenbacher, Bar-Sagi and Philips 2009, Sorkin and von Zastrow 2009). For example, it was shown that ERK signaling may be enhanced in the presence of signaling components present in endosomes (Kim, Taylor and

Bar-Sagi 2007, Teis and Huber 2003, Teis et al. 2006). Goh et al. showed that continuous AKT activity requires EGFR internalization (Goh et al. 2010), and the endosomal adaptor protein WDFY2 was found to be required for maintaining insulin-stimulated AKT2 phosphorylation (Walz et al.). In line with this hypothesis, 5KR and K256R, which are both defective in internalization, activate JAK2 but show attenuated activation of the ERK and AKT pathways. An alternative hypothesis is that ubiquitination of the EpoR may activate novel signaling pathways that contribute to mitogenic activity. A mutant mouse strain containing a severely truncated EpoR with no tyrosine residues in the cytoplasmic domain was viable with only slight alterations in erythropoiesis, contrary to the EpoR or JAK2 knockout animals that die embryonically (Zang et al. 2001, Wu et al. 1995, Neubauer et al. 1998, Parganas et al. 1998), indicating that novel non-tyrosine-based signaling pathways essential for erythropoiesis are yet to be identified. Interestingly, the severely truncated EpoR preserves 3 out of the 5 cytoplasmic lysines including K256. Understanding the precise mechanisms underlying receptor endocytosis and signaling will provide insights into the regulation of these important molecules and diseases that are caused by excessive receptor signaling.

4.2 UBIQUITINATION OF P85 BY CBL

Our second study identifies a novel PI3K kinase activity-independent function of p85, namely to recruit endocytic protein epsin-1 via its ubiquitination for EpoR internalization. This adds to the emerging concept that p85 has functions besides regulating PI3K kinase activity, (Garcia et al. 2006, Ueki et al. 2003, Jimenez et al. 2000, Chamberlain et al. 2008) and represents the first recognition of cytokine receptors as cargos of epsins. Other cytokine

receptors could be regulated by similar mechanisms, because most cytokine receptors activate PI3K, often through recruitment of p85. Epo-induced p85 ubiquitination appears to be a mono-ubiquitination. Interestingly, because higher molecular weight ubiquitinated p85 species were observed under denaturing cell lysis conditions, and because Cbl ubiquitinates multiple sites on p85 *in vitro*, it is possible that a deubiquitinating enzyme is involved in this process. However, so far, no deubiquitinating enzyme has been implicated in EpoR signaling.

We found that Cbl is an essential mediator in Epo-induced EpoR down-regulation and p85 is a novel Cbl substrate. This is consistent with the notion that Cbl constrains signal transduction for surface receptors. Cbl-b, another member in the Cbl family, was also reported to ubiquitinate p85. In that case, proteolysis-independent poly-ubiquitination of p85 affects its recruitment to CD28 or T cell antigen receptor ζ. (Fang and Liu 2001) Ubiquitination via different E3 Ub ligases regulates multiple steps in the process of EpoR endocytosis. Besides Cbl that mediates Epo-induced EpoR internalization via p85 ubiquitination, β-Trcp (β-transducin repeat-containing protein) was shown to mediate ubiquitination of EpoR itself and facilitate its degradation. (Bulut et al. 2011, Meyer et al. 2007) These steps may cooperatively ensure the proper down-regulation of EpoR upon stimulation.

Recently, Cbl mutants have been found in human myeloid leukemias and neoplasms. (Kales et al. 2010) Interestingly, studies of these mutants implicated that Cbl may possess both tumor suppressor and oncogenic functions. However, underlying mechanisms and their dependency on Cbl's E3 ubiquitin ligase activity, remain unresolved. Our study adds to the

understanding of E3-dependent functions of Cbl and Cbl substrates, which aids in elucidating its role under different situations.

Although the knowledge of mechanisms underlying Epo-induced receptor down-regulation has furthered our understanding of the molecular pathogenesis of PFCP, many key issues are unresolved. For example, upon Epo stimulation, we do not know how and where activation and termination of different signaling cascades are compartmentalized, nor how PFCP receptors and wild-type receptors differ. The relative importance of the various mechanisms of receptor down-regulation and the recruitment of phosphatases in controlling EpoR signaling duration are not known. In addition, it is not known whether mutations in p85 or epsin-1 exist in erythrocytosis patients.

In summary we identified a new role for Cbl and a novel PI3K kinase activity-independent function of p85, both in promoting EpoR endocytosis and down-regulation. Because p85 is recruited to other cytokine receptors, such as the thrombopoietin receptor, c-Mpl, as well, it will be of interest to determine if there is a similar ligand-induced negative regulatory mechanism for those receptors. This novel Cbl/p85/epsin-1 mediated EpoR down-regulation pathway critically controls EpoR signaling, and when defective contributes to red blood cell hyperproliferation in PFCP. Because there is increasing association of ubiquitin-mediated pathways in pathological conditions, the identification and functional characterization of E3 ligases and their contribution to diseases are important to uncover their function under physiological and pathological conditions.

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