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Signaling through the Erythropoietin Receptor is Promoted by
Dense Packing of the Transmembrane Domain
and Regulated by Rapid Receptor Internalization

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Summary

The fine-tuned balance of self-renewal and rapid adaptation in the hematopoietic system are regulated by cytokines. Cytokine receptors are single membrane-spanning proteins that lack intrinsic enzymatic activity and therefore associate with cytoplasmic tyrosine kinases to initiate signal transduction. The key regulator of erythropoiesis is the erythropoietin receptor (EpoR) that shows low cell surface expression and a partially punctuated subcellular localization. Efficient signaling through the preformed homodimeric receptor is facilitated by self-assembly of the transmembrane (TM) domain. Moreover, the sensitivity of signal transduction depends on the extent of receptor accessible for ligand binding and therefore on the trafficking kinetics for transport to and removal from the plasma membrane.

By using single-particle tracking, we demonstrated that trafficking of EpoR-containing vesicle-like structures critically relies on active transport along microtubules, leading to enhanced diffusion in the crowded cytoplasm. A TM domain mutant EpoR-T242N was identified that is not detected in punctuated structures. Surprisingly, EpoR-T242N showed cell surface expression as well as maturation and internalization kinetics comparable to wild-type EpoR, but deficiencies in selective signal amplification of downstream signal pathways. All-atom molecular modeling revealed an increased interhelical distance for the EpoR-T242N TM dimer, suggesting a link between packing density of the TM domain and the formation of visible dynamic higher oligomeric structures as well as efficient activation of signaling.

To gain insight into the dynamic behavior of receptor turnover and internalization, a systems biology approach was applied. Upon ligand stimulation, the EpoR was rapidly internalized, but remarkably the amount of ligand-bound receptor at the plasma membrane recovered after approximately four hours. Nevertheless, activation of EpoR was restrained upon prolonged stimulation, revealing that internalization does not mediate long-term attenuation of receptor signaling. Dynamic modeling of receptor endocytosis showed that the majority of internalized ligand was recycled to the medium, whereas only 20% were degraded. This mechanism permits EpoR signaling without depletion of the ligand in the extracellular environment, being especially important for low physiological Epo levels in the hematopoietic stem cell niche. Sensitivity analysis uncovered the parameters receptor turnover, $k_{on}$ for ligand binding, and internalization as critical for generating the steep rise and rapid decline in forming Epo-EpoR complexes, whereas the dissociation constant $K_D$ commonly used to characterize Epo derivatives for clinical applications had essentially no influence.

In conclusion we propose two mechanisms regulating signal activation at the receptor level. Rapid internalization of ligand-bound EpoR shapes the kinetics of signaling-competent ligand-receptor complex formation. Dynamic oligomerization beyond the dimer may permit control of selective amplification of downstream signal pathways and biological responses.
Zusammenfassung


Ein systembiologischer Ansatz wurde zur Untersuchung des dynamischen Verhaltens von Receptorumsatz und Internalisierung angewandt. Nach Ligandenstimulation wurde der EpoR schnell internalisiert, wobei die Menge an ligandengebundenem Rezeptor an der Plasmamembran bemerkenswerterweise nach ungefähr vier Stunden regeneriert war. Da die Aktivierung des EpoR nach anhaltender Stimulation trotz Präsenz des Receptors an der Zelloberfläche unterdrückt war, ist die Rezeptorinternalisierung nicht für die langfristige Abschwächung der Signalleitung verantwortlich. Die dynamische Modellierung der Rezeptorendozytose zeigte, dass der Großteil des internalisierten Liganden in das Medium rücktransportiert wurde, während nur 20% intrazellulär degradiert wurden. Dieser Mechanismus erlaubt eine Aktivierung des EpoR, ohne den Liganden im extrazellulären Medium aufzubrauchen, was vor allem bei den niedrigen physiologischen Epo-Konzentrationen der hämatopoetische Stammzellen nisch bedeutend ist. Eine Sensitivitätsanalyse identifizierte die Parameter Receptorumsatz, die Assoziationsrate \( k_{on} \) des Liganden sowie die Internalisierung als entscheidend, um den steilen Anstieg und die schnelle Abnahme bei der Bildung von Liganden-Rezeptor-Komplexen zu formen. Die Dissoziationskonstante \( K_D \), die im Allgemeinen zur Charakterisierung von Epo-Derivaten für klinische Anwendungen herangezogen wird, hatte dagegen keinen Einfluss auf diese Kinetik.

1. Introduction

Extracellular signals regulate a variety of cellular activities including growth, proliferation, survival, migration, and differentiation. Signaling molecules such as growth factors and cytokines are not capable to pass the plasma membrane and therefore bind to specific cell surface receptors to initiate signal transduction cascades within the cell. This process finally leads to modification of gene expression and thus regulates biological responses of the cell. Cytokines control highly adaptive developmental processes such as proliferation and differentiation of hematopoietic cells. Cytokine receptors consist of a signal-receiving extracellular domain, a single transmembrane (TM) domain, and a signal-transducing cytoplasmic domain that lacks intrinsic enzymatic activity and therefore has to associate with members of the Janus kinase (JAK) family of nonreceptor tyrosine kinases to initiate signal transduction (Ihle et al., 1994). Recent studies demonstrated the existence of preformed homodimers in the absence of ligand for cytokine receptors such as the erythropoietin receptor (EpoR) (Livnah et al., 1999), the growth hormone receptor (GHR) (Gent et al., 2002), and the leptin receptor (Devos et al., 1997). Preformed dimers facilitate the formation of ligand-receptor complexes and thus permit efficient receptor activation, especially for receptors showing low expression levels at the cell surface such as the EpoR (Yoshimura et al., 1990).

The sensitivity of the cell to respond to ligands is determined by the amount of specific receptors on the plasma membrane. Therefore, maturation and internalization kinetics of receptor proteins shape the cellular response towards extracellular stimuli. Furthermore, the onset of signal transduction through cell surface receptors is critically influenced by structural properties determining self-interaction and orientation of their TM domains (Jiang and Hunter, 1999) as examined for the EpoR (Constantinescu et al., 2001a; Constantinescu et al., 2001b; Kubatzky et al., 2001), the GHR (Brown et al., 2005), and ErbB2 (Fleishman et al., 2002). Receptor signaling is terminated by recruitment of inhibitory molecules such as phosphatases as well as activation of negative feedback loops (Hilton, 1999; Schlessinger, 2000). Moreover, ligand-mediated receptor endocytosis is proposed to be involved in downregulation of cell surface receptors, thus providing a mechanism for long term attenuation of signals emanating from the cell surface (Waterman and Yarden, 2001) (Fig. 1).

1.1 Transmembrane Domains and Receptor Function

Genomic analysis of archaens, eubacteria, and eukaryotes predict that 20-30% of all open reading frames encode for membrane proteins (Wallin and von Heijne, 1998). In general, two
classes of membrane proteins can be distinguished. Proteins belonging to the \( \alpha \)-helical class include cell surface receptors, ion channels, transporters, and redox proteins, whereas proteins of the \( \beta \) class form large transmembrane pores.

Figure 1. Schematic representation of maturation and internalization of preformed dimeric cell surface receptors. After maturation in the rough endoplasmic reticulum (ER) and Golgi complex, cell surface receptors traffic to the plasma membrane where they can bind to extracellular ligands, undergo a conformational change and thus get activated. Constitutive and ligand-induced receptor internalization to early and recycling endosomes enables the cell to exchange the plasma membrane pool of the receptor. After trafficking through late endosomes, the receptor as well as ligand-receptor complexes are subjected to lysosomal degradation. This mechanism is proposed to mediate receptor downregulation and thereby terminate signaling.

Membrane proteins of the \( \alpha \)-helical class that possess a single TM domain are classified as bitopic membrane proteins. These proteins can in principal form homo-oligomeric or hetero-
oligomeric TM helical bundles. In contrast, membrane proteins of the α-helical class that consist of more than one TM domain are termed polytopic (Arkin, 2002).

In general, the TM domain of cell surface receptors not only anchors the protein to the plasma membrane, but self-interaction and orientation of TM helices also critically influence the onset of signal transduction (Jiang and Hunter, 1999).

Motifs determining the folding of transmembrane helices

To understand the assembly of transmembrane proteins, two energetically distinct processes can be distinguished (Popot and Engelman, 1990). The first process describes the formation of a stable TM α-helix in the lipid bilayer, while the second step defines the association of these independently stable TM helices to form an oligomeric TM bundle.

Assemblies of TM helices are stabilized by van der Waals packing and infrequent hydrogen bonds as well as electrostatic interactions of side chains. Weak hydrogen bonding also occurs involving the Cα atom, but their contribution to TM helix assembly is still unclear. Mutational analysis revealed several motifs playing an essential role in stabilizing interactions of oligomeric TM domains including the GxxxG motif, leucine zippers, interhelical hydrogen bonding as well as proline-based motifs (Arkin, 2002; Senes et al., 2004).

GxxxG motif: The first dimerization motif LIxxGVxxGVxxT was found by mutational analysis of the erythrocyte protein glycophorin A (Lemmon et al., 1994), which could be further minimized to GxxxG (Langosch et al., 1996). This motif turned out to be common in TM domains as shown by bioinformatical analysis including the ErbB family of growth factor receptor tyrosine kinases as well as many members of G protein-coupled receptors (Senes et al., 2004).

Leucine zipper: Another common oligomerization motif is the leucine zipper that can drive oligomerization of artificial TM segments in bacterial membranes (Gurezka et al., 1999). This motif is built of repeated heptad motifs (abcdefg) in which amino acid residues at position a and d form a hydrophobic core of the helical interface. A leucine zipper based motif is predicted for TM domains of several proteins including the Friend spleen focus-forming virus envelope protein, Xenopus laevis E-cadherin, and the murine EpoR (Gurezka et al., 1999).

Polar amino acids: The fraction of strongly hydrophilic amino acid residues in TM domain sequences is much smaller compared to the composition of proteins in general. However, despite their rare occurrence in TM helices, they are often highly conserved, indicating a structural or functional role of strongly hydrophilic residues for TM helix assembly and transmembrane protein function (Arkin and Brunger, 1998). Polar amino acids such as
asparagine, glutamine, aspartic acid, glutamic acid, as well as histidine are simultaneously hydrogen bond donors and acceptors and thus are capable to drive strong interhelical association of polyleucine TM helices. In contrast, substitutions to serine, threonine, or tyrosine within polyleucine TM segments do not increase TM association (Zhou et al., 2001). Although single interactions of serine residues do not contribute significantly to TM association, cooperative interactions between multiple serine residues are proposed to suffice to stabilize assembly of TM helices (Adamian and Liang, 2002). In general, the stabilizing contribution of polar amino acids depends on the position within the TM helix resulting from a polarity gradient within the plasma membrane (Subczynski et al., 1994). Therefore, the effect on TM association is stronger when polar amino acids are located in the hydrophobic core of the lipid bilayer compared to a position at the interface of the lipid bilayer with the surrounding polar environment (Lear et al., 2003).

Since polar amino acids can stabilize interhelical interactions and thereby promote TM assembly, they are capable to lock a transmembrane protein in a specific conformation. This might be the cause of constitutive activation of cell surface receptors upon single residue mutations in the TM domain as observed for ErbB2 (V664E) (Bargmann et al., 1986), the insulin receptor (V938D) (Longo et al., 1992), the granulocyte colony-stimulating factor (G-CSF) receptor (T617N) (Forbes et al., 2002), or the thrombopoietin (TPO) receptor (S498N) (Onishi et al., 1996).

Tools to study TM assemblies
Several approaches have been applied to elucidate the structure of TM domains and to identify motifs for TM assembly, including experimental methods as well as prediction of TM helix structures by computational modeling (Arkin, 2002).

Detection of oligomerization: The detection of oligomers within membranes is classically performed by cross-linking studies, while more recent studies employ fluorescence resonance energy transfer (FRET) as reported for the epidermal growth factor receptor (EGFR) (Gadella and Jovin, 1995), members of the cytokine receptor family including the leptin receptor (Biener et al., 2005) and the GHR (Brown et al., 2005), glycophorin A (Adair and Engelman, 1994), as well as G protein-coupled receptors in yeast (Overton and Blumer, 2002).

Reporter-based assays in bacterial systems have been developed including the ToxR assay to examine self-interaction of the glycophorin A (Langosch et al., 1996) as well as EpoR TM helices (Kubatzky et al., 2001; Ruan et al., 2004). This system makes use of chimeric proteins inserted into the inner bacterial of an E. coli reporter strain and consist of the E. coli periplasmic domain of the maltose-binding protein (MalE), the TM segment of interest and
the cytoplasmic domain of the *Vibrio cholerae* ToxR protein (Fig. 2). Upon dimerization of the TM segments, the ToxR domain binds the *cholera toxin* (*ctx*) promoter and thus activates transcription of a lacZ reporter gene. The TOXCAT system is based upon the ToxR assay and utilizes the transcription of antibiotic resistance genes and subsequent genetic selection (Russ and Engelman, 1999).

In addition, oligomers have been detected in detergents by polyacrylamide gel electrophoresis (PAGE) that is suitable if the oligomerization state of the protein remains stable during detergent treatment as reported for glycophorin A (Lemmon et al., 1992) or the cardiac ion channel protein phospholamban (Arkin et al., 1994). Analytical ultracentrifugation has also been successfully applied to examine TM oligomerization of glycophorin A in detergents (Fleming et al., 1997).

**Figure 2. Reporter-based bacterial ToxR assay.** The ToxR assay makes use of a chimeric protein inserted into the inner bacterial membrane of an *E. coli* reporter strain. The cytoplasmic domain of ToxR is linked to the periplasmic MalE domain via the TM segment of interest. Upon self-assembly of the TM domains, ToxR binds to the *ctx* promoter and thus activates transcription of a reporter gene. MalE, maltose-binding protein; *ctx*, *cholera toxin*.

**Structural methods:** X-ray and electron crystallography diffraction studies of TM assemblies have successfully been applied to solve the structures of transmembrane proteins including for example bacterial rhodopsins (Grigorieff et al., 1996; Pebay-Peyroula et al., 1997), bovine rhodopsin (Palczewski et al., 2000), and cytochrome c oxidase (Iwata et al., 1995). Crystallographic approaches to access the structure of bitopic membrane proteins have not yet been reported. However, high-resolution solution nuclear magnetic resonance has been successfully applied to elucidate the structure of the bitopic glycophorin A TM dimer in a detergent micelle (MacKenzie et al., 1997).
**Computational prediction methods:** To predict the structure of α-helical TM bundles by computational analysis, helices can be described by three parameters (Fig. 3a). These are the tilt angle $\beta$ defining the relative helix tilt in respect to the bundle axis and the rotational angle $\phi$. The crossing angle $\Omega$ can be calculated by adding the tilt angles of the respective helices. The helix register $r$ representing the relative vertical position of the helix is expected to equal zero if the helices are symmetrically positioned (Arkin, 2002). Based on this principle, monte carlo and molecular dynamics simulations in vacuum have been performed to predict the TM structure of several proteins including the ErbB2 TM dimers (Kim et al., 2003) as well as the Influenza A M2 channel (Forrest et al., 2000) and the EpoR TM dimers (Seubert et al., 2003), respectively. However, since TM segments contain polar amino acids, models taking into account the plasma membrane environment are desirable to predict whether polar amino acids are pointing away from or are part of the helical interface. A five-slab modeling approach that distinguishes between the solvent, head-group and core regions of a membrane has been established (Fig. 3b) and was successfully applied to reproduce the experimentally determined tilt angle of glycophorin A (Sengupta et al., 2005).

![Figure 3. Computational prediction of TM helix assemblies.](image)

(a) The assemblies of TM helices can be described by parameters defining the tilt angle ($\beta$), the rotational angle ($\phi$) and the helix register ($r$). The crossing angle $\Omega_{ij}$ can be calculated by adding the tilt angles $\beta_i$ and $\beta_j$ (adapted from Arkin, 2002). (b) A modeling approach that takes into account the plasma membrane environment utilizes a five-slab representation of a biological membrane depicting the solvent (water, dielectric constant $\varepsilon=80$) as well as the head-group ($\varepsilon=10$) and the core regions ($\varepsilon=2$) of the plasma membrane. The widths of the membrane slabs are given in Å (adapted from Sengupta et al., 2005).

The development and further improvement of tools to study TM assemblies can expand our knowledge about structural requirements of signal activation at the receptor level.
1.2 Endocytosis of Cell Surface Receptors

The sensitivity of signaling through cell surface receptors relies on the amount of receptor proteins on the plasma membrane and therefore accessibility to ligand binding. Thus, the trafficking kinetics of transport vesicles as well as the rates of receptor maturation and internalization have a critical impact on signal transduction. Besides the activation of negative regulatory feedback loops and the recruitment of phosphatases (Hilton, 1999; Schlessinger, 2000), ligand-mediated receptor internalization is proposed to provide a mechanism for long-term attenuation of signals emanating from the plasma membrane by downregulation of cell surface receptors (Waterman and Yarden, 2001). Receptor endocytosis can target activated ligand-receptor complexes to either lysosomal or proteasomal degradation or to the site of phosphatase activity as demonstrated by fluorescence lifetime imaging of the interaction between internalized EGFR and the protein tyrosine phosphatase PTP-1B on the surface of the endoplasmic reticulum (ER) (Haj et al., 2002).

Besides macropinocytosis and phagocytosis, two major mechanisms of internalization from the plasma membrane can be distinguished. The best-studied way of entry into the cell is the formation and budding of clathrin-coated pits. Upon recruitment from the cytoplasm, clathrin assembles to cage-like structures enclosing membrane vesicles. The formation of clathrin-coated vesicles, which are 100-150 nm in diameter, is regulated by binding of adaptor molecules such as AP-2 as well as the GTPase dynamin. After pinching off, the clathrin-coated vesicles are uncoated and their fusion with early endosomes is controlled by the GTPase Rab5 (Robinson et al., 1996). Non-clathrin mediated endocytosis is sensitive to cholesterol withdrawal and mainly relies on caveolae, which exhibit a size of 50-80 nm in diameter. Caveolae are flask-like invaginations of the plasma membrane and constitute a morphologically identifiable type of lipid rafts enriched in sphingolipids, cholesterol, and caveolin (Le Roy and Wrana, 2005). In general, endocytosis can deliver molecules to diverse intracellular compartments, including the ER, the Golgi complex, as well as endosomal and lysosomal compartments.

Members of the Rab GTPases participate in formation, motility, docking, and fusion of transport vesicles (Zerial and McBride, 2001). By using live cell imaging of fluorescently tagged Rab GTPases, diverse endosomal compartments and subcompartments can be defined. Rab4 and Rab5 show overlapping but distinct distribution on early endosomes, while Rab4 and Rab11 occupy discrete domains on recycling endosomes (Sönnichsen et al., 2000; de Renzis et al., 2002). Late endosomes display distinct distribution of the markers Rab7 and Rab9 (Barbero et al., 2002). A subset of late endosomes exhibits a multivesicular appearance. Therefore, these endosomes are termed multivesicular bodies, which eventually
fuse with lysosomes and subject their luminal cargo to lysosomal degradation (Katzmann et al., 2002).

In addition to downregulation of receptor signaling, the concept of signaling endosomes has recently been discussed to mediate amplification of signal transduction cascades through receptor tyrosine kinases (Miaczynska et al., 2004). For the EGFR, a FRET approach showed the association of the receptor with the adapter growth factor receptor-bound protein Grb2 in intracellular endosomal compartments (Sorkin et al., 2000). Endosomal activity of the platelet-derived growth factor receptor (PDGFR) recruits various signaling molecules such as Grb2 and the regulatory subunit p85 of the phosphatidylinositol 3-kinase (PI3K) (Wang et al., 2004).

Thus, both the temporal as well as spatial organization of receptor activation play a key role in initiating downstream signaling, thereby enabling the organism to implement a specific and precisely timed program of cellular responses.

1.3 The Erythropoietin Receptor

Erythropoiesis

Cytokines and their cognate receptors regulate the development of several cell types. A well-studied example for cytokine signaling is the fine-tuned balance of continuous renewal of short-lived hematopoietic cells from a single pluripotent hematopoietic stem cell (HSC) and rapid adaptation to environmental changes. This process termed hematopoiesis is additionally controlled by various growth factors and cell-cell contacts within the cellular microenvironment. Cytokine regulation of hematopoiesis has to ensure hemostasis by regeneration of approximately $10^{10}$ blood cells per hour. On the other hand, signaling through cytokines has to enable the organism to rapidly and appropriately react in a highly adaptive manner upon environmental changes such as infection or blood loss. The HSC gives rise to the lymphoid lineage forming the B and T lymphocytes and natural killer cells as well as to the myeloid lineage including platelets and erythrocytes, as well as mast cells, granulocytes (basophils, eosinophils, neutrophils) and macrophages (Weissman et al., 2001).

