REGULATION OF ERYTHROPOIETIN ENDOCYTOSIS AND SIGNALING

APPROVED BY SUPERVISORY COMMITTEE

Lily Huang, Ph.D.

Lawrence Lum, Ph.D.

Michael Roth, Ph.D.

Michael White, Ph.D.

REGULATION OF ERYTHROPOIETIN ENDOCYTOSIS AND SIGNALING

by

RITA SULAHIAN

DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2008

ACKNOWLEDGEMENTS

I would like to thank Dr. Lily Huang, my graduate mentor, for giving me the opportunity to work and learn along her side. Her tremendous and constant enthusiasm for science has been a major driving force enabling me to complete my thesis. I would also like to thank my committee members Drs. Mike Roth, Mike White and Lawrence Lum for their guidance and support during the course of my projects. I also send many thanks to my friends and colleagues in the Huang lab and in the Cell Biology department. Moreover several faculty members of the Cell Biology department, Drs Peter Michaely, Kate Luby-Phelps, Bill Snell, Fred Grinnell and Joachim Seemann were tremendously helpful in different aspects of my projects and I will always be grateful for all their useful suggestions that made my work possible.

Finally, I would like to dedicate this thesis to my husband Ayman for his unconditional love, patience, support and the inspiration to pursue all our dreams.

REGULATION OF ERYTHROPOIETIN ENDOCYTOSIS AND SIGNALING

Rita Sulahian, PhD.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Supervising Professor: Lily Huang PhD.

Erythropoietin (Epo) and its receptor (EpoR) play an essential role in the survival, proliferation and differentiation of precursor red blood cells into mature erythrocytes. EpoR lacks intrinsic enzymatic activity essential to mediate downstream signaling cascades, instead, it associates with Janus tyrosine kinase 2 (JAK2), which upon Epo binding is auto-phosphorylated and activated. Activated JAK2 phosphorylates many of the tyrosines in the EpoR which recruit SH2-domain containing proteins that will carry on the signaling event. EpoR -/- and JAK2 -/- mice die during embryogenesis due to the absence of definitive erythropoiesis. Once activated, the EpoR is internalized and degraded through unidentified mechanisms. Sustained EpoR signaling and aberrant JAK2 activation are observed in hematological malignancies.

Here we show that cell-surface EpoR is internalized via clathrin-mediated endocytosis. Ligand-dependent EpoR internalization requires both JAK2 kinase activity and EpoR cytoplasmic tyrosines. In addition, phosphorylated Y429, Y431, and Y479 in the EpoR cytoplasmic domain binds p85 subunit of phosphoinositide 3-kinase (PI3-kinase) upon Epo stimulation and individually is sufficient to mediate Epo-dependent

EpoR internalization. Knockdown of p85α and p85β but not inhibition of PI3-kinase activity dramatically impaired EpoR internalization, indicating that p85α and p85β may recruit proteins in the endocytic machinery upon Epo stimulation. We carried on a candidate siRNA screen for endocytic proteins involved in EpoR endocytosis. c-Cbl, a E3 ubiquitin ligase associated with p85, was identified to be essential in mediating EpoR internalization. Ligase-deficient c-Cbl mutants dramatically diminished ligand-induced EpoR internalization. Consistent with this finding, c-Cbl knockout animals exhibit erythroid hyperplasia. We further demonstrate that ubiquitination of the EpoR itself is not required for internalization but may be important for endosomal sorting and degradation, and K428 in the EpoR cytoplasmic domain may be the primary ubiquitination site. Further studies will uncover the role of c-Cbl in EpoR down-regulation. These results provide the first characterization of EpoR downregulation mechanisms.

TABLE OF CONTENTS

Prior Publications

Bazarbachi A, Abou Merhi R, Gessain A, Talhouk R, El-Khoury H, Nasr R, Gout O, Sulahian R, Homaidan F, de Thé H, Hermine O and El-Sabban ME. Human T-cell lymphotropic virus type I-infected cells extravasate through the endothelial barrier by a local angiogenesis-like mechanism. *Cancer Research 64, 2039-2046, March 15, 2004*

Sulahian R, Sikder D, Johnston SA and Kodadek T. The proteasomal ATPase complex is required for stress-induced transcription in yeast. *Nucleic Acids Research 2006 34(5):1351-1357*

Tong W, Sulahian R, Gross AW, Hendon N, Lodish HF, Huang LJ. The membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms. *J Biol Chem. 2006 Dec 15;281(50):38930-40.*

Present Work

Sulahian R, Huang LJ. p85 subunit of PI3 kinase mediates ligand-induced EpoR internalization by binding to Y429, Y431, or Y479 in the EpoR cytoplasmic domain. *Submitted Blood*.

LIST OF FIGURES

LIST OF DEFINITIONS

μg	microgram
μl	microliter
μM	micromolar
EEA1	early-endosomal antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Epo	Erythropoietin
EpoR	Erythropoietin receptor
KO	knockout
siRNA	small interfering RNA

Chapter1. Introduction

1.1 Endocytosis

The cell plasma membrane tightly controls all ingoing and outgoing processes required to preserve cell survival, proliferation and differentiation as well as to prevent cell death. The nature and size of the transferred substance dictate the path to cross the membrane. Small molecules (O_2, H_2O, Na^+, K^+ etc…) have been shown to be transported into and from the cell through various methods such as lipid diffusion, osmosis, passive transport and active transport (Conner and Schmid, 2003). To enter the cell, large molecules (proteins, hormones, etc...) have to rely on a different mechanism called endocytosis. There are several described forms of endocytosis: phagocytosis, pinocytosis, macropinocytosis and receptor-mediated endocytosis (Conner and Schmid, 2003). The word phagocytosis literally means cell eating and is the most primitive form of food uptake illustrated in simple organisms such as amoebas (Tjelle et al., 2000). In phagocytosis, a vacuole forms from the plasma membrane and engulfs the material into the cytoplasm. Once in the cytoplasm, the vacuole, now a phagosome fuses with the lysosome which is an acidic organelle with a high content of hydrolases. These lysosomal enzymes undertake the digestion of the macromolecule content (Tjelle et al., 2000). Pinocytosis (cell drinking) is the uptake of small amounts of fluids through vacuoles and is thought to occur in almost all cells. In contrast, macropinocytosis allows the engulfment of larger amount of fluids through the formation of actin dependent large vesicles. This process is best observed in dentritic cells as part of their function to screen our immune system (Norbury, 2006).

The plasma membrane also maintains a steady pool of receptors which allow the cell to respond to extracellular stimuli. Receptor-mediated endocytosis is by far the most intricate endocytic scheme. In this form of uptake, a ligand binds its specific receptor on the plasma membrane, and initiates the inward budding of the plasma membrane vesicles containing the ligand-receptor complex. An extensive portion of endocytosis literature has been dedicated to the role of endocytosis in signal attenuation through the destruction of the receptor and its ligand in the lysosomes after internalization (Bonifacino and Traub,

2003). Indeed, many receptors that belong to different receptor families such as the receptor tyrosine kinase family (Haglund et al., 2003) or the cytokine receptor family (Walrafen et al., 2005) have been shown to be degraded shortly after ligand induction.

Supporting this important role to attenuate signaling, numerous observations in different diseases involve aberrant receptor down-regulation. The deregulation can affect different stages of the endocytic process. Abnormal regulation of a panel of receptors such as the erbB/Her receptors is believed to be critical in promoting excess cell proliferation in breast, colorectal, ovarian and lung cancers (Roskoski, 2004). These receptors are highly active and appear to escape endocytosis (Roskoski, 2004). In other cases, the problem lies in the endocytic proteins. Oncogenic forms of the E3 ubiquitin ligase c-Cbl fail to properly ubiquitinate the epidermal growth factor receptor (EGFR), a process required for receptor endosomal sorting, resulting in activated receptors being trapped on the surface (Andoniou et al., 1994; Thien et al., 2001).

Recent studies reveal that endocytosis and subsequent endosomal sorting also play a positive role in signal transduction. Internalization allows the activated receptors to recruit signaling proteins present in intracellular compartments inaccessible from the cell surface (Baass et al., 1995). For instance, internalized EGFR recruits Src tyrosine kinase in EEA1 positive endosomes (early endosomes) to initiate the ERK pathway (Donepudi and Resh, 2008). In cells expressing a dynamin mutant (K44A) which blocks EGFR internalization, EGFR was unable to activate the PI3K pathway and the ERK pathway (Vieira et al., 1996). Poor or loss of tyrosine phosphorylation of signaling proteins in the EGF stimulated K44A cells was observed when compared to wild type cells. Interestingly, phosphorylation of EGF was also decreased in K44A-expressing cells, indicating that EGFR is located in intracellular compartments other than on the cell surface (Vieira et al., 1996).

Currently, there are two major pathways for receptor-mediated endocytosis: 1) clathrin independent endocytosis and 2) clathrin-dependent endocytosis. This introductory chapter will devote its attention to clathrin-dependent endocytosis, since the alternative but equally fascinating endocytic pathway is beyond the scope of this dissertation.

Clathrin dependent endocytosis

Coated vesicles were long known to be associated with secretory processes. The structure of coated vesicles was observed in 1976 in pig and bullock brain specimens through electron microscopy (Pearse, 1976) and the major component of these vesicles was identified to be clathrin (Pearse, 1976). Clathrin interacts with different adaptor proteins such as AP2, synaptotagmin, Epsin and AP180 to form the observed coated vesicles (Mousavi et al., 2004).

Clathrin mediated receptor endocytosis consists of the following steps: recruitment of clathrin, sequestration of the receptor into clathrin-coated pits and receptor internalization. After its formation, the clathrin-coated pit invaginates and pinches off to form a clathrin-coated vesicle. Once in the cytoplasm, the vesicle is rapidly uncoated, a process thought to be necessary for subsequent fusion with other membrane structures such as late endosomes or lysosomes (Mousavi et al., 2004).

Many proteins work in tandem and coordinate the endocytic process. The extensively studied EGFR has proven to be a good model to illustrate the whole pathway and introduce all the participating players. Most of the described mechanisms in the following sections are employed by different receptors in clathrin-mediated endocytosis.

The EGFR is auto-inhibited in its basal state. Upon EGF binding, the receptor undergoes autophosphorylation on several tyrosines (Y992, Y1045, Y1068, Y1148 and Y1173) located on its cytoplasmic tail. Some of these phospho-tyrosines recruit key proteins such as Grb2 and c-Cbl (Huang and Sorkin, 2005) and initiate the endocytic process. Mutations or truncations of these tyrosines dramatically affects receptor down-regulation from the surface (Jiang et al., 2003; Sorkin et al., 1992).

Multi-ubiquitination of the cytoplasmic domain of EGFR by c-Cbl recruits several proteins essential for the formation of clathrin coated pits

The first member of the Cbl E3 ubiquitin ligase protein family was discovered in 1989 (Langdon et al., 1989a). The oncogene v-Cbl (Casitas B-lineage lymphoma) is transduced by the mouse Cas-NS-1

retrovirus and is the cause of several lymphomas as well as fibroblast transformation (Langdon et al., 1989a). The main role of E3 ubiquitin ligases such as Cbl is to ubiquitinate a substrate and target it primarily for degradation (Pickart and Eddins, 2004).

Ubiquitin is a 76 amino acid polypeptide. It is covalently linked to its substrate via an isopeptide bond between the C-terminal glycine residue of ubiquitin and a specific lysine residue on the target protein(reviewed in (Pickart and Fushman, 2004) and (Pickart and Eddins, 2004)). Several forms of ubiquitination have been described: a substrate can be monoubiquitinated, multi-monoubiquitinated and polyubiquitinated (reviewed in (Pickart and Eddins, 2004)). The nature of the appended ubiquitin moiety dictates the fate of the targeted protein and regulates related cellular events (reviewed in (Pickart and Fushman, 2004)).

To date, there are three proto-oncogenic proteins that are part of the Cbl family: c-Cbl (Langdon et al., 1989b), Cbl-b (Keane et al., 1995) and the shorter Cbl-c/Cbl-3 (Fiore et al., 2001) and there is a high degree of sequence homology between the three proteins. Cbl family proteins share a phosphotyrosine binding domain (PTB) (also known as the tyrosine kinase binding domain (TKB)) (Roxrud et al., 2008), a short linker region, a ring finger domain to recruit ubiquitin conjugating enzymes (Joazeiro et al., 1999; Zheng et al., 2000) and two proline rich regions that are followed by a variable C-terminal portion. In comparison, v-Cbl has a premature stop codon after amino acid 357and lacks the linker and the ring finger domain which explains its transforming potential (Blake et al., 1991).

The crucial role of c-Cbl in regulating receptor tyrosine kinases was originally discovered in Caenorhabditis elegans (Jongeward et al., 1995). The original aim of these early experiments was to screen for suppressors of hypomorphic mutations of let-23, a receptor tyrosine kinase responsible for vulvar induction (Koga and Ohshima, 1995). One of the hits that were found was sli-1 (suppressor of linage defect). The sli-1 gene encodes a protein homologous to c-Cbl and contains the ring finger domain for E3 ligase activity and multiple consensus binding sites for Src homology 3 (SH3) domains (Jongeward et al., 1995; Yoon et al., 1995).

In mammalian cells, the growth factor receptor binding protein 2 (Grb2) binds to Y1068 and Y1086 of the activated EGFR and couples the receptor to the Ras pathway (Li et al., 1993; Rozakis-Adcock et al., 1993). Apart from its role in signaling, knockdown of Grb2 through siRNA or mutation of both Y1068 and Y1086 abolish EGFR internalization (Jiang et al., 2003). Grb2 was shown to co-localize in the endosomes with EGFR and overexpression of mutated Grb2 proteins (P49L and G203R) that cannot bind c-Cbl and dynamin severely affected receptor internalization (Jiang et al., 2003). Therefore, Grb2 is essential for recruiting c-Cbl to the EGFR (Huang and Sorkin, 2005) and to phosphorylated Y1045 (Grovdal et al., 2004).Subsequently, c-Cbl with its E2 ligase UBCH7, ubiquitinates the receptor (Yokouchi et al., 1999) (Figure 1.1).

The nature of the ubiquitin signal has been initially thought to be a polyubiquitination event (Galcheva-Gargova et al., 1995). However, a set of experiments performed by two groups in 2003 used several antibodies that can recognize different form of ubiquitin chains and showed that EGFR is monoubiquitinated on multiple sites (multi-ubiquitination) (Haglund et al., 2003). Moreover, overexpression of a mutant ubiquitin that cannot form polymeric chains maintained normal EGFR ubiquitination, internalization and degradation in HEK 293T cells (Haglund et al., 2003; Mosesson et al., 2003). Then again more direct experiments using tandem mass spectroscopy implicated EGFR polyubiquitination (Huang et al., 2006). Regardless of the nature of ubiquination, the EGF receptor is clearly ubiquitinated and this ubiquitination is important for its down-regulation. The 2003 studies are considered as ground breaking since they inaugurated a whole new field for ubiquitin (mono-,poly- or multi-ubiquitination) in endosomal sorting and other signaling events (Haglund and Dikic, 2005; Tanaka et al., 2008). In the particular case of EGFR, a ubiquitination event on the receptor is required for EGFR

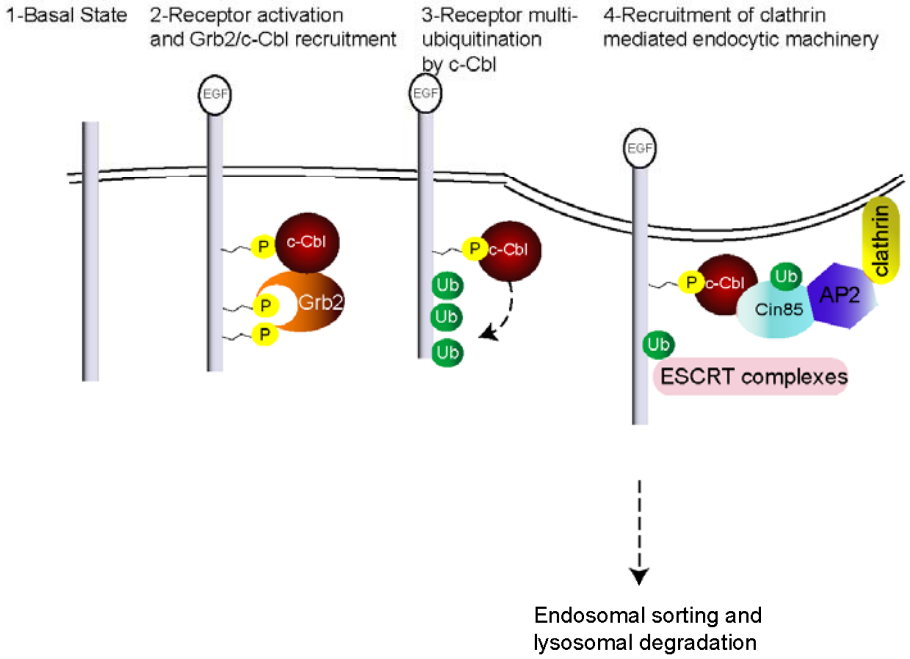

Figure 1.1 A model for EGFR endocytosis.

recruitment into clathrin coated pits (Stang et al., 2004) and endosomal sorting (Ravid et al., 2004) but not for internalization (Huang et al., 2007).

Aside from its E3 ligase activity, c-Cbl can recruit endophilin and AP2 of the endocytic machinery through interaction with Cin85 (Schmidt and Dikic, 2005). c-Cbl can also recruit AP2 and clathrin through the Eps15 protein which is another molecule recruited to activated EGFR and is a substrate of the receptor (Torrisi et al., 1999). Eps15 is a EH domain protein. It regulates clathrin coat assembly via its interaction with the NPF motif of the clathrin adaptor protein AP180 (Morgan et al., 2003). In order for Eps15 to recruit clathrin, EGFR needs to be ubiquitinated by c-Cbl as Eps15 binds to the ubiquitin moiety with its ubiquitin interacting motif (UIM) (de Melker et al., 2004) (Figure 1.1).

Finally, phophatidyl inositols and a couple of other proteins such as dynamins also play a role in clathrin pit formation as reviewed in (Clague and Urbe, 2001) and (Heath et al., 2003) but have not been shown to be directly linked to ubiquitination or Cbl proteins.

Endosomal sorting

Once in the endosome, ubiquitination of the receptor dictates its endosomal sorting and its delivery to the lysosomes or in some cases its recycling to the membrane (Schmidt and Dikic, 2005). Hepatocyte growth factor regulated tyrosine kinase substrate (HRS) is one of the main components of early endosomes. Like Eps15, the UIM domain of HRS is necessary to recognize the ubiquitinated receptor to support proper receptor sorting (Raiborg et al., 2002; Urbe et al., 2003). HRS also interacts with Eps15b, an Eps15 isoform that localizes exclusively in HRS-positive vesicles (Roxrud et al., 2008). Depletion of Eps15b was shown to delay EGFR degradation in the lysosomes while causing a re-routing of the receptor to the cell surface (Roxrud et al., 2008). Recycling of EGFR has also been observed with elevated ALIX protein levels (Schmidt et al., 2004). ALIX or AIP1 binds EGFR and distrupt Cbl/Cin85 complex by competitively binding to Cin85 obstructing efficient internalization (Schmidt et al., 2004).

1.2 Erythropoietin

Erythropoeitin (Epo) was discovered in 1953 by Allan Erslev as a humoral factor that can promote erythrocytosis (Erslev, 1953) a "process by which multipotential hematopoietic stem cells differentiate into mature, non nucleated erythrocytes" (Richmond et al., 2005). Erslev's data showed that injection of plasma from anemic rabbits into healthy rabbits stimulated red blood cell production. Later, it was suggested that that this factor is a hormone produced in the kidneys (Erslev, 1953; Erslev et al., 1980).After many years of trials, erythropoietin was finally cloned in 1985 (Lin et al., 1985) followed by its receptor in 1989 (D'Andrea et al., 1989).

The erythropoietin gene is localized on human chromosome 7 and its product is a protein of 193 amino acids of which 27 are cleaved upon secretion (Lin et al., 1985). The final product is a heavily glycosylated 30kDa glycoprotein. Glycosylation dictates Epo's biological activity and its affinity for the Epo receptor. For example, when terminal sialic acids were removed by sialidase, Epo's half life is dramatically shortened as it is cleared rapidly by the asialoglycoprotein receptor in the liver (Elliott et al., 2004; Stubiger et al., 2005). During embryonic development, Epo is expressed in the liver (Juul et al., 1998). In adults, Epo's primary source are the peritubular cells of the renal cortex (Lacombe et al., 1988). Epo is produced in response to anemic or hypoxic stress to help increase oxygen-carrying capacity in the blood by increasing the number of red blood cells (RBCs) through erythrocytosis (Aispuru et al., 2008). Because of the latter, synthetic Epo is used as a crucial therapeutic agent to boost RBC production during anemia, anemia induced by chemotherapy, chronic renal failure and bone marrow transplantation (Arcasoy, 2008a; Chen et al., 2008; Klaesson, 1999). The glycosylation property of Epo has been investigated extensively by drug companies to produce a more stable synthetic form of Epo for patient administration (Sinclair and Elliott, 2005; Wish and Coyne, 2007).

Apart from its beneficial role, recombinant Epo has also been misused as a doping agent in several sports. By increasing the oxygen-carrying capacity of blood, Epo improves the endurance of athletes. The first major Epo scandal happened during the famed cycling event of the tour de France in 1998. Several teams were found to use a cocktail of doping agents that included Epo to boost the

endurance of their riders. The tour ended with half of its original number of racers since most of them dropped from the race either from disqualification or fear of getting caught. This led to the creation of World Anti-Doping Agency (WADA) whose task is to constantly screen athletes at major events and develop advanced methods to uncover any doping activity (Birkeland et al., 2000; Lundby et al., 2008).

1.3 Erythropoietin receptor

The EpoR is a member of the type I cytokine receptor superfamily characterized by a single hydrophobic transmembrane spanning region, a highly variable cytoplasmic portion and an extracellular portion with conserved cysteine residues. Functionally, all members of this family lack kinase activity and are constitutively bound to cytosolic Janus tyrosine kinases (JAK) which initiate signaling events upon ligand binding (Gadina et al., 2001).

Aside from erythrocytosis, EpoR is also expressed in various non-hematopoietic cells such as gastrointestinal cells, skeletal muscle cells, neural cells and cardiac cells (reviewed in (Arcasoy, 2008b). The function of Epo and EpoR in all these systems is not entirely defined. For instance, EpoR is found in the cardiac tissue where it promotes angiogenesis (reviewed in (Arcasoy, 2008b)). Epo itself has been shown to wield cardioprotection during ischemia though the underlying mechanism is not clear (Arcasoy, 2008b). The EpoR is expressed in many different cell types such as endothelial, epithelial and decidual in the uterine endometrium (Yasuda et al., 2001). It is still not clear if signaling from the EpoR oscillates during a reproductive cycle and what are the consequences apart from local angiogenesis in the endometrium (Yasuda et al., 2001).

The extracellular portion:

Type I cytokine receptors share a WSXWS motif in the extracellular domain. In EpoR, this corresponds to a WSAWS motif critical for receptor folding and surface expression (Furmanek et al., 2003; Hilton et al., 1996).Mutation of the alanine residue in WSAWS with a glycine disrupts the structure and affects the function of the receptor whereas replacing the alanine with glutamic acid resulted in a

receptor that is folded and processed better than the wild type EpoR(Hilton et al., 1995; Yoshimura et al., 1992).The extracellular portion has two major fibronectin type 2 subdomains that are separated by a hinge domain. Crystallographic data shows that the receptor is dimerized on the surface through the four residue hinge domain (Livnah et al., 1999; Syed et al., 1998).Upon Epo binding, the major conformational change affects all four subdomains bridging together the c-terminal membrane proximal region from 76A° to 30A°, a crucial rearrangement proved to be vital for any subsequent signal transduction (Frank, 2002; Remy et al., 1999).

The cytoplasmic portion:

The EpoR like most type I cytokine receptors lacks enzymatic activities in the cytoplasmic portion. To compensate the absence of this critical characteristic, the membrane proximal region is decorated with two motifs: Box1 and Box 2. The Box 1 motif and its flanking amino acids are essential for the binding and activation of JAK2 (Miura et al., 1993; Tanner et al., 1995) whereas Box2 modulates EpoR mitogenic activity. The first study to look at the domains of JAK2 which may be required to bind the conserved Box1 motif concluded that individual JAK2 domains fail to recognize Box 1 and it seems that more than one domain is needed for efficient binding (Tanner et al., 1995). More recent data show clearly that the N-terminal domains of JAK2 are responsible for receptor recognition and specificity (Huang et al., 2001). A carboxyl-terminal truncated EpoR, with only the Box 1 motif is sufficient to induce Epo-dependent cell proliferation (Miura et al., 1993) and a truncated receptor with cytoplasmic sequences not much more than just Box1 and Box2 is capable of inducing normal erythroid development (Miura et al., 1993; Zang et al., 2001).

There are 8 tyrosine residues in the EpoR cytoplasmic domain which upon phosphorylation act as docking sites for a plethora of SH2 domain containing proteins. To name a few: Stat5 a/b, Shp1, SOCS1, SOCS3, Lyn, PLC gamma and p85 alpha the regulatory subunit of PI3K (Figure 1.2) (Richmond et al., 2005).

JAK2 activation

The EpoR relies on Janus tyrosine kinase 2 (JAK2) for signal transduction. Four members constitute the JAK kinase family, JAK1, JAK2, JAK3 andTYK2. These kinases are characterized by seven JAK homology domains (Radtke et al., 2005): A C-terminal kinase domain (JH1), a pseudokinase or kinase like domain thought to regulate kinase activity (JH2) and the N terminal JH3-JH7 domains that play an important task in binding the EpoR and for receptor surface expression (Huang et al., 2001; Radtke et al., 2005).

Several lines of evidence suggested that the EpoR/JAK2 complex assembled in the endoplasmic reticulum (ER) before reaching the cell surface. Cohen et al. found that EpoR can be phosphorylated in the ER (Cohen et al., 1997). Later it was shown that JAK2, specifically its N-terminal domain (JH3-7) is necessary and sufficient to bind to the EpoR in the ER to promote its surface expression (Huang 2001). Therefore, the EpoR/JAK2 complex should also be looked at as one functional entity. Consistent with this notion, JAK2 -/- mice and EpoR -/- mice die during embryogenesis at E12.5 and E13.5 respectively and due to the absence of definitive erythropoiesis (Neubauer et al., 1998; Parganas et al., 1998).

