Intracellular Trafficking of G-CSF Receptor;

Mechanisms and Implications for Signaling Function

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Intracellulaire Trafficking van G-CSF Receptor;

Mechanismen en Implicaties voor Signaal Functie

Proefschrift

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Excellence can be obtained if you:

Care more than others think is wise;
Risk more than others think is safe;
Dream more than others think is practical;
Expect more than others think is possible.

To my family

Contents

CHAPTER 1

General introduction

1. HEMATOPOIESIS

Hematopoiesis is the formation and development of blood cells. This process takes place in humans prenatally in the embryonic yolk sac in the first 3 to 6 weeks after gestation (Dzierzak et al., 1998; Palis and Yoder, 2001). By the third month of development, the fetal liver is the predominant hematopoietic organ and after birth, the bone marrow becomes the major site of hematopoiesis. All blood cells develop from hematopoietic stem cells (HSCs). An HSC has the ability to undergo asymmetric cell division, producing one identical stem cell daughter cell (self renewal) and one progenitor cell that has the potential to give rise to progeny of all blood cell lineages (pluripotency). The direct descendants of the pluripotent progenitors are the lineage restricted or committed progenitor cells, which proliferate and differentiate into distinct mature blood cells, i.e., granulocytes (neutrophils, eosinophils and basophils), lymphocytes, erythrocytes, monocytes (macrophages), natural killer cells, mast cells, platelets and dendritic cells (Figure 1).

Hematopoiesis is tightly controlled by hematopoietic growth factors (HGFs), which function through the activation of specific hematopoietic receptors. Interaction between a growth factor and its receptor promotes signal transmission and activates interacellular signaling cascades leading to cell proliferation and survival, while certain HGFs in addition control

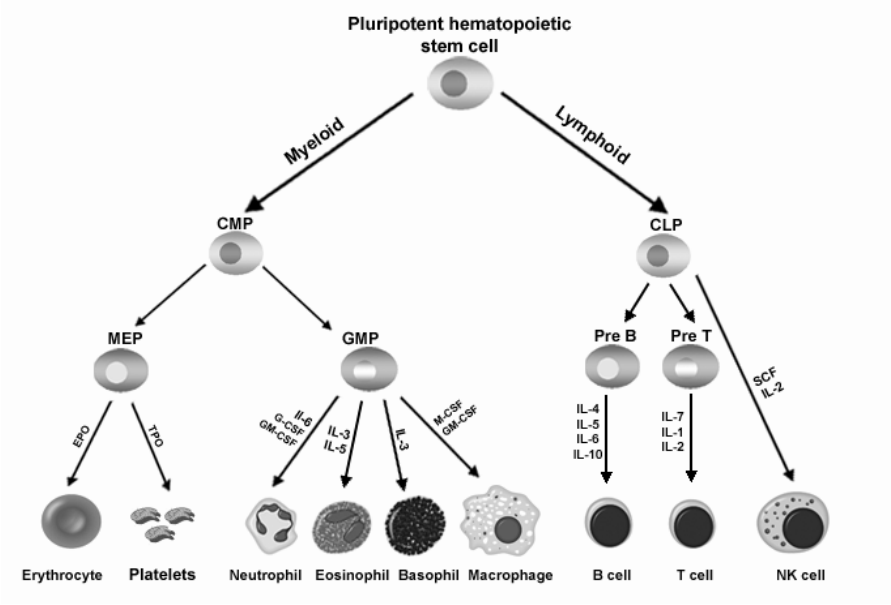

Figure 1. Schematic representation of the hematopoietic system

Pluripotent hematopoietic stem cells give rise to myeloid or lymphoid progenitor cells which develop into distinct mature blood cells. This process is regulated by various cytokines of which the major factors directing the last step of maturation are depicted. CMP: common myeloid progenitor; CLP: common lymphoid progenitor; MEP: megakaryocyte erythroid progenitor; GMP: granulocyte macrophage progenitor.