Erythropoiesis provides the body with red blood cells, which transport oxygen from the lung into the tissues. Erythrocytes possess a rather limited lifespan of approximately 120 days in human and 40 days in mice, respectively. During murine development, erythropoiesis can be classified into spatially, temporally, and morphologically distinct stages. Primitive erythropoiesis occurs in the blood islands of the yolk sac at 7.5 days postconception (d7.5) and produces large nucleated erythrocytes. In the second wave of erythropoiesis, the
production of red blood cells shifts to the fetal liver at d12.0, generating non-nucleated definitive erythrocytes. At d15.0 to d16.0, definitive erythropoiesis relocates to the bone marrow and the spleen where it occurs throughout life (Hoffman et al., 1995). During definitive erythropoiesis in mice (Fig. 4), the HCS cell gives rise to the multipotent colony-forming unit granulocytes, erythrocytes, monocytes, macrophages (CFU-GEMM), which undergoes commitment to the erythroid lineage by differentiating into the burst-forming unit-erythroid (BFU-E). In the presence of stem cell factor (SCF), interleukin-3 (IL-3), and erythropoietin (Epo), the BFU-E form erythroid clusters of up to 500 cells after 7-10 days (mice) or 17-20 days (human) of cultivation in semisolid medium. The BFU-E cells further develop into the colony-forming unit-erythroid (CFU-E), which after 2 days (mice) or 7 days (human) of \textit{in vitro} cultivation with Epo gives rise to small colonies consisting of 8-64 hemoglobin-positive cells. Under control of Epo, the CFU-E sequentially mature through four distinct stages of normoblasts, showing a gradual decrease in cell size as well as an increase in hemoglobin content. After ejection of the nucleus, the reticulocytes enter the blood stream and mature to oxygen-carrying erythrocytes (Hoffman et al., 1995).

![Figure 4. Schematic overview depicting the formation of erythrocytes from the HSC during murine definitive erythropoiesis.](image)

The generation of knockout mice (Fig. 5) demonstrated that Epo and its cognate receptor EpoR are the crucial regulators of red blood cell production (Wu et al., 1995). Commitment to the erythroid lineage does not depend on Epo. However, signaling through the EpoR
promotes survival, proliferation, and terminal differentiation of erythroid progenitor cells and the absence of Epo or the EpoR is embryonically lethal between d12.5 and d13.5 due to a block of CFU-E maturation and thus severe anemia.

The renewal of erythrocytes is a highly dynamic and at the same time accurately regulated process. Therefore, the precise initiation and termination of signal transduction through the EpoR is critical to maintain the balance of loss and formation of erythrocytes and thus to sustain the integrity of the erythropoietic compartment within the hematopoietic system. Dysregulation of EpoR signaling leads to erythroleukemia and more frequently anemia.

**Erythropoietin**

**Erythropoietin production**

Human Epo is a glycoprotein with a molecular mass of 30.4 kDa that acts as humoral cytokine and folds into a compact globular structure consisting of 4 α-helical bundles. Its carbohydrate chains are required for both secretion and full biological activity in vivo, whereas the peptide core of Epo is sufficient to stimulate in vitro erythropoiesis (Jelkmann, 2004). During embryogenesis, Epo is produced in the fetal liver whereas in the adult, Epo is primarily expressed in the tubular, juxtatubular endothelial, and interstitial cells of the adult kidney as well as extrarenally in hepatocytes and Kupffer cells (Krantz, 1991).

Low oxygen tension in the tissue due to blood loss, high altitude, or increased oxygen affinity for hemoglobin strongly induces expression of the Epo gene via members of the hypoxia-inducible transcription factors, HIF-1 and HIF-2. Epo induction results in an increase in erythrocyte formation. Subsequently, oxygen tension is improved and thus Epo expression decreases, representing a classic negative feedback loop (Ebert and Bunn, 1999).
Clinical use of erythropoietin

Recombinant human Epo (rhEpo) is successfully used to treat anemia associated with chronic renal failure, cancer therapy, or AIDS. However, rhEpo has a relative short serum half-life of about 8.5 hours requiring frequent dosing. Therefore, derivatives with a longer half-life have been developed including the hyperglycosylated novel erythropoiesis stimulating protein (NESP, darbepoetin alfa) (Macdougall et al., 1999) as well as the continuous erythropoietin receptor activator (CERA) harboring a 30 kDa methoxy-polyethylene glycol polymer chain (Brandt et al., 2006). Both Epo derivatives exhibit a lower affinity to the receptor.

Patients with malignant tumors often develop anemia either due to the activity of inflammatory cytokines (Means and Krantz, 1992) or due to side effects of chemotherapy and radiotherapy. To overcome anemia, treatment with rhEpo improves both prognosis (Thomas, 2001; Bohlius et al., 2005) and life quality of the patients (Glaspy, 1997). However, recent studies proposed that hypoxia conditions induce EpoR expression in human cancer cells (Acs et al., 2001; Arcasoy et al., 2003; Batra et al., 2003). Therefore, overtreatment with rhEpo or Epo derivatives may promote tumor growth as indicated for metastatic breast cancer (Leyland-Jones et al., 2005) and head and neck cancer (Henke et al., 2003). Thus, rhEpo should be carefully applied to not counteract the beneficial effects of treating malignancy-associated anemia.

Several studies demonstrated that Epo not only exerts hematopoietic activity but in addition also possesses a tissue-protective potential as well as angiogenic and neurotrophic functions. This non-hematopoietic function of Epo is suggested to be mediated by anti-apoptotic signaling through the signal transducer and activator of transcription STAT5 as well as PI3K (Jelkmann, 2004). To overcome side effects such as elevated hematocrit and thrombosis, carbamylated Epo (CEPO) has been developed that does not exhibit hematopoietic activities, but retains its cytoprotective capacity in neuronal cells (Leist et al., 2004) as well as in cardiomyocytes (Moon et al., 2006).

Signaling through the erythropoietin receptor

Structure of the erythropoietin receptor

The murine EpoR consists of 507 amino acids including the signal peptide (D'Andrea et al., 1989) and is a member of the type I cytokine receptor superfamily with a single membrane-spanning domain (Fig. 6). The ligand-binding extracellular domain possesses two fibronectin type II domains D1 and D2 and displays four cysteine residues with characteristic spacing and a WSXWS in proximity to the TM domain that are conserved among the hematopoietic
cytokine receptor superfamily (Bazan, 1990; Cosman et al., 1990). The signal-transducing cytoplasmic domain lacks intrinsic enzymatic activity and therefore associates with the cytoplasmic tyrosine kinase JAK2. Two proline-rich motifs Box1 and Box 2 are required for JAK2 function (Miura et al., 1993; Huang et al., 2001).

![Figure 6. Schematic representation of conserved features and the domain structure of the EpoR.](image)

Activation of cytokine receptors was thought to be mediated by hormone-induced receptor dimerization and subsequent activation of associated cytoplasmic JAK kinases. However, crystallographic studies demonstrated that the unliganded EpoR exists as a preformed dimer at the cell surface (Livnah et al., 1999). Ligand-independent receptor dimerization could also been shown by immunofluorescence co-patching analysis (Constantinescu et al., 2001b). Preformed dimers facilitate the formation of ligand-receptor complexes, especially if binding of the ligand to the first receptor subunit is of high affinity whereas binding to the second receptor subunit shows lower affinity as suggested for the EpoR (Philo et al., 1996). Thus, initiation of receptor signaling by a ligand-induced conformational switch of the preformed EpoR dimer (Remy et al., 1999) permits efficient activation despite low expression levels of the receptor at the cell surface (Yoshimura et al., 1990). Recently, it was proposed that JAK2 acts as a chaperone, binding the EpoR in the ER and thereby enhancing Golgi processing and cell surface expression of the receptor (Huang et al., 2001).
**The erythropoietin receptor transmembrane domain**

Sequence analysis of the EpoR predicted interaction of the TM domains based on a degenerated leucine zipper (Gurezka et al., 1999). Applying the bacterial ToxR assay, the existence of a leucine zipper motif was further supported by asparagine-scanning that revealed a heptad repeat pattern of increased EpoR TM self-interaction (Ruan et al., 2004). This approach was also employed to demonstrate self-interaction of the TM domain as critical for full activation of the preformed EpoR homodimer (Kubatzky et al., 2001). Insertion of a defined number of alanine residues within the TM domain or the juxtamembrane domain showed that efficient signaling through the EpoR is supported by proper orientation of the TM and juxtamembrane domain (Constantinescu et al., 2001a). Although it is well established that self-assembly of the EpoR TM domain facilitates receptor activation, it is unknown whether the EpoR undergoes oligomerization beyond the dimer. Clustering of the EpoR was speculated to mediate amplification of biological responses upon chemical pretreatment of erythroleukemia cells (Chern et al., 1990), but so far chemical cross-linking studies could not provide clear evidence for higher oligomeric structures (Damen et al., 1992; Takahashi et al., 1995).

Modeling of the EpoR TM domain has been performed using molecular dynamics simulations in vacuum, identifying two clusters that correspond to a functional active and inactive interface of receptor variants in which the extracellular domain of the EpoR was substituted by a coiled coil Put3 domain (Seubert et al., 2003). Recently, two independent studies revealed that mutation to cysteine of one of the first two EpoR TM residues L226 or I227 resulted in constitutive activation of the receptor (Kubatzky et al., 2005; Lu et al., 2006), probably by locking the receptor through disulfide bonds in an active conformation similar to an orientation the wild-type EpoR TM domain would adopt upon ligand binding.

**Signaling through the erythropoietin receptor**

EpoR activation is induced by ligand binding to the preformed receptor dimer and a subsequent conformational change bringing the cytoplasmic domains of the EpoR into close proximity.

**JAK2:** Upon these structural rearrangements, the receptor-associated JAK2 undergoes transphosphorylation and thereby gets activated (Witthuhn et al., 1993). In turn, JAK2 phosphorylates several tyrosine residues within the EpoR cytoplasmic domain, which then serve as docking sites for Src homology (SH) 2 domain-containing signaling molecules (Fig. 7). Analysis of knockout mice revealed the essential role of JAK2 for definitive erythropoiesis (Neubauer et al., 1998; Parganas et al., 1998).

**STAT5:** Signaling by STAT5 represents the most direct pathway to transmit receptor signaling to the nucleus. Binding of STAT5 to the phosphorylated residues Y343 and Y401 of
the EpoR is mediated by its SH2 domain and is sufficient for maximal STAT5 phosphorylation, whereas binding to phosphotyrosines 429 and 431 only results in partial STAT5 activation (Klingmüller et al., 1996). Upon phosphorylation by JAK2, STAT5 dissociates from the receptor, dimerizes, and enters the nucleus where it activates target gene expression including the negative regulator cytokine-inducible SH2 domain-containing protein (CIS) (Matsumoto et al., 1997). Rapid nucleocytoplasmic cycling of STAT5 has been shown to act as remote sensor continuously coupling receptor activation and gene expression (Swameye et al., 2003). STAT5 is proposed to mediate anti-apoptotic signaling through the EpoR (Socolovsky et al., 1999; Menon et al., 2006).

Figure 7. Schematic representation of positive and negative signal cascades activated through the EpoR. Upon Epo binding to the preformed receptor dimer, JAK2 gets activated by transphosphorylation and in turn phosphorylates the EpoR on several cytoplasmic tyrosine residues. Several signaling pathways emanate from the EpoR, including the RAF/Mek/ERK and the Akt/PKB cascade. The most direct way of signal transduction through the EpoR is the activation of the transcription factor STAT5. Induction of CIS represents a negative feedback loop for STAT5 activity. In addition to SOCS proteins, downregulation of EpoR signaling is mediated by recruitment of protein tyrosine phosphatases including SHP-1. Abbreviations, see text.
**MAPK**: Upon Epo binding, several members of the mitogen-activated protein kinase (MAPK) family including the extracellular-regulated kinases ERK1/2 are activated. There are several routes leading to phosphorylation of the MAPK signaling cascade. The classical route involves activation of Ras by either recruitment of SHC, Grb2 and the guanine-nucleotide exchange factor Sos to the receptor (Ravichandran et al., 1995) or JAK2 (He et al., 1995). An alternative route to activate MAPK signaling is proposed through activation of the protein kinase C (PKC) via the PI3K pathway (Toker et al., 1994; Karnitz et al., 1995). The MAPK cascade is critical for the differentiation of erythroid progenitor cells (Johnson et al., 1997).

**PI3K**: Activation of PI3K by either binding of its regulatory domain p85 to phosphorylated Y479 of the EpoR (Damen et al., 1995; Klingmüller et al., 1997) or by indirect recruitment and activation of the Grb2-associated binder Gab1 and Gab2 (Ravichandran et al., 1995) triggers phosphorylation of the protein kinase B (Akt/PKB) as well as PKC\(_\varepsilon\) (Toker et al., 1994). The PI3K pathway has been shown to promote Epo-induced maturation of erythroid progenitor cells (Ghaffari et al., 2006; Zhao et al., 2006).

**SHP-2**: The protein tyrosine phosphatase SHP-2 has been assigned as a positive mediator of EpoR signal transduction. Upon binding to phosphotyrosine 401 of the EpoR (Tauchi et al., 1996), SHP-2 is phosphorylated and thus can couple receptor activation to the MAPK cascade by recruitment of Grb2/Sos (Barber et al., 1997).

**Signal termination**: Several members of the suppressor of cytokine signaling (SOCS) family have been discovered to mediate termination of EpoR signaling. The negative regulators CIS (Ketteler et al., 2003) and SOCS-3 (Hortner et al., 2002) bind to the EpoR on phosphorylated Y401 and thereby compete for receptor binding with STAT5 and SHP-2. Furthermore, SOCS proteins are capable to target proteins for ubiquitin-dependent proteasomal degradation as shown for SOCS-1 that binds to activated JAK2 and thereby regulates kinase downregulation (Ungureanu et al., 2002). Recruitment of the protein tyrosine phosphatase SHP-1 to phosphotyrosine Y429 of the EpoR results in dephosphorylation and inactivation of JAK2 (Klingmüller et al., 1995). Two additional tyrosine phosphatases CD45 (Irie-Sasaki et al., 2001) and PTP-1B (Myers et al., 2001; Cohen et al., 2004) are discussed to negatively regulate JAK2 activity.

**Endocytosis and trafficking of the erythropoietin receptor**

Besides the recruitment of negative regulators, endocytosis is thought to play a critical role in the termination of signaling through receptor downregulation and lysosomal degradation. Ligand-mediated EpoR internalization depends on the WSXWS motif of the extracellular domain (Quelle et al., 1992). Furthermore, studies applying truncated receptor versions revealed the importance of cytoplasmic residues 268-276 (FEGLFTTHK) for Epo-mediated receptor endocytosis (Levin et al., 1998). Especially, F272 was shown to be crucial for ligand
uptake. However, additional cytoplasmic motifs were proposed since a full-length EpoR lacking residues 268-276 was not impaired in Epo-mediated internalization (Flint-Ashtamker et al., 2002). Furthermore, EpoR endocytosis does not strictly depend on both JAK2 activity as well as receptor tyrosine phosphorylation (Beckman et al., 1999). Blocking intracellular EpoR degradation by inhibiting cysteine proteinases results in an increase in the amount of cell surface receptor (Neumann et al., 1996). Downregulation of activated receptor in UT-7 cells is proposed to be mediated by lysosomal degradation (Walrafen et al., 2005). Recently, internalization and degradation kinetics of Epo bound to the human EpoR have been studied by kinetic modeling, showing that about 40% of ligand is degraded after uptake, while 60% of internalized Epo is resecreted intact (Gross and Lodish, 2006). However, so far little is known about how ligand-induced internalization determines the activation kinetics of the EpoR. Furthermore it is not understood if and to what extent endocytosis mediates amplification of downstream signaling through cytokine receptors in endosomal compartments as discussed for the EGFR and PDGFR (Miaczynska et al., 2004).

Immunofluorescence (Neumann et al., 1993) and live cell imaging studies (Ketteler et al., 2002b) indicated that the EpoR localizes to punctuated structures in unstimulated cells. These structures have been partially identified as lysosomes (Neumann et al., 1993). Several other cytokine receptors have also been observed in punctuated structures such as the leptin receptor (Couturier and Jockers, 2003), the prolactin receptor (Perrot-Applanat et al., 1997) and the common interleukin (IL)-2 gamma chain (γc) (Royer et al., 2005). However, it is unknown if a subset of EpoR-containing punctuated structures is part of the endosomal network, with what kinetics these punctuated structures move and whether active transport processes are involved to ensure efficient receptor trafficking in the crowded cytoplasm (Weiss et al., 2004).

1.4 Systems Biology

Much information about components of the EpoR signaling pathway has been accumulated. However, the functional properties of a system result from the dynamic interaction of network components. To uncover the dynamic behavior of a system such as signal transduction, systems biology approaches combine quantitatively acquired data with mathematical modeling, thereby elucidating general design principles. These systems’ properties include the capacity of rapid adaptation to environmental changes or robustness to internal perturbations in the amount and function of specific components (Kitano, 2002; Alberghina and Westerhoff, 2005). Applying a systems biology approach to EpoR internalization is thus desirable to gain insight into the regulation of receptor activation beyond the level of static
interaction schemes. Moreover, mathematical models allow *in silico* simulations to predict the dynamic behavior and thereby are valuable to point to new experiments for further understanding of a system. Additionally, by identifying control mechanisms of a network, systems biology approaches enables the identification of potential drug targets, thus being a new and fascinating tool of biomedical research.

1.5 Objective

The extent of receptors at the cell surface and thus the accessibility for ligand binding critically influences the sensitivity of signal transduction. Therefore, the rates of EpoR maturation and internalization as well as the trafficking kinetics of receptor-containing vesicle-like structures are of interest to gain insight into the regulation of signal initiation as well as termination at the receptor level. In addition to receptor trafficking, self-interaction of the TM domains of the preformed homodimeric EpoR facilitates efficient receptor activation despite its low cell surface expression. Although predictions of the EpoR TM structure have been proposed by in vacuum modeling, a deeper understanding of the influence of the EpoR TM domain on signaling lacks the correlation of a modeling approach with functional analysis of the mutant receptor proteins.

The goal of this work was to determine the influence of increased self-interaction of the EpoR TM domain on receptor trafficking and signal transduction by screening asparagine mutants for biological activity in the context of a full-length receptor. Further, this study aimed to understand the dynamic behavior of ligand-induced EpoR internalization by combining quantitative data for constitutive and Epo-stimulated receptor endocytosis with dynamic mathematical modeling, thereby uncovering determinants regulating receptor activation.
2. Results

2.1 Dense Packing of the Transmembrane Domain Promotes Selective Signal Amplification through the Epo Receptor

EpoR-containing vesicular structures move actively along microtubules

Trafficking of cell surface receptors controls their availability on the plasma membrane and thus receptor-mediated signal transduction. To monitor localization and trafficking of the EpoR in living cells, a variant of a C-terminally green fluorescent protein (GFP)-tagged receptor was used (Ketteler et al., 2002b). In line with immunofluorescence studies in NIH3T3 and BaF3 cells (Neumann et al., 1993), expression of wild-type EpoR in NIH3T3 cells revealed punctuated structures (Fig. 8, left panels). These structures were further characterized by coexpression of GFP-tagged wild-type EpoR and mRFP1-tagged markers for early and recycling endosomes, Rab4a and Rab5a. Both endosomal proteins showed a partial colocalization with wild-type EpoR (Fig. 8), indicating that a fraction of punctuated structures positive for wild-type EpoR are part of the endosomal network.

Figure 8. Wild-type EpoR colocalizes with endosomal markers. EpoR-positive punctuated structures in NIH3T3 cells stably expressing GFP-tagged wild-type HA-EpoR showed a partial colocalization with mRFP1-tagged Rab4a or Rab5a (indicated by arrows) as analyzed by spinning disc confocal microscopy (scale bar, 20 µm).

To determine if these punctuated structures were mobile and capable of supporting active transport of the EpoR, we utilized single-particle tracking of individual dots in NIH3T3 cells
expressing GFP-tagged EpoR. In particular, we determined the temporal variation of the average diffusion coefficient \(D(T)\) of a population of dots in several cells. In cells lacking microtubules due to treatment with nocodazole we did not observe active transport. Instead, all structures showed a strongly anomalous subdiffusion \(D(T) \sim T^{-0.5}\) (Fig. 9a; data provided by S. Heinzer and M. Weiss, DKFZ Heidelberg) in agreement with previous reports on anomalous diffusion in the crowded cytoplasm (Weiss et al., 2004). In contrast, a more dynamic motion was seen in untreated cells: the majority of structures showed normal diffusion with \(D(T)\) = const., while a subset of spots even showed super-diffusive behavior, \(D(T)\) = \(T\), which is indicative of active transport. From these data, the typical time \(\Delta t\) can be estimated that an EpoR-positive structure needs to travel a distance of \(\Delta x = 1\ \mu m\). Without microtubules we obtained \(\Delta t = 80\) min, while microtubule-mediated diffusion and super-diffusion yield a much more efficient traveling time of within \(\Delta t = 20\) s and \(\Delta t = 3\) s, respectively. Thus, active transport of EpoR-containing structures is critical to permit rapid receptor trafficking in the crowded cytoplasm and thereby facilitates exchange of the receptor on the cell surface.