Upon Epo binding, conformational changes in the EpoR resulted in JAK2 trans-phosphorylation and activation. Among these phosphorylated tyrosine residues on JAK2 are Y1007 and Y1008 which are located in the activation loop and are critical for kinase activity, whereas Y972 was shown to be important for optimal kinase activation (Argetsinger et al., 2004; Feng et al., 1997; Kurzer et al., 2004; McDoom et al., 2008). Activated JAK2 then phosphorylates many of the eight tyrosines in the EpoR cytoplasmic domain, thereby providing a platform for the recruitment and activation of signaling mediators through SH2 domain-mediated interactions. It is not yet known how many of the 8 tyrosines in the EpoR cytoplasmic domain JAK2 phosphorylates, however, JAK2 prefers to phosphorylate substrates

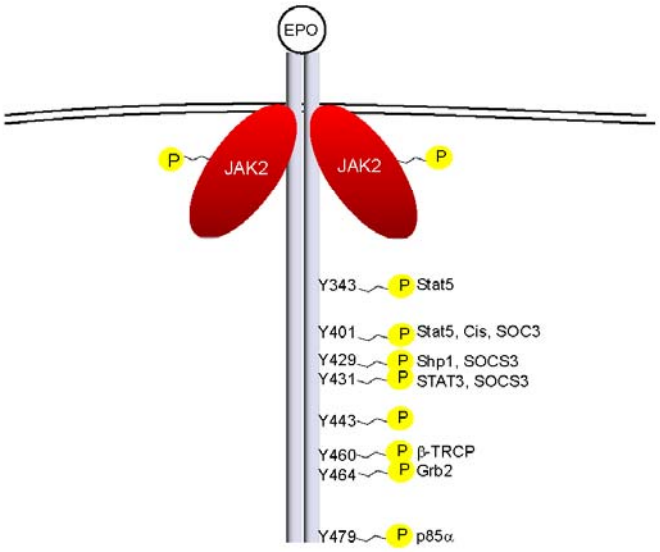

Figure 1.2 Association between activated EpoR and SH2-domain containing proteins

that have the YXX [L/I/V] motif (O'Brien et al., 2003). Four out of the eight tyrosines of EpoR fit this criterion: Y343, Y401, Y429 and Y431. In addition to JAK2, other tyrosine kinases such as Lyn are also recruited to activated EpoR and can phosphorylate the receptor upon Epo stimulation making it hard to decipher the sole contribution of JAK2 (Chin et al., 1998).

The signaling mediators recruited to the phosphorylated tyrosines include the STAT5 transcription factor and the p85 regulatory subunit of Phosphoinositide 3-Kinase (PI3K) (Figure 1.2). Recruited STAT5 protein is phosphorylated by JAK2. Phosphorylated STA5 dimerizes and is translocated into the nucleus and binds to the promoter of specific genes to regulate their transcription (Damen et al., 1997; Li et al., 2003). For example, activated Stat5 induces the expression of the anti-apoptotic factor Bcl-x (Socolovsky et al., 1999). The expression of anti-apoptotic factors during erythroid maturation is crucial to help the immature cells reach full differentiation (Dolznig et al., 2002; Grad et al., 2000).

Upon stimulation, the p85 regulatory subunit of Phosphoinositide 3-Kinase (PI3K) binds to the EpoR and recruits and activates the catalytic subunit of PI3K, which in turn activates Akt (Bouscary et al., 2003; Klingmuller et al., 1997; Zhao et al., 2006). One downstream substrate of Akt is the transcription factor Foxo3a. Foxo3a regulates the transcription of B cell translocation gene 1 (BTG1) to promote erythroid proliferation and maturation (Bakker et al., 2004).

The ERK pathway is also turned on by Epo through the engagement of phospholipase C gamma 1 through phosphorylated Y479 (Halupa et al., 2005).

1.4 Consequences of an aberrant EpoR signaling

Latest work on Epo and EpoR show a growing interest in their participation in cancer and other diseases.

Several lines of evidence suggest that deregulation of EpoR signaling can lead to leukemia. The expression of a mutant form of the EpoR, EpoR($Arg^{129}Cys$), in mouse bone marrow cells results in leukemia (Longmore and Lodish, 1991). Moreover, erythroleukemia can be induced by expressing gp55

in mouse erythroid progenitor cells, because this envelope protein of the Friend Spleen Focus Forming Virus (SFFV) binds and dimerizes the transmembrane domain of the EpoR and causes ligand-independent activation (Constantinescu et al., 1999b; Ruscetti, 1995). Another type of EpoR mutations that displays abnormal and increased signaling is observed with primary familial and congenital polycythemia (PFCP), a proliferative disorder of the red cell lineage characterized by increased red blood cell mass in afflicted patients (Hookham et al., 2007). These receptors are truncated by 59 to 110 amino acids in their cytoplasmic C-terminal domain due to a premature stop codon. The missing portions in these mutants include Y429 which has been described to recruit SHP-1, a phosphatase whose primary role is to dephosphorylate and deactivate JAK2 (Klingmuller et al., 1995).As a result, cells with PFCP EpoRs exhibit hypersensitivity to Epo and prolonged activation of the JAK/STAT pathway (Arcasoy, 2008b; Hookham et al., 2007).

In addition, samples from various cancer patients revealed that Epo and EpoR are over-expressed in breast cancer, non-small cell lung carcinoma, melanomas and ovarian cancer (Acs et al., 2001; Dunlop et al., 2007; Jeong et al., 2008; Kumar et al., 2006). On the other hand, a very serious concern dominates the current clinical field. As mentioned earlier in this chapter, recombinant EpoR is given to patients during the course of their chemotherapy to help boost their red blood cell production to correct drug induced anemia. If Epo and its receptor are also part of promoting the survival and invasion of cancer cells, the safety of recombinant Epo should be questioned. The latest studies addressing the issue do not seem to agree and more studies will be needed to understand Epo's input (Hardee et al., 2005; Solar et al., 2008; Tovari et al., 2008).

Finally, constitutive JAK2 activation has also been implicated in hematological malignancies (Ward et al., 2000). Constitutive activation of JAK2 by a chromosomal translocation with the transcription factor TEL has been shown to be associated with childhood T-cell leukemia, pre-B acute lymphoblastic leukemia (ALL), and atypical chronic myeloid leukemia (CML) (Lacronique et al., 1997; Peeters et al., 1997). Directed expression of TEL-JAK2 fusion in transgenic mice causes the development of CD8+ T-cell leukemia (Carron et al., 2000). Recently, chromosomal translocation between PCM1 and

JAK2 was found in atypical CML and acute leukemia (Bousquet et al., 2005), whereas translocation between BCR and JAK2 resulted in CML (Griesinger et al., 2005). In 2005, many groups implicated a somatic JAK2 mutation (V617F) that leads to the constitutive activation in myeloproliferative diseases (MPD) such as polycythemia vera, essential thrombocythemia, and idiopathic myelofibrosis (Baxter et al., 2005; Goerttler et al., 2005; James et al., 2005; Jones et al., 2008; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). Consistent with the notion that the EpoR/JAK2 complex functions as one entity, the transformation ability of JAK2V617F depends on its binding to receptors such as the EpoR (Lu 2005).

1.5 Attenuation and downregulation of the EpoR

SOCS and tyrosine phosphatases

SOCS proteins target their substrates for ubiquitin-mediated degradation through the SOCS box by recruiting the elongin BC complex which in turns interacts with Von Hippel Landau tumor suppressor protein to form a potent E3 ligase complex (Kamura et al., 1998; Piessevaux et al., 2008). SOCS1, SOCS3 and CIS are induced by Stat5 following Epo induction (Sasaki et al., 2000). SOCS3 binds to phosphorylated Y401 (Sasaki et al., 2000), and to phosphorylated Y429 and phosphorylated Y431(Hortner et al., 2002). However, it is not known if the EpoR and/or JAK2 are its substrates. SOCS1 negatively regulates EpoR signaling by binding phosphorylated Y1007 of JAK2 through its SH2 domain, and results in JAK2 polyubiquitination (Jegalian and Wu, 2002). The polyubiquinated JAK2 is believed to be degraded by the proteasome since treatment of the cells with the proteasome inhibitor MG132 prolongs JAK2 half life (Ungureanu et al., 2002). The ubiquitinated lysine (s) of JAK2 has not been identified. Cytokine inducible protein SH2 domain (Cis) binds to phosphorylated Y401 on the EpoR, however, its course of action remains elusive (Ketteler et al., 2003).

Tyrosine phosphatases also inhibit Epo signaling (Klingmuller et al., 1995). SHP1 binds to phosphorylated Y401 of the EpoR and inactivates JAK2 to terminate Epo signaling (Klingmuller et al., 1995). Overexpression of Shp1 reduces erythroid differentiation in culture and diminishes Epo-induced gene expression (Bittorf et al., 1999).

Endocytosis and degradation

Upon Epo stimulation, cell-surface EpoR is internalized and degraded as few, if any, EpoR molecules recycle back to the cell surface (Beckman et al., 1999; Levin et al., 1998; Walrafen et al., 2005). Neumann *et al.* showed that intermediates of glycosylated EndoH-resistant receptors are detected in the lysosomes when lysosomal function is inhibited by NH_4Cl, chloroquine or leupeptin (Neumann et al., 1993). In the presence of proteasome inhibitors, polyubiquitinated EpoR was enriched after three hours of Epo treatment (Yen et al., 2000). Consistent with these results, cells treated with proteasome inhibitors exhibited prolonged EpoR phosphorylation and signaling (Verdier et al., 2000). A follow-up study by the same group confirmed that both pathways are required for receptor degradation, likely by the E3 ligase beta transducing repeat containing protein (β TRCP) (Walrafen et al., 2005). However, if β-TRCP is depleted from the cells, EpoR still reaches the lysosomes. The study concluded that the polyubiquitination is a signal for additional proteasomal degradation to ensure the removal of all traces of activated EpoR (Meyer et al., 2007). Mechanisms underlying these processes are not clear.

Besides β-TRCP, another E3 ligase, $p33^{rul}$ was shown to ubiquitinate the EpoR. $p33^{rul}$ was identified in a yeast three-hybrid experiment that was conducted with the cytoplasmic portion of EpoR and JAK2 as baits(Friedman et al., 2003). This E3 ligase is phosphorylated upon Epo induction and its activity is required to mediate a mitogenic response (Friedman et al., 2003). Despite the fact that $p33^{rul}$ ubiquitinates EpoR when over-expressed in cells, there is no evidence supporting a role in EpoR degradation (Friedman et al., 2003).

Because increased EpoR signaling creates an imbalance in the cell and causes consequences such as cancer, regulatory mechanisms are employed by the cell to tightly control the amplitude and the length of the signal. Unfortunately, molecular mechanisms underlying ligand-induced EpoR endocytosis are not well defined and are the focus of this thesis.

1.6 Research Aims

As discussed in the preceding sections, the fate of the EpoR following receptor activation is ill-defined. This dissertation intends to further unravel the molecular mechanisms underlying Epo-dependent EpoR endocytosis and down-regulation.

Two major research aims are investigated:

1- Since the EpoR/JAK2 complex is one functional entity, I aim to determine the role of JAK2 in ligand-induced EpoR endocytosis.

2- EpoR is polyubiquitinated upon Epo induction (Yen et al., 2000). . I aim to characterize the role of ubiquitinated EpoR in endocytosis and down-regulation

Chapter 2. p85 subunit of PI3 kinase mediates ligand-induced EpoR internalization by binding to Y429, Y431, or Y479 in the EpoR cytoplasmic domain

Abstract

Erythropoietin (Epo) is essential for mature red blood cells production, and several lines of evidence support a causal role of truncated Epo receptor (EpoR) mutations in primary familial and congenital polycythemia (PFCP). Epo-induced endocytosis of EpoR plays important roles in the down-regulation of EpoR signaling and is the primary means that regulates circulating Epo concentrations. Here we show that cell-surface EpoR is internalized via clathrin-mediated endocytosis. Ligand-dependent EpoR internalization requires both JAK2 kinase activity and EpoR cytoplasmic tyrosines. In addition, phosphorylated Y429, Y431, and Y479 in the EpoR cytoplasmic domain binds p85 subunit of PI3 kinase upon Epo stimulation and individually is sufficient to mediate Epo-dependent EpoR internalization. Knockdown of p85α and p85β or expression of their dominant-negative forms dramatically impaired EpoR internalization, indicating that p85α and p85β may recruit proteins of the endocytic machinery upon Epo stimulation. Furthermore, mutated EpoRs from PFCP patients lacking the three important tyrosines do not bind p85β or internalize upon stimulation. Addition of residues encompassing Y429 and Y431 to these truncated receptors restored p85β binding and Epo sensitivity. Our results identify a novel PI3 kinase activity-independent function for p85β in EpoR internalization and also suggest that defect of this function in truncated EpoRs from PFCP patients may contribute to Epo hypersensitivity and prolonged signaling.

2.1 Introduction

Erythropoietin (Epo) is the primary cytokine regulating red blood cell production and its function is mediated through the Epo receptor (EpoR). Like most cytokine receptors, EpoR lacks intrinsic enzymatic activities and relies on the cytosolic tyrosine kinase JAK2 for signal transduction. Epo, EpoR, or JAK2 deficient mice die embryonically due to severe anemia. The binding of Epo to the EpoR activates JAK2 kinase activity. Activated JAK2 then phosphorylates many of the eight tyrosines in the EpoR cytoplasmic domain, thereby providing a platform for the recruitment and activation of signaling mediators through SH2 domain-mediated interactions. These signaling events ultimately result in the survival, proliferation, and differentiation of erythroid progenitor cells (Constantinescu et al., 1999a; Richmond et al., 2005). The signaling mediators include the STAT5 transcription factor and the p85 regulatory subunit of Phosphoinositide 3-Kinase (PI3K). p85 binds to phosphorylated Y479 of the EpoR upon stimulation (Klingmuller et al., 1997). This recruits and activates the catalytic subunit of PI3K, which in turn activates AKT, promoting erythroid proliferation and maturation (Bouscary et al., 2003; Klingmuller et al., 1997; Zhao et al., 2006). In addition to erythropoiesis, Epo and EpoR also function in non-hematopoietic tissues. In neurons, binding of Epo to the EpoR induces expression of anti-apoptotic genes that protects them from environmental insults such as ischemia and trauma. Epo also induces proliferation of cells of skeletal muscle, kidney, and intestine (Arcasoy, 2008b).

To ensure proper amplitude and duration of Epo signaling, mechanisms are turned on upon activation to attenuate EpoR signal transduction. For example, phosphorylated Y429 in the EpoR recruits the tyrosine phosphatase SHP-1 (SH2 domain-containing protein-tyrosine phosphatase-1) which inactivates JAK2 (Klingmuller et al., 1995). In addition, the synthesis of Suppressor Of Cytokine Signaling (SOCS) family proteins is induced which inactivates JAK2 and/or block access of STAT5 to receptor binding sites (Krebs and Hilton, 2001). Moreover, Epo binding promotes endocytosis and degradation of the EpoR (Beckman et al., 1999; Levin et al., 1998; Sawyer and Hankins, 1993; Walrafen et al., 2005).The importance of these regulations is underscored by the association of EpoR mutations with primary familial and congenital polycythemia (PFCP), a proliferative disorder of the red cell lineage

characterized by increased red blood cell mass (Hookham et al., 2007). The EpoR variants associated with PFCP have deletions that remove 59 to 110 amino acids of the cytoplasmic C-terminal domain, including Y429 that recruits SHP-1, and exhibit hypersensitivity to Epo and prolonged activation of the JAK/STAT pathway (Arcasoy, 2008b; Hookham et al., 2007). Consistently, a murine model where the EpoR gene was replaced with one of the human PFCP EpoR mutant shows marked polycythemia (Divoky et al., 2001).

Epo-induced endocytosis is a rapid and efficient way to decrease Epo responsiveness, as the cell-surface level of EpoR controls cellular Epo sensitivity (Suzuki et al., 2002). It also may bring about destruction of activated protein complexes and terminate signaling. Moreover, EpoR endocytosis plays a critical role in the clearance of Epo and thus regulates circulating Epo concentrations and its bioactivity (Gross and Lodish, 2006). Upon Epo stimulation, cell surface EpoR is internalized and degraded as few, if any, EpoR molecules recycle back to the cell surface (Beckman et al., 1999; Levin et al., 1998; Walrafen et al., 2005). Degradation of the EpoR is sensitive to inhibitors of both proteasomal and lysosomal function and requires ubiquitination by β-Trcp (Meyer et al., 2007; Walrafen et al., 2005). Mechanisms underlying these processes are not well understood.

In addition to its contribution to signaling, JAK2 is required for the EpoR to exit the endoplasmic reticulum and for EpoR expression on the cell surface (Huang et al., 2001). Therefore, JAK2 is an essential subunit of the EpoR. Because the EpoR/JAK2 complex essentially functions as a receptor tyrosine kinase, we hypothesize that JAK2 might regulate EpoR endocytosis. Here we show that following Epo stimulation, cell surface EpoR is internalized via clathrin-mediated endocytosis. This process requires both JAK2 kinase activity and EpoR cytoplasmic tyrosines. We further show that (phospho)Y429, (phospho)Y431, or (phospho)Y479 in the EpoR cytoplasmic domain is sufficient to mediate Epo-dependent EpoR internalization, and that p85β binding to these tyrosines plays an important role in this process. In addition, we show that mutated EpoRs from PFCP patients lacking the three important tyrosines do not bind p85β and do not internalize upon stimulation. This defect may contribute to their hypersensitivity to Epo and prolonged signaling.

2.2 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. Expression vectors of p85α and p85β were from OriGene. cDNAs corresponding to the SH2 domains for p85α and p85β as described in (Holt et al., 1994) were isolated by PCR and subcloned into the pcDNA3.1 vector. γ2A and Ba/F3 cells stably expressing wild type or mutant HA-EpoRs and various forms of JAK2 were generated as previously described (Huang et al., 2001). Antibodies were from the following sources: HA, Covance; JAK2, phospho-JAK2, Millipore; p85β, EEA1, clathrin heavy chain, Santa Cruz; V5, Invitrogen; actin, Sigma; SOCS3, Abcam; p85α, phospho-Akt, Cell Signaling. HA affinity resin was from Roche Applied Science. Horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence system were from Amersham Biosciences. Wortmannin was from Sigma.

Labeling and immuno-precipitation of cell surface EpoR (Surface IP)

γ2A cells stably expressing HA-EpoR with various JAK2 constructs were incubated with blocking buffer (PBS with 5% normal mouse serum) for 30 min at 4°C. Subsequently, cells were incubated with 10 μg/mL of anti-HA antibodies in blocking buffer for 60 min at 4°C. After washing with PBS, cells were lysed in 1% NP-40 lysis buffer containing 5 μg/mL of HA peptide in order to block any residual anti-HA antibodies. After incubation with protein A/G agarose for 90 min at 4°C, immuno-precipitants were washed three times with PBS containing 1% TritonX-100 and 0.1% SDS, run on SDS-PAGE, and immuno-blotted with anti-HA antibodies. In experiments where Epo treatment was performed, cells were stimulated with Epo (30 units) at 37°C for the duration indicated.

Flow cytometry and data analysis

Surface expression of the wild type or mutant HA-EpoRs was measured in the presence of wild-type or mutant JAK2 in one million γ2A cells as described previously.(Tong et al., 2006) For Epo

stimulation, cells were incubated with 30 units of Epo at 37°C. The median fluorescence of allophycocyanin (APC), which is conjugated to secondary antibodies that recognize anti-HA antibodies, was used to quantify the level of receptor on the cell surface. For each sample, the EpoR surface expression was normalized to that from samples co-expressing wild-type JAK2 prior to Epo stimulation. Each point represents data from three independent experiments. To inhibit PI3K activities, cells were incubated with wortmannin at concentrations indicated for 3 hours prior to Epo stimulation.

Glycosidase digestion of EpoR and MTT cell proliferation assay

Glycosidase treatment of the EpoR with EndoH (New England Biolabs), or with PNGaseF (New England Biolabs) and neuraminidase (New England Biolabs), and MTT cell proliferation assay were performed as described (Tong et al., 2006).

Immuno-precipitation and immuno-blotting

γ2A cells transiently transfected with p85β in the vector pCMV6-XL6 were starved for 12 hours in DMEM media with 1% BSA followed by Epo induction for the appropriate time as indicated. Cells were then lysed with 1% NP-40 lysis buffer with protease and phosphatase inhibitors. Lysates were immuno-precipitated with HA affinity resin or anti-p85β antibodies. The precipitates were eluted with SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-p85β antibodies or antibodies to HA. Bound antibodies were detected by the ECL chemiluminescence system after incubation with horseradish peroxidase-coupled secondary antibodies. The lysates were also immuno-blotted with antibodies to HA, p85β or phosphorylated JAK2.

Immunofluorescence

γ2A cells stably expressing HA-EpoR were seeded on glass coverslips. Coverslips were blocked with PBS containing 3% BSA and 5% normal goat serum for 30 min at –4°C and incubated with 20 μg/mL anti-HA antibodies for 1h. Coverslips were then washed 3 times in cold PBS and put back in the

incubator for Epo stimulation. Subsequently, cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with methanol at 4°C for 15 min. Coverslips were then incubated with antibodies for EEA1 (1:100) or clathrin heavy chain (1:300) for 45 min in blocking buffer. After washing in PBS, coverslips were incubated with Alexa Fluor 555-conjugated goat-anti mouse secondary antibodies, washed 3 times with PBS, and then incubated with Alexa Fluor 647-conjugated goat anti-rabbit secondary antibodies. Coverslips were washed three times with PBS and mounted onto coverslides with a semi-permanent Mowiol mounting medium (Calbiochem). Fluorescent images were taken on a Leica TCS SP5 confocal microscope with 40X oil objective lenses with numeric aperture of 1.25N. Confocal section images were acquire by Leica acquisition software and analyzed with Image J and Adobe Photoshop.

siRNA knockdown

Synthetic siRNAs to knockdown the different human genes were ordered from Dharmacon Inc., and delivered to the cells at 100nM using Dharmafect as a transfection agent. Sequences of siRNAs used for SOCS3: CCG CUU CGA CUG CGU CGU CAA and UCG GGA GUU CCU GGA CCA GUA, and for clathrin heavy chain: UCC AA UU CG AAG ACC AAUU. On-target plus Duplex siRNA pools from Dharmacon Inc. were used for p85α and p85β J-00302017 and J-003021-12). Knockdown efficiency was determined by immuno-blotting with the indicated antibodies 48 hours after treatment with siRNAs.

2.3 RESULTS

2.3.1 JAK2 tyrosine kinase activity is required for ligand-induced EpoR internalization

The role of JAK2 in ligand-induced EpoR down-regulation was examined in JAK2-null γ2A cells that stably co-expressed HA-tagged EpoR with JAK2, kinase-deficient JAK2 (JAK2KD), or vector. In these cells, the EpoR existed in two forms on SDS-PAGE: a faster migrating immature form that was Endo H sensitive and a slower migrating mature form that was Endo H resistant (Figure 2.1A left panels). As previously shown (Huang et al., 2001), little mature EpoR was detected in the absence of JAK2. JAK2 co-expression markedly increased the mature Endo H resistant form of the EpoR, and this JAK2-dependent maturation of EpoR did not depend on JAK2 kinase activity (Figure 2.1A and (Huang et al., 2001)). Stimulation with Epo down-regulated the EpoR and JAK2 kinase activity was essential for this process (Figure 2.1A right panels and (Walrafen et al., 2005)). The presence of the HA tag on the extracellular domain allowed mature cell-surface EpoR species to be specifically isolated by incubating non-permeabilized cells with an anti-HA antibody followed by immuno-precipitation (surface IP). Consistent with previous results, cell surface EpoRs were detected in the presence but not in the absence of JAK2 (Figure 2.1 B, - stimulation). Upon Epo stimulation, the majority of mature EpoR disappeared from the cell surface in the presence of JAK2 (Figure 2.1 B, + stimulation). In contrast, in the presence of JAK2KD, mature EpoR remained on the cell surface upon stimulation (Figure 2.1 B). These results show that JAK2 kinase activity is essential for ligand-induced EpoR internalization and degradation.

We next used flow cytometry to determine the kinetics of Epo-induced receptor internalization. In these assays, cell surface HA-EpoRs were detected by staining non-permeabilized cells with an anti-HA antibody followed by allophycocyanin (APC)-conjugated secondary antibodies. Because HA-EpoR was 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 HA-EpoR at the cell surface, the median APC fluorescence intensity was used.