cell differentiation and function. HGFs are glycoproteins that can be produced by multiple cell types, for instance fibroblasts, lymphocytes, monocytes/macrophages, stromal and endothelial cells. Where the different HGFs act in the process of blood cell development is shown in Figure 1. Major HGFs are erythropoietin (Epo) driving erythropoiesis, the colony-stimulating factors (CSFs) involved in myeloid cell development, various interleukins (IL-1 to IL-13), the megakaryocytic growth factor thrombopoietin (TPO), and stem-cell factor (SCF) and FLT3 ligand which are growth factors for pluripotent progenitor cells. The CSFs include granulocyte-CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), multipotential CSF (multi-CSF, also known as IL-3), and monocyte/macrophage CSF (M-CSF, also known as CSF-1).

Most of HGFs support the proliferation of early progenitors and have limited ability to induce differentiation. G-CSF, however, regulates both proliferation and differentiation of myeloid progenitors to neutrophils, which are crucial blood cells for host defense system against infections.

2. G-CSF AND ITS RECEPTOR

G-CSF is a member of the cytokine class I superfamily. The *G-CSF* gene (now referred to as *CSF3*) is located on human chromosome 17 at position q11-22 (Kanda et al., 1987). G-CSF is produced by a variety of cells, of which monocytes and macrophages are the most prominent examples (Demetri and Griffin, 1991). G-CSF plays crucial roles in myeloid progenitor cell proliferation, survival and maturation in steady state conditions as well as during stages of bacterial infections, when neutrophil levels need to be increased ("emergency" granulopoiesis). Increased production and release of G-CSF is observed following stimulation with bacterial lipopolyssacharide (Kolb-Maurer et al., 2004; Lieschke et al., 1994) and some cytokines such as TNF (Koeffler et al., 1987), IL-1 (Fibbe et al., 1988; Zsebo et al., 1988) and IL-3 (Oster et al., 1989). G-CSF-deficient mice (c*sf3*-/-) are viable, fertile, and superficially healthy, but have a chronic neutropenia and are unable to control bacterial infection such as Listeria monocytogenes, supporting the essential role of G-CSF in "emergency" situations (Lieschke et al., 1994).

Recombinant G-CSF treatment has been used clinically to reduce the duration of neutropenia and infection risk following chemotherapy. More importantly, G-CSF treatment restores neutrophil production in patients with severe forms of chronic or congenital neutropenia, thereby reducing the risk of severe recurrent infections and related mortality (Dale et al., 1993; Welte and Boxer, 1997). Finally, because G-CSF administration to stem cell donors results in mobilization of the HSCs into the peripheral blood, it is now routinely used in

hematopoietic stem cell harvest protocols. Since its introduction about 17 years ago, G-CSF has become one of the most successful recombinant proteins in clinical use.

The biological activities of G-CSF are mediated via the G-CSF receptor (G-CSFR), a member of the hematopoietin receptor superfamily. G-CSF binds to its receptor with high affinity (Kd: 100-500pM). Human G-CSFR (CD114, also known as CSF3R) is encoded by a single gene (*CSF3R*) located on the short arm of chromosome 1. Murine and human G-CSFR proteins are single transmembrane polypeptides of 812 and 813 amino acids (130kDa), respectively, with an overall homology of 72.5% at the nucleotide level and 62.5% at the amino acid level (Fukunaga et al., 1990). The extracellular portion of the receptor has 603 amino acids and is composed of an immunoglobulin-like (Ig-like) domain, a cytokine receptor homology (CRH) domain with four conserved cysteine residues and a WSXWS motif that is essential for ligand binding, and three fibronectin type III (FN III) modules (Anaguchi et al., 1995; Fukunaga et al., 1991). The cytoplasmic domain of G-CSFR contains regions with limited structural similarity with other hematopoietic receptors termed box1, box2 and box3. The membrane proximal region that contains box1 and box2 is involved in transduction of proliferation signals. The membrane distal domain that contains box3 is essential for ligand-induced differentiation signals of myeloid progenitor cell lines and is implicated in the transduction of phagocytic signals in mature neutrophils (Dong et al., 1993; Fukunaga et al., 1993; Santini et al., 2003).