![Figure 9. EpoR-containing punctuated structures move actively along microtubules.](image)

We were able to quantitatively reproduce and explain our observations in terms of a simple mathematical model (Fig. 9b). This model assumed that each punctuated structure shows a subdiffusive motion until binding to a randomly oriented microtubule with the rate \(k_{on}\). The attached punctuated structure is subject to directed transport along the microtubule with a velocity \(v = 1.5\ \mu m/s\), a typical value for kinesin-driven transport (Howard, 2001), until
detaching with the rate \( k_{\text{off}} \). For \( k_{\text{on}} = k_{\text{off}} = 0 \), the model reproduces the subdiffusive motion observed for nocodazole-treated cells. Normal diffusion was obtained for \( k_{\text{on}} = 7/\text{s}, k_{\text{off}} = 1/\text{s} \), while \( k_{\text{on}} = 20/\text{s}, k_{\text{off}} = 10/\text{s} \) yielded super-diffusive motion. Here, the different rates \( k_{\text{on}} \) and \( k_{\text{off}} \) derived for diffusive and super-diffusive motion may reflect the different amounts of motor proteins associated with the respective punctuated structures. We would like to emphasize that in agreement with the experimental data the model predicts that normal diffusion of the EpoR-containing structures relies on active transport. Thus, while the diffusion appears to be a solely thermally induced process at first glance it is a true nonequilibrium phenomenon.

**Altered subcellular detection of EpoR-T242N**

To test whether increased self-interaction of the EpoR TM domain influences receptor trafficking, we concentrated on the residues L240 to A245 since this region comprises the residues with the highest self-interaction (Ruan et al., 2004) as well as the double leucine motif L240/L241 shown to be critical for TM self-assembly and receptor activation (Kubatzky et al., 2001). Investigation of EpoR TM mutants exhibited no correlation of increased TM self-interaction and constitutive receptor activity (data not shown). Analysis of cell surface expression of mutants with increased TM self-interaction by flow cytometry revealed that only EpoR-T242N was expressed at the plasma membrane to a similar extent as wild-type EpoR (Fig. 10a). As demonstrated by live cell imaging, expression of the EpoR mutants L240N, L241N, V243N, L244N, and A245N in NIH3T3 cells resulted in a punctuated localization pattern (Fig. 10c) comparable to wild-type EpoR (Fig. 10b). The extent of

![Figure 10. EpoR-T242N is not detectable in punctuated structures.](image_url)

(a) Cell surface expression of GFP-tagged wild-type HA-EpoR and HA-EpoR TM domain mutants stably expressed in BaF3 cells was assessed by flow cytometry using a rat anti-HA antibody followed by a Cy5-coupled anti-rat IgG antibody. Live cells were gated using forward and side scatter and cell surface expression is shown as the percentage of the wild-type EpoR (mean ± S.D., n=3). Experiments were performed two times with similar results. (b,c) Expression of GFP-tagged wild-type HA-EpoR or HA-EpoR TM mutants in NIH3T3 cells was analyzed by spinning disc confocal microscopy (scale bar, 20 µm).
punctuated structures varied, partly correlating with the amount of cell surface receptor. Surprisingly, despite cell surface expression at similar levels as wild-type EpoR, the mutant EpoR-T242N exhibited a pattern indicative of primarily ER and Golgi complex localization and punctuated structures were not detectable (Fig. 10c).

**Wild-type EpoR and EpoR-T242N do not localize to lipid rafts**

Dynamic partitioning of proteins in lipid rafts has been suggested to promote receptor clustering and signal transduction (Simons and Toomre, 2000). The size of lipid rafts is discussed controversially (Anderson and Jacobson, 2002), however the punctuated localization pattern of the EpoR may reflect vesicles internalizing from lipid rafts as have been shown for the transforming growth factor (TGF)β receptor type II (Di Guglielmo et al., 2003) and the EGFR (Puri et al., 2005). Therefore we examined whether wild-type EpoR resides in lipid rafts and whether the altered detection pattern for EpoR-T242N is correlated to impaired lipid raft localization. Following detergent lysis and sucrose gradient fractionation of NIH3T3 cell lysates, samples were analyzed by immunoblotting. As positive and negative marker for lipid rafts, the distribution of Flotillin-1 and the transferrin receptor (TfR) was determined, respectively. Analysis of protein concentration revealed that approximately 85% of overall protein was detected in the pellet fractions #9/10 (data not shown). The positive marker Flotillin-1 was not exclusively found in the detergent-resistant membrane (DRM).

![Figure 11. Wild-type EpoR and EpoR-T242N do not localize to lipid rafts.](image)

NIH3T3 cells stably expressing GFP-tagged wild-type HA-EpoR or HA-EpoR-T242N were stimulated with 50 U/ml Epo or left unstimulated, lysed with 0.5% Triton X-100 and fractionated on a sucrose gradient. Total cellular lysates were separated by 10% SDS-PAGE. Blots were probed with anti-EpoR antibodies and reprobed with antibodies raised against Flotillin-1 as positive or the transferrin receptor (TfR) as a negative lipid raft marker.
fractions #2/3 but additionally in the pellet fractions, while the negative marker TfR was only detected in the pellet fractions (Fig. 11). Both wild-type EpoR and EpoR-T242N were enriched in the pellet fractions and this distribution was independent of Epo stimulation. Thus, although the fractionation procedure was inefficient regarding marker distribution, the detection of wild-type EpoR in punctuated structures does not correlate with lipid raft localization.

**EpoR-T242N maturation and internalization are comparable to wild-type EpoR**

Since the mutant receptor EpoR-T242N was not detected in punctuated structures by live cell imaging, but was present at the cell surface to a similar extent as wild-type EpoR (Fig. 10), we analyzed the steady state of cell surface expression in detail by determining processing and internalization kinetics of EpoR-T242N. We examined receptor processing in BaF3-EpoR and BaF3-EpoR-T242N cells that were pulse-labeled with \[^{35}\text{S}\]-methionine/cysteine and chased for up to 2 h. For each time point, immunoprecipitated EpoR was digested with EndoH or left untreated (Fig. 12a). For samples not digested with EndoH, wild-type EpoR showed a half-life of 36 min ± 3 min (Fig. 12b), whereas EpoR-T242N revealed a significantly longer half-life of 103 min ± 11 min. This increase in half-life of EpoR-T242N might contribute to its prominent ER staining as observed by live cell imaging (Fig. 10c). In line with cell surface expression levels, the EndoH-resistant form of both wild-type EpoR and EpoR-T242N (glycosylated form, EndoH-digested samples) were transported to the cell surface to a similar extent and degraded with comparable degradation kinetics, showing a half-life of approximately 60 min (Fig. 12a).

**Figure 12. Maturation of EpoR-T242N is comparable to wild-type EpoR.** (a) BaF3 cells (1x10^6) stably expressing HA-tagged wild-type EpoR or EpoR-T242N were labeled with \[^{35}\text{S}\]-methionine/cysteine and chased for up to 2 h. After immunoprecipitation with anti-EpoR antibodies, immunoprecipitates were left untreated or digested with EndoH. Proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and detected on a phosphoimager. Closed and open arrows indicate the glycosylated and unglycosylated EpoR, respectively. The experiment was performed three times with similar results. (b) Protein amounts of the total wild-type EpoR (blue circles) or EpoR-T242N (red triangles) of undigested immunoprecipitates were quantified and expressed as the percentage of the protein amount at the beginning of the chase. An exponential decay curve was fitted to three independent data sets.
Results

To analyze whether EpoR-T242N is capable to bind Epo with a rate similar to wild-type EpoR, stably transduced BaF3 cells were incubated with $[^{125}]$-Epo for 4 h at room temperature and specifically bound ligand was plotted versus free ligand. Scatchard analysis was additionally performed. Surprisingly, the amount of binding sites for $[^{125}]$-Epo on cells expressing EpoR-T242N was two-fold increased relative to wild-type EpoR (Fig. 13). Since both receptor variants are expressed to a similar extent at the cell surface (Fig. 10a) and the EpoR is known to be a preformed homodimer (Livnah et al., 1999), these results suggest that EpoR-T242N binds $[^{125}]$-Epo as a monomer at non-physiological room temperature conditions. In accordance, the dissociation constant $K_D$ of $[^{125}]$-Epo for EpoR-T242N (310 ± 20 pM) is slightly decreased in comparison to dimeric wild-type EpoR (235 ± 34 pM).

Figure 13. Scatchard analysis indicates monomeric ligand binding of EpoR-T242N at room temperature conditions. (a) BaF3 cells ($1 \times 10^6$) stably expressing GFP-tagged wild-type HA-EpoR (blue circles) or HA-EpoR-T242N (red triangles) were incubated with rising concentrations of $[^{125}]$-Epo for 4 h at room temperature. Specifically bound $[^{125}]$-Epo was plotted versus free $[^{125}]$-Epo and a one-site saturation regression was calculated for three independent data sets, indicating $B_{max}$ and $K_D$. (b) Scatchard analysis showed a linear regression for wild-type EpoR (blue circles) and EpoR-T242N (red triangles).

Even after ligand stimulation, receptor-containing endosomes were not detectable in NIH3T3 cells expressing EpoR-T242N (data not shown). Therefore we asked whether this mutant is able to internalize upon Epo binding. BaF3 cells stably expressing wild-type EpoR or EpoR-T242N were starved and subsequently incubated with $[^{125}]$-Epo for up to 120 min at 37°C. After separation of unbound ligand, cell surface bound $[^{125}]$-Epo and internalized $[^{125}]$-Epo were separated by acid stripping and the amount of internalized ligand was expressed as the percentage of total cell-associated radioactivity. This analysis revealed that EpoR-T242N showed internalization kinetics comparable to wild-type EpoR (Fig. 14) with 50% of the receptor internalized after about 25 min. Moreover, the total amount of specifically bound and
Results

internalized [125I]-Epo was similar for both wild-type EpoR and EpoR-T242N (data not shown), indicating that EpoR-T242N is dimeric at physiological conditions.

Thus, although punctuated structures could not be detected for EpoR-T242N, this mutant is fully capable of undergoing maturation and internalization with kinetics similar to wild-type EpoR.

Decreased activation of ERK and Akt/PKB signaling through EpoR-T242N

Since wild-type EpoR and EpoR-T242N were expressed at similar levels on the cell surface (Fig. 10a) and showed comparable maturation as well as internalization kinetics (Fig. 12, 14), both receptors should have the same prerequisite to activate signal transduction upon ligand stimulation. To analyze signaling, we stimulated BaF3 cells stably expressing wild-type EpoR or EpoR-T242N with increasing Epo concentrations. In line with metabolic labeling studies, analysis by anti-EpoR immunoblotting revealed that the overall expression level of the mutant receptor was consistently increased in comparison to wild-type EpoR (Fig. 15). A dose-dependent increase in tyrosine phosphorylation of wild-type EpoR and JAK2 was detected, but even at high Epo concentrations phosphorylation levels of receptor and JAK2 tyrosine were reduced for EpoR-T242N.

Figure 14. Epo-induced internalization of EpoR-T242N is comparable to wild-type EpoR. BaF3 cells (1x10⁶) stably expressing GFP-tagged wild-type HA-EpoR (blue circles) or HA-EpoR-T242N (red triangles) were starved and subsequently labeled with [125I]-Epo for the indicated time. After separation of free ligand and acid stripping, cell surface bound and internalized [125I]-Epo were measured and internalized ligand was plotted as the percentage of the total of cell surface and internalized radioactivity for three independent data sets. The experiment was performed two times with similar results.

Figure 15. Activation of EpoR-T242N is decreased. BaF3 cells (1x10⁶) stably expressing GFP-tagged wild-type HA-EpoR or HA-EpoR-T242N were stimulated with increasing concentrations of Epo or left unstimulated. Immunoprecipitates with anti-EpoR and anti-JAK2 antibodies were separated by 10% SDS-PAGE. Activated proteins were analyzed by immunoblotting with anti-phosphotyrosine antibodies. The blots were reprobed with anti-EpoR or anti-JAK2 antibodies. The experiment was performed three times with similar results.
Surprisingly, at elevated Epo concentrations activation of STAT5 was comparable for both wild-type EpoR and EpoR-T242N, whereas activation of ERK1/2 through EpoR-T242N was significantly reduced (Fig. 16). Furthermore, phosphorylation of Akt/PKB in EpoR-T242N expressing BaF3 cells was dramatically decreased and did not reach saturation even at elevated Epo concentrations.

Figure 16. Activation of ERK and Akt/PKB through EpoR-T242N is impaired. (a) BaF3 cells (1x10^7) stably expressing HA-tagged wild-type EpoR or EpoR-T242N were stimulated with increasing concentrations of Epo or left unstimulated. The total cellular lysates (TCL) were separated by 15% SDS-PAGE and immunoblotted with anti-phospho-Akt and subsequently with anti-phospho-ERK antibodies. Total Akt/PKB and ERK were detected by reprobing the membrane with anti-Akt or anti-ERK antibodies. The rest of the lysate was subjected to immunoprecipitation with anti-EpoR and subsequently anti-STAT5 antibodies and separated by 10% SDS-PAGE. Activated proteins were analyzed by immunoblotting with anti-phosphotyrosine antibody. The blots were reprobed with anti-EpoR or anti-STAT5 antibodies. (b) Phosphorylation levels of EpoR, STAT5, ERK1, and Akt/PKB for HA-tagged wild-type EpoR (blue circles) and EpoR-T242N (red triangles) were analyzed by LuminImager quantification. A sigmoidal curve was fitted through arbitrary units representing five independent data sets (pEpoR, pSTAT5) or three independent data sets (pERK1, pAkt/PKB).
Additionally, signal transduction through the mutant EpoR-T242N showed delayed activation kinetics of the MAPK and PI3K cascade compared to wild-type EpoR (Fig. 17).

Co-immunoprecipitation experiments revealed that EpoR-T242N is fully capable to associate with various downstream signaling molecules including the p85 subunit of the PI3K as well as the adapter molecule Grb2 (data not shown). Thus, the deficiency in amplification of the
MAPK and the PI3K signaling cascade by the mutant EpoR-T242N is unlikely to be caused by an incapability to interact with key mediators of these signaling cascades. Furthermore, analysis of EpoR TM asparagine mutants exhibiting increased self-interaction (Ruan et al., 2004) showed that in contrast to EpoR-T242N at elevated Epo levels activation of STAT5 and particularly ERK1/2 and Akt/PKB was not impaired despite low receptor phosphorylation (data not shown).

Altogether, the onset and extent of ERK1/2 and Akt/PKB activation is reduced in EpoR-T242N expressing cells, whereas activation of STAT5 is significantly less affected by lower levels of EpoR-T242N phosphorylation.

**Reduced capacity of EpoR-T242N to support proliferation and differentiation**

Next, we examined whether a reduced capacity to amplify certain signaling pathways results in deficiencies in stimulating biological responses. To compare proliferative responses, BaF3 cells expressing wild-type EpoR or EpoR-T242N were grown in 1 U/ml Epo for 3 days. While BaF3-EpoR cells reached up to $(92 \pm 4) \times 10^4$ cells/ml, BaF3-EpoR-T242N cells only reached $(21 \pm 4) \times 10^4$ cells/ml after 3 days of culture (Fig. 18a, upper panel). To determine whether the reduced cell numbers were due to a reduction in proliferation or an increase in apoptosis the fraction of apoptotic cells was determined as the number of Annexin V-positive and 7-AAD negative cells and a representative overlay for day 3 is shown (Fig. 18a, lower panel). This analysis revealed that the fraction of apoptotic cells was low and remained comparable for BaF3 cells expressing wild-type EpoR and EpoR-T242N as indicated by $(1.6 \pm 0.5)\%$ and $(2.2 \pm 0.4)\%$ apoptotic cells, respectively. Furthermore, the defect in proliferative responses observed for BaF3-EpoR-T242N cells could not be compensated by increasing Epo concentrations (Fig. 18b). Thus, in line with efficient STAT5 activation, the reduction in cell numbers obtained for BaF3-EpoR-T242N cells in comparison to BaF3-EpoR cells is not due to accelerated apoptosis but to a decrease in cell proliferation.

To address the consequences of inefficient signal activation for expansion and differentiation of erythroid progenitor cells, we transduced fetal liver cells from d12.5 old EpoR-/- embryos to reconstitute the expression of wild-type EpoR or mutant EpoR-T242N (Fig. 18c). As expected, mock-transduced fetal liver cells formed no CFU-E colonies, whereas expression of wild-type EpoR supported the formation of $86 \pm 13$ colonies. In contrast, transduction of EpoR-T242N resulted in a significantly reduced number of $36 \pm 6$ CFU-E colonies suggesting that maximal proliferation and differentiation of erythroid progenitor cells depend on efficient activation of the MAPK and PI3K signaling cascades.
Increased interhelical distance of the EpoR T242N TM dimer

To gain insight into molecular mechanisms underlying the severe deficiencies of EpoR T242N in eliciting biological responses, we modeled the structures of EpoR TM dimers in atomic detail in a membrane environment and determined the structural differences upon mutation of T242 to asparagine. In contrast to modeling approaches in vacuum, models taking into account the plasma membrane environment are desirable to predict whether polar amino acids are pointing away from or are part of the helical interface. Furthermore, *in vacuo* modeling tends to overestimate electrostatic interactions, affecting the helical interface and the interhelical distance. In our approach, the modeled structure of the wild-type EpoR TM
dimer exhibited two low-energy interfaces (Fig. 19a; modeling data provided by D. Sengupta and J.C. Smith, IWR Heidelberg). The model with the lowest energy (structure I) was found to be a left-handed helix-helix pair with a crossing angle of 20° showing an interhelical distance of 12 Å and volume of 16.1 nm$^3$ (Fig. 19b). For the second-lowest energy model (structure II) the interhelical distance was 10 Å. In both models the polar residues T229, S231, and T242 were not involved in interhelical hydrogen bonding, which could be explained by their position in the polar head-group region or at the interface of the polar head-group and hydrophobic region of the lipid bilayer, respectively.

**Figure 19.** All-atom structures modeled in a membrane environment reveals increased interhelical distance for EpoR-T242N TM dimer. (a) The lowest-energy (structure I) and second lowest-energy structure (structure II) of wild-type EpoR (upper panel) and EpoR-T242N (lower panel) TM dimers are depicted as ribbon diagrams (front view) and amino acids lining the helical interface are indicated. For structure II of the EpoR-T242N TM dimer, N242 is also marked. For structure I, the cartoon representation (side view) depicting the crossing angle and the solvent-accessible surface area (front view) are shown in addition. (b) The values for interhelical distance and volume are given for wild-type EpoR TM dimers as well as mutant EpoR TM dimers L240N, L241N, T242N, V243N, L244N, and A245N. Modeling data provided by D. Sengupta and J.C. Smith, IWR Heidelberg.
The modeled EpoR-T242N TM dimer also exhibited two low-energy interfaces (Fig. 19a), both with a crossing angle of 20° (Fig. 19b). However, in contrast to the EpoR TM dimers the helices in both structures were further apart revealing an interhelical distance of 14 Å and a volume of 17.0 nm³ and 17.1 nm³, respectively. The lowest energy structure I represented a unique orientation with just three amino acid residues L228, V235, and N242 lining the helix-helix interface. N242 was involved in hydrogen bonding and therefore stabilized the conformer in the absence of favorable side-chain packing. For structure II, the helical interface consisted of I227, S231, L234, S238, L241, and A245. Though N242 was not situated in the helix interface, its side chain adopted a rotameric state such that it bends back to the helix-helix interface.

In contrast to the EpoR-T242N TM dimer, the EpoR mutants L240N, L241N, V243N, L244N, and A245N that are detectable in punctuated structures displayed at least one structure with an interhelical distance and surface volume similar or even lesser compared to the wild-type EpoR TM dimer (Fig. 19b, 20).

Figure 20. All-atom models of mutant EpoR TM dimers. The lowest-energy (structure I) and second lowest-energy structure (structure II) of the mutant EpoR TM dimers L240N, L241N, V243N, L244N, and A245N are depicted as ribbon diagrams (front view) and amino acids lining the helical interface are indicated. Modeling data provided by D. Sengupta and J.C. Smith, IWR Heidelberg.)
Molecular modeling indicates dense packing of the TM dimer as crucial for EpoR signaling

To test whether high packing density of the EpoR TM dimer correlates with detection of the receptor in punctuated structures and efficient signal amplification, we analyzed EpoR mutants T242Q and T242A. Comparable to asparagine, glutamine is a strong hydrogen donor and acceptor whereas alanine is an apolar residue. Molecular modeling of the EpoR-T242Q TM dimer showed an interhelical distance of 14 Å (Fig. 21a,b; modeling data provided by D. Sengupta and J.C. Smith, IWR Heidelberg) equal to the EpoR-T242N TM dimer, while the mutant EpoR-T242A TM dimer displayed an interhelical distance of 10 Å. In line with the modeled structures, GFP-tagged EpoR-T242Q showed no punctuated structures in NIH3T3 cells resembling EpoR-T242N, whereas distribution of EpoR-T242A was similar to that of wild-type EpoR revealing punctuated localization (Fig. 21c).