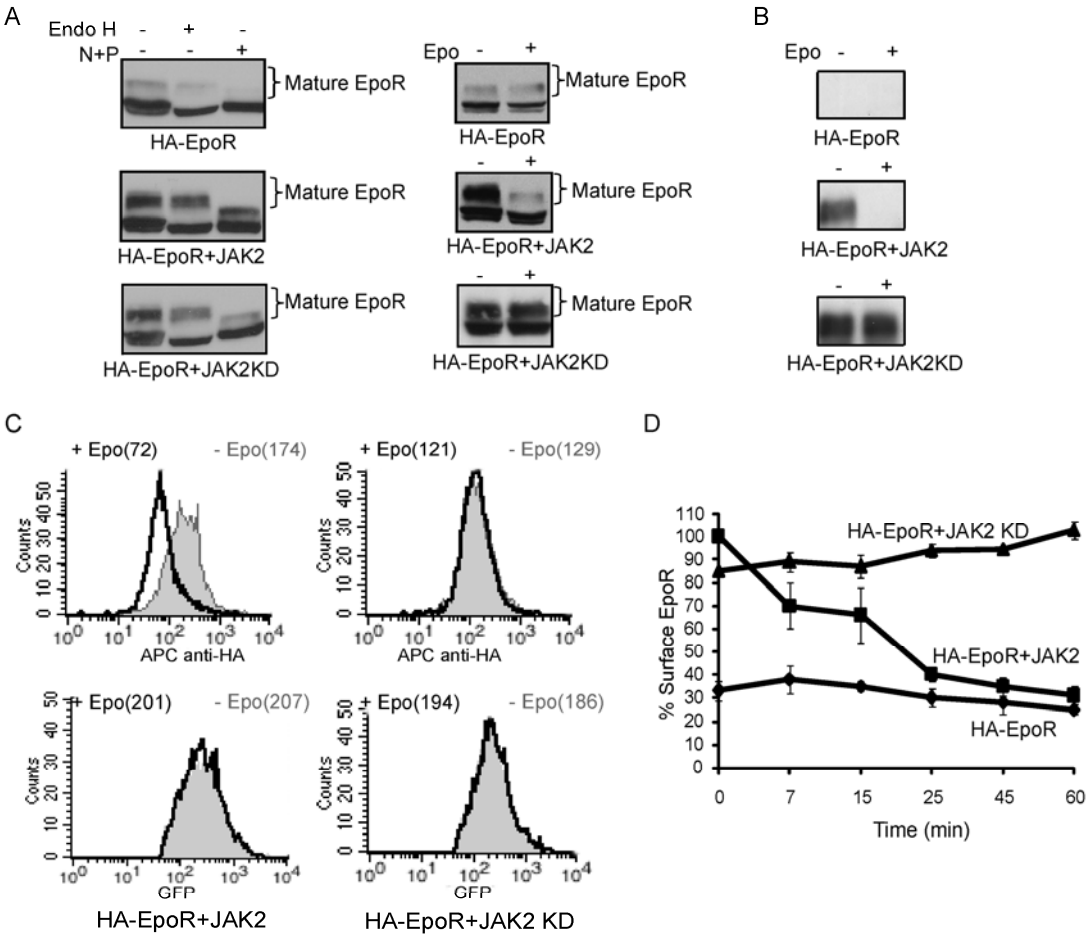

Figure 2.1: JAK2 tyrosine kinase activity is required for ligand-induced EpoR downregulation. (A) Mature HA-EpoR species were detected in cells stably expressing HA-EpoR with wild type or kinase-deficient JAK2 (JAK2KD) by their resistance to Endo H treatment (left). 45 min post Epo induction, mature HA-EpoR was degraded, and this degradation requires JAK2 kinase activity (right). (B) HA-EpoR at the cell surface was detected by surface-IP. Upon Epo induction, surface EpoR disappeared when co-expressed with wild type but not JAK2KD. (C) Cell surface HA-EpoR was quantified by flow cytometry using APC-conjugated anti-HA antibodies in non-permeabilized γ2A cell. Representative histograms of total receptor expression levels (GFP) and cell surface receptor expression levels (APC) are shown. In each histogram, the un-induced sample is in gray and the induced sample is in black.Median fluorescence of each sample is in parenthesis.(D) Kinetics of ligand-induced EpoR internalization. Levels of cell surface HA-EpoR were analyzed by flow cytometry at various time points after induction as described in (C). All data represent results from at least three independent experiments.
EndoH: endoglycosidase H. N+P: neuraminidase and PNGaseF. IB: immuno-blot. V: vector.

γ2A cells stably expressing HA-EpoR with JAK2 or JAK2KD expressed similar total EpoR protein (as judged by median GFP fluorescence) and their cell surface EpoR expression levels were similar (as judged by median APC fluorescence pre-stimulation, Figure 2.1C). Consistent with previous results (Figure 2.1 A, B), Epo-induced EpoR internalization was detected as early as 7 minutes and reached the maximum by 60 min in JAK2 cells (Figure 2.1 C, D). In contrast, no receptor was lost from the cell surface in JAK2KD cells (Figure 2.1C, D). Similar Epo-induced internalization kinetics were observed for the EpoR in the hematopoietic cell line Ba/F3 (data not shown).

2.3.2 The EpoR internalizes via clathrin-coated pits

The fate of cell surface EpoRs upon Epo induction was investigated by confocal immuno-fluorescence microscopy. Non-permeabilized cells were labeled with anti-HA antibodies prior to Epo induction. Cells were stimulated with Epo for 25 min and subsequently permeabilized and visualized with fluorescence-conjugated secondary antibodies. This approach thus followed only surface tagged receptors prior to Epo treatment. In JAK2 cells, Epo treatment caused the EpoR staining to shift from the plasma membrane to an internal compartment that co-localized with the early endosomal marker, EEA1 (early endosome antigen 1). Importantly, no internalized receptor was observed in JAK2KD cells (Figure 2.2).

We next examined the role of clathrin in ligand-induced EpoR internalization. As shown in Figure 2.3 A, cell surface EpoRs co-localized with clathrin on the plasma membrane 7 min after Epo addition (Figure 2.3 A), suggesting that Epo-dependent internalization of the EpoR involves clathrin coated pits. Consistent with this possibility, siRNA-mediated knockdown of clathrin heavy chain (to about 10% of controls, Figure 2.3 D) dramatically impaired receptor internalization as measured by flow cytometry, while control siRNA had no detectable effect on EpoR endocytosis (Figure 2.3 B, C, D). In contrast, EpoR did not co-localize with clathrin in JAK2KD cells (Figure 2.3 A). These results show that EpoR internalizes via clathrin-mediated endocytosis, and that JAK2 kinase activity is required for EpoR internalization.

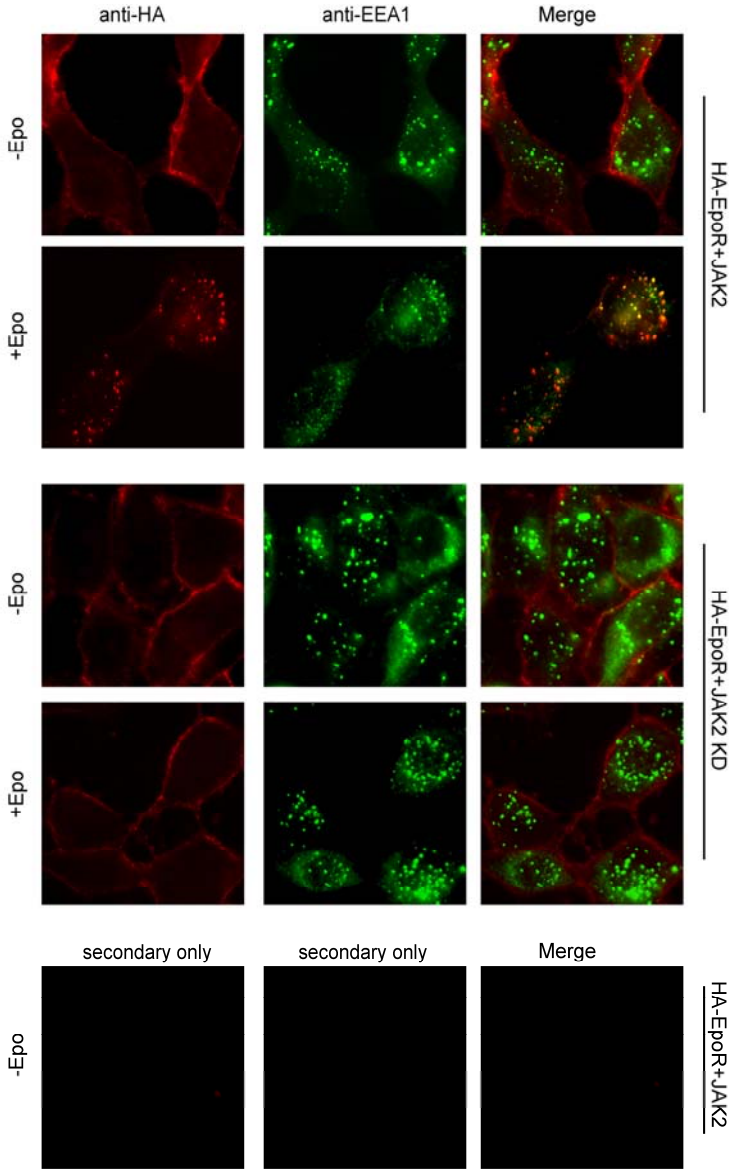

Figure 2.2. JAK2 kinase activity is required for ligand-induced EpoR co-localization with early endosomal marker EEA1. Cell surface HA-EpoRs were labeled with anti-HA antibodies prior to Epo stimulation of 25 min. Cells were fixed and immuno-stained with anti-EEA1 antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocal images (single section) for different conditions are presented. Images from negative controls with secondary antibodies alone are shown. Original magnification 40X (Leica TCS SP5).

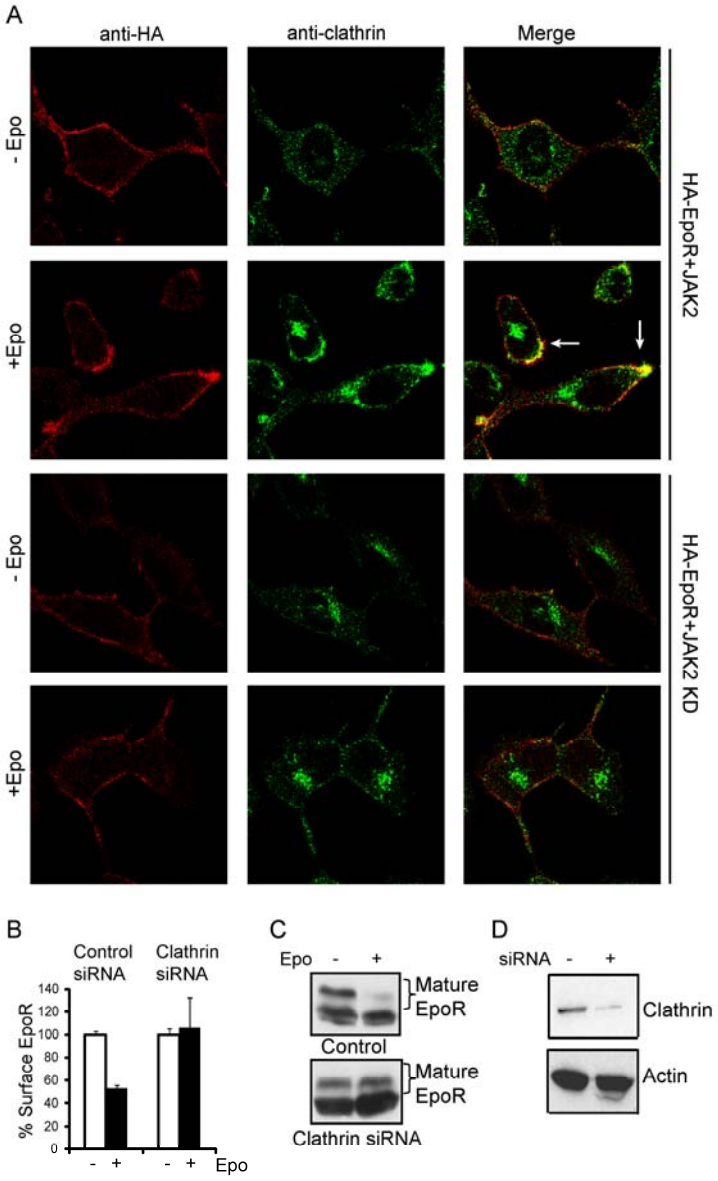

Figure 2.3: EpoR internalization is through a clathrin-dependent pathway.
(A) γ2A cells expressing HA-EpoR with JAK2 or JAK2KD were seeded on glass coverslips. Cell surface HA-EpoRs were labeled with anti-HA antibodies prior to Epo induction. 7 min post induction, cells were fixed and immuno-stained with anti-clathrin antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocalimages (single section) are presented. Selected areas of co-localization are indicated with arrows. Original magnification 40X (Leica TCS SP5). (B) Knockdown of the clathrin heavy chain abolished ligand-induced EpoR internalization. γ2A cells were transfected with siRNAs to the clathrin heavy chain and surface EpoR was analyzed by flow cytometry. (C) Knockdown of the clathrin heavy chain abolishedEpo-induced EpoR degradation. (D) Knockdown efficiency of the clathrin heavy chain shown by immuno-blotting. Immuno-blotting of the same blot with actin was shown as a control.

2.3.3 Y429, Y431 and Y479 in the EpoR cytoplasmic domain mediate EpoR internalization

Upon Epo stimulation, JAK2 phosphorylates multiple tyrosines in the EpoR cytoplasmic domain. These phospho-tyrosines serve as docking sites for proteins essential for signaling. We hypothesized that these phosphorylated tyrosines also recruit proteins critical for receptor internalization. To test this hypothesis, we examined an HA-tagged mutant EpoR with all eight tyrosines substituted by phenylalanines (HA-F8). In the presence of JAK2, little internalization of HA-F8 was detected and HA-F8 failed to degrade after Epo stimulation (Figure 2.4 A). HA-F8 also did not co-localize with clathrin or EEA1 in the presence of Epo (Figure 2.4 B). These results indicated that tyrosine residue(s) are essential for EpoR internalization and subsequent degradation, possibly by the recruitment of proteins in the endocytic machinery. Similar results were observed in the hematopoietic cell line Ba/F3 (data not shown).

In order to determine which of the eight tyrosines are important for EpoR internalization, individual tyrosine residues were added back to the HA-F8 receptor. Results from surface-IP (Figure 2.4 C) and flow cytometry analyses (Figure 2.4 D, E) demonstrated that Y429, Y431 and Y479 (as in HA-F7Y429, HA-F7Y431 and HA-F7Y479) restored EpoR internalization, while Y343, Y401, Y443, Y460 and Y464 did not. To determine whether Y429, Y431 and Y479 are necessary for internalization in a wild-type receptor background, we mutated them individually to phenylalanine. As shown in Figure 2.4 F, mutating any one of the three important tyrosine residues did not affect receptor internalization, suggesting that these tyrosines are functionally redundant. Consistent with these results, simultaneously replacing Y429 and Y431 (as in Y2F) affected EpoR internalization, although the effect was not statistically significant. Replacing all three tyrosines (as in Y3F) significantly impaired receptor internalization (Figure 2.4 F, $P=0.025$).

2.3.4 p85 is important for ligand-induced EpoR internalization

We investigated whether known binding proteins for Y429, Y431 or Y479 played a role in EpoR internalization. Phosphorylated Y429 binds to SHP1 (Klingmuller et al., 1995), however, SHP1 was not

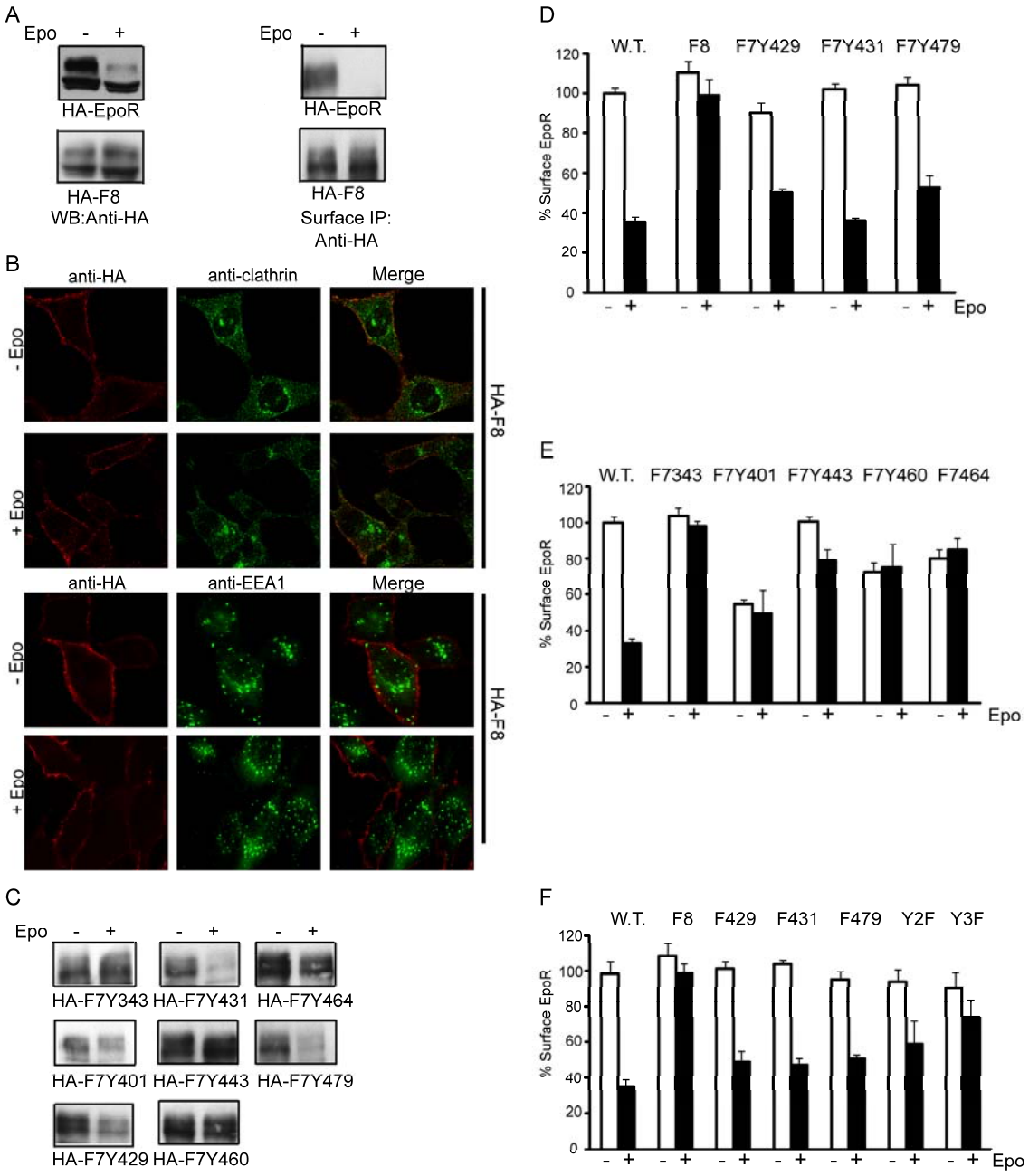
A
Epo - +
HA-EpoR
HA-F8
WB:Anti-HA
Epo - +
HA-EpoR
HA-F8
Surface IP:
Anti-HA
B
anti-HA
anti-clathrin
Merge
- Epo
+ Epo
HA-F8
anti-HA
anti-EEA1
Merge
- Epo
+ Epo
HA-F8
C
Epo - + - + - +
HA-F7Y343 HA-F7Y431 HA-F7Y464
HA-F7Y401 HA-F7Y443 HA-F7Y479
HA-F7Y429 HA-F7Y460
D
W.T. F8 F7Y429 F7Y431 F7Y479
% Surface EpoR
120
100
80
60
40
20
0
- + - + - + - + - + Epo
E
W.T. F7343 F7Y401 F7Y443 F7Y460 F7464
% Surface EpoR
120
100
80
60
40
20
0
- + - + - + - + - + - + Epo
F
W.T. F8 F429 F431 F479 Y2F Y3F
% Surface EpoR
120
100
80
60
40
20
0
- + - + - + - + - + - + - + Epo

Figure 2.4. Y429, Y431 and Y479 mediate ligand-induced EpoR internalization.
(A) Wild-type EpoR but notF8 is degraded (left) upon Epo stimulation. Wild-type EpoR but not F8 is internalized upon Epo stimulationas detected by surface-IP (right). (B) F8 does not co-localize with clathrin or EEA1 upon stimulation. Original magnification 40X (Leica TCS SP5). (C) Y429, Y431 or Y479 mediates ligand-induced EpoR internalization. Surface-IP was performed on γ2A cells stably expressing HA-EpoR constructs with individual cytosolic tyrosine. (D) and (E), Y429, Y431 or Y479 but not other tyrosines is sufficient for ligand-induced EpoR internalization. Ligand-induced receptor internalization was measured by flow cytometry. (F) Replacing Y429, Y431 or Y479individually on the wild-type EpoR (F429, F431 and F479) or replacing both Y429 and Y431 (Y2F) did not affect receptor internalization by flow cytometry, but simultaneously mutating all three tyrosines (Y3F) significantly reduced Epo-induced receptor internalization.
* indicates P= 0.025 (unpaired t-test) vs. control.

detected in γ2A cells, and SHP1 siRNA had no effect on EpoR internalization (data not shown). Phosphorylated Y429 and Y431 bind SOCS3 (Hortner et al., 2002), and phosphorylated Y479 binds to the p85α regulatory subunit of PI3K (Klingmuller et al., 1997). Knockdown of SOCS3 or p85α by siRNA also did not affect EpoR internalization (Figure 2.5). Because the residues around Y429 and Y431 resemble the binding consensus sequence for the p85β C-terminal SH2 domain (Huang et al., 2008), we next tested whether p85β was required for EpoR internalization. Knockdown of p85β alone caused a small but statistically significant decrease in EpoR internalization (Figure 2.5, P=0.001), and simultaneously knocking down p85α and p85β impaired EpoR internalization (Figure 2.5, P=0.001). Therefore, p85α and p85β share redundant roles in EpoR internalization. The residual internalization may be due to low knockdown efficiency.

2.3.5 Characterization of the mechanism of p85-dependent EpoR internalization

Because binding of p85α to the EpoR recruits and activates the catalytic subunit of PI3K, we tested whether PI3K kinase activity played a role in ligand-induced EpoR internalization. Treatment of JAK2 cells with the PI3K inhibitor, wortmannin, inhibited PI3K, as indicated by loss of AKT phosphorylation, but did not impair EpoR internalization (Figure 2.6 A). These results indicate that p85 does not promote EpoR internalization through a PI3K kinase activity-dependent mechanism.

We next tested whether p85α and p85β act as adaptors for proteins other than the catalytic subunit of PI3K that are important in receptor internalization. We generated vectors expressing either the N- or the C-terminal SH2 domain from p85α and p85□.respectively. We reasoned that these SH2 domains would interact with signaling partners and phospho-tyrosines in the EpoR and function as dominant-negative proteins. γ2A cells stably expressing HA-EpoR and JAK2 were transfected with individual or combinations of these SH2 domains and Epo-induced EpoR internalization was examined. Interestingly, simultaneous expression of the N-terminal SH2 domains from P85α and p85β significantly decreased EpoR internalization (Figure 2.6 B, P=0.0002). The N-SH2 domains of p85α and p85β have been shown

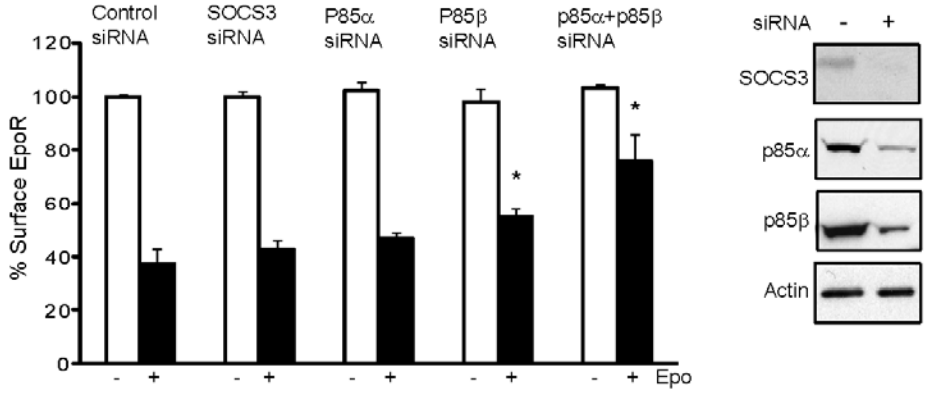

Figure 2.5. Concurrent knockdown of p85α and p85β diminishes receptor internalization. Receptor internalization was detected by flow cytometry in γ2A cells stably expressing EpoR and JAK2 and transfected with 100 nM of siRNAs to SOCS3, p85α, p85β, or p85α and p85β together. Scrambled siRNA to GFP was used as a negative control. Immuno-blots of each targeted protein demonstrate knockdown efficiency. Representative immuno-blotting with actin was shown as a control. * indicates P=0.001 (unpaired t-test) vs. control.