In the hematopoietic system, G-CSFR is primarily expressed in neutrophil progenitors and mature neutrophils. The average receptor numbers per cell increase with maturation of the myeloid cells, with the highest numbers being expressed on peripheral neutrophils. However, G-CSFR can also be found on monocytes, myeloid and lymphoid leukemia cells, normal B and T lymphocytes and various non-hematopoietic cells and tissues, including endothelial cells, placenta and fetal organ tissues (Boneberg et al., 2000; Budel et al., 1989; Corcione et al., 1996; Hanazono et al., 1990; Morikawa et al., 2002; Shimoda et al., 1993). Interestingly, recent studies have indicated that G-CSFR is upregulated on cardiomyocytes and neuronal cells after ischemic heart failure and stroke, respectively, and that G-CSF treatment in these conditions may be of clinical benefit (Harada et al., 2005; Li et al., 2006; Takano et al., 2003; Takano et al., 2006). G-CSFR deficient mice (*csf3r*-/-) show severe reduction in neutrophil levels which is approximately 15% of wild type (WT) littermates. Furthermore, myeloid progenitor cells are significantly decreased in the bone marrow of these mice (Liu et al., 1996).

3. G-CSF SIGNALING

Similar to other members of the hematopoietin receptor superfamily, G-CSFR lacks intrinsic kinase activity and recruits members of the Janus tyrosine kinase (JAK) family to phosphorylate tyrosine (Y) residues residing in its cytoplasmic domain. Human G-CSFR contains four conserved tyrosines located at positions 704, 729, 744, and 764.

Activation of the G-CSFR results in the activation of three members of the JAK family, JAK1, JAK2 and Tyk2 and of members of the Src kinase family (Nicholson et al., 1995; Nicholson et al., 1994; Shimoda et al., 1997; Shimoda et al., 1994; Ward et al., 1998). JAKs have seven defined regions of homology (JH1-JH7), among which JH1 and JH2 comprise the kinase and pseudokinase domains, respectively (Wilks et al., 1991; Ziemiecki et al., 1994). The N-terminal FERM domain (JH4-JH7) is necessary for binding to box1 and box2 regions of cytokine receptors (He et al., 2003; Hilkens et al., 2001; Zhou et al., 2001). Although the exact nature of JAK association with receptor remains to be resolved (Murray, 2007), it has been shown that tryptophan 650 (W650) located in the G-CSFR membrane-proximal region is necessary for JAK2 activation and proliferation signals (Barge et al., 1996).

Upon activation by G-CSF, G-CSFR is phosphorylated at four conserved tyrosines that serve as docking sites for SH2 domain-containing signaling proteins such as signal transducer and activator of transcription 3 (STAT3) and a variety of other down stream signaling proteins (Figure 2). Mutation of these tyrosines to phenylalanine revealed that they are differentially involved in growth regulatory functions. For instance, while tyrosine 764 (Y764) played an important role in promoting colony growth, Y729 showed inhibitory effects (Hermans et al., 2003).

Among different members of the STAT protein family, STAT1, STAT3 and STAT5 are activated by the G-CSFR. Binding of STATs to an activated receptor complex brings them in proximity of the JAKs, leading to their tyrosine phosphorylation, homo- or hetero-dimerization and translocation to the nucleus for binding to target gene promoters. Whereas activation of STAT1 and STAT5 requires the membrane-proximal region of G-CSFR, STAT3 activation depends on its recruitment to Y704 and Y744 of receptor (Chakraborty et al., 1999; Ward et al., 1999c). However, it was shown that tyrosine null G-CSFR can still activate STAT3 at high concentrations of G-CSF, suggesting that STAT3 activation by G-CSF in steady state versus emergency granulopoiesis is differentially regulated. (Akbarzadeh et al., 2002; Nicholson et al., 1996; Ward et al., 1999a)