Figure 21. Dense packing of the EpoR TM domain correlates with the detection of punctuated structures. (a) The lowest-energy structure of EpoR-T242Q (left panel) and EpoR-T242A (right panel) TM dimers is depicted as ribbon diagrams (front view) and amino acids lining the helical interface are indicated. The cartoon representation (side view) depicting the crossing angle and the solvent-accessible surface area (front view) are shown in addition. (b) The values for interhelical distance and volume are given for the wild-type EpoR TM dimer and for the mutant EpoR TM dimers T242N, T242Q, and T242A. Modeling data provided by D. Sengupta and J.C. Smith, IWR Heidelberg. (c) Expression of GFP-tagged wild-type HA-EpoR, HA-EpoR-T242N, HA-EpoR-T242Q, and HA-EpoR-T242A in NIH3T3 cells was analyzed by spinning disc confocal microscopy (scale bar, 20 µm).
To examine if increased interhelical distance of the EpoR-T242Q TM dimer and the absence of detectable punctuated structures correlates with inefficient signaling of the receptor, BaF3 cells expressing wild-type EpoR, EpoR-T242Q or EpoR-T242A were stimulated with increasing concentrations of Epo. Comparable to EpoR-T242N, receptor and JAK2 activation as well as phosphorylation of ERK1/2 and Akt/PKB through EpoR-T242Q were dramatically reduced, whereas activation of STAT5 at elevated Epo levels was similar to wild-type EpoR (Fig. 22b). Though cell surface expression of EpoR-T242Q was reduced relative to wild-type EpoR (Fig. 22a), this cannot entirely account for inefficient signaling since EpoR-L241N, which is expressed at the plasma membrane to similar levels as EpoR-T242Q, is

Figure 22. Efficient signal transduction through the EpoR depends on dense packing of the TM domain. (a) Cell surface expression of GFP-tagged wild-type HA-EpoR (blue area) and HA-EpoR TM domain mutants stably expressed in BaF3 cells was assessed by flow cytometry using a rat anti-HA antibody followed by a Cy5-coupled anti-rat IgG antibody. Live cells were gated using forward and side scatter and cell surface expression is shown as a representative overlay (left panel) and as the percentage of the wild-type EpoR (mean ± S.D., n=3) (right panel). (b) BaF3 cells (1x10^7) stably expressing GFP-tagged wild-type HA-EpoR, HA-EpoR-T242Q, or HA-EpoR-T242A were stimulated with increasing concentrations of Epo or left unstimulated. The total cellular lysates (TCL) were separated by 15% SDS-PAGE and immunoblotted with anti-phospho-Akt and subsequently with anti-phospho-ERK antibodies. Total Akt/PKB and ERK were detected by reprobing the membrane with anti-Akt or anti-ERK antibodies. The rest of the lysate was subjected to immunoprecipitation with anti-EpoR, anti-JAK2, and subsequently anti-STAT5 antibodies and separated by 10% SDS-PAGE. Activated proteins were analyzed by immunoblotting with anti-phosphotyrosine antibody. The blots were reprobed with anti-EpoR, anti-JAK2, or anti-STAT5 antibodies.
nevertheless capable of mediating efficient signaling at elevated Epo levels (Ruan et al., 2004). In contrast, the mutant EpoR-T242A is expressed at the cell surface to similar levels as wild-type EpoR. Consistent with dense TM packing, activation of JAK2 and downstream signaling molecules STAT5, ERK1/2, and Akt/PKB through EpoR-T242A was slightly more efficient at low Epo levels compared to wild-type EpoR, while maximal phosphorylation levels were similar to wild-type EpoR (Fig. 22b).

Thus, an increase in the interhelical distance of the EpoR TM dimer correlates with the absence of detectable punctuated structures and with selective deficiencies in amplifying the MAPK and the PI3K signaling cascade.
2.2 Internalization Controls Early Phase Kinetics of Epo Receptor Activation

Dynamic model of ligand-induced EpoR internalization

The sensitivity of signal transduction is critically determined by the amount of receptor accessible to ligand binding at the plasma membrane. Thus, the dynamic behavior of EpoR signaling relies on the rate of receptor endocytosis upon Epo binding. To gain insight into the kinetics of ligand-induced EpoR internalization, a dynamic mathematical model was compiled comprising 10 reactions and 8 species (Fig. 23; in collaboration with M. Schilling, DKFZ Heidelberg). Unbound EpoR at the plasma membrane is subjected to constant turnover, production, and cell surface transport, being regulated by the turnover rate and the maximal amount of receptor on the cell surface $B_{\text{max}}$. Constitutive endocytosis and degradation of the EpoR depend on the amount of receptor on the cell surface and on the turnover rate. Therefore, in the absence of ligand, the amount of cell surface EpoR is constant and equal to $B_{\text{max}}$. Free functional Epo in the medium binds to EpoR with the on-rate $k_{\text{on}}$ and dissociation occurs with the off-rate $k_{\text{off}}$. As the dissociation constant $K_D$ can be experimentally

![Figure 23. Graphic representation of the dynamic mathematical model describing ligand-induced EpoR internalization. The EpoR undergoes constant turnover. Upon Epo-binding, the receptor is internalized and recycles to the cell surface with a delay. Internalized Epo can as well recycle to the medium or gets degraded before being released to the medium. Measured observables are represented by orange shading (Epo in medium), blue shading (Epo on surface), and green shading (Epo in cells). In collaboration with M. Schilling, DKFZ Heidelberg.](image-url)
Results
determined, \( k_{\text{off}} \) is expressed as \( k_{\text{off}} \times K_D \). Epo bound to the receptor is internalized and finally dissociates from the EpoR during trafficking through endosomal and lysosomal compartments. Internalized EpoR can recycle to the surface with a delay, while internalized Epo can proceed by two routes. It is either recycled to the medium or degraded within intracellular compartments and afterwards released to the medium, unable to re-bind to the receptor. These steps comprise the minimal model that can both describe our data and is consistent with biological knowledge. The delay for EpoR recycling is absolutely necessary to describe our data, whereas delays at other steps in the model including Epo binding and internalization as well as Epo recycling and release proved to be not necessary. Additionally, degradation and release of Epo are inevitably serial, not parallel steps.

To determine the parameters \( B_{\text{max}} \) and \( K_D \), BaF3-HA-EpoR cells were incubated with increasing concentrations of \([^{125}\text{I}]-\text{Epo}\) and free as well as the specifically bound \([^{125}\text{I}]-\text{Epo}\) was measured (Fig. 24a; data provided by M. Schilling, DKFZ Heidelberg). We calculated the parameters by one-site saturation regression analysis, revealing a dissociation constant \( K_D = 164 \pm 28 \text{ pM} \) that is comparable to published data (D’Andrea et al., 1991) and \( B_{\text{max}} = 129 \pm 9 \text{ pM} \). A kinetic measurement of Epo-induced receptor internalization was performed for BaF3 HA-EpoR cells at 37°C. Cells were stimulated with 2100 pM (approximately 5 U/ml) \([^{125}\text{I}]-\text{Epo}\) for up to 240 min. Unbound \([^{125}\text{I}]-\text{Epo}\) in the medium was removed, and surface-bound \([^{125}\text{I}]-\text{Epo}\) was separated from internalized \([^{125}\text{I}]-\text{Epo}\) by acid stripping (Fig. 24b).

**Figure 24. Scatchard analysis and Epo-induced internalization of the EpoR.** (a) To calculate the parameters \( B_{\text{max}} \) and \( K_D \), BaF3-HA-EpoR cells \((1 \times 10^6)\) were incubated with increasing concentrations of \([^{125}\text{I}]-\text{Epo}\) and specifically bound \([^{125}\text{I}]-\text{Epo}\) is plotted versus free \([^{125}\text{I}]-\text{Epo}\). A one-site saturation regression was calculated (solid line), indicating \( B_{\text{max}} \) (maximal binding, long dash) and \( K_D \) (free \([^{125}\text{I}]-\text{Epo}\) concentration for half-maximal binding, short dash). Scatchard analysis shows a linear curve (inset). Data provided by M. Schilling, DKFZ Heidelberg. (b) To measure the kinetic of \([^{125}\text{I}]-\text{Epo}\) internalization, BaF3-HA-EpoR cells \((4 \times 10^6)\) were incubated with 2100 pM Epo (approximately 5 U/ml) \([^{125}\text{I}]-\text{Epo}\) at 37°C for the times indicated. Unbound \([^{125}\text{I}]-\text{Epo}\) was measured (Epo in medium, orange). Cells were acid-stripped to remove cell surface-bound \([^{125}\text{I}]-\text{Epo}\) and supernatants (Epo on surface, blue) as well as cell pellets (Epo in cells, green) were measured. Error bars represent standard deviation of triplicates.
Results

Ligand-receptor complexes were rapidly internalized, while $[^{125}\text{I}]-\text{Epo}$ in the medium was not depleted within the observed time. Remarkably, after a decline the extent of cell surface-bound $[^{125}\text{I}]-\text{Epo}$ recovered at 240 min, indicating that the amount of EpoR at the plasma membrane is not depleted for prolonged ligand stimulation. Measurements were done in triplicates, the standard deviations being small enough for parameter estimation techniques.

**Dynamic model of constitutive receptor internalization**

To determine the turnover rate of the EpoR in unstimulated cells, we made use of a streptavidin-binding peptide-tagged EpoR (SBP-EpoR) and incubation with $[^{125}\text{I}]-\text{streptavidin}$ in medium depleted of biotin. To test whether the use of biotin-free medium in the experimental setup is comparable to standard medium, we assessed the amount of receptor at the plasma membrane of BaF3 cells expressing HA-EpoR using anti-HA-antibodies. Flow cytometry analysis revealed no difference in viability and EpoR cell surface expression of cells washed and starved either in standard cultivation medium (+ biotin) or in biotin-free medium (- biotin) (Fig. 25a). Furthermore, while SBP-EpoR was activated upon Epo stimulation, streptavidin bound to the receptor without inducing EpoR phosphorylation even at high concentrations (Fig. 25b). Therefore, the use of streptavidin allows determination of constitutive receptor internalization and turnover.

**Figure 25. Streptavidin does not induce EpoR phosphorylation.** (a) BaF3-HA-EpoR cells were washed and starved for 1 h in standard RPMI 1640 medium (+ biotin; blue area) or biotin-free medium (- biotin; red line). HA-EpoR cell surface expression was determined by flow cytometry using anti-HA antibodies as first antibody and Cy5-coupled secondary antibodies. Life cells were gated by forward and side scatter and a representative overlay is shown. (b) Mock-transduced BaF3 cells (ctrl) or BaF3-SBP-EpoR cells ($1\times10^7$) were stimulated with 50 U/ml Epo or with increasing concentrations of streptavidin or left unstimulated. Immunoprecipitations were performed with anti-EpoR antibodies. Samples were separated by 10% SDS-PAGE and activated receptor was analyzed by immunoblotting with anti-phosphotyrosine antibodies. Blots were reprobed with anti-EpoR antibodies.
The model for constitutive EpoR internalization is analogous to the model describing Epo-induced receptor endocytosis (Fig. 26; in collaboration with M. Schilling, DKFZ Heidelberg). The main difference between these two models is that constitutive EpoR endocytosis is expressed with a turnover rate that is independent of bound streptavidin. In the unstimulated cell, the amount of cell surface EpoR is assumed to be constant ($B_{\text{max}}$). Therefore, it is not important whether the constitutively internalized receptor is recycled to the plasma membrane or whether it is newly produced. Internalized streptavidin is recycled to the medium intact or is released to the medium after degradation. The parameter release was assumed to be same for both Epo and streptavidin, as parameter estimation resulted in very similar values and it is likely that degraded peptides are processed with similar reactions within intracellular compartments.

![Figure 26. Graphic representation of the dynamic mathematical model describing constitutive EpoR internalization.](image)

To determine the rates for $B_{\text{max, strep}}$ and $K_{D, \text{strep}}$, BaF3-SBP-EpoR cells were treated with increasing concentrations of $[^{125}\text{I}]$-streptavidin. $[^{125}\text{I}]$-Streptavidin did not efficiently bind to SBP-EpoR, therefore the dissociation constant $K_{D, \text{strep}}=2964 \pm 1189$ pM showed a high
standard deviation (Fig. 27a). However, the binding affinity of $[^{125}\text{I}]$-streptavidin for the SBP-tagged receptor was within the reported nanomolar range (Keefe et al., 2001). A kinetic measurement of constitutive EpoR internalization was performed for BaF3-SBP-EpoR cells stimulated with 1000 pM $[^{125}\text{I}]$-streptavidin at 37°C for up to 60 min. Free $[^{125}\text{I}]$-streptavidin in the medium was removed and $[^{125}\text{I}]$-streptavidin on the cell surface was separated from intracellular $[^{125}\text{I}]$-streptavidin by acid stripping (Fig. 27b). The amount of cell surface-bound $[^{125}\text{I}]$-streptavidin increased within the observed time, indicative of the inefficient binding to the receptor. Measurements were done in triplicates, showing standard deviations that were small enough for parameter estimation techniques.

![Figure 27](image_url)

**Figure 27. Scatchard analysis and constitutive internalization of SBP-EpoR.** (a) To calculate $B_{\text{max}}$, $K_D$, $B_{\text{max}}$, and $K_D$, BaF3-SBP-EpoR cells ($1\times10^6$) were incubated with increasing concentrations of $[^{125}\text{I}]$-streptavidin and specifically bound $[^{125}\text{I}]$-streptavidin is plotted versus free $[^{125}\text{I}]$-streptavidin. A one-site saturation regression was calculated (solid line), indicating $B_{\text{max}}$ (maximal binding) and $K_D$ (for half-maximal binding). (b) To measure the kinetic of constitutive receptor internalization, BaF3-SBP-EpoR cells ($1\times10^6$) were incubated with 1000 pM $[^{125}\text{I}]$-streptavidin at 37°C for the times indicated. Unbound $[^{125}\text{I}]$-streptavidin was measured (streptavidin in medium, orange). Cells were acid-stripped to remove surface-bound $[^{125}\text{I}]$-streptavidin and supernatants (streptavidin on surface, blue) and cell pellets were measured (streptavidin in cells, green). Error bars represent standard deviation of triplicates.

**Combined modeling approach for ligand-induced and constitutive EpoR internalization**

For parameter estimation, we converted the models describing Epo-induced and constitutive receptor endocytosis into two sets of ordinary differential equations (ODE) (see Appendix 6.5; in collaboration with M. Schilling, DKFZ Heidelberg). The delay for receptor recycling to the plasma membrane was modeled by using a 10-step compartmentalization reaction. All start values were set to zero, except for cell surface EpoR set to $B_{\text{max}}$ or $B_{\text{max}}$, as well as Epo and strep set to the initial amount ± 100 pM used for the internalization experiments. $B_{\text{max}}$ or $B_{\text{max}}$, were fixed to the determined values. Parameters for both models were...
estimated simultaneously and possessed two shared parameters turnover and release. Parameter estimation was performed for 500 times with different starting conditions and the trajectories for the best fit are depicted (Fig. 28; in collaboration with M. Schilling, DKFZ Heidelberg). The combined model captured the dynamics of Epo-dependent internalization very well, including the steep initial decrease of Epo in the medium followed by a slower decline, the rapid peak of Epo bound on the cell surface that is followed by a decline before rising again, as well as the accumulation of internalized Epo in the cells. The data for streptavidin were equally well represented. Low $\chi^2$-values indicate accurate explanation of the data by the two dynamic mathematical models. To gain insight into the systems properties, we additionally plotted the trajectories of all variables of the Epo-induced receptor

![Figure 28. Parameter estimation for mathematical models of Epo-induced and constitutive EpoR internalization. Data trajectories of the best fit are indicated by solid lines and experimental data are represented by hollow symbols with standard deviations. Parameter estimation was performed simultaneously for both models, with common parameters turnover and release having the same value. $\chi^2$-values lower than number of data points indicate good agreement of the model with the experimental data, as seen for both Epo-induced (a,b,c) and constitutive internalization (d,e,f). In collaboration with M. Schilling, DKFZ Heidelberg.](image-url)
internalization model (Fig. 29; in collaboration with M. Schilling, DKFZ Heidelberg). Modeling revealed that free Epo is in large excess in the medium, however, unbound EpoR on the cell surface is not completely occupied by the ligand. Rather, the receptor is rapidly depleted to about one third of the initial concentration and then slowly replenished by recycling. Receptor-bound Epo, both on the cell surface and within the cell, displays a remarkable kinetics. A sharp peak in the first minutes is followed by a sharp descent, the concentration rising again after 120 min. Internalized Epo is either rapidly recycled or degraded and released to the medium.

![Graphs showing concentration vs. time for Epo, EpoR, Epo-EpoR, Epo_recycling, Epo_degradation, strep_dissociation, strep_recycling, and strep_degradation](image)

**Figure 29. Unobserved variables of the ligand-induced EpoR internalization model.** After parameter estimation, the unobserved parameters were plotted for the model describing EpoR-induced receptor internalization. In collaboration with M. Schilling, DKFZ Heidelberg.

**Identifiability of estimated parameters**

To investigate the identifiability of the estimated parameter values, we calculated the standard deviation of the parameters for the best 65% of the calculated 500 fits (see Appendix 6.6; data provided by S. Hengl, T. Maiwald, and J. Timmer, FDM, University of Freiburg). Five out of 16 parameters exhibited standard deviations larger than 25% and were therefore defined as non-identifiable (Epo_recycling, Epo_degradation, strep_dissociation, strep_recycling, and strep_degradation). As these parameters were highly correlated in two groups, we could fix one of the parameters for each correlation group and thereby re-estimate the other parameters. Applying this approach, all estimated 14 parameters exhibited standard deviations smaller than 25% and were therefore defined as identifiable. The estimated parameters allowed gaining insight into the kinetics of different steps of receptor internalization. By comparing the parameters turnover and internalization, it became evident
that internalization of Epo-bound receptor is accelerated by approximately 3.5-fold in comparison to constitutive EpoR endocytosis. Moreover, studying the analytically correlated parameters \( \text{Epo\_recycling} \) and \( \text{Epo\_degradation} \) showed that approximately 80% of Epo are recycled to the medium intact where it can re-bind to the receptor, while only 20% of ligand are degraded within intracellular compartments.

**Sensitivity analysis reveals turnover, \( k_{on} \), and internalization as critical for the combined kinetics of cell surface and internalized ligand-bound EpoR**

To analyze what determines the kinetic behavior of ligand-bound EpoR, we performed a sensitivity analysis by investigated the sum of the two populations of cell surface and internalized ligand-receptor complexes that are in principle capable of initiating signal transduction. For sensitivity analysis, three quantities were defined. The peak amplitude is the concentration at the first maximum, the peak time is the time at the first maximum, and the extrema amplitude is the concentration at the first maximum minus the concentration at the first minimum (Fig. 30a). The control coefficients of the parameters of the Epo-induced EpoR internalization model for these quantities were determined. Positive control coefficients indicate that values for peak amplitude, extrema amplitude, and peak time increase with higher parameter values, while negative control coefficients indicate that higher parameters lead to lower values for these three quantities. Higher absolute values of control coefficients thereby represent a larger influence of the parameter (Fig. 30b; data provided by S. Hengl, T. Maiwald, and J. Timmer, FDM, University of Freiburg). The turnover rate has a minor positive impact on peak amplitude and peak time, but a massive negative effect on extrema amplitude. The effect of \( k_{on} \) is positive for both peak and extrema amplitude and negative for peak time, indicating that faster binding leads to more rapid and thus probably more pronounced signaling. On the other hand, the dissociation constant \( K_D \) has little control on all parameters, demonstrating that the on-rate kinetics is more important than the affinity of Epo binding to its receptor. Internalization possesses negative control on peak amplitude, minor positive control on extrema amplitude, and strong negative control on peak time. Dissociation has a minor negative impact on all quantities. The rest of the parameters have essentially no control on the kinetics of ligand-receptor complex formation. As can be shown by summation theorems, the sum of all control coefficients equals to 0 for peak and extrema amplitude and -1 for peak time (Hornberg et al., 2005b). Since these control coefficients were calculated for 1% changes, we tested whether the results are also applicable for larger parameter variations for the parameters turnover and internalization. The trajectories for the sum of cell surface and internalized Epo-EpoR complexes were plotted depending on parameter changes (Fig. 30c). As expected from the calculation of control coefficients, increased turnover led to a slightly higher peak but a shallower kinetic for the cell surface and
internalized ligand-bound EpoR population, while increased internalization caused a sharper but lower peak. Thus, sensitivity analysis allowed the identification of the factors causing the steep rise and rapid decline of signaling-competent receptor-ligand complexes.