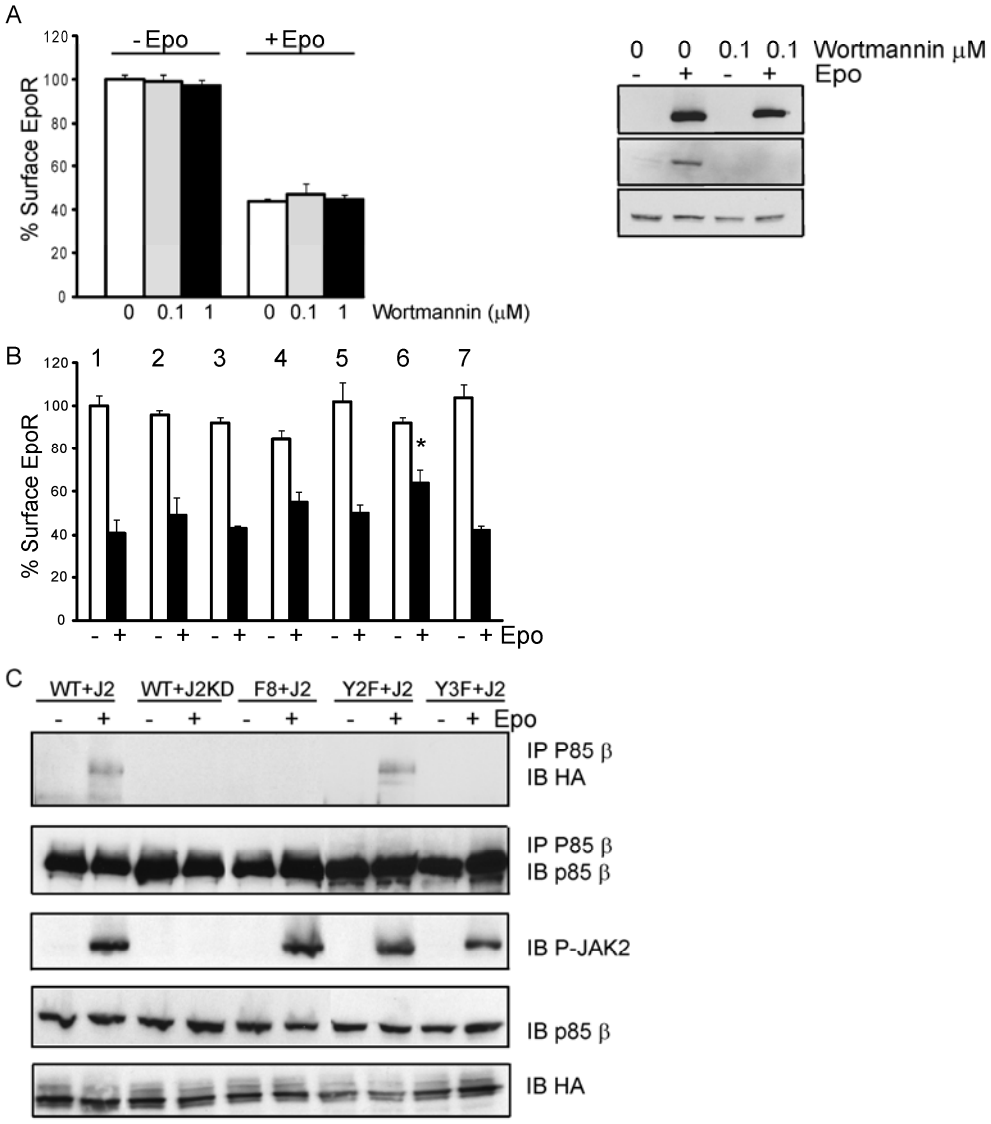

Figure 2.6. p85α and p85β are important in mediating EpoR internalization.
(A) γ2A cells stably expressing HA-EpoR and JAK2 were treated with wortmannin for 2 hrs at indicated concentrations followed by Epo stimulation. 45 minutes post Epo stimulation, surface EpoRs were quantified by flow cytometry. Wortmannin inhibited AKT activation as detected by phospho-AKT antibodies. (B) Epo-induced EpoR internalization was measured by flow cytometry in γ2A cells stably expressing HA-EpoR and JAK2 transiently transfected with vectors expressing the N or C terminal-SH2 domains from p85α and p85β. 1: vector control; 2: p85α N-terminal SH2; 3: p85α C-terminal SH2; 4: p85β N-terminal SH2; 5: p85β C-terminal SH2; 6: p85α and p85β N-terminal SH2s; 7: p85α and p85β C-terminal SH2s. * indicates P= 0.0002 (unpaired t-test) vs. control.
(C) γ2A cells stably expressing HA-EpoR and JAK2 were transiently transfected with vectors expressing full-length p85β. 48 hrs post transfection, cells were starved overnight and treated with Epo for 10 min. Cell lysates were subjected to immuno-precipitation by p85β antibodies and immuno-blotted with anti-HA antibody for the receptor. Cell lysates were also subjected to immuno-blotting with antibodies to active JAK2 (P-JAK2), p85β and HA. IP: immuno-precipitation. IB: immuno-blot.

to bind to YXXM motif (Funaki et al., 2000) found in proteins like c-Cbl (Hartley et al., 1995), which plays important roles in clathrin-mediated endocytosis of receptor tyrosine kinases(Haglund et al., 2003). Together these observations suggest that p85 proteins act as adaptors linking the EpoR to the endocytic machinery.

p85α binds to phosphorylated Y479 in the EpoR cytoplasmic domain upon Epo stimulation (Miura et al., 1994), however, whether p85β also interacts with the EpoR is not clear. Upon Epo induction, p85β interacted with wild type EpoR but not F8, and this interaction is lost when JAK2KD rather than JAK2 was co-expressed (Figure 2.6 C). Therefore, p85β interacts with the EpoR, and this binding likely requires phosphorylated tyrosine(s) in the EpoR cytoplasmic domain. We thus examined the ability for p85β to interact with Y2F or Y3F upon induction. Y2F and Y3F activated JAK2 to the same degree as wild type EpoR or F8 as shown by antibodies specifically recognize activated JAK2 (P-JAK2) (Figure 2.6 C). Consistent with our results showing that internalization is defective in Y3F but not Y2F (Figure 2.6 G), p85β was able to bind Y2F but not Y3F (Figure 2.6 C). Therefore, Y479 is likely a p85β-binding site. We also tested binding between p85β and EpoR harboring a single tyrosine Y429 or Y431. However, no interaction was detected (data not shown), possibly because both tyrosines are required for optimal p85β binding.

2.3.6 Truncated EpoRs from PFCP patients do not internalize upon Epo stimulation

C-terminal deletions of the EpoR are associated with PFCP. These EpoR variants exhibit increased Epo sensitivity and prolonged signal transduction compared to wild-type EpoR (Arcasoy, 2008b; Hookham et al., 2007). Interestingly, all truncated PFCP receptors lack Y429, Y431 and Y479. We hypothesized that the deletions associated with PFCP impair EpoR internalization and that this defect contributes to the prolonged signaling from these truncated receptors. To test this hypothesis, we engineered two EpoR truncations based upon truncations identified in PFCP patients. The first construct, Stop1, lacks residues after S409 and corresponds to the most extensive EpoR truncation identified from PFCP patients (Motohashi et al., 2001). The second construct, Stop2, lacks residues after L427 and is

analogous to the minimal EpoR truncation identified from PFCP patients (Figure 2.7 A). The ability of γ2A cells stably expressing these receptors to internalize EpoR was tested by our flow cytometry internalization assay. As shown in Figure 2.7 B, Epo-induced internalization was virtually absent with Stop1 whereas only 20% of the receptors internalized with Stop2 compared to wild-type EpoR. Interestingly, when KYLYL, the residues surrounding Y429 and Y431, were appended to the C-terminus of Stop2, Epo-stimulated internalization was restored. Furthermore, Stop2+KFLFL, where the two Ys (corresponding to Y429 and Y431) in the KYLYL sequence were mutated to F, showed an internalization rate similar to Stop2 (Figure 2.7 B). These results suggest that truncated EpoRs from PFCP patients have defective internalization, and that the Y429 and Y431 in KYLYL are essential for Stop2+KYLYL internalization. These results are also consistent with our data showing that Y429 and Y431 are sufficient for EpoR internalization (Figure 2.4.F).

Because our results suggest a role of p85β in EpoR internalization, we examined the interaction between p85β and truncated EpoRs based on PFCP patients. p85β did not interact with Stop1 or Stop2 (Figure 2.7 C). Importantly, we detected Epo-inducible interaction between p85β and Stop2+KYLYL, which contains Y429 and Y431, whereas no interaction was detected between p85β and Stop2+KFLFL where Y429 and Y431 were mutated (Figure 2.7 C). Therefore, p85β likely binds via Y429 and/or Y431 in Stop2+KYLYL.

The added KYLYL motif bears a lysine residue. In some receptors such as the leptin receptor ubiquitinated lysine residues are essential for internalization (Belouzard and Rouille, 2006). Therefore, we mutated the lysine residue in the KYLYL motif into arginine. Figure 2.8 A shows that the internalization rate in the Stop2+RYLYL mutant is decreased by 12% when compared to Stop2+KYLYL. This decrease was not statistically significant ($p=0.08$, unpaired t-test versus Stop3+KYLYL). Binding to p85β in Stop3+RYLYL was not disrupted. We conclude that ubiquitination on this lysine residue is not important for internalization.

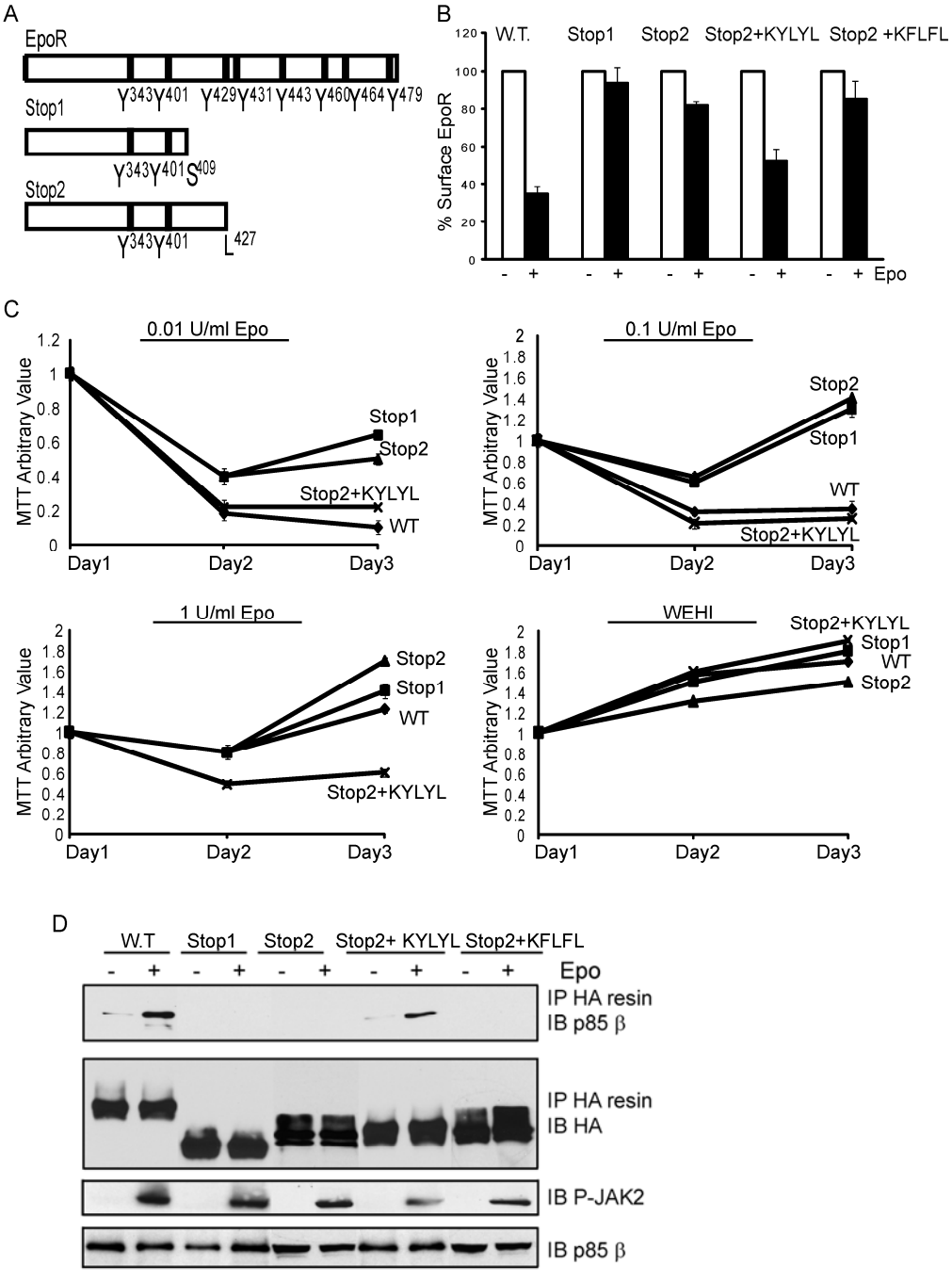
A
EpoR
Y343Y401 Y429Y431Y443Y460Y464Y479
Stop1
Y343Y401S409
Stop2
Y343Y401 L427
B
W.T. Stop1 Stop2 Stop2+KYLYL Stop2 +KFLFL
% Surface EpoR
- + - + - + - + - + Epo
C
0.01 U/ml Epo
MTT Arbitrary Value
Stop1
Stop2
Stop2+KYLYL
WT
Day1 Day2 Day3
0.1 U/ml Epo
Stop2
Stop1
WT
Stop2+KYLYL
1 U/ml Epo
Stop2
Stop1
WT
Stop2+KYLYL
WEHI
Stop2+KYLYL
Stop1
WT
Stop2
D
W.T Stop1 Stop2 Stop2+ KYLYL Stop2+KFLFL
- + - + - + - + - + Epo
IP HA resin
IB p85 β
IP HA resin
IB HA
IB P-JAK2
IB p85 β

Figure 2.7. Truncated EpoR mutants fail to bind p85β or internalize upon Epo stimulation. (A) Schematic diagram for truncated EpoR mutants used in our studies based on those from PFCP patients. (B) Truncated EpoR mutants did not internalization upon Epo treatment by flow cytometry. (C) Stop2+KYLYL but not Stop1, Stop2 or Stop2+KFLFL interacted with p85β upon Epo induction. (D) Truncated EpoR mutants are hypersensitive to Epo. Ba/F3 cells stably expressing wild type or truncated EpoR mutants were grown under different Epo concentrations. Cell numbers were measured by MTT assays every 24 hrs for 3 days. Cell numbers from cultures in WEHI media as a source of IL-3 were shown as controls. IP: immunoprecipitation. IB: immuno-blot. P-JAK2: phosphorylated active JAK2.

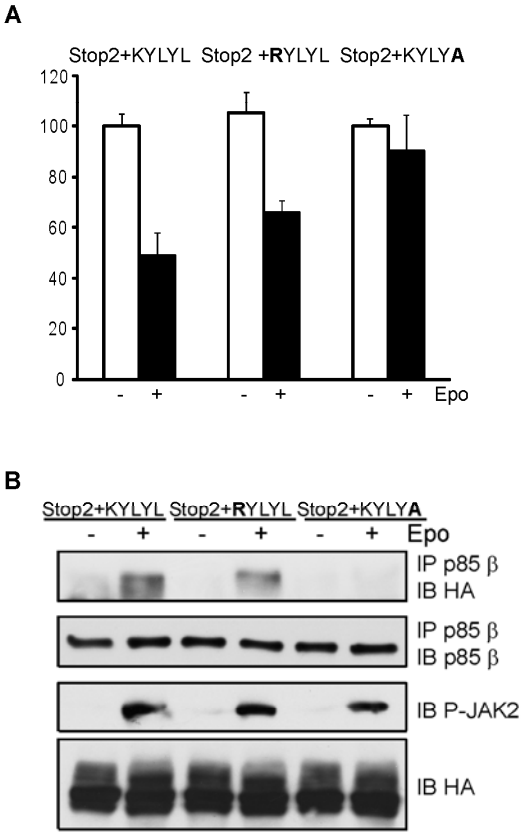

Figure 2.8. **Function of the non-phosphorylated residues in the KYLYL motif in the truncated PFCP mutant**s. (A) Epo-induced EpoR internalization was measured by flow cytometryin γ2A cells stably expressing Stop2+KYLYL (or designated point mutations) and JAK2 transiently transfected with a vector expressing full lenght wild type p85β. Truncated EpoR mutant Stop2+ RYLYL still internalized but not Stop2+ KYLYA upon Epo treatment. (B) Stop2+KYLYL but not Stop2+KYLYA retain its binding capacity with p85β upon Epo induction.

The proposed binding consensus sequence for the p85β C-terminal SH2 domain is Y(V/I/E/**L**)(N/I/M/**L**)(M/V/**L**/T) (Huang et al., 2008).We generated a Stop2+KYLYA construct. This receptor failed to internalize (Figure 2.8 A). Moreover, we did not detect an interaction with this mutant to p85β (Fig. 2.8B). These data reinforce the important role of p85β as the main link to internalization and suggest that YLYL acts as an endocytic motif for EpoR (Figure 2.8 B).

Together with data from Figures 2.5 and 2.6, our results indicate that p85α, together with p85β, play a role in ligand-induced EpoR internalization via a PI3K kinase activity-independent mechanism. It does so by binding to Y429 and/or Y431, and Y479 in the EpoR cytoplasmic domain upon stimulation, and act as an adaptor to recruit proteins in the endocytic machinery.

Previous reports demonstrated that truncated EpoRs from PFCP patients display an increased sensitivity to Epo compared to wild-type EpoR (Arcasoy, 2008b). We examined our EpoR truncations for Epo sensitivity in the IL-3 dependent hematopoietic Ba/F3 cells. Ba/F3 cells stably expressing the different truncated 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 2.7 D, cells expressing Stop1 and Stop2 grew normally in Epo at 1 U/mL. However, cells expressing Stop1 and Stop2 were able to grow in 0.1 U/mL or 0.01 U/mL of Epo, whereas cells expressing wild-type receptor did not (Figure 2.7 D). Importantly, cells expressing Stop2+KYLYL, which internalized after Epo stimulation similar to wild type EpoR, showed similar Epo sensitivity compared to wild-type EpoR. As controls, cells expressing all constructs grew similarly in WEHI IL-3 conditioned media (Figure 2.7 D).

These results suggest that upon Epo stimulation, JAK2-dependent phosphorylation of Y429, Y431 and Y479 results in the recruitment of p85, which promotes EpoR internalization by acting as an adaptor to engage the endocytic machinery. Moreover, truncated EpoRs in PFCP patients lacking these tyrosines have impaired internalization, which contributes to hypersensitivity to Epo.

2.4 DISCUSSION

Receptor endocytosis is a major mechanism whereby cells control the magnitude and duration of signaling induced by extracellular ligands. For the EpoR, this process also controls cellular sensitivity to Epo (Suzuki et al., 2002), and is the primary means that regulates circulating Epo concentrations (Gross and Lodish, 2006). Our results demonstrate that cell surface EpoR is internalized via clathrin-mediated endocytosis. Ligand-dependent EpoR internalization requires both JAK2 kinase activity and EpoR cytoplasmic tyrosines. We further show that phosphorylated Y429, Y431, and Y479 in the EpoR cytoplasmic domain share redundant functions and individually is sufficient to mediate Epo-dependent EpoR internalization. These phosphotyrosines bind p85 upon Epo stimulation and may recruit proteins in the endocytic machinery. In addition, mutated EpoRs from PFCP patients lacking the three important tyrosines do not bind p85β and do not internalize upon stimulation. This defect may in part explain their hypersensitivity to Epo and prolonged signaling.

We show that Epo induces EpoR internalization via clathrin-mediated endocytosis, similar to other cytokine receptors such as the prolactin receptor, thrombopoietin receptor, growth hormone receptor, and gp130 (Hitchcock et al., 2008; Tanaka et al., 2008; van Kerkhof et al., 2000; Vincent et al., 1997). Importantly, Epo-induced EpoR internalization requires both JAK2 kinase activity and the tyrosine residues of the EpoR cytoplasmic domain. These results are consistent with studies demonstrating that ligand stimulated internalization of the prolactin receptor (Swaminathan et al., 2008) and the thrombopoietin receptor (Hitchcock et al., 2008) also depends on associated JAK2 kinase activities. Moreover, as the EpoR/JAK2 complex functions as a unit that is equivalent to a receptor tyrosine kinase, these results are consistent with experiments showing that kinase activity is necessary for maximal rate of internalization and down-regulation of receptor tyrosine kinases (Glenney et al., 1988; Sorkin et al., 1991; Sorokin et al., 1994).

Our results differ from two previous reports. One study showed that a mitogenically inactive EpoR mutant EpoR(W282R) can still internalize radio-labeled Epo, and that a tyrosine-null EpoR still internalizes Epo albeit less efficiently (Beckman et al., 1999). Because W282 is part of the JAK2

interaction site and changing it to Ala abolishes JAK2-dependent EpoR surface expression (Huang et al., 2001), the mutated receptors that reached the plasma membrane in that study are not likely to have formed a normal complex with JAK2. The other study showed that inhibiting JAK2 kinase activity with the AG490 inhibitor impaired internalization of radio-labeled Epo, and monensin treatment partially restored internalization (Walrafen et al., 2005). It was concluded that EpoR was internalized when JAK2 activity was blocked but that internalized receptors recycled to the plasma membrane. In addition, proteasomal degradation removes the portion of the EpoR cytoplasmic domain containing all of the tyrosine residues before the receptor is internalized (Walrafen et al., 2005). We did not observe any recycling of EpoR in our JAK2KD cells (data not shown). We reason that the residual JAK2 activity after AG490 treatment may be sufficient for receptor internalization. In addition, monensin is known to have pleiotropic effects on vesicular trafficking. Alternatively, the differences between these studies may have resulted from differences in cell lines and methodology involved. At an Epo concentration much higher than used herein, we did observe some internalization of F8 (data not shown), although at a level less than 50% compared to wild-type EpoR. In contrast, no internalization was detected in the presence of JAK2KD under all Epo concentrations tested (data not shown). Therefore, there may exist an internalization mechanism that depends on JAK2 but not EpoR tyrosines at high Epo concentrations. This mechanism may involve proteasomal removal of part of the EpoR cytoplasmic domain. As the endocytosis of EGF receptors vary with EGF concentrations (Haglund et al., 2003), EpoR may also employ different internalization mechanisms depending on Epo concentrations.

Our characterization of the mechanism of EpoR internalization suggests a novel function for p85 that is independent of PI3K kinase activities. Upon receptor activation, p85α binds to Y479 (Klingmuller et al., 1997), while p85β binds to Y429/Y431 and Y479 in the EpoR cytoplasmic domain (Figures 2.6,2.7). p85 may act as a scaffold that recruits proteins of the endocytic machinery. p85α and p85β interact directly (Hartley et al., 1995), or indirectly through Grb2 (Donovan et al., 1994; Jiang et al., 2003; Wang et al., 1995),with the ubiquitin ligase c-Cbl, which recruits endophilin and AP-2 (through Cin85) of the endocytic machinery (Schmidt and Dikic, 2005). Both c-Cbl and Grb2 have previously been shown to

be important for recruitment of EGFRs into clathrin-coated pits (Jiang et al., 2003). p85 may also bind dynamin, a protein that plays important roles in clathrin-mediated endocytosis (Booker et al., 1993; Gout et al., 1993), or interact through the Grb2-SOS-Ras linkage to activate Rab5 (Heath et al., 2003), a protein implicated in the formation of clathrin-coated vesicles and fusion of these vesicles to early endosomes (Gorvel et al., 1991; McLauchlan et al., 1998). Alternatively, p85 may exert GAP activities toward GTPases critical for clathrin-mediated endocytosis thus regulating their function. This hypothesis is based on the observation that p85α has GAP activity for Rab5, and for Cdc42 which was implicated in vesicle movement (Chamberlain et al., 2004). Although no GAP activities have yet been demonstrated for p85β, p85β shares the GAP domain of p85α.

Our results add to the emerging concept that p85 has functions independent of its regulatory function of PI3K kinase activity (Chamberlain et al., 2008; Garcia et al., 2006; Jimenez et al., 2000; Taniguchi et al., 2006; Ueki et al., 2003). For example, cytokinesis defects observed in p85α-deficient cells are restored by expression of a p85α mutant that does not bind the PI3K catalytic subunit (Garcia et al., 2006), and that a p85α mutant with defective GAP activities caused cellular transformation via a kinase-independent mechanism (Chamberlain et al., 2008). PI3K is activated by most cytokine receptors, and a common step in the activation involves the recruitment and interaction of p85 with components of the cytokine receptor complex. Whether p85 also contributes to ligand-dependent internalization of other cytokine receptors is currently being tested.

Y429, Y431 and Y479 in the EpoR cytoplasmic domain are individually sufficient to support Epo-stimulated EpoR internalization. Mutations of all these tyrosines to phenylalanines or deletions that remove these tyrosines such as those found in PFCP patients prevent Epo-induced EpoR internalization. Consistent with a role of p85β in EpoR internalization, both Stop1 and Stop2 failed to interact with p85β. Importantly, fusion of KYLYL but not KFLFL, residues encompassing Y429 and Y431, to Stop2 restored both p85β binding and ligand-induced internalization. Moreover, the hypersensitivity to Epo of cells expressing Stop2 was reversed when KYLYL was fused to Stop2. Therefore, the PFCP EpoR truncations not only prevent recruitment of SHP-1 that inactivates JAK2, but also impair endocytosis of EpoR. Both

defects prolong the duration of signaling. To our knowledge, this is the first report linking internalization to signaling in polycythemia. Consistent with the notion that defects in internalization prolong signaling, mutations in the granulocyte colony-stimulating factor receptor that inhibit ligand-induced internalization lead to prolong receptor activation and acute myeloid leukemia (Hunter et al., 1999; Ward et al., 1999). In addition, oncogenic forms of human EGFR are less efficiently internalized (Grandal et al., 2007). Understanding the precise mechanism responsible for attenuating receptor signaling will provide insight into PCFP and other diseases that are caused by excessive receptor signaling.

Chapter3. c-Cbl is essential for ligand-mediated EpoR internalization

Abstract

A candidate siRNA screen for proteins that are part of the endocytic machinery and for proteins that participate in EpoR downstream signaling revealed that c-Cbl is essential in mediating ligand-mediated EpoR internalization. Consistent with a role in EpoR down-regulation, c-Cbl knock-out mice display hemopoietic hyperplasia, characterized by splenomegaly primarily of erythroid hyperplasia, fibrosis and extensive extramedullary hemopoiesis. Studies performed on the EGFR showed that c-Cbl mediated ubiquitination is needed for EGFR internalization and proper endosomal sorting. In addition to its E3 ligase activity, c-Cbl acts as an adaptor that is capable of recruiting a variety of endocytic proteins. In this study, we show that c-Cbl is phosphorylated upon Epo induction and that JAK2 can phosphorylate c-Cbl in vitro. c-Cbl phosphorylation is a pre-requisite for its E3 ligase activity. Accordingly, we demonstrate that c-Cbl E3 ligase activity is critical for ligand-dependent EpoR internalization through the use of c-Cbl mutants that have defective ligase activities. We propose that in addition to recruiting endocytic proteins, c-Cbl may ubiquitinate the p85 subunit of the PI-3 kinase to mediate EpoR internalization upon stimulation.

3.1 Introduction

In the previous chapter, we provided evidence for a novel function for the regulatory subunit of PI3K, p85β and p85α, in mediating ligand-induced EpoR internalization. We also described that p85β binds to Y429 and/or Y431, and Y479 in the EpoR cytoplasmic domain upon stimulation. We hypothesize that p85β and p85α act as adaptors to recruit proteins from the endocytic machinery.

There are several described endocytic proteins known to interact with different domains of p85 (α,β). More importantly, some of them have been shown to participate in erythropoiesis through a direct or an indirect association with an activated EpoR/JAK2 unit.

The first and most relevant candidate is the E3 ligase c-Cbl. c-Cbl is an important component of the endocytic machinery as it down-regulates signaling of active tyrosine kinase receptors such as the EGFR by targeting them for lysosomal degradation (Ravid et al., 2004). Phosphorylated c-Cbl associates specifically with p85β in stimulated B lymphocytes (Kim et al., 1995) and in Jurkat T cells (Hartley et al., 1995). This interaction relies on phosphorylated Y731 in the pYXXM motif of c-Cbl and the N-terminal SH2 domain of p85β (Standaert et al., 2004). Phosphorylation on other tyrosine residues such as Y368 and Y371 has been reported to activate the E3 ligase activity of c-Cbl by inducing a conformational change that will allow the transfer of ubiquitin to the targeted substrate (Kassenbrock and Anderson, 2004; Ryan et al., 2006). c-Cbl is phosphorylated upon Epo induction, however, the phosphorylation site(s) have not yet been characterized (Barber et al., 1997b; Odai et al., 1995). In addition, the function of the phosphorylated c-Cbl upon Epo stimulation is not known. c-Cbl knock-out mice display hemopoietic hyperplasia, characterized by splenomegaly primarily of erythroid lineage, fibrosis and extensive extramedullary hemopoiesis, indicating that it plays a role in erythropoiesis (Murphy et al., 1998) (Naramura et al, PNAS 1998). Besides being recruited by p85, c-Cbl also can bind indirectly to the activated receptor via Grb2 and recruits Crk which in turn links the receptor to the Rap1/ERK signaling pathway (Barber et al., 1997b).