Introduction of dominant negative forms of STAT3 interferes with growth arrest and neutrophil differentiation of 32D cells (de Koning et al., 2000; Shimozaki et al., 1997). Expression of G-CSFR mutant Y704F in mouse models revealed that STAT3 contributes in transduction of both proliferation and differentiation signals (McLemore et al., 2001). However, mice lacking STAT3 in their hematopoietic progenitors showed neutrophilia (Lee et al., 2002),

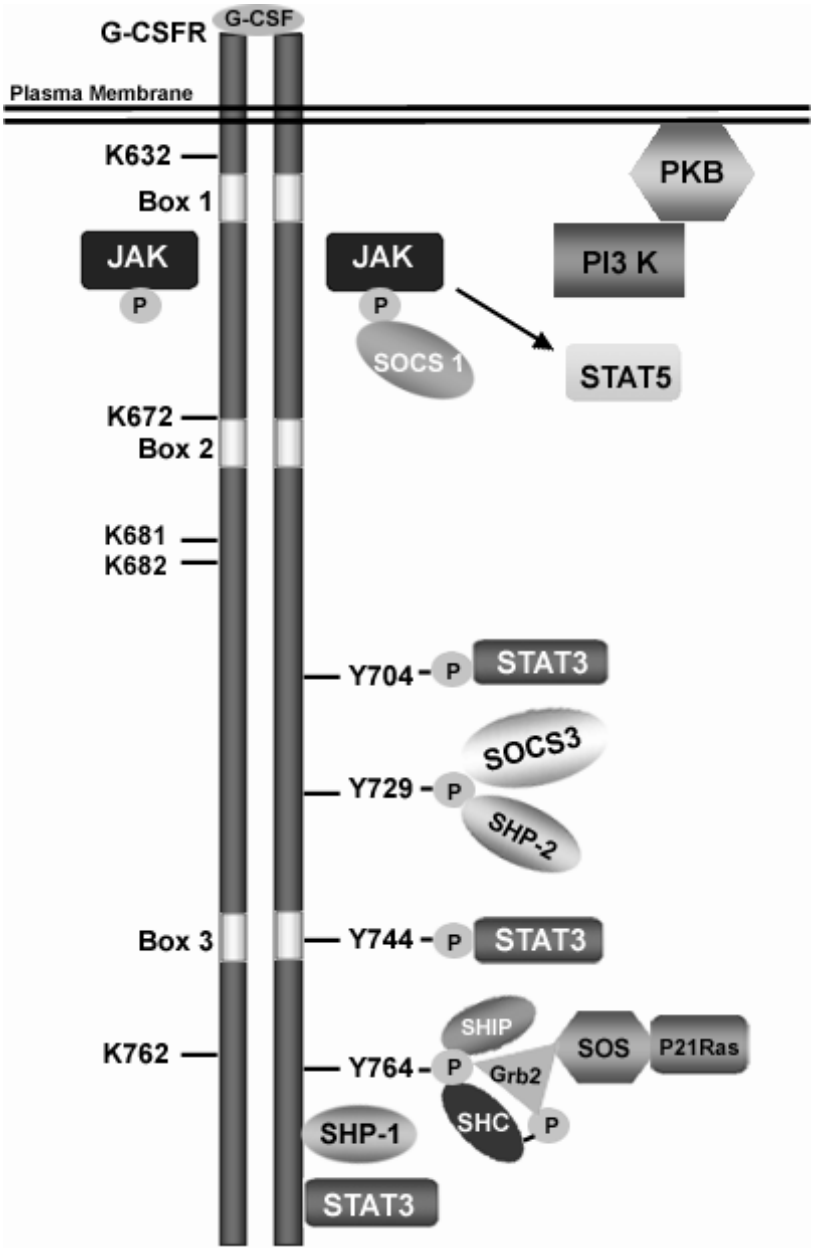

Figure 2. Signaling pathways coupled to G-CSFR

Upon G-CSF stimulation tyrosines located at cytoplasmic domain of G-CSFR become phosphorylated By JAKs and serve as docking sites for various signaling molecules. Activation of different signaling pathways leads to positive or negative regulation of G-CSFR. See main text for functional implications.