**Figure 30.** Sensitivity analysis reveals impact of turnover and internalization on ligand binding. (a) The trajectory for the sum of EpoR-bound Epo and internalized EpoR-bound Epo is represented by a solid line. The three quantities analyzed are peak amplitude (concentration at first maximum, orange), extrema amplitude (concentration at first maximum minus concentration at first minimum, green) and peak time (time at first maximum, blue). (b) Control coefficients for the parameters of the Epo-induced internalization model on the sum of surface and internalized EpoR-bound Epo. Positive control coefficients indicate higher values for peak amplitude (orange), extrema amplitude (green), and peak time (blue) for increasing parameters, while negative control coefficients indicate decreasing values for the quantities with increasing parameter values. Higher absolute values of control coefficients represent larger control. (c) Trajectories are depicted for the sum of surface and internalized EpoR-bound Epo depending on relative parameter changes in turnover and internalization are predicted. Time course simulations are shown in green for increased parameters, in black for unchanged parameters and in orange for decreased parameters. Data provided by S. Hengl, T. Maiwald, and J. Timmer, FDM, University of Freiburg.
Long-term EpoR activation is restrained despite receptor cell surface prevalence

Mathematical modeling showed that the cell surface population of ligand-bound EpoR recovers after approximately 240 min. Therefore, unless negative regulators prevent signal initiation one would assume that EpoR and JAK2 activation follow this kinetic behavior and phosphorylated proteins are detectable upon prolonged ligand stimulation. To examine the kinetics of receptor activity, BaF3 cells stably expressing HA-EpoR were stimulated with 5 U/ml Epo for up to 240 min and the amount activated receptor and JAK2 were quantified (Fig. 31; data provided by J. Bachmann, DKFZ Heidelberg). Phosphorylated EpoR showed peak intensity at 10 min, followed by a sharp descent until EpoR activation was reduced to basal levels between 60 and 120 min (Fig. 31a). Despite recovery of the plasma membrane pool of receptor-ligand complexes at 240 min of stimulation (Fig. 29), phosphorylated EpoR could not be detected at later time points. Analysis of JAK2 revealed that the activated kinase exhibits similar kinetics to phosphorylated EpoR (Fig. 31b).

Figure 31. Long-term EpoR activation is restrained despite ligand-receptor complex prevalence at the cell surface. BaF3 cells (1x10^7) stably expressing HA-EpoR were stimulated with 5 U/ml Epo for up to 240 min. Immunoprecipitates with anti-EpoR antibodies (a) or anti-JAK2 antibodies (b) were separated by 10% SDS-PAGE and analysis of activated receptor was performed by immunoblotting with anti-phosphotyrosine antibodies. Data provided by J. Bachmann, DKFZ Heidelberg.

Thus, ligand-induced receptor internalization does not mediate long-term attenuation of EpoR activation but rather shapes the initial kinetics of ligand-receptor complex formation. Probably, negative regulators take over to terminate EpoR signaling upon prolonged stimulation.
3. Discussion

In this study, we identified active transport of the EpoR along microtubules as critical for achieving efficient trafficking kinetics of receptor-containing vesicular structures and dense packing of the TM domain as a prerequisite for selective signal amplification through the EpoR. Remarkably, analysis of ligand-induced EpoR endocytosis revealed that long-term attenuation of receptor activation is not mediated by downregulation of the receptor pool at the plasma membrane. By applying a systems biology approach, we further uncovered the parameters that determine the kinetics of ligand-receptor complex formation and thus activation of EpoR signaling.

3.1 Dense Packing of the Transmembrane Domain Promotes Selective Signal Amplification through the Epo Receptor

Receptor trafficking
The extent of cell surface expression regulates the sensitivity of receptor-mediated signal transduction. After synthesis in the ER and the Golgi complex, receptor proteins are transported to and internalized from the plasma membrane by transport vesicles. To circumvent the bottleneck of inefficient subdiffusion in the crowded cytoplasm (Weiss et al., 2004), EpoR-positive punctuated structures use transient association with microtubules. From our calculations, we can infer that this strategy allows EpoR-containing punctuated structures to move in a wide spectrum of modes, depending on the amount and type of attached motors. To cover a distance of 1 \( \mu \text{m} \), receptor-containing vesicular structures not associated with microtubules require approximately 80 min. However, the traveling time for microtubule-mediated diffusion and super-diffusion is within the range of seconds. Microtubules and motors therefore facilitate transport of the receptor to and from the cell surface in the crowded cytoplasm and thus enable the cell to efficiently exchange the cell surface pool of receptor proteins on a feasible time scale.

Dynamic higher oligomeric receptor structures
The TM mutant EpoR-T242N shows processing and internalization kinetics comparable to wild-type EpoR, but punctuated structures including receptor-positive endosomes were not detectable by live cell imaging for EpoR-T242N as well as EpoR-T242Q. Molecular modeling revealed that the structures for both mutants EpoR-T242N and EpoR-T242Q exhibit an increase in interhelical distance. Therefore, we propose that dense packing of the TM domain could promote the formation of dynamic higher oligomers and thus enable detection
of EpoR in punctuated structures. Our results further indicate that dense packing of the EpoR TM domain is required for selective signal amplification through the EpoR. Previously, it was suggested that clustering of the EpoR mediates amplification of biological responsiveness in erythroleukemia cells pretreated with dimethyl sulfoxide (Yonekura et al., 1991). Since it has been shown that dimethyl sulfoxide changes the structure of the lipid bilayer (Gordeliy et al., 1998), this treatment could promote higher order oligomerization of the EpoR mediated by dynamic interaction of the TM domain. Cross linking studies with \[^{125}\text{I}\]-Epo have not revealed clear evidence for higher oligomeric structures (Damen et al., 1992; Takahashi et al., 1995), but this could be due to the choice of the cross-linker as well as low levels of EpoR at the cell surface.

Homodimeric cytokine receptors related to the EpoR also localize to punctuated structures, including the leptin receptor (Couturier and Jockers, 2003) and the prolactin receptor (Perrot-Applanat et al., 1997). Evidence by Devos et al. suggests that the leptin receptor forms higher oligomeric structures (Devos et al., 1997). Therefore, the concept to regulate selective signaling by the dynamic formation of higher oligomeric structures may also be applicable to other cytokine receptors.

Recently, derivatives of recombinant human Epo (rhEpo) with increased serum half-life have been developed for clinical applications. The continuous erythropoietin receptor activator (CERA) exhibits lower receptor affinity compared to rhEpo (Brandt et al., 2006). Possibly, dynamic higher order oligomerization of the EpoR could compensate for the reduced affinity of this Epo derivative and thus support continuous receptor activation.

All-atom molecular modeling of the EpoR TM dimer

Determinants and motifs that promote the assembly of TM helices have been assessed by several experimental approaches (Arkin, 2002). The EpoR TM assembly has been studied applying the ToxR assay, which is based on insertion of a chimeric protein in the inner bacterial membrane. By using this approach, it has been shown that the EpoR TM possesses a strong capacity to self-interact, an ability that is important for maximal signaling responses in the context of the full-length receptor (Kubatzky et al., 2001). Asparagine-scanning of the EpoR TM utilizing the ToxR assay showed that the TM assembly relies on a degenerated leucine zipper motif (Ruan et al., 2004). Unexpectedly, analysis of EpoR TM mutants with the highest tendency of self-assembly within the heptad repeat pattern revealed no correlation between an increase in self-interaction and constitutive activation (Ruan et al., 2004). However, the insertion of bulky side chains such as asparagine is likely to introduce structural changes in the TM helix.

Thus, to determine the effect of mutations on the structure of the EpoR TM domain dimer, we applied an implicit five-slab membrane model (Sengupta et al., 2005) that allows exposure of
OH groups on the molecular surface of a helix dimer. Our results revealed a positive crossing angle for the structures of the wild-type EpoR TM dimer as suggested by the leucine zipper nature of EpoR TM assembly (Ruan et al., 2004). In line with our model that identifies L226 or I227 as residues lining the two low-energy interfaces of the wild-type EpoR TM dimer, recent evidence showed that independent replacement of these two residues by cysteine results in constitutively active EpoR receptor variants (Kubatzky et al., 2005; Lu et al., 2006). Additionally, the two interfaces of the wild-type EpoR TM dimer we identified by all-atom modeling were both reported to be functionally active in biological assays applying receptor variants in which the extracellular domain of the EpoR has been substituted by a coiled coil Put3 domain (Seubert et al., 2003). In vacuo modeling by Seubert et al. identified two clusters that have been proposed to correspond to a functionally active as well as inactive interface. This discrepancy to our findings could be due to the difference in considering the membrane environment as we include in our five-slab model.

In addition to an increased interhelical distance, the helices of the lowest-energy structure for the EpoR-T242N TM dimer are hydrogen-bonded by asparagine. Besides glutamine, glutamic acid and aspartic acid, inclusion of asparagine in TM helices can promote strong interhelical association (Zhou et al., 2000). Interhelical hydrogen bonding of the EpoR-T242N TM dimer in our model suggests that elevated self-assembly as detected in the ToxR assay could reflect stabilization of the dimeric form of the EpoR-T242N. For cytokine receptors, substitution of a single residues by asparagine within the TM domain has been reported to result in constitutive activation of the G-CSF receptor (Forbes et al., 2002) and the TPO receptor (Onishi et al., 1996). However, an EpoR asparagine mutant L241N exhibiting increased self-interaction of the TM did not reveal ligand-independent signaling of the receptor (Ruan et al., 2004). This indicates that additional structural requirements of the TM domain including rotational flexibility and proper orientation of the TM and juxtamembrane domain (Constantinescu et al., 2001a) have to be fulfilled to constitutively activate the EpoR. Modeling of further EpoR TM mutants revealed that only the EpoR-T242N as well as EpoR-T242Q TM dimers exhibited increased interhelical distances, whereas the other receptor variants showed at least one structure with a packing density similar or even better compared to the wild-type EpoR TM dimer.

**Selective signal amplification through the EpoR**

Signaling through the EpoR promotes proliferation, survival, and differentiation of erythroid progenitor cells (Wu et al., 1995). Based on in vivo mouse experiments it has been proposed that STAT5 controls survival of erythroid cells (Socolovsky et al., 1999; Menon et al., 2006). We observe that EpoR-T242N efficiently activates STAT5 and promotes survival of the hematopoietic cell line BaF3 despite reduced levels of receptor activation. In accordance with
Discussion
these results, analysis of EpoR activation by the erythropoiesis-stimulating protein NESP (darbepoetin alfa), showed that STAT5 activation is robust even at suboptimal levels of receptor phosphorylation (Elliott et al., 2004). In contrast to STAT5 activation, EpoR-T242N is strongly impaired in activating ERK1 and Akt/PKB, key components of the MAPK and PI3K signaling cascades, respectively. This correlates directly with reduced proliferative and differentiation capacities of the mutant receptor in erythroid progenitors. Coexpression of constitutively active Akt/PKB and MEK resulted in prolonged Epo-independent proliferation of erythroid progenitor cells and in a block in erythroid differentiation (Zhang and Lodish, 2004). In line with these observations, the MAPK pathway is constitutively activated in most cases of human acute myelogenous leukemia and in leukemic blast (Platanias, 2003). Epo-mediated PI3K signaling has been shown to promote Epo-induced maturation of erythroid progenitor cells (Ghaffari et al., 2006) and activation of Akt/PKB signaling has been linked to tissue-protective effects of Epo in neuronal cells (Chong et al., 2003).

We propose that the dynamic formation of higher oligomeric structures is promoted by dense packing of the TM domain, facilitating selective signaling through the EpoR (Fig. 32).

Figure 32. Schematic representation of selective signal amplification through dynamic formation of higher oligomeric EpoR structures. In the absence of ligand binding, the EpoR can dynamically switch between dimeric or higher oligomeric structures at the plasma membrane. Upon Epo stimulation, the dimeric EpoR preferentially signals through the transcription STAT5, thereby triggering survival of the cell. In contrast, higher oligomeric EpoR structures are capable to activate the MAPK as well as PI3K cascade in addition to STAT5 signaling. By making full use of the potential downstream signaling cascades, the oligomeric EpoR promotes proliferation, survival, and differentiation. Thus, by regulating the fraction of higher oligomeric receptor structures, the cell is capable to vary the repertoire of cellular decisions upon external stimuli.
3.2 Internalization Controls Early Phase Kinetics of Epo Receptor Activation

**Long-term attenuation of receptor activation**

Receptor endocytosis has been proposed to control downregulation of activated receptor complexes and thus terminate signals emanating from the plasma membrane. However, while the EGFR is rapidly internalized depending on autophosphorylation of the cytoplasmic domain and subsequently downregulated by lysosomal degradation (Sorkin and Waters, 1993), ErbB3 endocytosis exhibits a significantly slower kinetics and recycles to the plasma membrane (Waterman et al., 1998). These studies indicate the existence of different strategies for attenuation of receptor signaling, even within one family of structurally related receptors and that internalization of cell surface receptors not necessarily correlates with long-term attenuation of signaling. For the human myeloid cell line UT-7 expressing high levels of EpoR, downregulation of the activated receptor is proposed to be mediated by both proteasomal and lysosomal degradation (Walrafen et al., 2005). However, our observations revealed that despite recovery of ligand-receptor complexes at the cell surface, EpoR activation is restrained upon prolonged stimulation, arguing against the assumption that long-term attenuation of EpoR signaling is mediated by internalization and downregulation of the receptor plasma membrane pool.

Therefore, other mechanisms have to terminate EpoR signaling, probably including the recruitment of phosphatases to the EpoR, the induction of negative feedback loops, as well as degradation of the receptor-associated JAK2. A prominent phosphatase involved in negative regulation of EpoR signaling is SHP-1 that is recruited to the phosphorylated tyrosine residues Y429 and Y431 of the EpoR cytoplasmic domain and subsequently inhibits JAK2 activation (Klingmüller et al., 1995; Yi et al., 1995). Since the induction of negative feedback loops requires protein synthesis, recruitment of the SOCS family members CIS (Ketteler et al., 2003) and SOCS-3 (Hortner et al., 2002) to tyrosine-phosphorylated EpoR residue 401 is likely to occur at later time points during EpoR activation. Recent studies proposed that upon Epo stimulation, JAK2 is autophosphorylated on a negatively regulating tyrosine within the receptor-binding FERM domain, followed by dissociation from the EpoR and subsequent kinase degradation (Funakoshi-Tago et al., 2006). This indicates that JAK2 availability for receptor phosphorylation may be the limiting factor for long-term EpoR activity. Assuming a constant extent of SHP-1 expression, we speculate that the stoichiometry of JAK2 and SHP-1 expression shifts to an excess of phosphatase levels, unfavorable for EpoR activation upon prolonged ligand stimulation (Fig. 33).
Recycling of the ligand and the receptor are key features of our EpoR internalization model. Approximately 80% of Epo recycles to the medium and therefore allows EpoR activation without depleting the ligand in the extracellular space, being especially important for low Epo levels present in the hematopoietic stem cell niche (Noe et al., 1999) that are approximately
500-fold lower relative to our experimental setup. Thus, Epo recycling enables erythroid progenitor cells to respond to Epo without affecting ligand concentration in the extracellular environment for adjacent cells. Recovery of the amount of ligand-receptor complexes at the plasma membrane upon prolonged stimulation critically depends on EpoR recycling. The overall intracellular receptor pool is depleted upon Epo stimulation (data not shown).

Association of JAK2 with the EpoR in the ER was reported to act as a chaperone-like mechanism for receptor maturation and cell surface expression of newly synthesized proteins (Huang et al., 2001). Therefore, we speculate that the decline in total receptor expression during ligand stimulation might correlate with decreased JAK2 levels, possibly resulting in an accelerated degradation of unprocessed EpoR protein, whereas receptor transport in recycling endosomes and therefore recovery of the cell surface pool is independent of JAK2 association.

While ligand-induced EpoR endocytosis does not require receptor phosphorylation (Beckman et al., 1999), autophosphorylation of EGFR on its cytoplasmic domain are a prerequisite for rapid internalization (Sorkin and Waters, 1993). Our results show that EpoR internalization is significantly accelerated upon ligand binding, indicating that conformational changes of the receptor may induce a higher affinity to localize to sites of endocytosis such as clathrin-coated pits, thereby enhancing internalization of the ligand-bound receptor.

**Parameters controlling the formation of signaling-competent ligand-receptor complexes**

Our modeling approach revealed that three parameters receptor turnover, the association rate $k_{on}$ for ligand binding to the receptor, as well as receptor internalization determined the sharp peak of ligand-receptor complex formation. We suggest that these parameters were evolutionary chosen to control the extent of peak levels in the formation of signaling-competent receptor complexes and simultaneously allow an immediate decline after peak levels. Analysis of EpoR and JAK2 phosphorylation levels indicates that rapid receptor internalization determines the initial kinetics of signaling at the receptor level. To further dissect the impact of EpoR endocytosis and recruitment of negative regulators for the early phase of receptor activity, it would be desirable to block receptor internalization. However, endocytosis inhibitors in general simultaneously exhibit phosphatase inhibitor activity. To distinguish between the effect of endocytosis and phosphatase recruitment on the kinetics of EpoR signaling during the early phase of receptor activation, comparative studies of wild-type EpoR and a mutant EpoR-YY429/431FF unable to recruit the key phosphatase SHP-1 (Klingmüller et al., 1995) could be applied.

The majority of the EpoR resides within the ER and the Golgi complex, while only a minor part is transported to the cell surface (Yoshimura et al., 1990). In contrast to a recent study...
modeling Epo trafficking and degradation for human EpoR (Gross and Lodish, 2006), our modeling approach includes the turnover rate of the receptor, revealing that receptor turnover is critical for the early phase of ligand-receptor complex formation. This observation indicates that the continuous exchange of the EpoR plasma membrane pool and sampling of the extracellular environment is essential for signal transduction.

Sensitivity analysis of parameters describing the ligand-stimulated EpoR internalization demonstrated that $k_{on}$ but not $K_D$ is critical for both the extent as well as the rate to form signaling-competent ligand-receptor complexes. Thus, for designing efficient Epo derivatives such as darbepoetin alfa (Macdougall et al., 1999) or CERA (Brandt et al., 2006) used in clinical applications it would be desirable to determine the on-rate kinetics rather than the dissociation constant of the ligand for receptor binding.

### 3.3 Conclusions and Perspective

In conclusion, trafficking of the EpoR critically relies on active transport through the crowded cytoplasm to rapidly exchange the pool of cell surface receptors. We propose that dense packing of the EpoR TM domain promotes the formation of dynamic higher oligomeric structures and thereby facilitates selective amplification of signaling pathways as well as biological responses. This concept may be applicable to other cytokine receptors since they have been reported to form preformed dimers (Devos et al., 1997; Gent et al., 2002) and are partially localized in punctuated structures (Perrot-Applanat et al., 1997; Couturier and Jockers, 2003). Thus, differences in the lipid composition of the cell membrane could influence the formation of cytokine receptor oligomers and thereby bias cellular decisions or support cell context-specific responses.

Tools to study structure and oligomerization of the EpoR TM domain are desirable. However, biochemical approaches of the dynamic interaction of proteins within the plasma membrane environment are not applicable. Analysis of the primary sequence of the EpoR TM domain and the TM domain of various cytokine receptors related to the EpoR does not reveal a comparable amino acid composition. Therefore, molecular modeling of the TM structure of these cytokine receptors such as the leptin receptor or the prolactin receptor should uncover whether these receptors show dense packing of the TM domain similar to the EpoR. Furthermore, the use and further development of imaging techniques such as fluorescence correlation spectroscopy promise to study the dynamic behavior of transmembrane proteins in live cells and thus to examine whether higher oligomeric receptor structures constitute a common mechanism of promoting and regulating selective signaling.
Internalization of the EpoR determines the early phase kinetics of ligand-receptor complex formation and therefore regulates the rapid receptor activation and deactivation. In contrast to other cell surface receptors, long-term attenuation of EpoR signaling is not mediated by receptor downregulation at the plasma membrane, indicating that different mechanisms have been evolved to control the responsiveness of cells towards external stimuli. Further studies should dissect the impact of rapid EpoR internalization and recruitment of negative regulators during the onset of receptor stimulation and reveal mechanisms of signal termination upon prolonged Epo stimulation. Moreover, analysis of the influence of receptor turnover and ligand-mediated internalization on signaling in primary erythroid progenitor cells may lead to a deeper understanding of how EpoR signaling at the receptor level regulates cellular decisions leading to survival, proliferation, and differentiation. These studies should help to gain insight into how erythropoiesis is tightly controlled and at the same time capable to rapidly adapt to environmental changes such as blood loss.