The second protein that interacts with p85 and that plays a critical role in bringing the endocytic machinery to the EGFR is Grb2 (Jiang et al., 2003). Grb2 is a cytoplasmic adaptor protein consisting of

one SH2 domain flanked by two SH3 domains and mediates the activation of the Ras signaling pathway. It was suggested that Grb2 interacts with EpoR through a consensus binding motif located atY464 on the EpoR (Barber et al., 1997a).

Finally, dynamin, one of the most closely associated endocytic proteins, binds to the SH3 domain of p85. However, it is not known if dynamin interacts with the EpoR. We used siRNA to knockdown these proteins and examine their role in ligand-induced EpoR endocytosis. Along with these three candidates, we picked an array of endocytic and signaling proteins and performed a candidate siRNA screen for proteins participating in ligand-induced EpoR endocytosis.

3.2 Materials and Methods

Plasmid constructs, cell lines, and reagents

Wt Cbl cloned in pSRα vector is a generous gift from Dr Joachim Herz. All Cbl mutants were generated in the pSRα vector using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. γ2A cells stably expressing wild type or mutant HA-EpoRs and various forms of JAK2 were generated as previously described (Huang et al., 2001). The same method was used to generate stable MEF cells expressing HA-EpoR. Cbl +/+, -/+, and -/- MEFS were a generous gift from Dr. Brian Druker (Oregon Health and Science University). Antibodies were from the following sources: HA, Covance; p85β, clathrin light chain, Cis1, Stat5 a, Stat5b, Santa Cruz; actin, Sigma; SOCS1, SOCS3, SOCS4,SOCS5, SOCS6 and SOCS7 Abcam; p85α, Shp2, Ship1, Ship2, PLCγ, Cell Signaling; Cbl, Lyn, , Crk, Crkl, Shc1, clathrin heavy chain, Grb2, Ap2, Caveolin, BD Bioscience; 4G10, Upstate; HRS, Alexis Biochemical; Cin85 and Dynamin Calbiochem. Horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence system were from Amersham Biosciences.

Flow cytometry and data analysis

Surface expression of the wild type or mutant HA-EpoRs was measured in the presence of wild-type or mutant JAK2 in one million γ2A cells or 10,000 MEF cells as described previously (Tong et al.,

2006). For Epo stimulation, cells were incubated with 30 units of Epo at 37°C. The median fluorescence of allophycocyanin (APC), which is conjugated to secondary antibodies that recognize anti-HA antibodies, was used to quantify the level of receptor on the cell surface. For each sample, the EpoR surface expression was normalized to that from samples co-expressing wild-type JAK2 prior to Epo stimulation. Each point represents data from three independent experiments.

Immuno-precipitation and immuno-blotting

γ2A cells transiently transfected with Cbl in the vector pSRα were starved for 12 hrs in DMEM media with 1% BSA followed by Epo induction for the appropriate time as indicated. Cells were then lysed with 1% NP-40 lysis buffer with protease and phosphatase inhibitors. Lysates were immuno-precipitated with anti-Cbl antibodies. The precipitates were eluted with SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-Cbl antibodies or antibodies to 4G10. Bound antibodies were detected by the ECL chemiluminescence system after incubation with horseradish peroxidase-coupled secondary antibodies.

Kinase assay

293T cells transiently transfected with GST-JAK2 kinase domain construct, (or GST-JAK2 kinase domain dead) were lysed 1% NP-40 lysis buffer with protease and phosphatase inhibitors. Lysates were incubates with glutathione beads to pull down JAK2 constructs. Cbl was immunoprecipitated as described in the previous section; however it was not eluted with SDS sample buffer. Both proteins were kept on their respective beads and were set for kinase assay, 60 min at 37°C in the following buffer: 10mM HEPES pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM Na_3VO_4,1mM DTT, 10 mM glycerophosphate, complete protease inhibitors and 10 mM ATP.

siRNA knockdown

Synthetic siRNAs to knockdown the different human genes were ordered from Dharmacon Inc., and delivered to the cells at 100nM using Dharmafect as a transfection agent. Sequences of siRNAs used for SOCS3: CCGCUUCGACUGCGUCGUCAA and UCGGGAGUUCCUGGACCAGUA; clathrin heavy chain: UCCAAUUCGAAGACCAAUU; BTRC L003463:UGACAACACUAUCAGAUUAUU, CACAUAAACUCGUAUCUUAUU, GACCUUAAAUGGACAAAUU, ACACCGAGCUGCUGUCAAUUU; CRKL L-012023: CCGAAGACCUGCCCUUUAAUU, GGUGAGAUCCUAGUGAUAAUU, UACGGACUCUGUAUGAUUUUU, CAGAAGAUAACCUGGAAUAUU; CRK L-010503: GGACAGCGAAGGCAAGAGAUU, GAAUAGGAGAUCAAGAGUUUU, GGUGAGCUGGUAAAGGUUAUU, GGACAAGCCUGAAGAGCAGUU; Shc1 L-018841: GACAAUCACUUGCCCAUCAUU, GAGUUGCGCUUCAAACAAUUU, CACGGGAGCUUUGUCAAUAUU, GACUAAGGAUCACCGCUUUUU; CISH L-017381: CCGACUACCUCCGACAGUAUU, GCUGAUACCCGAAGCGACAUU, GAAUCUGGCUGGUAUUGGGUU, GACAACACCUGCAGAAGAUUU;
SOCS1: CCCGCCGUGCACGCAGCAUUA, CUGGUUGUUGUAGCAGCUUAA; Lyn: CGGACGACUUGUCUUUCA;
SOCS3 CCGCUUCGACUGCGUCGUCAA, UCGGGAGUUCCUGGACCA
GUA; Cin85: GAGGAUAAGGAGGAACACAUU, GGAUUACUGCAAAGUAAUAUU; Clathrin: UCC AA UU CG AAG ACC AAUU; Intersectin GG AC AUA GUU GUA CUG AAA UU; c-Cbl: GGA GACA CAU UUC GGA UUA, GAU CUG ACC UGC AAU GAUU,
GAC AAU CCC UCA CAA UAAA, CCA GAAA GCU UUG GUC AUU;
Dynamin 2: CAA GCACGUCUUCGCCAUC, GAGCCGAGUUUGAAGUGUG

On-target plus Duplex siRNA pools from Dharmacon Inc. were used for p85α J-00302017, p85β J-003021-12), PDCD6IP or HP95/ALIX (M-004233), PTPN6 or Shp1 (M-009778), HGS/HRS (M-

016835), SOCS3 (M-004299), c-Cbl (M-003003), and Grb2 (M-019220). Knockdown efficiency was determined by immuno-blotting with the indicated antibodies 48 hrs after treatment with siRNAs.

3.3 Results

3.3.1 siRNA candidate screen identifies c-Cbl and Lyn in mediating EpoR ligand mediated internalization

To identify targets involved in ligand mediated internalization of EpoR, we screened a selective pool of siRNAs. The effect of each siRNA on internalization was measured through flow cytometry. Figure 3.1 summarizes results from our screen. The majority of proteins we chose to study did not affect internalization, however, not all the target proteins have commercially available antibodies. It is thus possible that a couple of hits were missed since we do not know if the knockdown was complete in these samples. Proteins whose knockdown affected ligand-induced EpoR internalization can be divided into two groups. One group affected ligand-mediated EpoR internalization, which includes Grb2, HP95/ALIX and p85 (please refer to chapter 2 for a detailed study on p85). The other group, including clathrin, c-Cbl and Lyn, completely abolishes ligand mediated EpoR internalization. The role of clathrin in ligand-induced EpoR internalization was discussed in Chapter 2. We will focus on the role of c-Cbl in EpoR internalization in this chapter.

3.3.2 c-Cbl is essential for ligand-induced EpoR internalization

As shown in Figure 3.2, knocking down c-Cbl to 90% of endogenous levels completely abolished EpoR internalization upon stimulation. As a control, we measured internalization of transferrin receptor in these knock-down cells. Fluorescent-labeled transferrin was added to the cells 48h after c-Cbl siRNA

Targeted genes	Internalization	Knockdown efficiency (WB)
Scrambled control	+++	N/A
AP2	++	partial
β-TRCP	+++	N/A
Caveolin	+++	partial
c-Cbl	-	good
Cin85	++	N/A
Cis1	+++	good
Clathrin	-	good
Crk	++	partial
CrkL	+++	N/A
Dynamin II	+++	good
Grb2	+	partial
HP95	+	good
HRS	++	good
Intersectin	+++	good
Lyn	-	partial
p85α	+++	good
p85β	++	good
p85 α+β	+	good
PLC γ	+++	partial
Shc	+++	good
Ship1	+++	N/A
Ship2	+++	partial
Shp1	+++	N/A
Shp2	+++	good
SOCS1	+++	good
SOCS2	N/A	N/A
SOCS3	+++	good
SOCS4	+++	N/A
SOCS5	+++	partial
SOCS6	+++	no knockdown
SOCS7	+++	N/A
Stat5a	+++	good
Stat5b	+++	good
SYK	+++	N/A
UBCH7	+++	N/A
ZAP70	++	N/A

Figure 3.1: Clathrin, c-Cbl and Lyn play a role in mediating EpoR internalization. EpoR internalization was examined by flow cytometry in γ2A cells stably expressing EpoR and JAK2 and transfected with100 nM of indicated siRNAs. Western blots for all targets with available commercial antibodies were done to demonstrate knockdown efficiency.
+++: normal internalization, ++: internalization rate < 50%,
+: internalization rate < 25%, - : no internalization. N/A not available.

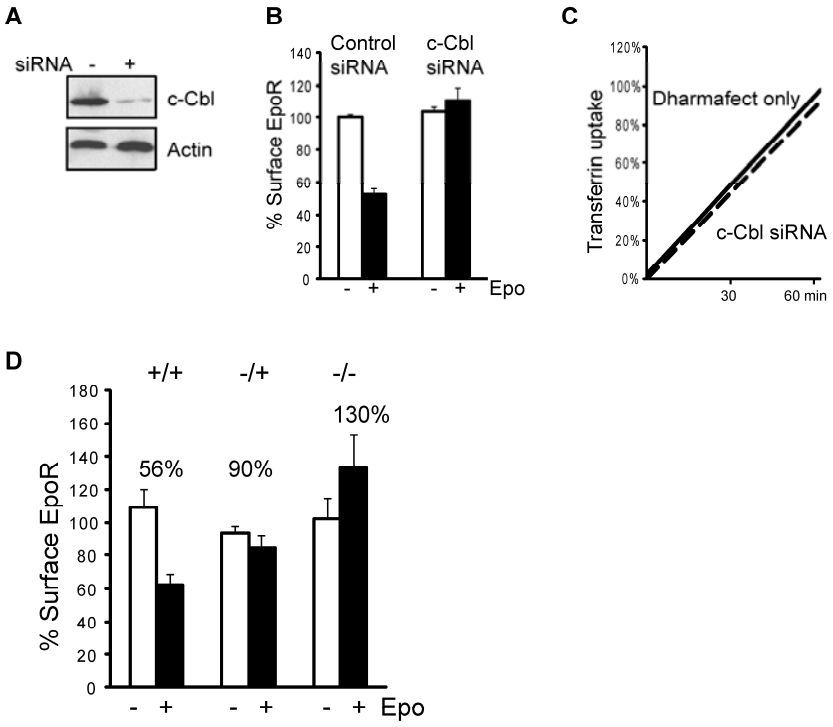

Figure 3.2: c-Cbl is required for EpoR internalization.
(A) Knockdown efficiency of c-Cbl is shown by immuno-blotting. Immuno-blotting of the same blot with actin was shown as a control. (B) γ2A cells were transfected with siRNAs to c-Cbl and surface EpoR was analyzed by flow cytometry. (C) Transferrin uptake is similar in control cells and in c-Cbl knockdown cells. (D) Ligand-induced EpoR internalization is abolished in c-Cbl +/- and -/- MEFs. Internalization was studied through flow cytometry.

transfection and internalization was measured through flow cytometry. Transferrin uptake was normal in all conditions (Figure 3.2 C). To further confirm our findings, we examined ligand-induced EpoR internalization in c-Cbl knockout mouse embryonic fibroblast cells (MEF). About 44% of the EpoR internalized upon Epo stimulation in wild-type MEFs. In contrast, internalization was dramatically diminished in +/- or -/- MEFs (Figure. 3.2 D). These results corroborate our previous findings to demonstrate that c-Cbl plays in important part in receptor internalization upon stimulation.

3.3.3 c-Cbl is phosphorylated by JAK2 upon ligand stimulation

To begin to understand the contribution of c-Cbl in EpoR internalization, we first confirmed that Epo induces c-Cbl phosphorylation (Barber et al., 1997b; Odai et al., 1995). Tyrosine phosphorylated c-Cbl was detected with 4G10, an anti-phosphotyrosine antibody, in cells stably expressing HA-EpoR with JAK2 but not JAK2KD (Figure 3.3 A). To validate our data, cells were transfected with c-Cbl siRNA. Knocking down c-Cbl resulted in the loss of phosphorylated c-Cbl (Figure 3.3 A). We further showed that JAK2 can phosphorylate c-Cbl in an in vitro kinase assay (Figure 3.3 B). Ongoing studies will address which tyrosine residues of c-Cbl are phosphorylated by JAK2.

3.3.4 c-Cbl E3 ligase activity is important for EpoR ligand induced internalization

Intact ring finger domain and linker regions are needed for functional c-Cbl E3 ligase activities (Kassenbrock and Anderson, 2004). In particular, it was shown that phosphorylation of Y368 and Y371 located in the linker regions are prerequisites for ubiquitin ligase activity (Kassenbrock and Anderson, 2004). It was suggested that these two tyrosines can regulate the positioning of the phosphotyrosine binding protein to the ring domain, facilitating the transfer of ubiquitin to this target (Kassenbrock and Anderson, 2004). The specific function of these tyrosines was further dissected by the Langdon laboratory. In cell lines expressing Y368F or Y371F c-Cbl mutants, the internalization rate of EGFR was normal (Kassenbrock and Anderson, 2004). Ubiquitination, on the other hand, was not detected in cells

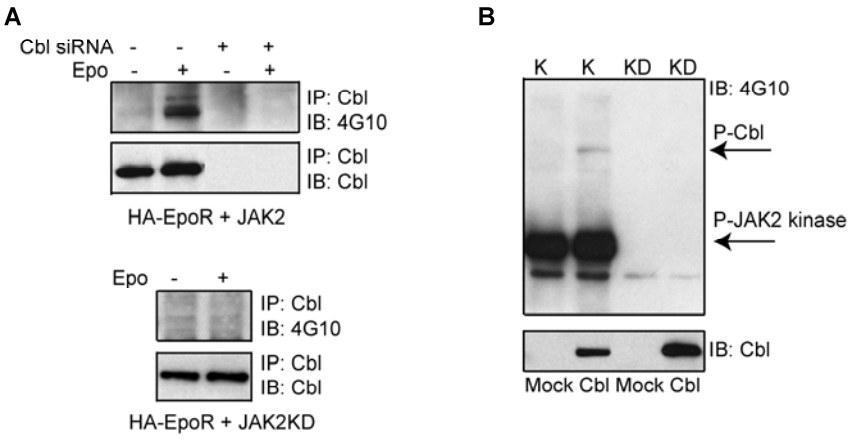

Figure 3.3: c-Cbl is phosphorylated by JAK2 upon ligand stimulation.
(A) γ2A cells stably expressing HA-EpoR and JAK2 or JAK2KD were transiently transfected with vectors expressing full-length c-Cbl. 48 hrs post transfection, cells were starved overnight and treated with Epo for 15 min. Cell lysates were subjected to immuno-precipitation by c-Cbl antibodies and immuno-blotted with 4G10 antibody for phosphorylated c-Cbl. To confirm 4G10 specificity, in addition to the transfected c-Cbl vectors, cells were also transfected with c-Cbl siRNA in lanes 3 and 4.
(B) JAK2 phosphorylates c-Cbl in vitro. JAK2 or JAK2KD was co-expressed with c-Cbl in 293T cells. Each protein is immunoprecipitated and set up for kinase assays. Phosphorylated c-Cbl was detected with 4G10 antibody. IP: immuno-precipitation. IB: immuno-blot. K: JAK2 kinase domain, KD: JAK2 Kinase dead.

expressing the Y371F mutant c-Cbl, indicating that Y371 may be more important than Y368 for E3 ligase activity. In addition, deletion of either tyrosine residue blocked EGFR ubiquitination and also severely affected receptor internalization (Thien et al., 2001). We generated c-Cbl mutant constructs which harbor deleted Y368 or Y371. γ2A cells stably expressing HA-EpoR and JAK2 were transfected with vectors expressing wild-type or mutant c-Cbl proteins, and ligand-induced EpoR internalization was studied through flow cytometry. Over-expressed c-Cbl constructs did not affect the amount of receptors on the surface prior to Epo treatment (Figure 3.4). Upon Epo stimulation, EpoR internalizes normally in cells expressing wild-type c-Cbl. In contrast, EpoR internalization was significantly reduced in cells expressing c-Cbl mutants Δ368 and Δ371. The less severe phenotype compared to c-Cbl knockdown cells may be due to endogenous c-Cbl expressed in γ2A cells.

Our results show that c-Cbl's E3 ligase activity plays an important role in EpoR internalization upon stimulation. Our current efforts are concentrated on finding its substrate. Surprising preliminary data indicates that p85β is mono-ubiquitinated upon Epo induction. We transfected γ2A cells stably expressing HA-EpoR and JAK2 with vectors expressing p85β and Flag-Ubiquitin. Cells were starved and stimulated with 30 units Epo for 40 min. As shown in Figure 3.5, mono-ubiquitinated p85β was detected only after Epo treatment. Since p85β can bind to c-Cbl (Kim et al., 1995), ongoing experiments will try to establish whether p85β is the intended substrate and if mono-ubiquitinated p85 is required for internalization. Another potential substrate for c-Cbl is the EpoR. It has been reported to be polyubiquitinated after Epo stimulation (Yen et al., 2000). Chapter 4 will be dedicated in deciphering the role of receptor ubiquitination.

3.4 Discussion

Our siRNA screen resulted in several hits. Interestingly, some of these proteins have previously been shown to play a role in receptor down-regulation, and many have been reported to interact with each other in different settings. More importantly, all of our hits are closely associated with c-Cbl. Although

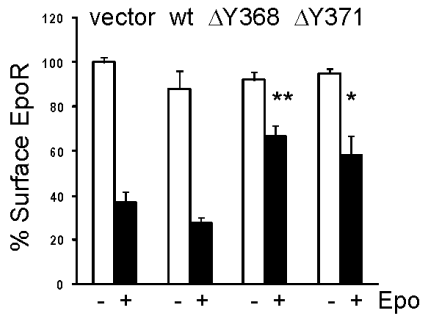

Figure 3.4: E3 ligase activity of Cbl is important for EpoR ligand-induced internalization.
γ2A cells stably expressing HA-EpoR and JAK2 were transiently transfected with vectors expressing wt c-Cbl or vectors expressing Δ368 or Δ371 that bear deficient E3 ligase activity. After 48h, ligand-induced receptor internalization was measured by flow cytometry. ** indicates P=0.0004 (unpaired t-test) vs. control.
* indicates P=0.0174 (unpaired t-test) vs. control.

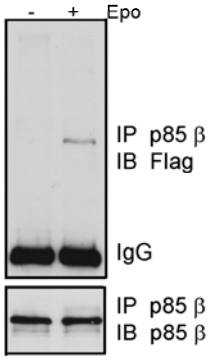

Figure 3.5. p85β is mono-ubiquitinated upon Epo induction. γ2A cells stably expressing HA-EpoR and JAK2 were transfected with vectors that express Flag-tagged ubiquitin and p85β. 48 hours post transfection,cells were stimulated for 40 min with Epo. p85β was immunoprecipitated with p85βantibodies,and samples were then probed for ubiquitinated p85β with anti-Flag antibodies. IP: immuno-precipitation. IB: immuno-blot

we were able to recapitulate the inducible c-Cbl phosphorylation upon Epo stimulation, our attempts to detect c-Cbl binding to the receptor have failed (data not shown). Specific phosphorylated tyrosine residues on c-Cbl are docking sites for different SH2 domain-containing proteins such as p85β. It is likely that c-Cbl may associate with the EpoR via other proteins. There are two pYXXM motifs in c-Cbl surrounding Y371 and Y731 that promote p85β binding (Standaert et al., 2004). Y731 is proposed to be phosphorylated by a Src kinase Fyn upon cytokine treatment (Hunter et al., 1999). Our siRNA screen did not include Fyn, as its course of action is best defined in mast cells and lymphocytes (but not erythrocyte progenitors) where they associate with the high-affinity IgE or IgM receptors and initiate signaling upon activation (Eiseman and Bolen, 1992). However, knockdown of another Src kinase, Lyn, dramatically diminished ligand-induced EpoR internalization (Figure 3.1).

Lyn has been shown to bind pY464 or pY479 on the EpoR where it phosphorylates Stat5 to enhance its transcriptional activity (Chin et al., 1998). Lyn also phosphorylates CrkL, a member of the Crk family of adaptor proteins. CrkL binds EpoR on pY460 and requires Lyn-mediated phosphorylation to initiate a Ras-dependent activation of the Raf/ERK pathway through Sos1 and C3G, two guanine nucleotide exchange proteins of the Ras family (Arai et al., 2001). More importantly, phosphorylated CrkL forms a complex with c-Cbl (Arai et al., 2001). CrkL was not detected in γ2A cells, and knocking down Crk, its counterpart, was not efficient probably due to a long protein half-life. Nevertheless, we have not ruled out Crks as adaptors required to recruit c-Cbl to the activated EpoR for internalization. In the same study that revealed a link between CrkL and Lyn, the authors showed that Lyn phosphorylates c-Cbl after Epo stimulation, however, they did not comment on the meaning of this data (Arai et al., 2001). Since Lyn siRNA inhibited EpoR internalization, we hypothesize that Lyn, similar to Fyn, may phosphorylate c-Cbl on Y731. This phosphorylation may allow p85β to interact with c-Cbl in order to be mono-ubiquitinated for EpoR internalization. Studies are underway to test this hypothesis.

Another means to link c-Cbl to the EpoR is through Grb2. Besides signaling, Grb2 initiates clathrin-mediated internalization by recruiting c-Cbl to receptors such as EGFR and c-Met ((Jiang et al.,

2003; Li et al., 2007)). EGFR receptor internalization is negatively affected in cells over-expressing a mutant form of Grb2 that cannot bind to c-Cbl (Jiang et al., 2003). c-Met tyrosine kinase does not internalize when cells are transfected with Grb2 siRNA (Jiang et al., 2003; Li et al., 2007). Moreover, a Grb2-c-Cbl chimera rescues ligand dependent c-Met tyrosine kinase receptor internalization in the presence of a ubiquitin ligase deficient form of c-Cbl (Li et al., 2007). Our siRNA screen strengthens the involvement of Grb2 in mediating receptor internalization since a moderate siRNA knock-down of Grb2 severely impacted EpoR internalization (Figure 3.1). Although there is no data supporting Grb2 binding to the EpoR, researchers believe that pY464 and surrounding amino acids on EpoR is the main binding site (Barber et al., 1997a). If Grb2 is recruited to the receptor directly, or indirectly via p85 (Radhakrishnan et al., 2008), Grb2 may bring c-Cbl to the EpoR since both proteins were shown to be constitutively bound in hematopoietic cell lines (Odai et al., 1995).

Our results also suggest that ALIX, a proline rich protein associated with neuronal cell death (Missotten et al., 1999), plays a role in ligand-induced EpoR internalization. ALIX interacts with different components of the endocytic machinery such as endophilins (Chatellard-Causse et al., 2002), Cin85 (an adaptor protein that binds Cbl to help regulate endocytosis) (Chen et al., 2000), the endosomal sorting complexes required for transport (ESCRTs) (Falguieres et al., 2008), and the lysobisphosphatidic acid (LBPA, a component of the late endosomal membranes) (Dikic, 2004). The first siRNA experiment targeting ALIX revealed that ALIX is necessary for proper cellular distribution of endosomes by affecting the actin organization of the cell (Cabezas et al., 2005). Early, recycling and late endosomes co-localized in the perinuclear region in ALIX depleted cells (Cabezas et al., 2005). However, transferrin endocytosis and recycling as well as EGFR endocytosis and degradation were not affected (Cabezas et al., 2005). Immunofluorescence assays suggest that ALIX links the endocytic machinery to the actin cytoskeleton (Cabezas et al., 2005). Another study suggests that increased levels of ALIX in the cell distrupt c-Cbl/Cin85 complexes and block EGFR internalization (Schmidt et al., 2004). Our preliminary data revealed that the EpoR is lost from the cell surface when ALIX is over-expressed prior to Epo induction. This effect is reverted with concurrent knockdown of ALIX. In addition, JAK2 phosphorylates ALIX in

our in vitro kinase assay (data not shown). More experiments will be needed to understand the role of ALIX in EpoR endocytosis.

Our siRNA screen discovered proteins important for ligand-induced EpoR internalization and degradation. Among these proteins, p85 (αβ), Grb2 and c-Cbl have been reported to interact with one another (Cerboni et al., 1998). The resulting complex is likely crucial for bringing the endocytic machinery to the EpoR upon stimulation.

Chapter 4. Ligand-mediated EpoR ubiquitination regulates its endosomal sorting and degradation in the lysosomes but not its internalization

Abstract

Ubiquitination is a major regulatory mechanism that controls receptor trafficking from the surface to the lysosomes. EpoR is ubiquitinated upon ligand stimulation. Here we show that ubiquitination of the EpoR itself is not essential for internalization as a lysineless EpoR (EpoR-ALLKR) is internalized normally. In contrast, we find that unlike wild-type EpoR that is delivered to EEA1-positive early endosomes and then to LAMP2-labeled lysosomes, EpoR-ALLKR co-localizes with EEA1 but does not reach LAMP2-labeled lysosomes. Therefore, ubiquitination of the EpoR is important for post-endosomal sorting and lysosomal degradation. In addition, we show that K428 appears to be the primary ubiquitination site on the EpoR.

4.1 Introduction

Similar to phosphorylation, ubiquitination is a post-translational modification that controls distinct cellular processes such as protein degradation (Blankenship et al., 2008), DNA repair (Lovly et al., 2008), histone regulation (Cao et al., 2008; Geng and Tansey, 2008), cellular trafficking (Putz et al., 2008) and receptor internalization (Belouzard and Rouille, 2006). Ubiquitin is a 76 amino acid polypeptide. It is covalently linked to its substrate via an isopeptide bond between the C-terminal glycine residue of ubiquitin and a specific lysine residue on the target protein. Ubiquitin itself can be ubiquitinated through any of its seven lysine residues resulting in the generation of polyubiquitinated chains (reviewed in (Pickart and Fushman, 2004) and (Pickart and Eddins, 2004)). Several forms of ubiquitination have been described: a substrate can be monoubiquitinated, multi-monoubiquitinated and polyubiquitinated (reviewed in (Pickart and Eddins, 2004)). The nature of the appended ubiquitin moiety dictates the fate of the targeted protein and regulates related cellular events (reviewed in (Pickart and Fushman, 2004)).

Three groups of enzymes transfer ubiquitin to its substrate through a multi-step reaction. First, the ubiquitin activating enzyme (E1) is conjugated to ubiquitin via an ATP-dependent reaction. Ubiquitin is then transferred to the cysteine residue of the ubiquitin conjugating enzyme (E2). The transfer of ubiquitin to the lysine of the substrate protein involves a ubiquitin ligase enzyme (E3) which dictates substrate selectivity and specificity (reviewed in (Ardley and Robinson, 2005)). These three enzymes were thought to be sufficient in the ubiquitination process until the discovery of conjugating factors E4 in yeast that assist in ubiquitin chain elongation (Koegl et al., 1999). Indeed, the E3 ligase Parkin associates with the E4 ligase CHIP (carboxyl terminus of the Hsc70-interacting protein) to promote efficient target polyubiquitination, a discovery that may provide new therapeutic venues for Parkinson disease (Imai et al., 2002).

Lysosomes and proteasomes control protein turnover in the cell (Wolf, 2004). A protein targeted for proteasomal degradation bears a polyubiquitin tag whereas both polyubiquitination and monoubiquitination are involved in delivering proteins to the lysosomes (Barriere et al., 2007). The latter

is best illustrated in receptor endocytosis since most of the steps that lead to receptor downregulation from the cell surface and their delivery to the lysosomes rely on subsequent yet unique ubiquitination events (Horak, 2003; Huang et al., 2006; Schmidt and Dikic, 2005).

Internalization of certain receptors such as the insulin-like growth factor receptor requires a monoubiquitin signal (Monami et al., 2008). For other receptors such as the EGFR, ubiquitination is important but not essential for internalization since knocking down c-Cbl, a E3 ligase which ubiquitinates EGFR at the cell surface, does not alter internalization (Duan et al., 2003). Once in the early endosomes, the ubiquitin signal will serve to sort the receptor into the multi-vesicular body pathway. Proteins from the sorting machinery such as HRS, STAM or Epsin contain ubiquitin binding motifs to recognize ubiquitinated receptors (Bache et al., 2003b). Some of these proteins undergo ligand induced phosphorylation and are decorated by monoubiquitin recognized by their counterparts to orchestrate efficient sorting (Komada and Kitamura, 2005). For example, upon EGF stimulation, HRS is first phosphorylated by EGFR and then ubiquitinated by c-Cbl (Bache et al., 2002; Stern et al., 2007). HRS traps the ubiquitinated receptor in a specialized area of the early endosomes that are coated by flat clathrin coats (Raiborg et al., 2002) and recruits ESCRT-1 (Bache et al., 2003a) and SNX-1 (Chin et al., 2001). There are three distinct ESCRT complexes that sequentially bind to the ubiquitinated proteins and move them further towards the lysosomes (reviewed in (Hurley, 2008)). It has also been shown that receptors such as EGFR are continuously ubiquitinated even after internalization to further assist their own degradation (Umebayashi et al., 2008).

To date, only a handful of papers addressed the role of ubiquitin in mediating the downregulation of the cytokine receptors. For instance, the leptin receptor short form becomes mono-ubiquitinated on K877 and K889, and ubiquitination is required for internalization via the clathrin-coated pits (Belouzard and Rouille, 2006). For the prolactin receptor, ligand-induced receptor ubiquitination initiates clathrin-mediated internalization. Moreover, polyubiquitination through K63 of the prolactin receptor is essential for late endosomal sorting as receptors that fail to become ubiquitinated do not reach the lysosomes (Varghese et al., 2008). The same group proposes that Ser349 phosphorylation of the prolactin receptor

precedes ubiquitination and this initial modification depends on JAK2 kinase activity (Swaminathan et al., 2008).

The EpoR belongs to the cytokine receptor family and is ubiquitinated upon stimulation (Walrafen et al., 2005). β-TRCP is the suggested E3 ligase that mediates the ubiquitination event (Meyer et al., 2007). It is thought that this polyubiquitination targets EpoR for proteasomal degradation (Walrafen et al., 2005).

Here we shown that ubiquitination is not required for EpoR internalization but is necessary for proper delivery to the lysosomes.

4.2 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. Ubiquitin was cloned into pCMV3Xflag vector (Sigma). pMX-IRES-GFP vector expressing the EpoR-Ub chimera was generated through overlapping extension PCR reactions. γ2A cells stably expressing wild-type or mutant HA-EpoRs and various forms of JAK2 were generated as previously described (Huang et al., 2001). Antibodies were from the following sources: HA, Covance; EEA1, LAMP2, Santa Cruz; Flag M2 agarose Sigma. Horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence system were from Amersham Biosciences. Lactacystin was purchased from Boston Biochem, Bafilomycin A from LC laboratories and Cycloheximide from MP biochemicals.

Labeling and immuno-precipitation of cell surface EpoR (Surface IP)

γ2A cells stably expressing HA-EpoR with various JAK2 constructs were incubated with blocking buffer (PBS with 5% normal mouse serum) for 30 min at 4°C. Subsequently, cells were incubated with 10 μg/mL of anti-HA antibodies in blocking buffer for 60 min at 4°C. After washing with PBS, cells were lysed in 1% NP-40 lysis buffer containing 5 μg/mL of HA peptide in order to block any

residual anti-HA antibodies. After incubation with protein A/G agarose for 90 min at 4°C, immuno-precipitants were washed three times with PBS containing 1% TritonX-100 and 0.1% SDS, run on SDS-PAGE, and immuno-blotted with anti-HA antibodies. In experiments where Epo treatment was performed, cells were stimulated with Epo (30 units) at 37°C for the duration indicated.

Flow cytometry and data analysis

Surface expression of the wild type or mutant HA-EpoRs was measured in the presence of wild-type or mutant JAK2 in one million γ2A cells as described previously (Tong et al., 2006). For Epo stimulation, cells were incubated with 30 units of Epo at 37°C. The median fluorescence of allophycocyanin (APC), which is conjugated to secondary antibodies that recognize anti-HA antibodies, was used to quantify the level of receptor on the cell surface. For each sample, the EpoR surface expression was normalized to that from samples co-expressing wild-type JAK2 prior to Epo stimulation. Each point represents data from three independent experiments. To inhibit PI3K activities, cells were incubated with wortmannin at concentrations indicated for 3 hrs prior to Epo stimulation.

Glycosidase digestion of EpoR

Glycosidase treatment of the EpoR with EndoH (New England Biolabs), or with PNGaseF (New England Biolabs) and neuraminidase (New England Biolabs) was performed as described (Tong et al., 2006).

Immuno-precipitation and immuno-blotting

γ2A cells transiently transfected with Flag-tagged ubquitin were starved for 12 hrs in DMEM media with 1% BSA followed by Epo induction for the appropriate time as indicated. Cells were then lysed with 1% NP-40 lysis buffer with protease and phosphatase inhibitors. Lysates were immuno-precipitated with anti-Flag resin (Sigma). The precipitates were eluted with SDS sample buffer, separated

by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-HA. Bound antibodies were detected by the ECL chemiluminescence system after incubation with horseradish peroxidase-coupled secondary antibodies. The lysates were also immuno-blotted with antibodies to HA.

Immunofluorescence

γ2A cells stably expressing HA-EpoR were seeded on glass coverslips. Coverslips were blocked with PBS containing 3% BSA and 5% normal goat serum for 30 min at –4°C and incubated with 20 μg/mL anti-HA antibodies for 1h. Coverslips were then washed 3 times in cold PBS and put back in the incubator for Epo stimulation. Subsequently, cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with methanol at 4°C for 15 min. Coverslips were then incubated with antibodies for EEA1 (1:100) or LAMP2 (1:50) for 45 min in blocking buffer. After washing in PBS, coverslips were incubated with Alexa Fluor 555-conjugated goat-anti mouse secondary antibodies, washed 3 times with PBS, and then incubated with Alexa Fluor 647-conjugated goat anti-rabbit secondary antibodies. Coverslips were washed three times with PBS and mounted onto coverslides with a semi-permanent Mowiol mounting medium (Calbiochem). Fluorescent images were taken on a Leica TCS SP5 confocal microscope with 40X oil objective lenses with numeric aperture of 1.25N. Confocal section images were acquire by Leica acquisition software and analyzed with Image J and Adobe Photoshop.

4.3 Results and Discussion

4.3.1 Ubiquitination of the EpoR is not required for ligand-induced internalization

The EpoR is ubiquitinated upon stimulation ((Walrafen et al., 2005)). To characterize the role of ubiquitination in ligand-induced EpoR endocytosis and degradation, we examined EpoR ubiquitination in γ2A cells stably expressing HA-EpoR with JAK2 or kinase deficient JAK2 (JAK2KD). Cells transfected with a vector expressing flag-tagged ubiquitin were treated with 10μM lactacystin and induced with Epo, and lysates were immunoprecipitated with anti-Flag antibodies to enrich for ubiquitinated proteins and probed with anti-HA antibodies to detect HA-EpoR. As shown in Figure 4.1, consistent with previous

reports (Walrafen et al., 2005), a smear representing polyubiquitinated EpoR was only detected in the presence of JAK2 but not in JAK2KD. Therefore, JAK2 kinase activity is required for receptor ubiquitination. This is also in line with previous results showing that JAK2 kinase activity is essential for down-regulation of mature EpoR.

Because there are five lysine residues in the EpoR cytoplasmic domain, we engineered a mutant EpoR, AllKR, in which all five lysines are replaced with arginines. This construct was expressed as a HA-tagged protein and tested for Epo-induced receptor internalization using flow cytometry. The majority of wild type EpoR was internalized 45 min post stimulation. Similar Epo-induced internalization kinetics were observed for AllKR (Figure 4.2).

4.3.2 Fusion of ubiquitin to the EpoR cytoplasmic domain is not sufficient for targeting to the endosome

Because it was demonstrated that fusing of ubiquitin to the EGFR was sufficient to target the chimeric protein to early endosomes (Mosesson et al., 2003), we fused ubiquitin to the c-terminus of the EpoR and examined its localization. This chimeric receptor failed to mature or express on the cell surface (Figure 4.3). We hypothesized that the fused ubiquitin moiety may cause artificial polyubiquitination and force the receptor to undergo ERAD degradation. We thus replaced the two most prominent lysines for

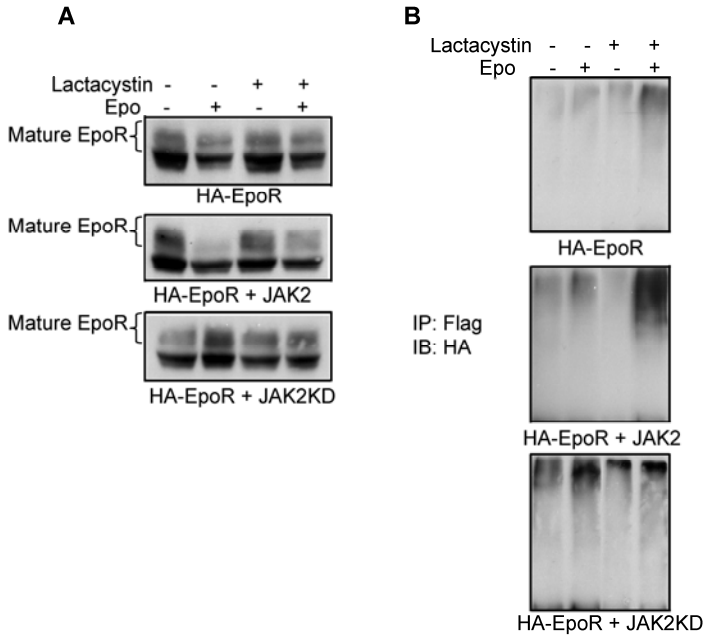

Figure 4.1: EpoR is ubiquitinated upon Epo stimulation.
(A) γ2A cells stably expressing HA-EpoR with vector, JAK2, or JAK2K were treated with 10 μM Lactacystin for 45 minutes followed by 45 minutes of Epo induction. Lysates were ran and probed with anti-HA antibodies for the receptor.
(B) γ2A cells stably expressing HA-EpoR with vector, JAK2, or JAK2KD were transfected with vectors that express Flag-tagged ubiquitin. 48 hours post transfection, cells were treated with 10 μM Lactacystin for 45 minutesfollowed by another 45 min of Epo stimulation.EpoR was immunoprecipitated with HA antibodies,and samples were then probed for ubiquitinated EpoR with anti-Flag antibodies.
IP: immuno-precipitation. IB: immuno-blot

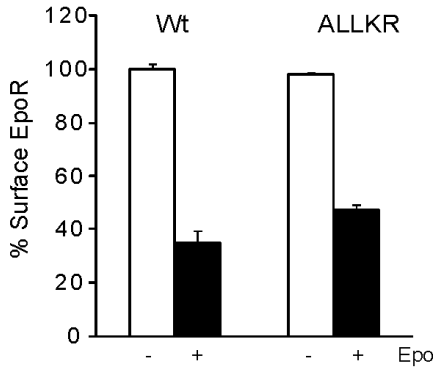

Figure 4.2: Lysineless EpoR maintains normal internalization. Levels of cell surface HA-EpoR were analyzed by flow cytometry at 45 min for wild type (Wt) and lysineless EpoR (ALLKR).

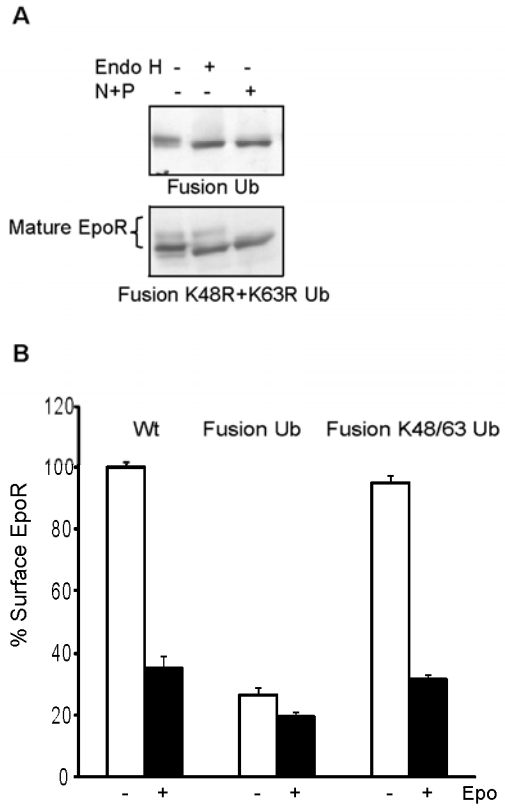

Figure 4.3: Fusion of ubiquitin to the EpoR cytoplasmic domain is not sufficient for targeting to the endosome
(A) EpoR construct fused with wild-type ubiquitin at the C terminus does not progress into a mature EpoR species as depicted by an absent Endo H resistant pool.On the other hand, EpoR construct fused with a mutated form of ubiquitin that cannot form a polyubiquitinated chain through two major lysines (K48R,K63R) undergoes complex glycosylation. (B) Internalization is normal for HA-EpoR-Ub(K48R,K63R).

polyubiquitination on ubiquitin to arginines (K48 and K63). This new chimera was capable of reaching the cell surface and EndoH-resistant mature receptors were detected (Figure 4.3). The number of chimeric receptors on the surface was similar to wild type EpoR and internalization was not affected (Figure 4.3.B). Therefore, fusion of ubiquitin to the EpoR, unlike the EGFR, is not sufficient to target the receptor to the endosome.

4.3.3 EpoR cytoplasmic lysine residues are essential for sorting to the lysosomes upon stimulation

We examined the fate of cell-surface HA-AllKR upon Epo induction by confocal immuno-fluorescence microscopy. Non-permeabilized cells were labeled with anti-HA antibodies prior to Epo induction. Cells were stimulated with Epo and subsequently permeabilized and visualized with fluorescence-conjugated secondary antibodies. This approach thus followed only surface-tagged receptors prior to Epo treatment. For HA-EpoR, EpoR staining was shifted from the plasma membrane to an internal compartment that co-localized with the early endosomal marker, EEA1 (early endosome antigen 1) 25 min post Epo treatment (Chapter 2, Figure 2.2). At 30 min post Epo stimulation, EpoR staining co-localizes with a lysosomal marker, LAMP2 (Figure 4.4), indicating that the EpoR has reached the lysosomes. Similar to wild-type EpoR, AllKR receptors co-localizes with EEA1 in endosomes 25 min post Epo stimulation (Figure 4.5). Interestingly, these AllKR receptors failed to sort to the lysosome, as no AllKR receptors co-localizes with LAMP2 30 min post Epo treatment (Figure 4.6). Consistent with these results, ligand-induced degradation of AllKR was dramatically reduced (Figure 4.7 A). Our results demonstrated that ubiquitination of the EpoR is not necessary for ligand-induced receptor internalization but may contribute to endosomal sorting and down-regulation.

4.3.4 K428 in the EpoR cytoplasmic domain mediates EpoR ubiquitination and degradation

In order to determine which of the five lysines are important for EpoR ubiquitination and degradation, we replaced individual lysine to arginine, and these mutated receptors were examined for

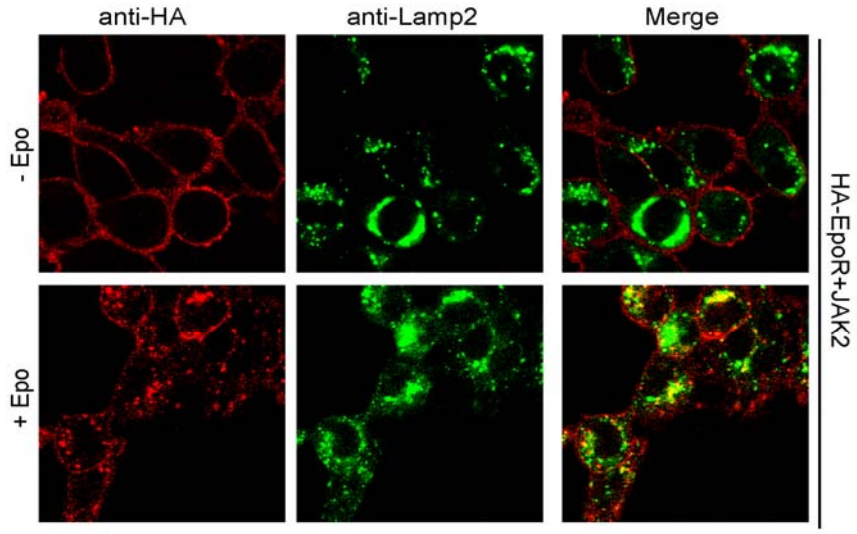

Figure 4.4: HA-EpoR colocalizes with lysosomal marker LAMP2 following ligand stimulation. Cell surface HA-EpoRs in γ2A cells stably expressing HA-EpoR and JAK2 were labeled with anti-HA antibodies prior to Epo stimulation of 35 min. Cells were fixed and immuno-stained with anti-Lamp2 antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocal images (single section) for different conditions are presented. Original magnification 40X (Leica TCS SP5).

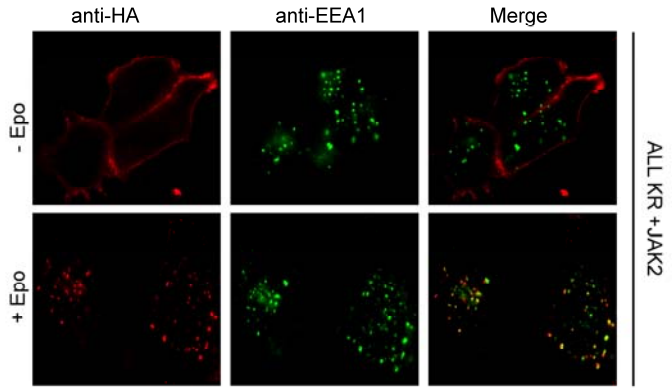

Figure 4.5: Lysineless EpoR colocalizes with early endosomal marker EEA1 upon ligand stimulation. Cell surface HA-EpoRs in γ2A cells stably expressing HA-EpoR-ALL KR and JAK2 were labeled with anti-HA antibodies prior to Epo stimulation of 25 min. Cells were fixed and immuno-stained with anti-EEA1 antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocal images (single section) for different conditions are presented. Original magnification 40X (Leica TCS SP5).

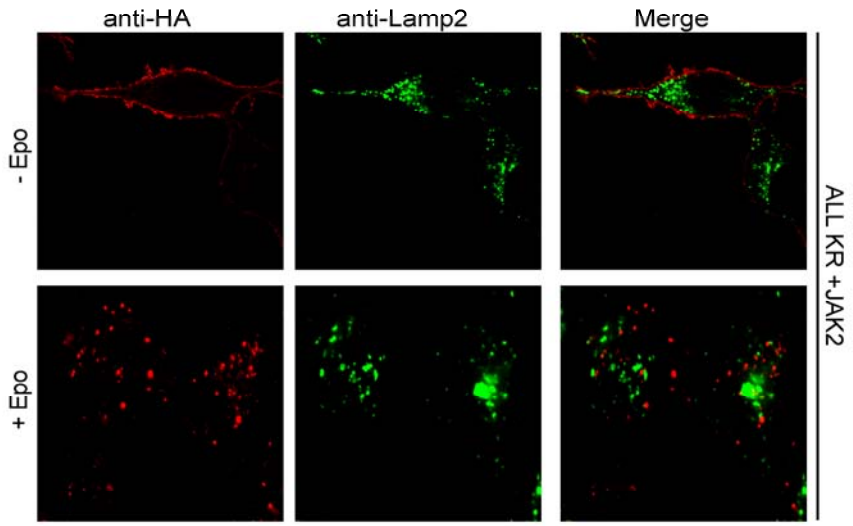

Figure 4.6: Lysineless EpoR is not sorted to the lysosomes.
Cell surface HA-EpoRs in γ2A cells stably expressing HA-EpoR-ALL KR+JAK2 were labeled with anti-HA antibodies prior to Epo stimulation of 35 min. Cells were fixed and immuno-stained with anti-Lamp2 antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocal images (single section) for different conditions are presented. Original magnification 40X (Leica TCS SP5).

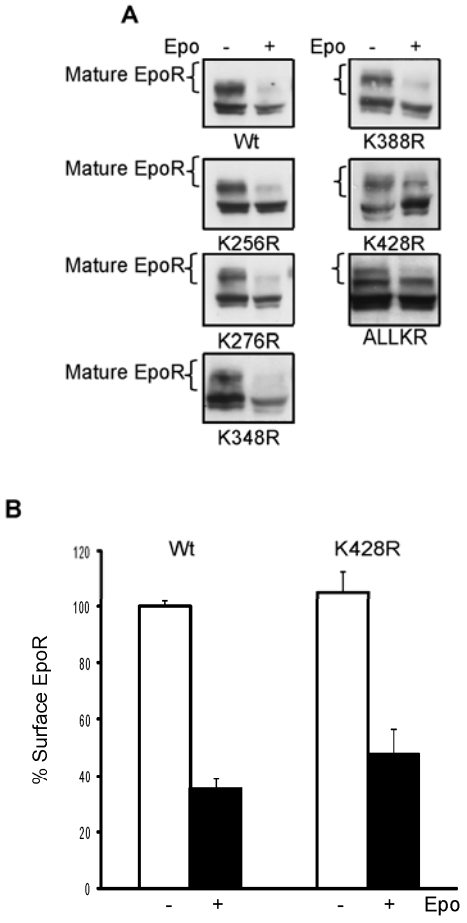

Figure 4.7: K428 in the EpoR cytoplasmic domain mediates EpoR degradation. (A) Wild type HA-EpoR and HA-EpoR mutants of lysine residues, except for K428R, are degraded upon ligand stimulation.(B) Flow cytometry shows normal Internalization for HA-EpoR-K428R.

ligand-induced degradation. As shown in Figure 4.7 A, mutation of K428R greatly impaired degradation, while mutations of K256, K276, K348, or K388 had no effect. Consistent with these results, no ubiquitinated species were detected in cells expressing K428R upon stimulation (preliminary data). In line with results from the AllKR receptor, HA-K428R internalized normally (Figure 4.7.B). In addition, K428R receptors were detected in LAMP2-positive lysosomes, despite at a much lower frequency compared to wild-type EpoR (Figure 4.8). Their detection in early endosomes was normal.

These results suggest that ubiquitination of the EpoR is essential for endosomal sorting, and that K428 may participate in this process. In conclusion, we demonstrate that mutation of all five lysine residues in the EpoR cytoplasmic domain resulted in a receptor that internalizes but fails to reach the lysosomes. Therefore, ubiquitination of the EpoR is vital for receptor lysosomal degradation. Although we show that ubiquitination of the EpoR itself is not essential for internalization, however, it is possible that ubiquitination of a EpoR associated protein may mediate internalization. JAK2 is polyubiquitinated upon ligand stimulation (Ungureanu et al., 2002),c-Cbl, is capable of undergoing auto-ubiquitination (Kassenbrock and Anderson, 2004), and p85β was found to be mono-ubiquitinated in our cells (Figure 3.5). Ongoing experiments will help decipher whether ubiquitination of these proteins play any role in EpoR endocytosis and degradation.