To cure anemia associated with chronic renal failure or cancer is a widely accepted beneficial aspect of rhEpo, although it should be cautiously applied to prevent a putative tumor-promoting activity. In addition to stimulating erythropoiesis, Epo promotes tissue-protection in neuronal cells and cardiomyocytes. To circumvent side effects such as thrombosis, specific Epo derivatives are desirable that either exhibit erythropoietic or tissue-protective activity. However, to understand what regulates these diverse processes in detail not only requires the detection of the respective pathway components. In fact, it is indispensable to understand and identify general mechanisms that allow selectively influencing the repertoire of cellular decisions. Thus, understanding and predicting the dynamic behavior of signal initiation and termination at the receptor level is a step forward to a goal-directed design of Epo mimetics and derivatives specifically driving either erythropoiesis or tissue protection. Thus, we still need to expand our knowledge about signaling through the EpoR to enable the targeted application of Epo as a therapeutic for anemia, cancer, as well as cerebral and cardiovascular ischemia.
4. Materials and Methods

If not stated otherwise, chemicals were purchased from Sigma and mammalian cell culture media and supplements were obtained from Gibco.

4.1 Molecular Cloning

Preparation of competent E. coli cells
For high-efficiency transformation of plasmid DNA, the E. coli strain DH5α dam+ (Stratagene) was used. To prepare chemically competent bacteria, DH5α cells were cultured in a volume of 500 ml LB medium up to an optical density of 0.6-0.8 as measured at 600 nm. After incubation on ice for 10 min, cells were sedimented with 4,100 g for 5 min at 4°C and resuspended in 150 ml TFBI (100 mM RbCl, 50 mM MnCl2, 10 mM CaCl2, 30 mM potassium acetate, 15% glycerol, pH 5.3). Following 20 min of incubation on ice, cells were centrifuged for 5 min at 1,400 g and 4°C, resuspended in 10-15 ml TFBII (10 mM RbCl, 75 mM CaCl2, 10 mM MOPS pH 7.0, 15% glycerol), and aliquots were stored at -80°C.

Transformation of E. coli DH5α dam+ cells
For transformation of competent DH5α dam+, cells were thawed on ice and 40 µl of cells were mixed with 0.5 µg of plasmid DNA or 5 µl of ligation reaction. After incubation for 20 min on ice, cells were subjected to a heat shock for 5 min at 37°C followed by an incubation for 10 min on ice. Subsequently, cells were diluted in 1 ml LB medium and incubated at 37°C for 30 min. Cells were plated in a total volume of 100 µl on TB-containing agar plates (Fluka). The plates were incubated o/n at 37°C and single colonies were picked for further cultivation in LB medium. For positive selection of transformed bacteria, the medium was supplemented either with 100 µg/ml ampicillin or with 25 µg/ml kanamycin.

Purification of plasmid DNA
Plasmid DNA was amplified in E. coli cultures either in small analytic or large preparative scale.
To isolate plasmid DNA in small scale, 1 ml of an E. coli culture was cultured o/n at 37°C in LB medium supplemented with the corresponding antibiotic. After sedimentation, cells were resuspended in 100 µl of remaining medium and lysed by alkaline lysis in 300 µl TENS buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaOH, 0.5% SDS). Proteins were precipitated by adding 150 µl sodium acetate (3 M, pH 5.2). After centrifugation for 2 min at 15,700 g, the supernatant was transferred to a new tube and DNA was precipitated using two
volumes of ice-cold ethanol. The DNA was washed with 70% ethanol and resuspended in 50 µl TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) supplemented with 40 µg/ml RNaseA (Roche Diagnostics). For sequencing grade, the QIAprep® Spin Miniprep Kit (Qiagen) was used according to the manufacturer’s instructions.

To prepare plasmid DNA in large scale, a single colony or 50 µl of an E. coli culture were cultured in 100 ml LB medium supplemented with the corresponding antibiotic for 16-18 h at 37°C. For purification, the JETSTAR 2.0 Maxi Kit (Genomed) was used according to the manufacturer’s instructions.

**Quantification of plasmid DNA**

The concentration of DNA solutions was determined by measuring the absorbance at 260nm (Ultrospec 3100 pro, GE Healthcare).

**DNA sequencing**

To verify DNA sequences, 1.5 µg of DNA of plasmid preparations were sent for sequencing to MWG Biotech AG, Martinsried, Germany.

**Molecular cloning of DNA fragments**

To generate specific DNA sequences, plasmid DNA was digested using a 3-5 fold excess of the corresponding restriction enzyme (New England Biolabs). To generate blunted DNA fragments, 500 µM dNTP (Roche Diagnostics) and 3 U T4 DNA Polymerase (New England Biolabs) were added to the digestion reaction. After incubation for 20 min at 16°C, the polymerase was heat-inactivated for 10 min at 65°C. For sequential digestion, DNA fragments were purified with the QIAquick® PCR Purification Kit (Qiagen). The resulting DNA fragments were separated on a 1-2% agarose gel (Invitrogen) supplemented with 100 ng/ml ethidiumbromide and excised from the gel using a scalpel. After elution and purification of the DNA fragments with the QIAEX®II Gel Extraction Kit (Qiagen), vector and an excess of insert DNA were ligated for 10-20 min at RT using 1 µl of Quick T4 DNA Ligase (New England Biolabs) and subsequently transformed into competent E. coli DH5α cells.

**Generation of double-stranded DNA adapters**

To produce double-stranded DNA adapters that contain specific restriction sites or linker sequences, 1 µM of complementary sense and anti-sense primers were heated in annealing buffer (10 mM Tris pH 8.0, 300 mM NaCl) for 5 min to 95°C. Subsequently, the mixture was cooled down for 30 min to RT, 10-fold diluted and used immediately for ligation into the corresponding plasmid.
Amplification of DNA fragments
To manipulate DNA by site-directed mutagenesis or by introducing restriction sites or linker sequences, DNA was amplified by PCR in a PTC-200 Thermo Cycler (MJ Research). The number and duration of cycles as well as the annealing temperatures were optimized for the expected product length and the corresponding primers. The annealing temperature of the PCR was generally chosen to be 4-8°C lower than the melting temperature \( T_M \) of the primers calculated by the formula \( T_M \text{ in } ^\circ\text{C} = \Sigma (2x(A+T) + 4x(G+C)) \). In general, PCR amplification was performed in a 100 \( \mu l \) reaction volume containing 50 ng plasmid template DNA, 100 \( \mu M \) of each dNTP, 1 \( \mu M \) of forward and reverse primer, 10% of DMSO, 2.5 units Cloned Pfu DNA Polymerase (Stratagene) and buffer according to the manufacturer’s manual.

Genotyping of the Balb/c EpoR\(^{-/-}\) mouse strain
Mouse tails were subjected to 500 \( \mu l \) lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl) supplemented with 100 \( \mu g/ml \) proteinase K o/n at 55°C. After centrifugation for 2 min at 15,700 g, the supernatants were mixed with an equal volume of isopropanol until the DNA became visible. The DNA was washed twice with 70% ethanol and dried at 37°C. The sedimented DNA was resuspended in 100 \( \mu l \) of a 1:1 mixture of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) and ddH\(_2\)O, heated to 95°C for 3 min, and stored at 4°C.

To genotype the Balb/c EpoR\(^{-/-}\) mouse strain (Wu et al., 1995), the genomic DNA was 20-fold diluted and amplified by PCR using 2 \( \mu M \) of allele-specific primers 1-3 (see Appendix), 50 \( \mu M \) of dNTP, 0.25 U Taq DNA Polymerase (Roche Diagnostics) in the appropriate buffer supplemented with 1.5 mM MgCl\(_2\). This amplification yields a 786 bp wild-type DNA fragment and a 417 bp knock-out DNA fragment for heterozygous mice.

Generation of plasmids
Retroviral expression vectors were pMOWS-puro or pMOWS-neo (Ketteler et al., 2002a). For generation of pMOWS-EpoR-GFP, a linker was added to GFP by PCR using the primer 4/5, the PCR fragment was digested with EcoRI and ligated into pMOWS-EpoR-GFP4 (Ketteler et al., 2002b), which had been digested with NotI, treated with T4 DNA polymerase, and subsequently digested with EcoRI. To yield pMOWS-HA-EpoR or pMOWS-HA-EpoR-GFP, a EcoRI and BamHI fragment from the vector pMX-EpoR-HA-IRES-GFP (provided by S. Constantinescu, Ludwig Institute for Cancer Research, Brussels, Belgium) was inserted into the PacI and BamHI restriction sites of pMOWS-EpoR (Swameye et al., 2003) or pMOWS-EpoR-GFP, respectively.

A Kozak consensus sequence was inserted 5’ of the HA-EpoR cDNA to yield pMOWS-Kz-HA-EpoR (used for experiments in Results, section 2.2) by using the primers 6/7 and
pMOWS-HA-EpoR as template and subcloning of the PCR fragment into the *PacI* and *PmlI* restriction sites of pMOWS-EpoR. To generate pMOWS-Kz-SBP-EpoR, the HA-tag from pMOWS-Kz-HA-EpoR was exchanged with an SBP-tag (Keefe et al., 2001).

The various EpoR TM domain mutants were constructed by PCR mutagenesis using pMX-puro-EpoR (Ketteler et al., 2003) as template and the 5’-primer 8 as well as 3’-primers 9-16. To enhance PCR efficiency, the 3’-primer 17 was added to the PCR reaction after 5 cycles. The PCR fragments were subcloned into the *SalI* and *BglII* restriction sites of a pBabe-adapter-EpoR constructed by adapter cloning using primers 18/19 and pBabe-EpoR (Klingmüller et al., 1996). pMOWS-EpoR TM mutants were generated by subcloning the *Xhol* and *EcoRI* fragment from pBabe-EpoR TM mutant vectors into the corresponding sites of pMOWS-EpoR. The various pMOWS-HA-EpoR TM mutants containing a C-terminal GFP-tag were established by subcloning the *PmlI* and *BglII* fragment of pBabe-EpoR TM mutants and GFP as a *BglII-EcoRI* fragment from pMOWS-EpoR-GFP into the corresponding sites of pMOWS-HA-EpoR (Ketteler et al., 2002b). pMOWS-HA-EpoR-T242N was generated by subcloning a *PmlI* and *EcoRI* fragment of pMOWS-EpoR-T242N into the corresponding sites of pMOWS-HA-EpoR.

To generate mRFP1-tagged human Rab4a and Rab5a, the GFP of pEGFP-Rab vectors provided by M. Zerial (Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) was exchanged with mRFP1 provided by R. Tsien (University of California, San Diego, USA). mRFP1 was amplified by using the primers 20/21. The *BamHI-Xhol* fragment containing mRFP1 and the *Xhol-PacI* fragment amplified by using primers 22-25 were subcloned into the corresponding sites of the pMOWS-neo expression vector. All clones were verified by sequence analysis. For primer sequences refer to Appendix – 6.2 Primers.

**4.2 Mammalian Cell Lines and Primary Cells**

**Cultivation of mammalian cell lines**

The packaging cell line Phoenix eco was cultured in DMEM medium supplemented with 10% FCS and 1% antibiotics (10,000 U/ml penicillin and 10,000 µg/ml streptomycin sulfate). For selection of Phoenix eco cells stably expressing Gag-Pol-Env, cells were treated with 2 µg/ml Diphtheria toxin (Calbiochem) and 200 µg/ml Hygromycin B (Roche Diagnostics). Phoenix eco cells were subcultured by treatment with 0.5 mg/ml Trypsin and 0.2 mg/ml EDTA and never cultured for longer than 2 weeks prior to transfection.
The IL-3 dependent murine pro B cell line BaF3 (Palacios and Steinmetz, 1985) was cultured in RPMI 1640 medium including 10% WEHI as a source of IL-3 and supplemented with 10% FCS and 1% antibiotics. Cells were subcultured after reaching a density of 5-8x10^5 cells/ml.

The murine fibroblast cell line NIH3T3 was cultured in DMEM medium supplemented with 10% calf serum and 1% antibiotics. Cells were grown to 85% confluency and subcultured by treatment with 0.5 mg/ml Trypsin and 0.2 mg/ml EDTA.

In general, cells were stored in liquid nitrogen in 90% serum and 10% DMSO at a density of 5x10^6 for Phoenix eco and BaF3 cells or 1x10^6 for NIH3T3 cells.

**Preparation of WEHI-conditioned medium**

To prepare IL-3 containing medium, WEHI-3B cells (Warner et al., 1969; Ralph and Nakoinz, 1977a; Ralph and Nakoinz, 1977b) were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics until confluency. After expansion of the cells in a total volume of 50 ml, WEHI-conditioned medium was harvested every 5 days by centrifugation and subsequent filtration through a 0.2 \( \mu \)m filter in order to remove cell debris. The remaining adherent cells as well as the sedimented cells were further supplied with 50 ml of fresh medium and adherent cells were subcultured after 4-6 weeks.

**Preparation of murine fetal liver cells**

Fetal liver cells (FLC) of EpoR^-/- mice were isolated at d12.5. Female pregnant mice were sacrificed, the embryos were extracted from the uterus, and all parts except of the liver were removed. The fetal liver was resuspended to a single cell suspension in ice-cold PBS/0.3% BSA and subsequently transduced (see Material and Methods: "Retroviral transduction of cells").

**Transient transfection of Phoenix eco cells**

Transient transfection of Phoenix eco cells was performed by calcium-phosphate precipitation. Cells were seeded at a density of 8x10^5 cells in 6-well plates 16-18 h before transfection. A mix of 10 \( \mu \)g of plasmid DNA and 12.5 \( \mu \)l CaCl\(_2\) (2.5 M) was precipitated together with 125 \( \mu \)l of 2x HBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na\(_2\)HPO\(_4\), pH 7.05) and dropwise transferred to the cells. To ensure an efficient uptake of DNA, cells were incubated for 6-8 h in standard medium supplemented with 25 \( \mu \)M chloroquine. Subsequently, the medium was replaced by IMDM supplemented with 30% FCS, 1% antibiotics, and 50 \( \mu \)M \( \beta \)-mercaptoethanol. The retrovirus-containing supernatant was harvested after 16-18 h of incubation and filtered through a 0.45 \( \mu \)m filter (Millipore). Supernatants were either directly used or stored at -80°C for up to 3 months. Transfection
efficiency was determined by measuring GFP expression in the FL-1 channel of a FACSCalibur (Becton Dickinson), in general yielding a GFP-positive population of 75-90%.

**Retroviral transduction of cells**

To stably transduce BaF3 cells, 250 µl retroviral supernatants generated with Phoenix eco cells were adjusted to 8 µg/ml polybrene, mixed with 1x10^5 BaF3 cells and centrifuged for 2 h at 340 g and 37°C in round bottom 2-ml microcentrifuge tubes. After centrifugation, cells were cultured for 48 h in standard medium and transduction efficiency was determined by measuring GFP expression in the FL-1 channel of a FACSCalibur (Becton Dickinson), in general yielding a GFP-positive population of 35-45%. Cells were subsequently selected and cultured with 1.5 µg/ml puromycin.

NIH3T3 cells were seeded at a density of 5x10^4 cells/well in a 6-well plate 16-18 h before transduction. For transduction, the medium was replaced with 250 µl retroviral supernatant supplemented with 8 µg/ml polybrene and 750 µl standard medium and centrifuged at RT for 3 h at 340 g. After centrifugation, cells were cultured in standard medium for 24 h and subsequently selected with 3 µg/ml puromycin or 600 µg/ml G418.

To transduce fetal liver cells (FLC) from Balb/c EpoR^+^ embryos (d12.5), 1x10^4 cells were centrifuged for 2 h at 430 g and 37°C with 200 µl of 10-fold diluted retroviral supernatants of Phoenix eco cells supplemented 8 µg/ml polybrene. To determine transduction efficiency, 5x10^4 FLC from embryos exhibiting a wild-type phenotype were transduced with GFP-encoding cDNA. After centrifugation, cells were cultured in IMDM supplemented with 30% FCS, 1% antibiotics, 50 µM β-mercaptoethanol, and 4 U/ml Epo (Janssen-Cilag) for 2 days and GFP expression was analyzed in the FL-1 channel of a FACSCalibur yielding a GFP-positive population of approximately 37%.

**4.3 Cell Biology Techniques**

**Starving and stimulation of BaF3 cells**

To investigate Epo-induced protein phosphorylation of BaF3 cells expressing EpoR receptor variants, the corresponding cells were washed three times with RPMI 1640 and starved for 3-5 h at 37°C in RPMI 1640 supplemented with 1 mg/ml BSA. After starving, the cells were sedimetned and resuspended at a concentration of 1x10^7 cells/250 µl in RPMI 1640. The cells were preincubated for 5 min at 37°C and subsequently stimulated with 0.5-50 U/ml Epo (Janssen-Cilag) for up to 240 min at 37°C.

To determine the activation of SBP-EpoR after incubation with streptavidin, BaF3 cells stably expressing SBP-EpoR were washed six times in biotin-free RPMI 1640 (PAN Biotech) and
starved for 3 h at 37°C in streptavidin binding medium (biotin-free RPMI 1640 supplemented with 1 mM L-glutamine and 25 mM HEPES pH 7.4) supplemented with 1 mg/ml BSA. After starvation, cells were sedimented and resuspended at a final concentration of 1x10^7 cells/250 µl in streptavidin binding medium. Cells were preincubated for 5 min at 37°C and subsequently stimulated with increasing concentrations of streptavidin for 20 min at 37°C.

**CFU-E colony assay**
After transduction of fetal liver cells derived from d12.5 EpoR^/-^ embryos, cells were plated in 0.8% methylcellulose supplemented with 4 U/ml Epo (Janssen-Cilag) and 20 ng/ml SCF (R&D Systems) and stained with 0.04% benzidine and 0.3% H2O2 diluted in 0.5 M acetic acid after 2 days.

**Proliferation assay**
For proliferation assays, BaF3 cells stably expressing HA-tagged EpoR variants were washed three times with RPMI 1640 and plated at densities of 4x10^4 cells/well in 24-well plates in the presence of Epo (Janssen-Cilag) concentrations ranging from 0.1 to 5 U/ml in RPMI 1640 supplemented with 10% FCS and 1% antibiotics or without Epo. After 4 days, cell numbers were determined using a Coulter Counter Z2 (Beckman, particle size 4.00-17.35 µm) and expressed as the percentage of growth obtained in a parallel well containing 5% WEHI-conditioned medium as a source for IL-3.

**Flow cytometry**
For analysis of cell surface expression, BaF3 cells stably expressing HA-tagged EpoR variants were incubated with anti-HA (Roche Diagnostics) as primary antibody and anti-rat IgG coupled to Cy5 (Dianova) as secondary antibody (Appendix – 6.1 Antibodies and Conjugates) and analyzed by flow cytometry using the FL-4 channel of a FACSCalibur (Becton Dickinson). Live cells were gated by forward and side scatter.
For analysis of apoptosis, BaF3 cells stably expressing EpoR variants were washed in RPMI 1640, seeded at a density of 4x10^4 cells/ml and grown in 1 U/ml Epo (Janssen-Cilag). Cells were counted in a Neubauer chamber 24, 48, and 72 h later and an equivalent of 3x10^5 cells was stained with Annexin V-PE and 7-AAD (Pharmingen) according to the manufacturer’s instructions and analyzed by flow cytometry using the FL-2 and FL-3 channel of a FACSCalibur. Annexin V-PE positive and 7-AAD negative cells were regarded as apoptotic cells. Cells grown without Epo or in 5% WEHI-conditioned medium as a source of IL-3 were taken as control.
**Fluorescence microscopy**

For fluorescence microscopy, stably transduced NIH3T3 cells were grown in 2-well chambered coverglass 1.0 borosilicate (Nalge Nunc) in DMEM supplemented with 10% calf serum and antibiotics. Analysis of subcellular localization of GFP-tagged wild-type HA-EpoR or HA-EpoR TM mutants (Nikon Plan Apo 60x/1.2 WI objective) as well as analysis of colocalization of GFP-tagged wild-type HA-EpoR with mRFP1-tagged human Rab proteins (Nikon Plan Apo 100x/1.4 oil immersion objective) was performed on a Nikon Eclipse TE2000 inverted microscope with a Perkin Elmer UltraView ERS spinning disc confocal unit on a heated stage (37°C). Images were acquired with a Hamamatsu EM-CCD camera (C9100-02) and UltraView ERS Imaging Suite software. Adjustments of brightness and contrast were performed using Adobe Photoshop 7.0. Image merge was performed with ImageJ 1.37.