There are three potential E3 ligases that may ubiquitinate EpoR after internalization: Cbl, $p33^{rul}$ and β-TRCP. We failed to detect ubiquitinated EpoR by c-Cbl in in vitro ubiquitin assays (data not shown). As for $p33^{rul}$, there is no evidence supporting its role in EpoR degradation (Friedman et al., 2003) but its plausible role merits to be further studied. Since β-TRCP was shown to bind mature EpoR after Epo induction, we will first study the role of βTRCP and ubiquitination for EpoR endosomal sorting. Finally, future projects will unravel the mechanism through which ubiquitin recruits the sorting machinery that will eventually guide the receptor to the lysosomes

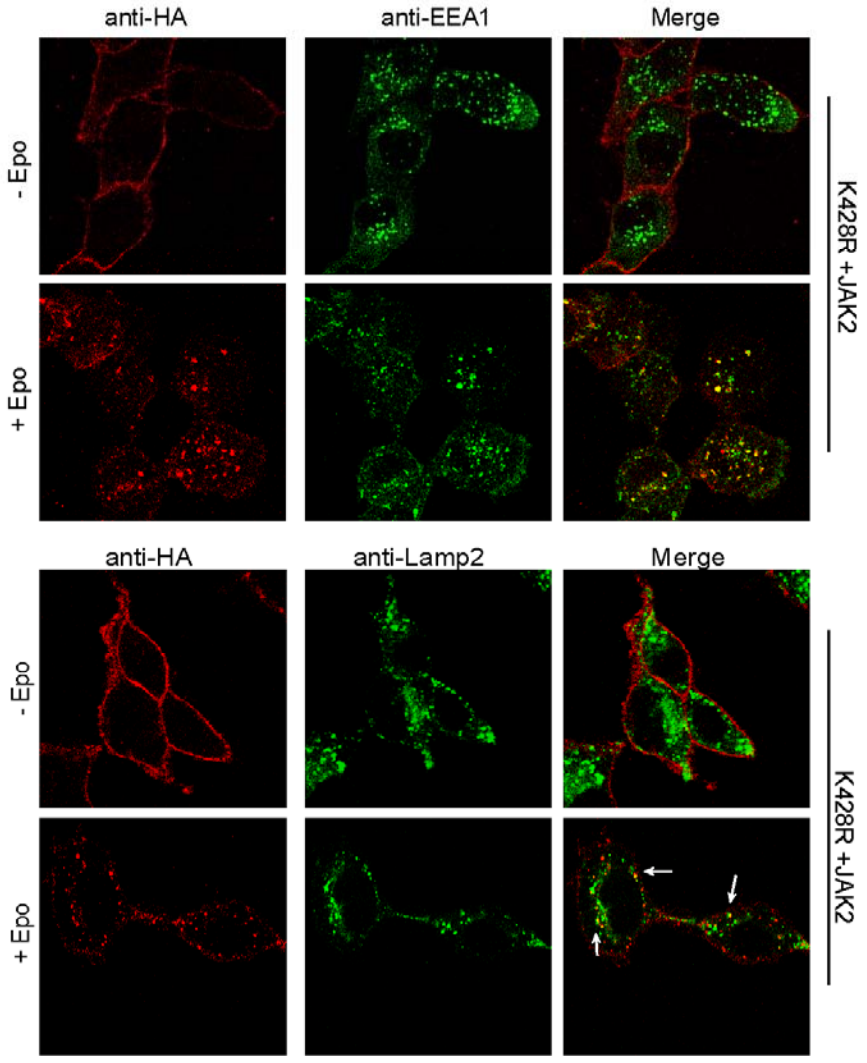

Figure 4.8: K428R is not delivered efficiently to the lysosomes upon ligand stimulation. Cell surface HA-EpoRs in γ2A cells stably expressing HA-EpoR-K428R and JAK2 were labeled with anti-HA antibodies prior to Epo stimulation of 25 min. Cells were fixed and immuno-stained with anti-EEA1 antibodies followed by appropriate fluorescence-conjugated secondary antibodies. Representative confocal images (single section) for different conditions are presented. Original magnification 40X (Leica TCS SP5).

Chapter 5. Conclusive remarks

Erythropoietin (Epo) is the primary cytokine regulating red blood cell production and its function is mediated through the Epo receptor (EpoR). Mechanisms that govern ligand-induced EpoR down-regulation are not entirely understood. In chapter 2, I report that JAK2 kinase activity is essential to mediate EpoR internalization via clathrin. JAK2 phosphorylates tyrosines on the EpoR cytoplasmic domain and in turn the phosphorylated tyrosines recruit proteins that are part of the endocytic as well as signaling machinery. Phosphorylated Y429, Y431, or Y479 on the EpoR mediates internalization by binding to the p85 subunit of PI3 kinase upon Epo stimulation. Knockdown of p85α and p85β or expression of their dominant-negative forms dramatically impaired EpoR internalization, whereas inhibition of PI3 kinase activity has no effect. My work provides the first evidence for p85 in receptor endocytosis, likely by recruiting proteins in the endocytic machinery upon Epo stimulation. In chapter 3, I described a siRNA candidate screen for proteins essential for EpoR endocytosis upon stimulation. From this screen, c-Cbl, Lyn, and Grb2 are identified that may play a role in EpoR internalization. Interestingly, these proteins have all been shown to either directly or indirectly bind to p85 (model summarized in figure 5.1). In particular, knocking down c-Cbl completely blocks EpoR internalization. I further show that E3 ligase activity is important for this process, as the expression of ligase-deficient c-Cbl mutants greatly reduced EpoR internalization. Because p85 is mono-ubiquitinated upon Epo stimulation, I hypothesize that c-Cbl may ubiquitinate p85 to regulate ligand-induced EpoR internalization

In chapter 4, I present data demonstrating that ubiquitination on the EpoR itself upon stimulation, is not required for internalization but for endosomal sorting. Specifically, K428 is the primary site of ubiquitination. Ongoing experiments will identify the E3 ubiquitin ligase which ubiquitinates the EpoR on K428.

Finally, my results contribute to our understanding of a hematological malignancy named primary familial and congenital polycythemia (PFCP). PFCP patients harbor truncated froms of the EpoR and experience increased erythrocytosis. These receptors lack our three important tyrosines for internalization

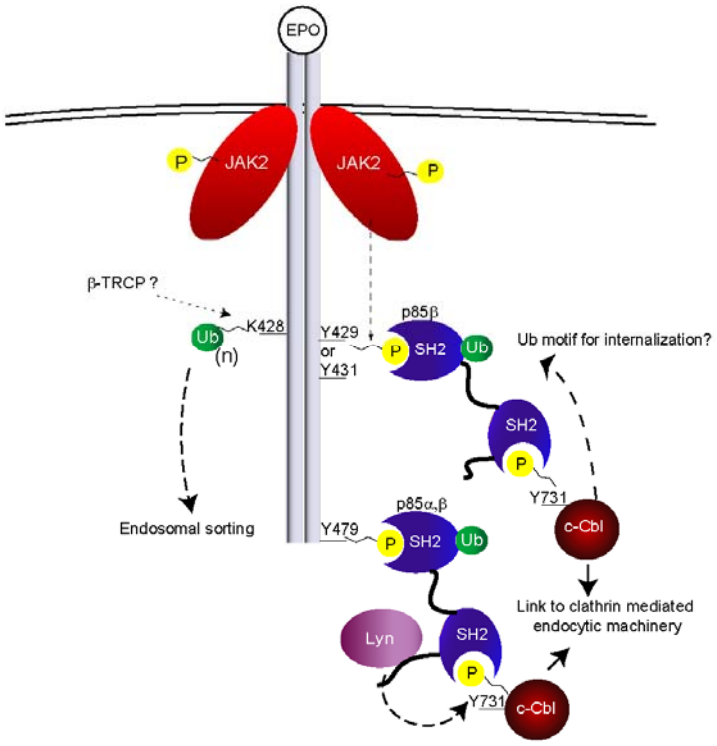

Figure 5.1: A model for the downregulation of EpoR

and do not bind p85β. More importantly, addition of residues encompassing Y429 and Y431 to these truncated receptors restored p85β binding and Epo sensitivity. Therefore, the defect in internalization and down-regulation of truncated EpoRs from PFCP patients may contribute to their hypersensitivity to Epo and prolonged signaling.

While I have identified novel components that contribute to EpoR internalization and degradation upon stimulation, many questions remain regarding the mechanism of these processes. Future work will address the targets of c-Cbl and Lyn, and the function of other endocytic proteins that are recruited to the receptor after internalization for endosomal sorting.

BIBLIOGRAPHY

Acs, G., Acs, P., Beckwith, S. M., Pitts, R. L., Clements, E., Wong, K., and Verma, A. (2001). Erythropoietin and erythropoietin receptor expression in human cancer. Cancer Res *61*, 3561-3565.

Aispuru, G. R., Aguirre, M. V., Aquino-Esperanza, J. A., Lettieri, C. N., Juaristi, J. A., and Brandan, N. C. (2008). Erythroid expansion and survival in response to acute anemia stress: the role of EPO receptor, GATA-1, Bcl-xL and caspase-3. Cell Biol Int *32*, 966-978.

Andoniou, C. E., Thien, C. B., and Langdon, W. Y. (1994). Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene. Embo J *13*, 4515-4523.

Arai, A., Kanda, E., Nosaka, Y., Miyasaka, N., and Miura, O. (2001). CrkL is recruited through its SH2 domain to the erythropoietin receptor and plays a role in Lyn-mediated receptor signaling. J Biol Chem *276*, 33282-33290.

Arcasoy, M. O. (2008a). Erythropoiesis-stimulating agent use in cancer: preclinical and clinical perspectives. Clin Cancer Res *14*, 4685-4690.

Arcasoy, M. O. (2008b). The non-haematopoietic biological effects of erythropoietin. Br J Haematol *141*, 14-31.

Ardley, H. C., and Robinson, P. A. (2005). E3 ubiquitin ligases. Essays Biochem *41*, 15-30.

Argetsinger, L. S., Kouadio, J. L., Steen, H., Stensballe, A., Jensen, O. N., and Carter-Su, C. (2004). Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity. Mol Cell Biol *24*, 4955-4967.

Baass, P. C., Di Guglielmo, G. M., Authier, F., Posner, B. I., and Bergeron, J. J. (1995). Compartmentalized signal transduction by receptor tyrosine kinases. Trends Cell Biol *5*, 465-470.

Bache, K. G., Brech, A., Mehlum, A., and Stenmark, H. (2003a). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. J Cell Biol *162*, 435-442.

Bache, K. G., Raiborg, C., Mehlum, A., Madshus, I. H., and Stenmark, H. (2002). Phosphorylation of Hrs downstream of the epidermal growth factor receptor. Eur J Biochem *269*, 3881-3887.

Bache, K. G., Raiborg, C., Mehlum, A., and Stenmark, H. (2003b). STAM and Hrs are subunits of a multivalent ubiquitin-binding complex on early endosomes. J Biol Chem *278*, 12513-12521.

Bakker, W. J., Blazquez-Domingo, M., Kolbus, A., Besooyen, J., Steinlein, P., Beug, H., Coffer, P. J., Lowenberg, B., von Lindern, M., and van Dijk, T. B. (2004). FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. J Cell Biol *164*, 175-184.

Barber, D. L., Corless, C. N., Xia, K., Roberts, T. M., and D'Andrea, A. D. (1997a). Erythropoietin activates Raf1 by an Shc-independent pathway in CTLL-EPO-R cells. Blood *89*, 55-64.

Barber, D. L., Mason, J. M., Fukazawa, T., Reedquist, K. A., Druker, B. J., Band, H., and D'Andrea, A. D. (1997b). Erythropoietin and interleukin-3 activate tyrosine phosphorylation of CBL and association with CRK adaptor proteins. Blood *89*, 3166-3174.

Barriere, H., Nemes, C., Du, K., and Lukacs, G. L. (2007). Plasticity of polyubiquitin recognition as lysosomal targeting signals by the endosomal sorting machinery. Mol Biol Cell *18*, 3952-3965.

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N.*, et al.* (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet *365*, 1054-1061.

Beckman, D. L., Lin, L. L., Quinones, M. E., and Longmore, G. D. (1999). Activation of the erythropoietin receptor is not required for internalization of bound erythropoietin. Blood *94*, 2667-2675.

Belouzard, S., and Rouille, Y. (2006). Ubiquitylation of leptin receptor OB-Ra regulates its clathrin-mediated endocytosis. Embo J *25*, 932-942.

Birkeland, K. I., Stray-Gundersen, J., Hemmersbach, P., Hallen, J., Haug, E., and Bahr, R. (2000). Effect of rhEPO administration on serum levels of sTfR and cycling performance. Med Sci Sports Exerc *32*, 1238-1243.

Bittorf, T., Seiler, J., Zhang, Z., Jaster, R., and Brock, J. (1999). SHP1 protein tyrosine phosphatase negatively modulates erythroid differentiation and suppression of apoptosis in J2E erythroleukemic cells. Biol Chem *380*, 1201-1209.

Blake, T. J., Shapiro, M., Morse, H. C., 3rd, and Langdon, W. Y. (1991). The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. Oncogene *6*, 653-657.

Blankenship, J. W., Varfolomeev, E., Goncharov, T., Fedorova, A. V., Kirkpatrick, D. S., Izrael-Tomasevic, A., Phu, L., Arnott, D., Aghajan, M., Zobel, K.*, et al.* (2008). Ubiquitin binding modulates IAP antagonist stimulated proteasomal degradation of c IAP1 and c IAP2. Biochem J.

Bonifacino, J. S., and Traub, L. M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem *72*, 395-447.

Booker, G. W., Gout, I., Downing, A. K., Driscoll, P. C., Boyd, J., Waterfield, M. D., and Campbell, I. D. (1993). Solution structure and ligand-binding site of the SH3 domain of the p85 alpha subunit of phosphatidylinositol 3-kinase. Cell *73*, 813-822.

Bouscary, D., Pene, F., Claessens, Y. E., Muller, O., Chretien, S., Fontenay-Roupie, M., Gisselbrecht, S., Mayeux, P., and Lacombe, C. (2003). Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood *101*, 3436-3443.

Bousquet, M., Quelen, C., De Mas, V., Duchayne, E., Roquefeuil, B., Delsol, G., Laurent, G., Dastugue, N., and Brousset, P. (2005). The t(8;9)(p22;p24) translocation in atypical chronic myeloid leukaemia yields a new PCM1-JAK2 fusion gene. Oncogene.

Cabezas, A., Bache, K. G., Brech, A., and Stenmark, H. (2005). Alix regulates cortical actin and the spatial distribution of endosomes. J Cell Sci *118*, 2625-2635.

Cao, Y., Dai, Y., Cui, S., and Ma, L. (2008). Histone H2B Monoubiquitination in the Chromatin of FLOWERING LOCUS C Regulates Flowering Time in Arabidopsis. Plant Cell.

Carron, C., Cormier, F., Janin, A., Lacronique, V., Giovannini, M., Daniel, M. T., Bernard, O., and Ghysdael, J. (2000). TEL-JAK2 transgenic mice develop T-cell leukemia. Blood *95*, 3891-3899.

Cerboni, C., Gismondi, A., Palmieri, G., Piccoli, M., Frati, L., and Santoni, A. (1998). CD16-mediated activation of phosphatidylinositol-3 kinase (PI-3K) in human NK cells involves tyrosine phosphorylation of Cbl and its association with Grb2, Shc, pp36 and p85 PI-3K subunit. Eur J Immunol *28*, 1005-1015.

Chamberlain, M. D., Berry, T. R., Pastor, M. C., and Anderson, D. H. (2004). The p85alpha subunit of phosphatidylinositol 3'-kinase binds to and stimulates the GTPase activity of Rab proteins. J Biol Chem *279*, 48607-48614.

Chamberlain, M. D., Chan, T., Oberg, J. C., Hawrysh, A. D., James, K. M., Saxena, A., Xiang, J., and Anderson, D. H. (2008). Disrupted RabGAP function of the p85 subunit of phosphatidylinositol 3-kinase results in cell transformation. J Biol Chem *283*, 15861-15868.

Chatellard-Causse, C., Blot, B., Cristina, N., Torch, S., Missotten, M., and Sadoul, R. (2002). Alix (ALG-2-interacting protein X), a protein involved in apoptosis, binds to endophilins and induces cytoplasmic vacuolization. J Biol Chem *277*, 29108-29115.

Chen, B., Borinstein, S. C., Gillis, J., Sykes, V. W., and Bogler, O. (2000). The glioma-associated protein SETA interacts with AIP1/Alix and ALG-2 and modulates apoptosis in astrocytes. J Biol Chem *275*, 19275-19281.

Chen, H. H., Tarng, D. C., Lee, K. F., Wu, C. Y., and Chen, Y. C. (2008). Epoetin alfa and darbepoetin alfa: effects on ventricular hypertrophy in patients with chronic kidney disease. J Nephrol *21*, 543-549.

Chin, H., Arai, A., Wakao, H., Kamiyama, R., Miyasaka, N., and Miura, O. (1998). Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. Blood *91*, 3734-3745.

Chin, L. S., Raynor, M. C., Wei, X., Chen, H. Q., and Li, L. (2001). Hrs interacts with sorting nexin 1 and regulates degradation of epidermal growth factor receptor. J Biol Chem *276*, 7069-7078.

Clague, M. J., and Urbe, S. (2001). The interface of receptor trafficking and signalling. J Cell Sci *114*, 3075-3081.

Cohen, J., Altaratz, H., Zick, Y., Klingmuller, U., and Neumann, D. (1997). Phosphorylation of erythropoietin receptors in the endoplasmic reticulum by pervanadate-mediated inhibition of tyrosine phosphatases. Biochem J *327 (Pt 2)*, 391-397.

Conner, S. D., and Schmid, S. L. (2003). Regulated portals of entry into the cell. Nature *422*, 37-44.

Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999a). The Erythropoietin Receptor: Structure, Activation and Intracellular Signal Transduction. Trends Endocrinol Metab *10*, 18-23.

Constantinescu, S. N., Liu, X., Beyer, W., Fallon, A., Shekar, S., Henis, Y. I., Smith, S. O., and Lodish, H. F. (1999b). Activation of the erythropoietin receptor by the gp55-P viral envelope protein is determined by a single amino acid in its transmembrane domain. Embo J *18*, 3334-3347.

D'Andrea, A. D., Lodish, H. F., and Wong, G. G. (1989). Expression cloning of the murine erythropoietin receptor. Cell *57*, 277-285.

Damen, J. E., Liu, L., Wakao, H., Miyajima, A., Rosten, P., Jefferson, A. B., Majerus, P. W., Krosl, J., Humphries, R. K., and Krystal, G. (1997). The role of erythropoietin receptor tyrosine phosphorylation in erythropoietin-induced proliferation. Leukemia *11 Suppl 3*, 423-425.

de Melker, A. A., van der Horst, G., and Borst, J. (2004). Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. J Biol Chem *279*, 55465-55473.

Dikic, I. (2004). ALIX-ing phospholipids with endosome biogenesis. Bioessays *26*, 604-607.

Divoky, V., Liu, Z., Ryan, T. M., Prchal, J. F., Townes, T. M., and Prchal, J. T. (2001). Mouse model of congenital polycythemia: Homologous replacement of murine gene by mutant human erythropoietin receptor gene. Proc Natl Acad Sci U S A *98*, 986-991.

Dolznig, H., Habermann, B., Stangl, K., Deiner, E. M., Moriggl, R., Beug, H., and Mullner, E. W. (2002). Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. Curr Biol *12*, 1076-1085.

Donepudi, M., and Resh, M. D. (2008). c-Src trafficking and co-localization with the EGF receptor promotes EGF ligand-independent EGF receptor activation and signaling. Cell Signal *20*, 1359-1367.

Donovan, J. A., Wange, R. L., Langdon, W. Y., and Samelson, L. E. (1994). The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor. J Biol Chem *269*, 22921-22924.

Duan, L., Miura, Y., Dimri, M., Majumder, B., Dodge, I. L., Reddi, A. L., Ghosh, A., Fernandes, N., Zhou, P., Mullane-Robinson, K., *et al.* (2003). Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. J Biol Chem *278*, 28950-28960.

Dunlop, E. A., Maxwell, A. P., and Lappin, T. R. (2007). Impaired downregulation following erythropoietin receptor activation in non-small cell lung carcinoma. Stem Cells *25*, 380-384.

Eiseman, E., and Bolen, J. B. (1992). Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. Nature *355*, 78-80.

Elliott, S., Egrie, J., Browne, J., Lorenzini, T., Busse, L., Rogers, N., and Ponting, I. (2004). Control of rHuEPO biological activity: the role of carbohydrate. Exp Hematol *32*, 1146-1155.

Erslev, A. (1953). Humoral regulation of red cell production. Blood *8*, 349-357.

Erslev, A. J., Caro, J., Kansu, E., and Silver, R. (1980). Renal and extrarenal erythropoietin production in anaemic rats. Br J Haematol *45*, 65-72.

Falguieres, T., Luyet, P. P., Bissig, C., Scott, C. C., Velluz, M. C., and Gruenberg, J. (2008). In vitro budding of intralumenal vesicles into late endosomes is regulated by Alix and Tsg101. Mol Biol Cell *19*, 4942-4955.

Feng, J., Witthuhn, B. A., Matsuda, T., Kohlhuber, F., Kerr, I. M., and Ihle, J. N. (1997). Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol Cell Biol *17*, 2497-2501.

Fiore, F., Ollendorff, V., and Birnbaum, D. (2001). Characterization of the mouse Cblc/Cbl3 gene. Biochem Biophys Res Commun *280*, 182-187.

Frank, S. J. (2002). Receptor dimerization in GH and erythropoietin action--it takes two to tango, but how? Endocrinology *143*, 2-10.

Friedman, A. D., Nimbalkar, D., and Quelle, F. W. (2003). Erythropoietin receptors associate with a ubiquitin ligase, p33RUL, and require its activity for erythropoietin-induced proliferation. J Biol Chem *278*, 26851-26861.

Funaki, M., Katagiri, H., Inukai, K., Kikuchi, M., and Asano, T. (2000). Structure and function of phosphatidylinositol-3,4 kinase. Cell Signal *12*, 135-142.

Furmanek, A., Hess, D., Rogniaux, H., and Hofsteenge, J. (2003). The WSAWS motif is C-hexosylated in a soluble form of the erythropoietin receptor. Biochemistry *42*, 8452-8458.

Gadina, M., Hilton, D., Johnston, J. A., Morinobu, A., Lighvani, A., Zhou, Y. J., Visconti, R., and O'Shea, J. J. (2001). Signaling by type I and II cytokine receptors: ten years after. Curr Opin Immunol *13*, 363-373.

Galcheva-Gargova, Z., Theroux, S. J., and Davis, R. J. (1995). The epidermal growth factor receptor is covalently linked to ubiquitin. Oncogene *11*, 2649-2655.

Garcia, Z., Silio, V., Marques, M., Cortes, I., Kumar, A., Hernandez, C., Checa, A. I., Serrano, A., and Carrera, A. C. (2006). A PI3K activity-independent function of p85 regulatory subunit in control of mammalian cytokinesis. Embo J *25*, 4740-4751.

Geng, F., and Tansey, W. P. (2008). Polyubiquitylation of histone H2B. Mol Biol Cell *19*, 3616-3624.

Glenney, J. R., Jr., Chen, W. S., Lazar, C. S., Walton, G. M., Zokas, L. M., Rosenfeld, M. G., and Gill, G. N. (1988). Ligand-induced endocytosis of the EGF receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies. Cell *52*, 675-684.

Goerttler, P. S., Steimle, C., Marz, E., Johansson, P. L., Andreasson, B., Griesshammer, M., Gisslinger, H., Heimpel, H., and Pahl, H. L. (2005). The Jak2V617F mutation, PRV-1 overexpression and EEC formation define a similar cohort of MPD patients. Blood.

Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991). rab5 controls early endosome fusion in vitro. Cell *64*, 915-925.

Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., and et al. (1993). The GTPase dynamin binds to and is activated by a subset of SH3 domains. Cell *75*, 25-36.

Grad, J. M., Zeng, X. R., and Boise, L. H. (2000). Regulation of Bcl-xL: a little bit of this and a little bit of STAT. Curr Opin Oncol *12*, 543-549.

Grandal, M. V., Zandi, R., Pedersen, M. W., Willumsen, B. M., van Deurs, B., and Poulsen, H. S. (2007). EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. Carcinogenesis *28*, 1408-1417.

Griesinger, F., Hennig, H., Hillmer, F., Podleschny, M., Steffens, R., Pies, A., Wormann, B., Haase, D., and Bohlander, S. K. (2005). A BCR-JAK2 fusion gene as the result of a t(9;22)(p24;q11.2) translocation in a patient with a clinically typical chronic myeloid leukemia. Genes Chromosomes Cancer.

Gross, A. W., and Lodish, H. F. (2006). Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP). J Biol Chem *281*, 2024-2032.

Grovdal, L. M., Stang, E., Sorkin, A., and Madshus, I. H. (2004). Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. Exp Cell Res *300*, 388-395.

Haglund, K., and Dikic, I. (2005). Ubiquitylation and cell signaling. Embo J *24*, 3353-3359.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol *5*, 461-466.

Halupa, A., Chohan, M., Stickle, N. H., Beattie, B. K., Miller, B. A., and Barber, D. L. (2005). Erythropoietin receptor Y479 couples to ERK1/2 activation via recruitment of phospholipase Cgamma. Exp Cell Res *309*, 1-11.

Hardee, M. E., Kirkpatrick, J. P., Shan, S., Snyder, S. A., Vujaskovic, Z., Rabbani, Z. N., Dewhirst, M. W., and Blackwell, K. L. (2005). Human recombinant erythropoietin (rEpo) has no effect on tumour growth or angiogenesis. Br J Cancer *93*, 1350-1355.

Hartley, D., Meisner, H., and Corvera, S. (1995). Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl. J Biol Chem *270*, 18260-18263.

Heath, C. M., Stahl, P. D., and Barbieri, M. A. (2003). Lipid kinases play crucial and multiple roles in membrane trafficking and signaling. Histol Histopathol *18*, 989-998.

Hilton, D. J., Watowich, S. S., Katz, L., and Lodish, H. F. (1996). Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor. J Biol Chem *271*, 4699-4708.

Hilton, D. J., Watowich, S. S., Murray, P. J., and Lodish, H. F. (1995). Increased cell surface expression and enhanced folding in the endoplasmic reticulum of a mutant erythropoietin receptor. Proc Natl Acad Sci U S A *92*, 190-194.

Hitchcock, I. S., Chen, M. M., King, J. R., and Kaushansky, K. (2008). YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation. Blood.