Tracking of GFP-tagged EpoR-positive structures was performed with a Leica SP2 confocal microscope (63x/1.4 oil objective) using an opened pinhole (2.6 Airy units). Time series of 150 frames (815 ms/frame) were acquired in 3 different, representative cells (15 µm x 15 µm zoom area). Acquired time series were analyzed with the freeware program RYTRACK (http://titan.iwu.edu/~gspaldin/rytrack.html) running under IDL (Interactive Data Language, Visual Numerics). For the evaluation, only spots were included that could be identified and tracked unambiguously over at least 10 subsequent frames. In untreated cells, 17 spots were difficult to track automatically due to fast movements; these were analyzed manually over 10 frames using ImageJ. From this analysis, we obtained the squared displacement (SD) $\Delta x^2$ for individual EpoR-positive structures between any two frames with lag time $T$. From this, we calculated for fixed lag times $T$ the cumulative distribution of SDs, $P(\Delta x^2)$, that included the motion of the entire spot population in a given cell. We then fitted $P(\Delta x^2)$ with an exponential $P(\Delta x^2) = 1 - \exp\left\{-\frac{\Delta x^2}{4DT}\right\}$ (for details see Guigas and Weiss, 2006) and assumed a lognormal distribution of diffusion coefficients $p(D) = \exp\left\{-\frac{(\ln D - \mu_L)^2}{2\alpha_L^2}\right\}\left/\left(D\alpha_L\sqrt{2\pi}\right)\right.$ to account for the heterogeneous motion and sizes of EpoR-positive structures. To account for the limited accuracy in the center-of-mass positions, we only fitted $P(\Delta x^2)$ in the range $\Delta x > 50$ nm. By optimizing $\mu_L$ and $\alpha_L$ we obtained good fits to the experimental data and determined the average diffusion coefficient $D(T)$ of the population of dots as $D(T) = \exp\left\{\mu_L + \alpha_L^2/2\right\}$. The value obtained for $D(T)$ showed only slight deviations between the different cells, therefore their average is shown. The traveling time needed to overcome a distance $\Delta x$ by any type of diffusion may be estimated via the formula for the mean square displacement in three dimensions, $\Delta x^2 = 6 \cdot D(T) \cdot \Delta t$. 
Scatchard analysis

To determine the affinity of Epo to the EpoR as well as the amount of receptor at the cell surface, $1 \times 10^6$ BaF3 cells stably expressing EpoR variants were incubated with 10 pM, 100 pM, 250 pM, 500 pM, or 2000 pM $[^{125}\text{I}]-\text{Epo}$ (GE Healthcare) in 100 µl RPMI 1640 supplemented with 10% FCS for 4 h at RT. Cells were centrifuged through FCS and cell-bound as well as free $[^{125}\text{I}]-\text{Epo}$ was measured in a Cobra gamma counter (Packard). Specific binding was determined by subtracting the mean value of radioactivity of cells incubated with both $[^{125}\text{I}]-\text{Epo}$ and 250 U/ml unlabelled Epo (Janssen-Cilag) (for three independent cell pools) from the radioactivity of cells incubated in the absence of unlabelled Epo. Results are presented either with fitting a one-site saturation curve or as a Scatchard analysis plotting the specifically bound $[^{125}\text{I}]-\text{Epo}$ against the ratio of specifically bound to free $[^{125}\text{I}]-\text{Epo}$. The negative reciprocal value of the slope of the linear regression fitted to the three data sets revealed the dissociation constant $K_D$ as well as the number of binding sites.

For determining the affinity of streptavidin to SBP-EpoR, $1 \times 10^6$ BaF3 cells stably expressing SBP-EpoR were washed with biotin-free RPMI 1640 (PAN Biotech) for six times and incubated with 100 pM, 250 pM, 1000 pM, 2500 pM, or 5000 pM $[^{125}\text{I}]-\text{streptavidin}$ (GE Healthcare) in 100 µl streptavidin binding medium (biotin-free RPMI 1640 supplemented with 1 mM L-glutamine and 25 mM HEPES pH 7.4) for 4 h at RT. Cells were washed three times with 500 µl biotin-free RPMI 1640 and pooled supernatants as well as the cells were measured in a Cobra gamma counter. To determine specific binding of $[^{125}\text{I}]-\text{streptavidin}$, control cells were additionally incubated with 2.5 µM unlabelled streptavidin.

Internalization assay

To measure Epo-induced endocytosis of the EpoR, $1 \times 10^6$ BaF3 cells stably expressing GFP-tagged HA-EpoR or HA-EpoR-T242N or $4 \times 10^6$ BaF3 cells stably expressing HA-EpoR were washed three times with RPMI 1640 and starved in RPMI 1640 supplemented with 1 mg/ml BSA for 1 h or 3 h at 37°C, respectively. Cells were incubated in 100 µl RPMI 1640 for up to 240 min at 37°C with 500 pM or with 2100 pM (equal to approximately 5 U/ml) $[^{125}\text{I}]-\text{Epo}$ (GE Healthcare), respectively. After incubation, receptor internalization was blocked on ice and cells were centrifuged through a layer of ice-cold FCS to remove free $[^{125}\text{I}]-\text{Epo}$. Subsequently, acid stripping was performed with 4% acetic acid for 5 min at 4°C and cells were centrifuged through ice-cold FCS to separate cell-bound and internalized $[^{125}\text{I}]-\text{Epo}$. The amount of radiolabelled Epo was measured in a Cobra gamma counter (Packard). Specific binding was determined by subtracting the mean value of radioactivity of cells incubated with both $[^{125}\text{I}]-\text{Epo}$ and 250 U/ml or 500 U/ml unlabelled Epo (Janssen-Cilag) (for three independent cell pools) from the radioactivity of cells incubated in the absence of unlabelled
Epo, respectively. Efficiency of acid stripping was about 98% and viability of cells after acid stripping was confirmed by Trypan Blue (Fluka) exclusion.

To measure constitutive endocytosis of the EpoR, 1x10^6 BaF3 cells stably expressing SBP-EpoR were washed six times with biotin-free RPMI 1640 (PAN Biotech) and starved for 1 h at 37°C in streptavidin binding medium supplemented with 1 mg/ml BSA. Cells were incubated with 1000 pM [125I]-streptavidin in 100 µl streptavidin binding medium (biotin-free RPMI 1640 supplemented with 1 mM L-glutamine and 25 mM HEPES pH 7.4) for up to 60 min at 37°C. After incubation, receptor internalization was blocked on ice and cells were washed three times with ice-cold biotin-free RPMI 1640 to remove free [125I]-streptavidin and supernatants were pooled. Subsequently, acid stripping was performed with 4% acetic acid for 5 min at 4°C and cells were washed three times with ice-cold biotin-free RPMI 1640 to separate cell-bound and internalized [125I]-streptavidin and supernatants were pooled. The amount of radiolabelled streptavidin was measured in a Cobra gamma counter. Specific binding was determined by subtracting the mean value of radioactivity of cells incubated with both [125I]-streptavidin and 2.5 µM unlabelled streptavidin (for three independent cell pools) from the radioactivity of cells incubated in the absence of unlabelled streptavidin.

**Metabolic labeling**

BaF3 cells stably expressing EpoR variants were washed with Cys/Met-free RPMI 1640, resuspended in Cys/Met-free RPMI 1640 including 10% dialyzed FCS, and preincubated for 45 min at 37°C. Labeling was performed with 0.5 mCi [35S] in vitro labeling mix (GE Healthcare)/1x10^7 cells for 15 min at 37°C. Subsequently, cells were chased by adding an access of unlabeled cysteine/methionine. For each time point, 1x10^6 cells were lysed with 2x RIPA buffer (1x buffer: 50 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 2 µg/ml aprotinin and 200 µg/ml AEBSF and lysates were precleared with CL4B-sepharose (GE Healthcare) before anti-EpoR immunoprecipitation (see Material and Methods: “Immunoprecipitation of proteins”). Immunoprecipitates were left untreated or digested with EndoH (New England Biolabs) for 1 h at 37°C and separated by 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Schleicher&Schuell) (see Material and Methods: “SDS-PAGE and immunoblot analysis”), incubated with Ponceau Red and Amplify™ (GE Healthcare) before detection on a Typhoon™ phosphoimager (GE Healthcare). Blots were quantified with the LumiAnalyst 3.1 software (Roche Diagnostics).
4.4 Protein Biochemistry

Preparation of total cellular lysates
Detergent lysates of cells were prepared either with 2x 1% NP40 buffer (1x buffer: 1% NP40, 150 mM NaCl, 20 mM Tris pH 7.4, 10 mM NaF, 1 mM EDTA pH 8.0, 1 mM ZnCl₂ pH 4.0, 1 mM MgCl₂, 1 mM Na₃VO₄, 10% Glycerol) or 2x 0.5% NP40-buffer (1x buffer: 0.5% NP40, 150 mM NaCl, 50 mM Tris pH 7.4, 5 mM NaF, 1 mM EDTA pH 8.0, 0.5 mM Na₃VO₄, 1 mM DTT), each supplemented with 2 µg/ml aprotinin and 200 µg/ml AEBSF. After 30 min incubation at 4°C, the lysate was centrifuged for 10 min at 20,000 g and 4°C. The supernatant was used for immunoprecipitation or total cellular lysates or was stored at -80°C.

Sucrose gradient fractionation
To isolate detergent-resistant membranes (DRM), NIH3T3 cells equivalent to two 100 mm-dishes with a confluency of approximately 70% were washed three times with DMEM and stimulated with 50 U/ml Epo (Janssen-Cilag) for 20 min at 37°C or left unstimulated. Cells were lysed in MBS buffer (25 mM MES, 150 mM NaCl, pH 6.5) including 0.5% Triton X-100 (Roche Diagnostics) as well as 1 µg/ml aprotinin and 100 µg/ml AEBSF for 45 min on ice. Cell debris was removed by centrifugation for 1 min at 430 g and 4°C. The supernatant (1 ml) was adjusted to 40% sucrose with 1 ml of 80% sucrose diluted in MBS buffer and placed at the bottom of a polyclear tube (SCI). The sample was overlaid with 6 ml 30% sucrose and 4.7 ml 5% sucrose diluted in MBS buffer and centrifuged for 19 h at 36,000 rpm and 4°C in a SW40 rotor in an ultracentrifuge (Beckman). Fractions were collected from top to bottom (1 ml each), thereby discarding the first 2.6 ml. For determining the DRM fraction, 40 µl of fractions 1-10 supplemented with SDS sample buffer were separated by SDS-PAGE and subjected to immunoblot analysis with Flotillin-1-antibodies as a positive marker for lipid rafts and transferrin receptor (TfR)-antibodies as a negative marker (see Material and Methods: “SDS-PAGE and immunoblot analysis”; Appendix – 6.1 Antibodies and Conjugates).

Quantification of proteins
Protein concentrations of total cellular lysates were measured with the Bradford Protein Assay Kit (Biorad) at a wavelength of 595 nm according to the manufacturer’s manual. Purified BSA dilutions (New England Biolabs) were used for calibration. To determine protein concentrations of NIH3T3 lysates after sucrose gradient fractionation, the Micro BSA Protein Assay Kit (Pierce) was used according to the manufacturer’s instructions. To simultaneously measure protein concentrations in the pellet fractions 9 and 10, the corresponding lysates were 10-fold diluted.
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**Immunoprecipitation of proteins**

Immunoprecipitation was performed with an equivalent of 1x10⁷ BaF3 cells by adding target-specific antibody (see Appendix – 6.1 Antibodies and Conjugates) and 25 µl of Protein A sepharose (GE Healthcare) to the lysate for 2-8 h or o/n at 4°C. The immunoprecipitates were washed twice with 1x 1% NP40 lysis buffer and once with TNE buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, pH 8.0, 100 µM Na₃VO₄) and were resuspended in 25 µl 2x SDS sample buffer (1x buffer: 2% SDS, 50 mM Tris pH 7.4, 10% glycerol, 5% β-mercaptoethanol, 100 mM DTT, 0.01% bromphenolblue). The immunoprecipitates were immediately subjected to protein gel electrophoresis or stored at -20°C. Immunoprecipitates of pulse-chase experiments were washed four times with 1x RIPA buffer and twice with TNE buffer. Samples generated for time course analysis of EpoR and JAK2 activation (Results, section 2.2, Figure 31) were prepared adding 40 ng of calibrator protein GST-EpoR (cytoplasmic domain) and 18 ng of SBP-JH2 to the lysates before immunoprecipitation and sample loading on SDS-PAGE (see Material and Methods: “SDS-PAGE and immunoblot analysis”) was randomized (Schilling et al., 2005b; Schilling et al., 2005a).

To immunoprecipitate proteins from sucrose gradient fractionations of NIH3T3 cells, 450 µl of total cellular lysates were supplemented with 10x solubilization buffer (350 mM Tris pH 8.0, 10% Triton X-100, 600 mM octylglucosides) before adding target-specific antibody and Protein A sepharose beads.

**SDS-PAGE and immunoblot analysis**

Proteins were separated according to their electrophoretic mobility in a denaturing SDS-PAGE (Laemmli, 1970). Protein samples were boiled for 3 min in SDS sample buffer. Protein samples were separated by 10% or 15% SDS-PAGE with low bis-acrylamide (GE Healthcare) (Table 1) and separated in an electric field in running buffer (192 mM glycine, 25 mM Tris, 0.1% SDS). Immunoprecipitates were centrifuged for 2 min at 15,700 g before loading. For total cellular lysates, an amount of 100 µg protein resuspended in 4x SDS sample buffer were separated by SDS-PAGE.

<table>
<thead>
<tr>
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<th>stacker gel</th>
<th>10% separating gel</th>
<th>15% separating gel</th>
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<tbody>
<tr>
<td>40% acrylamide</td>
<td>1 ml</td>
<td>5 ml</td>
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</tr>
<tr>
<td>2% w/v methylenebisacrylamide</td>
<td>0.5 ml</td>
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<tr>
<td>1M Tris-HCl, pH 6.8</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td></td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
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<td>0.2 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>7.15 ml</td>
<td>8.5 ml</td>
<td>6.42 ml</td>
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Table 1: SDS-Page for 10% and 15% polyacrylamide gels.
Immunoblotting was performed in semi-dry chambers (GE Healthcare) on nitrocellulose membranes with a pore size of 0.2 µm (Schleicher&Suell) for immunoprecipitation or analysis of sucrose gradient fractionation samples. For analysis of Akt/PKB and ERK1/2, proteins were transferred on PVDF membranes with a pore size of 0.45 µm (Millipore). Blotting was performed in transfer buffer (192 mM glycine, 25 mM Tris, 0.075% SDS, 0.5 mM Na3VO4, 15% methanol) for 1 h at approximately 1.3 mA/cm². Proteins were reversibly stained fixed with Ponceau Red. After blocking unspecific antibody binding with 2-5% BSA diluted in TBS-T (10 mM Tris pH 7.4, 150 mM NaCl, 0.2% Tween-20), membranes were incubated with the appropriate first and secondary antibodies (see Appendix – 6.1 Antibodies and Conjugates) and proteins were visualized with the ECL or ECL Advance Western Blotting Detection Reagents (GE Healthcare) and subsequently detected on a Lumi-Imager F1™ (Roche Diagnostics). Quantification was performed using the LumiAnalyst 3.1 software (Roche Diagnostics). To evaluate total protein levels, membranes were reprobed by incubation in stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 20-25 min at 65°C.

4.5 Modeling Approaches

Model calculations for the motion of EpoR-containing structures

A set of 50 'particles' of GFP-positive structures that initially showed a subdiffusive motion in a three-dimensional volume. This mode of motion was simulated by using the Weierstrass-Mandelbrot function with a Hurst coefficient of H=0.25 (for details see Weiss et al., 2003) for a random walk (fractional Brownian motion) in each dimension. Prefactors were chosen in such a way that a good overlap with the experimental observations in nocodazole-treated cells was accomplished. In particular, the time between two diffusive steps was defined to be Δt=20ms. The influence of microtubules was mimicked by switching with probability p_on=Δt·k_on to a directed motion with velocity v=1.5 µm/s. The direction of movement was taken as a random unit vector in three-dimensional space (i.e. a randomly oriented microtubule) and motion along this direction was calculated via an explicit Euler scheme. With probability p_off=Δt·k_off, the particle stopped its directed motion (dissociation from the microtubule) and resumed its anomalous diffusion. The tracked positions obtained from the simulation were subject to the same statistical analysis as the experimental data. To match the experimental data, k_on and k_off were varied.
All-atom structures of the EpoR TM dimer modeled in a membrane environment

The most probable structure of a transmembrane dimer is that with the lowest solvation energy, \( \Delta G_{\text{solv}} \), defined as the free energy change for transferring the molecule from vacuum to its position in the membrane (Leach, 2001). The solvation energy was calculated using an all-atom model for the transmembrane dimer together with the Poisson-Boltzmann equation for the electrostatic solvation energy and a term representing the cost of cavity formation in the aqueous solvent as described (Sengupta et al., 2005). A five-slab continuum dielectric model that distinguishes between the solvent, head-group, and core regions was used to describe the membrane environment. For a dimer in the membrane, the structure was described by four parameters: the inter-helical distance, \( d \), the crossover angle, \( \theta \), the membrane insertion, \( \nu \), and helix rotation, \( \tau \). \( d \) is defined as the minimum distance between the two helix axes and \( \theta \) the angle between them. \( \nu \) is the distance of the center of mass of each helix from the center of the membrane. \( \tau \) is the angle of rotation of each helix about its axis. The transmembrane segment of EpoR [LILTLSLILVLISLLLTVLALLS] was modeled as an ideal \( \alpha \)-helix with \( \psi = -47^\circ \) and \( \phi = -57^\circ \) (Branden and Tooze, 1999). Values were assigned to \( d \), \( \theta \), \( \nu \), and \( \tau \) systematically so as to sample the relevant conformational space of the homodimer. The side-chains were positioned using the software SCWRL (Canutescu et al., 2003) (version 3.0), which determines the most probable rotameric conformations from a backbone-dependent rotamer library by an algorithm-based graph theory. The dimers were energy minimized in a dielectric medium of \( \varepsilon = 2 \) using the CHARMM potential (Brooks et al., 1983) (version 30b1) with 1000 steps of Steepest Descent followed by 1000 steps of Newton-Raphson minimization with 1 kcal/mol harmonic constraints on the backbone atoms. The structures were then introduced in the five-slab membrane model and scored based on the value of the solvation energy. The volume of a dimer was calculated as the volume defined by rolling a sphere of radius 2.2 Å on the molecular surface to fill all cavities in the dimer interface. The lowest-energy conformers along with the conformers with energies accessible at 300K (KBT) were chosen.

Mathematical modeling and sensitivity analysis of EpoR internalization

Modeling was performed using PottersWheel (Maiwald et al., unpublished, http://www.potterswheel.de/). Parameter estimation was performed in logarithmized parameter space using trust region optimization method. For each fit, 100 iterations were performed with \( \chi^2 \) tolerance of \( 10^{-7} \) and fit parameters tolerance of \( 10^{-7} \). Using the best fit as starting value, 500 fits were computed, each time varying all parameters with a disturbance strength of 0.4. The boundaries for \( K_D \) and \( K_{D_\text{strep}} \) were confined by the standard deviations of the measurements, and \( B_{\text{max}} \) and \( B_{\text{max, strep}} \) were fixed to the measured values. All other parameters were estimated with boundaries between \( 10^{-7} \) and \( 10^3 \), and none of the
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estimated parameters lay on these boundaries. The initial values for EpoR were set to the experimentally determined $B_{\text{max}}$ or $B_{\text{max, strep}}$, while the initial values for Epo and strep were set to the concentrations $\pm 100$ pM used for the experiments.

Sensitivity analysis was applied to investigate relative changes of derived system variables $K$ as a result of relative changes in parameter values $p_i$:

$$S_{p_i}^K = \frac{p_i}{K} \frac{\partial K}{\partial p_i}$$

Hornberg et al. (Hornberg et al., 2005a) derived summation laws for sensitivies of derived system variables like signal amplitude, signal duration, and area under curve. The proofs for the summation laws (Hornberg et al., 2005b) can easily be extended to show the existence of summation laws for the system variables investigated in our approach.

$$\sum_{i} S_{p_i}^{\text{peak amplitude}} = 0$$

$$\sum_{i} S_{p_i}^{\text{peak time}} = -1$$

$$\sum_{i} S_{p_i}^{\text{extrema amplitude}} = 0$$

Sensitivity analysis is a local approach, because derivations are evaluated at a certain point in parameter space (local sensitivity analysis).