Holt, K. H., Olson, L., Moye-Rowley, W. S., and Pessin, J. E. (1994). Phosphatidylinositol 3-kinase activation is mediated by high-affinity interactions between distinct domains within the p110 and p85 subunits. Mol Cell Biol *14*, 42-49.

Hookham, M. B., Elliott, J., Suessmuth, Y., Staerk, J., Ward, A. C., Vainchenker, W., Percy, M. J., McMullin, M. F., Constantinescu, S. N., and Johnston, J. A. (2007). The myeloproliferative disorder-associated JAK2 V617F mutant escapes negative regulation by suppressor of cytokine signaling 3. Blood *109*, 4924-4929.

Horak, J. (2003). The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. Biochim Biophys Acta *1614*, 139-155.

Hortner, M., Nielsch, U., Mayr, L. M., Heinrich, P. C., and Haan, S. (2002). A new high affinity binding site for suppressor of cytokine signaling-3 on the erythropoietin receptor. Eur J Biochem *269*, 2516-2526.

Huang, F., Goh, L. K., and Sorkin, A. (2007). EGF receptor ubiquitination is not necessary for its internalization. Proc Natl Acad Sci U S A *104*, 16904-16909.

Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006). Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Mol Cell *21*, 737-748.

Huang, F., and Sorkin, A. (2005). Growth factor receptor binding protein 2-mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis. Mol Biol Cell *16*, 1268-1281.

Huang, H., Li, L., Wu, C., Schibli, D., Colwill, K., Ma, S., Li, C., Roy, P., Ho, K., Songyang, Z., *et al.* (2008). Defining the specificity space of the human SRC homology 2 domain. Mol Cell Proteomics *7*, 768-784.

Huang, L. J., Constantinescu, S. N., and Lodish, H. F. (2001). The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol Cell *8*, 1327-1338.

Hunter, S., Burton, E. A., Wu, S. C., and Anderson, S. M. (1999). Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. J Biol Chem *274*, 2097-2106.

Hurley, J. H. (2008). ESCRT complexes and the biogenesis of multivesicular bodies. Curr Opin Cell Biol *20*, 4-11.

Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K. I., and Takahashi, R. (2002). CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell *10*, 55-67.

James, C., Ugo, V., Le Couedic, J. P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., *et al.* (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature *434*, 1144-1148.

Jegalian, A. G., and Wu, H. (2002). Differential roles of SOCS family members in EpoR signal transduction. J Interferon Cytokine Res *22*, 853-860.

Jeong, J. Y., Feldman, L., Solar, P., Szenajch, J., and Sytkowski, A. J. (2008). Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells. Int J Cancer *122*, 274-280.

Jiang, X., Huang, F., Marusyk, A., and Sorkin, A. (2003). Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell *14*, 858-870.

Jimenez, C., Portela, R. A., Mellado, M., Rodriguez-Frade, J. M., Collard, J., Serrano, A., Martinez, A. C., Avila, J., and Carrera, A. C. (2000). Role of the PI3K regulatory subunit in the control of actin organization and cell migration. J Cell Biol *151*, 249-262.

Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science *286*, 309-312.

Jones, A. V., Cross, N. C., White, H. E., Green, A. R., and Scott, L. M. (2008). Rapid identification of JAK2 exon 12 mutations using high resolution melting analysis. Haematologica *93*, 1560-1564.

Jongeward, G. D., Clandinin, T. R., and Sternberg, P. W. (1995). sli-1, a negative regulator of let-23-mediated signaling in C. elegans. Genetics *139*, 1553-1566.

Juul, S. E., Yachnis, A. T., and Christensen, R. D. (1998). Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. Early Hum Dev *52*, 235-249.

Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W. G., Jr., Conaway, R. C., and Conaway, J. W. (1998). The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. Genes Dev *12*, 3872-3881.

Kassenbrock, C. K., and Anderson, S. M. (2004). Regulation of ubiquitin protein ligase activity in c-Cbl by phosphorylation-induced conformational change and constitutive activation by tyrosine to glutamate point mutations. J Biol Chem *279*, 28017-28027.

Keane, M. M., Rivero-Lezcano, O. M., Mitchell, J. A., Robbins, K. C., and Lipkowitz, S. (1995). Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene. Oncogene *10*, 2367-2377.

Ketteler, R., Moghraby, C. S., Hsiao, J. G., Sandra, O., Lodish, H. F., and Klingmuller, U. (2003). The cytokine-inducible Scr homology domain-containing protein negatively regulates signaling by promoting apoptosis in erythroid progenitor cells. J Biol Chem *278*, 2654-2660.

Kim, T. J., Kim, Y. T., and Pillai, S. (1995). Association of activated phosphatidylinositol 3-kinase with p120cbl in antigen receptor-ligated B cells. J Biol Chem *270*, 27504-27509.

Klaesson, S. (1999). Clinical use of rHuEPO in bone marrow transplantation. Med Oncol *16*, 2-7.

Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995). Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell *80*, 729-738.

Klingmuller, U., Wu, H., Hsiao, J. G., Toker, A., Duckworth, B. C., Cantley, L. C., and Lodish, H. F. (1997). Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors. Proc Natl Acad Sci U S A *94*, 3016-3021.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635-644.

Koga, M., and Ohshima, Y. (1995). Mosaic analysis of the let-23 gene function in vulval induction of Caenorhabditis elegans. Development *121*, 2655-2666.

Komada, M., and Kitamura, N. (2005). The Hrs/STAM complex in the downregulation of receptor tyrosine kinases. J Biochem *137*, 1-8.

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., Tichelli, A., Cazzola, M., and Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med *352*, 1779-1790.

Krebs, D. L., and Hilton, D. J. (2001). SOCS proteins: negative regulators of cytokine signaling. Stem Cells *19*, 378-387.

Kumar, S. M., Yu, H., Fong, D., Acs, G., and Xu, X. (2006). Erythropoietin activates the phosphoinositide 3-kinase/Akt pathway in human melanoma cells. Melanoma Res *16*, 275-283.

Kurzer, J. H., Argetsinger, L. S., Zhou, Y. J., Kouadio, J. L., O'Shea, J. J., and Carter-Su, C. (2004). Tyrosine 813 is a site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-B beta. Mol Cell Biol *24*, 4557-4570.

Lacombe, C., Da Silva, J. L., Bruneval, P., Fournier, J. G., Wendling, F., Casadevall, N., Camilleri, J. P., Bariety, J., Varet, B., and Tambourin, P. (1988). Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. J Clin Invest *81*, 620-623.

Lacronique, V., Boureux, A., Valle, V. D., Poirel, H., Quang, C. T., Mauchauffe, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J., and Bernard, O. A. (1997). A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science *278*, 1309-1312.

Langdon, W. Y., Hartley, J. W., Klinken, S. P., Ruscetti, S. K., and Morse, H. C., 3rd (1989a). v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci U S A *86*, 1168-1172.

Langdon, W. Y., Hyland, C. D., Grumont, R. J., and Morse, H. C., 3rd (1989b). The c-cbl proto-oncogene is preferentially expressed in thymus and testis tissue and encodes a nuclear protein. J Virol *63*, 5420-5424.

Levin, I., Cohen, J., Supino-Rosin, L., Yoshimura, A., Watowich, S. S., and Neumann, D. (1998). Identification of a cytoplasmic motif in the erythropoietin receptor required for receptor internalization. FEBS Lett *427*, 164-170.

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., Boggon, T. J., Wlodarska, I., Clark, J. J., Moore, S., *et al.* (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell *7*, 387-397.

Li, K., Miller, C., Hegde, S., and Wojchowski, D. (2003). Roles for an Epo receptor Tyr-343 Stat5 pathway in proliferative co-signaling with kit. J Biol Chem *278*, 40702-40709.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature *363*, 85-88.

Li, N., Lorinczi, M., Ireton, K., and Elferink, L. A. (2007). Specific Grb2-mediated interactions regulate clathrin-dependent endocytosis of the cMet-tyrosine kinase. J Biol Chem *282*, 16764-16775.

Lin, F. K., Suggs, S., Lin, C. H., Browne, J. K., Smalling, R., Egrie, J. C., Chen, K. K., Fox, G. M., Martin, F., Stabinsky, Z., and et al. (1985). Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci U S A *82*, 7580-7584.

Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999). Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. Science *283*, 987-990.

Longmore, G. D., and Lodish, H. F. (1991). An activating mutation in the murine erythropoietin receptor induces erythroleukemia in mice: a cytokine receptor superfamily oncogene. Cell *67*, 1089-1102.

Lovly, C. M., Yan, L., Ryan, C. E., Takada, S., and Piwnica-Worms, H. (2008). Regulation of Chk2 ubiquitination and signaling through autophosphorylation of serine 379. Mol Cell Biol *28*, 5874-5885.

Lundby, C., Achman-Andersen, N. J., Thomsen, J. J., Norgaard, A. M., and Robach, P. (2008). Testing for recombinant human erythropoietin in urine: problems associated with current anti-doping testing. J Appl Physiol *105*, 417-419.

McDoom, I., Ma, X., Kirabo, A., Lee, K. Y., Ostrov, D. A., and Sayeski, P. P. (2008). Identification of tyrosine 972 as a novel site of Jak2 tyrosine kinase phosphorylation and its role in Jak2 activation. Biochemistry *47*, 8326-8334.

McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M., and Smythe, E. (1998). A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. Curr Biol *8*, 34-45.

Meyer, L., Deau, B., Forejtnikova, H., Dumenil, D., Margottin-Goguet, F., Lacombe, C., Mayeux, P., and Verdier, F. (2007). beta-Trcp mediates ubiquitination and degradation of the erythropoietin receptor and controls cell proliferation. Blood *109*, 5215-5222.

Missotten, M., Nichols, A., Rieger, K., and Sadoul, R. (1999). Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. Cell Death Differ *6*, 124-129.

Miura, O., Cleveland, J. L., and Ihle, J. N. (1993). Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. Mol Cell Biol *13*, 1788-1795.

Miura, O., Nakamura, N., Ihle, J. N., and Aoki, N. (1994). Erythropoietin-dependent association of phosphatidylinositol 3-kinase with tyrosine-phosphorylated erythropoietin receptor. J Biol Chem *269*, 614-620.

Monami, G., Emiliozzi, V., and Morrione, A. (2008). Grb10/Nedd4-mediated multiubiquitination of the insulin-like growth factor receptor regulates receptor internalization. J Cell Physiol *216*, 426-437.

Morgan, J. R., Prasad, K., Jin, S., Augustine, G. J., and Lafer, E. M. (2003). Eps15 homology domain-NPF motif interactions regulate clathrin coat assembly during synaptic vesicle recycling. J Biol Chem *278*, 33583-33592.

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J., and Yarden, Y. (2003). Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem *278*, 21323-21326.

Motegi, A., Liaw, H. J., Lee, K. Y., Roest, H. P., Maas, A., Wu, X., Moinova, H., Markowitz, S. D., Ding, H., Hoeijmakers, J. H., and Myung, K. (2008). Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication forks. Proc Natl Acad Sci U S A *105*, 12411-12416.

Motohashi, T., Nakamura, Y., Osawa, M., Hiroyama, T., Iwama, A., Shibuya, A., and Nakauchi, H. (2001). Increased cell surface expression of C-terminal truncated erythropoietin receptors in polycythemia. Eur J Haematol *67*, 88-93.

Mousavi, S. A., Malerod, L., Berg, T., and Kjeken, R. (2004). Clathrin-dependent endocytosis. Biochem J *377*, 1-16.

Murphy, M. A., Schnall, R. G., Venter, D. J., Barnett, L., Bertoncello, I., Thien, C. B., Langdon, W. Y., and Bowtell, D. D. (1998). Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. Mol Cell Biol *18*, 4872-4882.

Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998). Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell *93*, 397-409.

Neumann, D., Wikstrom, L., Watowich, S. S., and Lodish, H. F. (1993). Intermediates in degradation of the erythropoietin receptor accumulate and are degraded in lysosomes. J Biol Chem *268*, 13639-13649.

Norbury, C. C. (2006). Drinking a lot is good for dendritic cells. Immunology *117*, 443-451.

O'Brien, K. B., Argetsinger, L. S., Diakonova, M., and Carter-Su, C. (2003). YXXL motifs in SH2-Bbeta are phosphorylated by JAK2, JAK1, and platelet-derived growth factor receptor and are required for membrane ruffling. J Biol Chem *278*, 11970-11978.

Odai, H., Sasaki, K., Iwamatsu, A., Hanazono, Y., Tanaka, T., Mitani, K., Yazaki, Y., and Hirai, H. (1995). The proto-oncogene product c-Cbl becomes tyrosine phosphorylated by stimulation with GM-CSF or Epo and constitutively binds to the SH3 domain of Grb2/Ash in human hematopoietic cells. J Biol Chem *270*, 10800-10805.

Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., *et al.* (1998). Jak2 is essential for signaling through a variety of cytokine receptors. Cell *93*, 385-395.

Pearse, B. M. (1976). Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. Proc Natl Acad Sci U S A *73*, 1255-1259.

Peeters, P., Raynaud, S. D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van den Berghe, H., and Marynen, P. (1997). Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood *90*, 2535-2540.

Pickart, C. M., and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta *1695*, 55-72.

Pickart, C. M., and Fushman, D. (2004). Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol *8*, 610-616.

Piessevaux, J., De Ceuninck, L., Catteeuw, D., Peelman, F., and Tavernier, J. (2008). Elongin B/C recruitment regulates substrate binding by CIS. J Biol Chem *283*, 21334-21346.

Putz, U., Howitt, J., Lackovic, J., Foot, N., Kumar, S., Silke, J., and Tan, S. S. (2008). Nedd4-family interacting protein 1 (Ndfip1) is required for the exosomal secretion of Nedd4-family proteins. J Biol Chem.

Radhakrishnan, Y., Maile, L. A., Ling, Y., Graves, L. M., and Clemmons, D. R. (2008). Insulin-like growth factor-I stimulates Shc-dependent phosphatidylinositol 3-kinase activation via Grb2-associated p85 in vascular smooth muscle cells. J Biol Chem *283*, 16320-16331.

Radtke, S., Haan, S., Jorissen, A., Hermanns, H. M., Diefenbach, S., Smyczek, T., Schmitz-Vandeleur, H., Heinrich, P. C., Behrmann, I., and Haan, C. (2005). The Jak1 SH2 domain does not fulfill a classical SH2 function in Jak/STAT signaling but plays a structural role for receptor interaction and up-regulation of receptor surface expression. J Biol Chem *280*, 25760-25768.

Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E., and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat Cell Biol *4*, 394-398.

Ravid, T., Heidinger, J. M., Gee, P., Khan, E. M., and Goldkorn, T. (2004). c-Cbl-mediated ubiquitinylation is required for epidermal growth factor receptor exit from the early endosomes. J Biol Chem *279*, 37153-37162.

Remy, I., Wilson, I. A., and Michnick, S. W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. Science *283*, 990-993.

Richmond, T. D., Chohan, M., and Barber, D. L. (2005). Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol *15*, 146-155.

Roskoski, R., Jr. (2004). The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun *319*, 1-11.

Roxrud, I., Raiborg, C., Pedersen, N. M., Stang, E., and Stenmark, H. (2008). An endosomally localized isoform of Eps15 interacts with Hrs to mediate degradation of epidermal growth factor receptor. J Cell Biol *180*, 1205-1218.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature *363*, 83-85.

Ruscetti, S. K. (1995). Erythroleukaemia induction by the Friend spleen focus-forming virus. Baillieres Clin Haematol *8*, 225-247.

Ryan, P. E., Davies, G. C., Nau, M. M., and Lipkowitz, S. (2006). Regulating the regulator: negative regulation of Cbl ubiquitin ligases. Trends Biochem Sci *31*, 79-88.

Sasaki, A., Yasukawa, H., Shouda, T., Kitamura, T., Dikic, I., and Yoshimura, A. (2000). CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2. J Biol Chem *275*, 29338-29347.

Sawyer, S. T., and Hankins, W. D. (1993). The functional form of the erythropoietin receptor is a 78-kDa protein: correlation with cell surface expression, endocytosis, and phosphorylation. Proc Natl Acad Sci U S A *90*, 6849-6853.

Schmidt, M. H., and Dikic, I. (2005). The Cbl interactome and its functions. Nat Rev Mol Cell Biol *6*, 907-918.

Schmidt, M. H., Hoeller, D., Yu, J., Furnari, F. B., Cavenee, W. K., Dikic, I., and Bogler, O. (2004). Alix/AIP1 antagonizes epidermal growth factor receptor downregulation by the Cbl-SETA/CIN85 complex. Mol Cell Biol *24*, 8981-8993.

Sinclair, A. M., and Elliott, S. (2005). Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. J Pharm Sci *94*, 1626-1635.

Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C., and Lodish, H. F. (1999). Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell *98*, 181-191.

Solar, P., Feldman, L., Jeong, J. Y., Businge, J. R., and Sytkowski, A. J. (2008). Erythropoietin treatment of human ovarian cancer cells results in enhanced signaling and a paclitaxel-resistant phenotype. Int J Cancer *122*, 281-288.

Sorkin, A., Helin, K., Waters, C. M., Carpenter, G., and Beguinot, L. (1992). Multiple autophosphorylation sites of the epidermal growth factor receptor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors. J Biol Chem *267*, 8672-8678.

Sorkin, A., Westermark, B., Heldin, C. H., and Claesson-Welsh, L. (1991). Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF beta-receptor. J Cell Biol *112*, 469-478.

Sorokin, A., Mohammadi, M., Huang, J., and Schlessinger, J. (1994). Internalization of fibroblast growth factor receptor is inhibited by a point mutation at tyrosine 766. J Biol Chem *269*, 17056-17061.

Standaert, M. L., Sajan, M. P., Miura, A., Bandyopadhyay, G., and Farese, R. V. (2004). Requirements for pYXXM motifs in Cbl for binding to the p85 subunit of phosphatidylinositol 3-kinase and Crk, and activation of atypical protein kinase C and glucose transport during insulin action in 3T3/L1 adipocytes. Biochemistry *43*, 15494-15502.

Stang, E., Blystad, F. D., Kazazic, M., Bertelsen, V., Brodahl, T., Raiborg, C., Stenmark, H., and Madshus, I. H. (2004). Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. Mol Biol Cell *15*, 3591-3604.

Stern, K. A., Visser Smit, G. D., Place, T. L., Winistorfer, S., Piper, R. C., and Lill, N. L. (2007). Epidermal growth factor receptor fate is controlled by Hrs tyrosine phosphorylation sites that regulate Hrs degradation. Mol Cell Biol *27*, 888-898.

Stubiger, G., Marchetti, M., Nagano, M., Grimm, R., Gmeiner, G., Reichel, C., and Allmaier, G. (2005). Characterization of N- and O-glycopeptides of recombinant human erythropoietins as potential biomarkers for doping analysis by means of microscale sample purification combined with MALDI-TOF and quadrupole IT/RTOF mass spectrometry. J Sep Sci *28*, 1764-1778.

Suzuki, N., Ohneda, O., Takahashi, S., Higuchi, M., Mukai, H. Y., Nakahata, T., Imagawa, S., and Yamamoto, M. (2002). Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. Blood *100*, 2279-2288.

Swaminathan, G., Varghese, B., Thangavel, C., Carbone, C. J., Plotnikov, A., Kumar, K. G., Jablonski, E. M., Clevenger, C. V., Goffin, V., Deng, L., *et al.* (2008). Prolactin stimulates ubiquitination, initial internalization, and degradation of its receptor via catalytic activation of Janus kinase 2. J Endocrinol *196*, R1-7.

Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslund, T. D., Chirino, A. J., Zhang, J., *et al.* (1998). Efficiency of signalling through cytokine receptors depends critically on receptor orientation. Nature *395*, 511-516.

Tanaka, N., Kyuuma, M., and Sugamura, K. (2008). Endosomal sorting complex required for transport proteins in cancer pathogenesis, vesicular transport, and non-endosomal functions. Cancer Sci *99*, 1293-1303.

Taniguchi, C. M., Tran, T. T., Kondo, T., Luo, J., Ueki, K., Cantley, L. C., and Kahn, C. R. (2006). Phosphoinositide 3-kinase regulatory subunit p85alpha suppresses insulin action via positive regulation of PTEN. Proc Natl Acad Sci U S A *103*, 12093-12097.

Tanner, J. W., Chen, W., Young, R. L., Longmore, G. D., and Shaw, A. S. (1995). The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. J Biol Chem *270*, 6523-6530.

Thien, C. B., Walker, F., and Langdon, W. Y. (2001). RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. Mol Cell *7*, 355-365.

Tjelle, T. E., Lovdal, T., and Berg, T. (2000). Phagosome dynamics and function. Bioessays *22*, 255-263.

Tong, W., Sulahian, R., Gross, A. W., Hendon, N., Lodish, H. F., and Huang, L. J. (2006). The membrane proximal region of the thrombopoietin receptor (TpoR) confers its high surface expression by JAK2-dependent and independent mechanisms. J Biol Chem.

Torrisi, M. R., Lotti, L. V., Belleudi, F., Gradini, R., Salcini, A. E., Confalonieri, S., Pelicci, P. G., and Di Fiore, P. P. (1999). Eps15 is recruited to the plasma membrane upon epidermal growth factor receptor activation and localizes to components of the endocytic pathway during receptor internalization. Mol Biol Cell *10*, 417-434.

Tovari, J., Pirker, R., Timar, J., Ostoros, G., Kovacs, G., and Dome, B. (2008). Erythropoietin in cancer: an update. Curr Mol Med *8*, 481-491.

Ueki, K., Fruman, D. A., Yballe, C. M., Fasshauer, M., Klein, J., Asano, T., Cantley, L. C., and Kahn, C. R. (2003). Positive and negative roles of p85 alpha and p85 beta regulatory subunits of phosphoinositide 3-kinase in insulin signaling. J Biol Chem *278*, 48453-48466.

Umebayashi, K., Stenmark, H., and Yoshimori, T. (2008). Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. Mol Biol Cell *19*, 3454-3462.

Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D. J., and Silvennoinen, O. (2002). Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. Mol Cell Biol *22*, 3316-3326.

Urbe, S., Sachse, M., Row, P. E., Preisinger, C., Barr, F. A., Strous, G., Klumperman, J., and Clague, M. J. (2003). The UIM domain of Hrs couples receptor sorting to vesicle formation. J Cell Sci *116*, 4169-4179.

van Kerkhof, P., Govers, R., Alves dos Santos, C. M., and Strous, G. J. (2000). Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. J Biol Chem *275*, 1575-1580.

Varghese, B., Barriere, H., Carbone, C. J., Banerjee, A., Swaminathan, G., Plotnikov, A., Xu, P., Peng, J., Goffin, V., Lukacs, G. L., and Fuchs, S. Y. (2008). Polyubiquitination of prolactin receptor stimulates its internalization, postinternalization sorting, and degradation via the lysosomal pathway. Mol Cell Biol *28*, 5275-5287.

Verdier, F., Walrafen, P., Hubert, N., Chretien, S., Gisselbrecht, S., Lacombe, C., and Mayeux, P. (2000). Proteasomes regulate the duration of erythropoietin receptor activation by controlling down-regulation of cell surface receptors. J Biol Chem *275*, 18375-18381.

Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996). Control of EGF receptor signaling by clathrin-mediated endocytosis. Science *274*, 2086-2089.

Vincent, V., Goffin, V., Rozakis-Adcock, M., Mornon, J. P., and Kelly, P. A. (1997). Identification of cytoplasmic motifs required for short prolactin receptor internalization. J Biol Chem *272*, 7062-7068.

Walrafen, P., Verdier, F., Kadri, Z., Chretien, S., Lacombe, C., and Mayeux, P. (2005). Both proteasomes and lysosomes degrade the activated erythropoietin receptor. Blood *105*, 600-608.

Wang, J., Auger, K. R., Jarvis, L., Shi, Y., and Roberts, T. M. (1995). Direct association of Grb2 with the p85 subunit of phosphatidylinositol 3-kinase. J Biol Chem *270*, 12774-12780.

Ward, A. C., Touw, I., and Yoshimura, A. (2000). The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood *95*, 19-29.

Ward, A. C., van Aesch, Y. M., Schelen, A. M., and Touw, I. P. (1999). Defective internalization and sustained activation of truncated granulocyte colony-stimulating factor receptor found in severe congenital neutropenia/acute myeloid leukemia. Blood *93*, 447-458.

Wish, J. B., and Coyne, D. W. (2007). Use of erythropoiesis-stimulating agents in patients with anemia of chronic kidney disease: overcoming the pharmacological and pharmacoeconomic limitations of existing therapies. Mayo Clin Proc *82*, 1371-1380.

Wolf, D. H. (2004). From lysosome to proteasome: the power of yeast in the dissection of proteinase function in cellular regulation and waste disposal. Cell Mol Life Sci *61*, 1601-1614.

Yasuda, Y., Fujita, Y., Musha, T., Tanaka, H., Shiokawa, S., Nakamatsu, K., Mori, S., Matsuo, T., and Nakamura, Y. (2001). Expression of erythropoietin in human female reproductive organs. Ital J Anat Embryol *106*, 215-222.

Yen, C. H., Yang, Y. C., Ruscetti, S. K., Kirken, R. A., Dai, R. M., and Li, C. C. (2000). Involvement of the ubiquitin-proteasome pathway in the degradation of nontyrosine kinase-type cytokine receptors of IL-9, IL-2, and erythropoietin. J Immunol *165*, 6372-6380.

Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A., and Baron, R. (1999). Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. J Biol Chem *274*, 31707-31712.

Yoon, C. H., Lee, J., Jongeward, G. D., and Sternberg, P. W. (1995). Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c-cbl. Science *269*, 1102-1105.

Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y., and Lodish, H. F. (1992). Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. J Biol Chem *267*, 11619-11625.

Zang, H., Sato, K., Nakajima, H., McKay, C., Ney, P. A., and Ihle, J. N. (2001). The distal region and receptor tyrosines of the Epo receptor are non-essential for in vivo erythropoiesis. Embo J *20*, 3156-3166.

Zhao, R., Xing, S., Li, Z., Fu, X., Li, Q., Krantz, S. B., and Zhao, Z. J. (2005). Identification of an acquired JAK2 mutation in polycythemia vera. J Biol Chem *280*, 22788-22792.

Zhao, W., Kitidis, C., Fleming, M. D., Lodish, H. F., and Ghaffari, S. (2006). Erythropoietin stimulates phosphorylation and activation of GATA-1 via the PI3-kinase/AKT signaling pathway. Blood *107*, 907-915.

Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533-539.