Most models are non-identifiable, i.e., there exist model parameters that cannot be determined unambiguously. Often, non-identifiability manifests itself in functionally related parameters (see linear and hyperbolic relationship, Appendix - Identifiability Analysis of Parameters for EpoR Internalization Models). For example, the output functions of the constitutive EpoR internalization model are invariant under parameter variations along the hyperbola. Thus, without prior knowledge, it can in principle not be determined statistically at which point in parameter space sensitivity analysis has to be performed. To deal with this problem, we took the following approach: The model was fitted $N$ times to data ($N = 500$). Each fit yielded different estimates for the non-identifiable parameters. Non-identifiabilities were detected with $NBI$ and sensitivies (1) were calculated at the actual point along the non-identifiability, here the linear and the hyperbolic relationship, respectively. For analytical non-identifiabilities, the derived system variables (peak amplitude, peak time, and extrema amplitude) are invariant to changes along the non-identifiabilities. Therefore, the results of the sensitivity analysis do not depend on the values we chose for the non-identifiable parameters. Sensitivities for non-identifiable parameters are not determinable, yet in our model, the non-identifiable parameters had essentially no control over the derived system variables independent of their values. Therefore, the control coefficients we determined varied by less than $10^{-4}$, which is due to numerical reasons.
5. References


References


## 6. Appendix

### 6.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>BFU-E</td>
<td>burst forming unit-erythroid</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>CEPO</td>
<td>carbamylated erythropoietin</td>
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<tr>
<td>CERA</td>
<td>continuous erythropoietin receptor activator</td>
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<tr>
<td>CFU-E</td>
<td>colony forming unit-erythroid</td>
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<td>CFU-GEMM</td>
<td>colony forming unit-granulocytes, erythrocytes, monocytes, macrophages</td>
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<tr>
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<td>D(T)</td>
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<td>Dulbecco’s modified eagle medium</td>
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<tr>
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<td>dimethyl sulfoxide</td>
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<tr>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>DRM</td>
<td>detergent-resistant membrane</td>
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<tr>
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<td>Escherichia coli</td>
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<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGFR</td>
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<td>enhanced green fluorescent protein</td>
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<td>EndoH</td>
<td>endoglycosidase H</td>
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<tr>
<td>EpoR</td>
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<td>endoplasmic reticulum</td>
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<tr>
<td>ERK</td>
<td>extracellular-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>FCS</td>
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<tr>
<td>FLC</td>
<td>fetal liver cells</td>
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<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<td>g</td>
<td>g force</td>
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<td>G418</td>
<td>geneticin</td>
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<tr>
<td>Gab</td>
<td>Grb2-associated binder</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GHR</td>
<td>growth hormone receptor</td>
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<td>Grb</td>
<td>growth factor receptor-bound protein</td>
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<td>h</td>
<td>hours</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
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<td>hypoxia-inducible transcription factor</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<td>IB</td>
<td>immunoblot</td>
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<td>immunoglobulin</td>
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<td>Isocove’s modified Dulbecco’s medium</td>
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<td>immunoprecipitation</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>K_d</td>
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<td>LB</td>
<td>Luria Bertani broth</td>
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<tr>
<td>M</td>
<td>molarity</td>
</tr>
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<td>MalE</td>
<td>maltose-binding protein</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<td>microliter</td>
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<td>non-parametric bootstrap-based algorithm for identifiability testing</td>
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<td>neo</td>
<td>neomycin resistance gene</td>
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<tr>
<td>NESP</td>
<td>novel erythropoiesis stimulating protein (darbepoetin alfa)</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>o/n</td>
<td>over night</td>
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</table>
ODE ordinary differential equations
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PCR polymerase chain reaction
PDGFR platelet-derived growth factor receptor
PE phycoerythrin
Pfu *Pyrococcus furiosus*
PI3K phosphatidylinositol 3-kinase
PKB protein kinase B
PKC protein kinase C
puro puromycin
rhEpo recombinant human erythropoietin
rpm rounds per minute
RT room temperature
SBP streptavidin-binding peptide
SCF stem cell factor
SDS sodium dodecyl sulfate
SH Src homology
SHP SH2 domain-containing protein tyrosine phosphatase
SOCS suppressor of cytokine signaling
Sos son of sevenless
STAT signal transducer and activator of transcription
strep streptavidin
TAE Tris-acetate-EDTA
Taq *Thermus aquaticus*
TB Terrific broth
TBS Tris buffered saline
TBST Tris buffered saline with Tween-20
TE Tris-EDTA
TfR transferrin receptor
TM transmembrane
TPO thrombopoietin
Tris Tris(hydroxymethyl)-aminomethane
U unit of enzyme activity
WEHI Walter and Eliza Hall Institute
w/v weight per volume
6.2 Antibodies and Conjugates

The following antibodies were used for immunoprecipitation (IP), immunoblot analysis (IB), or flow cytometry (F); HRP, horseradish peroxidase.

<table>
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<tr>
<th>primary antibodies</th>
<th>use</th>
<th>specification</th>
<th>company</th>
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<tr>
<td>mouse anti-Flotillin-1</td>
<td>IB (1:250)</td>
<td>monoclonal, 18</td>
<td>BD Biosciences</td>
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<tr>
<td>mouse anti-phosphoTyr</td>
<td>IB (1:10000)</td>
<td>monoclonal, 4G10</td>
<td>UBI</td>
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<tr>
<td>mouse anti-Transferrin receptor</td>
<td>IB (1:500)</td>
<td>monoclonal, H68.4</td>
<td>Zymed</td>
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<tr>
<td>rabbit anti-Akt/PKB</td>
<td>IB (1:1000)</td>
<td>polyclonal</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>rabbit anti-phospho-Akt/PKB (Ser473)</td>
<td>IB (1:1000)</td>
<td>monoclonal, 193H12</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>rabbit anti-EpoR</td>
<td>IP (3 µl)</td>
<td>polyclonal, M-20</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>rabbit anti-JAK2 (Fig. 31)</td>
<td>IB (1:10000)</td>
<td>monoclonal, 24B11</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>rabbit anti-JAK2 (Fig. 31)</td>
<td>IP (5 µl)</td>
<td>polyclonal, HR-758</td>
<td>Santa Cruz</td>
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<td>rabbit anti-JAK2 (Fig. 15,22)</td>
<td>IP (1.5 µl)</td>
<td>polyclonal, serum</td>
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<td>IB (1:10000)</td>
<td>polyclonal</td>
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<td>rabbit anti-phospho-MAPK (p44/42) (Thr202/Tyr204)</td>
<td>IB (1:5000)</td>
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<tr>
<td>rabbit anti-STAT5</td>
<td>IP (5-10 µl)</td>
<td>polyclonal, C-17</td>
<td>Santa Cruz</td>
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<tr>
<td>rat anti-HA</td>
<td>F (1:100)</td>
<td>monoclonal, 3F10</td>
<td>Roche Diagnostics</td>
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</table>

<table>
<thead>
<tr>
<th>conjugates</th>
<th>use</th>
<th>specification</th>
<th>company</th>
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</thead>
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<td>donkey anti-rabbit IgG HRP</td>
<td>IB (1:10000; 1:15000 for MAPK)</td>
<td>polyclonal</td>
<td>GE Healthcare</td>
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<td>sheep anti-mouse IgG HRP</td>
<td>IB (1:10000)</td>
<td>polyclonal</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Protein A HRP</td>
<td>IB (1:10000)</td>
<td>polyclonal</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>donkey anti-rat IgG Cy5</td>
<td>F (1:100)</td>
<td>F(ab’)2 (H+L)</td>
<td>Dianova</td>
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6.3 Primers

Primers were ordered at MWG Biotech, Martinsried. GT, genotyping of EpoR\(^{-/-}\) mouse strain; for, forward; rev, reverse; PCR, polymerase chain reaction; Ad, adaptor.

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<td>EpoR_KO_1</td>
<td>GT</td>
<td>5'-GCACTGAGTGTGTCTG-3'</td>
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<td>2</td>
<td>EpoR_KO_2</td>
<td>GT</td>
<td>5'-GCCTCACACCTCACC-3'</td>
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<td>3</td>
<td>EpoR_KO_3</td>
<td>GT</td>
<td>5'-GCTGCTAAAGCGCATGT-3'</td>
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<td>4</td>
<td>5'EpoR-GFP</td>
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<td>5'-CTCAGCGGCAGCGGCAGCTGAGCGCGGCAGCATGGTG AGCAAGGG-3'</td>
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<td>5</td>
<td>3'EpoR-GFP</td>
<td>rev PCR</td>
<td>5'-GCATCGAATTCTTACTTGTACAGCTC-3'</td>
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<tr>
<td>6</td>
<td>5'PacI_Kozak_EpoR</td>
<td>for PCR</td>
<td>5'-CTACCTTTAATTAGGGCCACCATTGAGCAAACCTAGG-3'</td>
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<td>7</td>
<td>3'PmlI_EpoR</td>
<td>rev PCR</td>
<td>5'-CAGCACCACGTTGAGGC-3'</td>
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<tr>
<td>8</td>
<td>EpoR-NSalBam</td>
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<td>5'-GCATGATCAGTCAGACCATGACCAAACTCAG-3'</td>
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<td>9</td>
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<td>GGGCCAGAACCAGTCGATTGCAAGGAGATGAGGAC-3’</td>
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<td>for Ad</td>
<td>5'-TCGACCGGAATTCCGGA-3’</td>
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<td>19</td>
<td>3’Ad. Sall-EcoRI-BglIII</td>
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<td>5'-GATCTTCCGAATTCCG-3’</td>
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<td>for PCR</td>
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<td>22</td>
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<td>25</td>
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<td>rev PCR</td>
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</table>
6.4 Erythropoietin Receptor Sequence

Depiction of the amino acid sequence of the HA-tagged murine erythropoietin receptor (Swiss-Prot entry P14753):

The signal peptide is highlighted in green, the HA epitope is marked in blue, the conserved WSXWS motif is underlined in black, and the amino acids of the putative transmembrane domain are highlighted in red. Amino acids L240, L241, T242, V243, L244, and A245 are underlined in red. Cytoplasmic tyrosine residues that are phosphorylated by JAK2 are highlighted in orange (Y343, Y401, Y429, Y431, Y443, Y460, Y464, Y479). Numbering of the amino acids refers to the untagged processed protein after signal peptide cleavage.
6.5 Ordinary Differential Equations

Ordinary differential equations (ODE) are shown for the Epo-induced EpoR internalization as well as for the constitutive EpoR internalization model. EpoR_recycling is modeled with a 10-step compartmentalization delay reaction (in collaboration with M. Schilling, DKFZ Heidelberg).

Ordinary differential equations: Epo-induced EpoR internalization

\[
\begin{align*}
\dot{x}_1 &= k_1 k_2 - k_1 x_1 - k_3 k_2 x_2 + k_3 k_4 x_3 + k_7 x_{17} \\
\dot{x}_2 &= -k_3 k_2 x_2 + k_3 k_4 x_3 + k_6 x_5 \\
\dot{x}_3 &= k_3 x_2 x_1 - k_2 k_4 x_3 - k_5 x_3 \\
\dot{x}_4 &= k_5 x_3 - k_6 x_4 \\
\dot{x}_5 &= k_6 x_4 - k_9 x_5 \\
\dot{x}_6 &= k_9 x_5 - k_5 x_6 \\
\dot{x}_7 &= k_2 x_6 - k_{10} x_7 \\
\dot{x}_8 &= k_{10} x_7 \\
\dot{x}_9 &= k_7 x_6 - k_7 x_9 \\
\dot{x}_{10} &= k_7 x_9 - k_7 x_{10} \\
\dot{x}_{11} &= k_7 x_{10} - k_7 x_{11} \\
\dot{x}_{12} &= k_7 x_{11} - k_7 x_{12} \\
\dot{x}_{13} &= k_7 x_{12} - k_7 x_{13} \\
\dot{x}_{14} &= k_7 x_{13} - k_7 x_{14} \\
\dot{x}_{15} &= k_7 x_{14} - k_7 x_{15} \\
\dot{x}_{16} &= k_7 x_{15} - k_7 x_{16} \\
\dot{x}_{17} &= k_7 x_{16} - k_7 x_{17}
\end{align*}
\]

Parameters

<table>
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<th>turnover:</th>
<th>( k_1 )</th>
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<td>( B_{\text{max}} ):</td>
<td>( k_2 )</td>
</tr>
<tr>
<td>( k_{\text{on}} ):</td>
<td>( k_3 )</td>
</tr>
<tr>
<td>( k_{\text{off}} ):</td>
<td>( k_4 )</td>
</tr>
<tr>
<td>internalization:</td>
<td>( k_5 )</td>
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<tr>
<td>dissociation:</td>
<td>( k_6 )</td>
</tr>
<tr>
<td>( \text{EpoR_recycling} ):</td>
<td>( k_7 )</td>
</tr>
<tr>
<td>( \text{Epo_recycling} ):</td>
<td>( k_8 )</td>
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<tr>
<td>( \text{Epo_degradation} ):</td>
<td>( k_9 )</td>
</tr>
<tr>
<td>release:</td>
<td>( k_{10} )</td>
</tr>
</tbody>
</table>

Initial values

| EpoR: | \( x_1 \) |
| Epo: | \( x_2 \) |

Observables

| Epo in medium: \( y_1 \) | \( x_2 + x_8 \) |
| Epo on surface: \( y_2 \) | \( x_3 \) |
| Epo in cells: \( y_3 \) | \( x_4 + x_5 + x_7 \) |
Ordinary differential equations: Constitutive EpoR internalization

\begin{align*}
\text{EpoR:} & \quad \dot{x}_1 = k_1 \cdot k_2 - k_1 \cdot x_1 - k_3 \cdot x_2 \cdot x_1 + k_5 \cdot k_4 \cdot x_3 \\
\text{strep:} & \quad \dot{x}_2 = -k_3 \cdot x_2 \cdot x_1 + k_5 \cdot k_4 \cdot x_3 + k_6 \cdot x_5 \\
\text{strep}_\text{EpoR:} & \quad \dot{x}_3 = k_5 \cdot x_2 \cdot x_1 - k_5 \cdot k_4 \cdot x_3 - k_1 \cdot x_3 \\
\text{strep}_\text{EpoR}_\text{internalized:} & \quad \dot{x}_4 = k_1 \cdot x_3 - k_2 \cdot x_4 \\
\text{strep}_\text{internalized:} & \quad \dot{x}_5 = k_6 \cdot x_4 - k_6 \cdot x_5 - k_7 \cdot x_5 \\
\text{strep}_\text{degraded:} & \quad \dot{x}_6 = k_7 \cdot x_5 - k_8 \cdot x_6 \\
\text{strep}_\text{released:} & \quad \dot{x}_7 = k_8 \cdot x_6
\end{align*}

Parameters

\begin{align*}
\text{turnover:} & \quad k_1 \\
B_{\text{max}} \text{_strep:} & \quad k_2 \\
k_{\text{on}} \text{_strep:} & \quad k_3 \\
K_{\text{D}} \text{_strep:} & \quad k_4 \\
strep \_dissociation: & \quad k_5 \\
strep \_recycling: & \quad k_6 \\
strep \_degradation: & \quad k_7 \\
release: & \quad k_8
\end{align*}

Initial values

\begin{align*}
\text{EpoR:} & \quad x_1 \\
\text{strep:} & \quad x_2
\end{align*}

Observables

\begin{align*}
\text{streptavidin in medium:} & \quad y_1 = x_2 + x_7 \\
\text{streptavidin on surface:} & \quad y_2 = x_3 \\
\text{streptavidin in cells:} & \quad y_3 = x_4 + x_5 + x_6
\end{align*}
6.6 Identifiability Analysis

Non-parametric bootstrap-based algorithm for identifiability testing (NBI) reveals dependent parameters. (a) Box plots of all estimated parameters are shown for the best 65% of 500 fits. Five parameters have a standard deviation larger than 25%. (b, c) NBI revealed parameter dependencies for a set of two and three parameters, respectively. \textit{Epo}_{\text{recycling}} and \textit{Epo}_{\text{degradation}} show a linear relationship, while \textit{strep}_{\text{dissociation}}, \textit{strep}_{\text{recycling}} and \textit{strep}_{\text{degradation}} are described by a skew hyperbola in space. (d) The parameters \textit{Epo}_{\text{degradation}} and \textit{strep}_{\text{dissociation}} were fixed and parameter estimation was repeated. Box plots of all estimated parameters are shown for the best 65% of 500 fits. All parameters show a standard deviation smaller than 25% (data provided by S. Hengl, T. Maiwald, and J. Timmer, FDM, University of Freiburg).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>turnover</td>
<td>0.0483 ± 5.44 x 10^-6 min^-1 (1%)</td>
</tr>
<tr>
<td>k_0</td>
<td>4.58 x 10^6 ± 2.46 x 10^-7 pm^3 min^-1 (1%)</td>
</tr>
<tr>
<td>K_D</td>
<td>171.13 ± 18.43 pm (11%)</td>
</tr>
<tr>
<td>internalization</td>
<td>0.162 ± 0.64 x 10^-6 min^-1 (1%)</td>
</tr>
<tr>
<td>dissociation</td>
<td>0.432 ± 0.05653 min^-1 (1%)</td>
</tr>
<tr>
<td>EpoR_recycling</td>
<td>0.0487 ± 2.67 x 10^-6 min^-1 (1%)</td>
</tr>
<tr>
<td>Epo_recycling</td>
<td>10.264 ± 0.0840 min^-1 (1%)</td>
</tr>
<tr>
<td>release</td>
<td>0.0103 ± 1.26 x 10^-6 min^-1 (1%)</td>
</tr>
<tr>
<td>Epo</td>
<td>2098.5 ± 0.0437 pm (0%)</td>
</tr>
<tr>
<td>k_0, etop</td>
<td>2.47 x 10^6 ± 1.47 x 10^-6 pm^3 min^-1 (1%)</td>
</tr>
<tr>
<td>K_D, etop</td>
<td>1810.9 ± 131.5 pm (7%)</td>
</tr>
<tr>
<td>etop_recycling</td>
<td>0.165 ± 0.0205 min^-1 (18%)</td>
</tr>
<tr>
<td>etop_degradation</td>
<td>0.0096 ± 0.0234 min^-1 (24%)</td>
</tr>
<tr>
<td>etop</td>
<td>0.0004 ± 0.00025 pm (0%)</td>
</tr>
</tbody>
</table>

Values (median = 1)
6.7 Curriculum Vitae

Verena Becker

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69120 Heidelberg
Germany
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e-mail: v.becker@dkfz-heidelberg.de

date of birth: August 26, 1977
place of birth: Lennestadt, Germany
nationality: German

Primary & Secondary School

1984 – 1988 Primary school, Schmallenberg, Germany
1988 – 1997 Secondary school “Maria Königin”, Lennestadt, Germany
June 1997 Final school examination

University

October 1997 – December 2002 Studies in biology (diploma) at the Albert-Ludwigs University of Freiburg
September 1999 Intermediate exams
May 2001 Diploma exam in neuropathology
November 2001 Diploma exams in molecular immunology, developmental biology, and biochemistry
December 2001 – December 2002 Diploma thesis at the Max-Planck Institute of Immunobiology, Freiburg, Germany
“Die Rolle der Transmembrandomäne für die Signalleitung durch den Erythropoetin-Rezeptor”
Supervisor: PD Dr. U. Klingmüller
December 2002 Diploma in biology
## PhD Thesis

<table>
<thead>
<tr>
<th>Period</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>March 2003 – June 2003</td>
<td>PhD thesis at the Max-Planck Institute of Immunobiology, Freiburg, Germany</td>
</tr>
<tr>
<td>July 2003 – March 2007</td>
<td>PhD thesis at the German Cancer Research Center, Heidelberg, Germany</td>
</tr>
<tr>
<td></td>
<td>“Signaling through the Erythropoietin Receptor is Promoted by Dense Packing of the Transmembrane Domain and Regulated by Rapid Receptor Internalization”</td>
</tr>
<tr>
<td></td>
<td>Supervisor: PD Dr. U. Klingmüller</td>
</tr>
<tr>
<td></td>
<td>Participation in the International PhD Study Program of the German Cancer Research Center</td>
</tr>
</tbody>
</table>

## Employments

<table>
<thead>
<tr>
<th>Period</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 2000 – April 2000</td>
<td>Research assistant in the group of Prof. Dr. W. Driever, Department of Developmental Biology, University of Freiburg, Germany</td>
</tr>
<tr>
<td>April 2000 – July 2000</td>
<td>Student assistant with teaching duties at the Institute of Zoology, University of Freiburg, Germany</td>
</tr>
<tr>
<td>April 2001 – November 2001</td>
<td>Research assistant in the group of PD Dr. U Klingmüller, Max-Planck Institute of Immunobiology, Freiburg, Germany</td>
</tr>
</tbody>
</table>

## Teaching Experiences

<table>
<thead>
<tr>
<th>Period</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 18 – 22, 2005</td>
<td>Assistant for practical course “Components and Mechanisms of Signal Transduction” (HP-F05; Master of Cell Biology, University of Heidelberg)</td>
</tr>
<tr>
<td>May 1 – 5, 2006</td>
<td>Assistant for practical course “Components and Mechanisms of Signal Transduction” (HP-F06; Master of Cell Biology, University of Heidelberg)</td>
</tr>
</tbody>
</table>

## Scientific Meetings

<table>
<thead>
<tr>
<th>Period</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 2002</td>
<td>EMBL/Salk/EMBO Conference on Oncogenes and Growth Control - Signaling and Cancer; Heidelberg, Germany; poster presentation</td>
</tr>
<tr>
<td>November 2002</td>
<td>Signal Transduction – Receptors, Mediators and Genes (STS); Weimar, Germany; poster presentation</td>
</tr>
<tr>
<td>March 2005</td>
<td>DGZ Annual Meeting; Heidelberg, Germany; poster presentation</td>
</tr>
</tbody>
</table>
May 2005  
CSHL Conference on Protein Phosphorylation & Cell Signaling; Cold Spring Harbor, USA; short talk

June 2006  
SBMC Conference on Systems Biology of Mammalian Cells; Heidelberg, Germany; poster presentation

Publications


6.8 Erklärung


Verena Becker