indicating that, *in vivo*, STAT3 is required for maintaining an appropriately balanced neutrophil production but is not essential for differentiation. The negative role of STAT3 on the

proliferation of myeloid progenitor cells has been attributed to two mechanisms: upregulation of cyclin-dependent kinase inhibitor $p27^{Kip1}$ (de Koning et al., 2000) and induction of the suppressor of cytokine signaling (SOCS) protein, SOCS3 (Lee et al., 2002; van de Geijn et al., 2004a; van de Geijn et al., 2004b).

Unlike STAT3, activation of STAT5 occurs independently of phosphorylated tyrosines of G-CSFR via binding to activated JAK kinases (Fujitani et al., 1997). However maximal activation needs an additional domain encompassing Y704. STAT5 promotes proliferation and survival in response to G-CSF (Dong et al., 1998). In agreement with this, bone marrow cells from mice lacking both STAT5A and STAT5B, the two isoforms of STAT5 showed reduced response to G-CSF (Teglund et al., 1998).

Similar to most other hematopoietin receptors, the G-CSFR activates p21Ras/Raf/MEK/MAPK and phosphatidylinositol 3-kinase (PI3-kinase) /protein kinase B (PKB) signaling pathways. These pathways transmit signals that contribute to proliferation and survival (de Koning et al., 1998; Dong and Larner, 2000; Hunter and Avalos, 1998). Tyrosine 764 of G-CSFR plays a prominent role in the activation of p21Ras and MAPK pathway owing to the fact that this tyrosine forms a unique docking site for the adaptor protein Shc (Akbarzadeh et al., 2002; de Koning et al., 1998; Hermans et al., 2003; Ward et al., 1999b) (Figure 2).

4. SIGNALING DEFECTS CAUSED BY C-TERMINAL TRUNCATIONS OF THE G-CSFR FOUND IN SEVERE CONGENITAL NEUTROPENIA PATIENTS

A number of independent mutations in the gene encoding the G-CSFR have been described in myeloid diseases, including severe congenital neutropenia (SCN). The most frequent mutations in SCN result in the introduction of stop codons in the G-CSFR C-terminal region, the region implicated in induction of maturation and growth arrest. Expression of these truncated forms of G-CSFR in SCN patients is associated with an increased risk of developing acute myeloid leukemia (AML) (Dong et al., 1995; Dong et al., 1997; Freedman and Alter, 2002). Introduction of an equivalent mutation (Δ715 G-CSFR) in mice results in hyperproliferative phenotype in response to G-CSF (Hermans 1998, McLemore 1998). Several signaling abnormalities have been linked to Δ715 G-CSFR. In cells expressing this mutant, activation of PI3K/PKB pathway is increased and STAT5 activity is significantly prolonged compared to WT G-CSFR. Moreover, endocytosis and signal attenuation are disturbed in cells expressing Δ715 G-CSFR, due to loss of internalization motifs present in the C-terminal region and the lack of recruitment sites for negative regulators of signaling such as SHIP1, SHP-1 and SOCS3(see below). Recently, is was shown that the Δ715 G-CSFR confers a strong proliferative advantage to pluripotent hematopoietic stem cells in vivo and that STAT5 is essential for

the clonal dominance of mutant stem cells over their normal counterparts.. Strikingly, this effect depended strongly on exogenous G-CSF (Liu et al., 2008).

5. NEGATIVE CONTROL OF G-CSF SIGNALING

Activation of growth factor-induced signaling cascades requires specific mechanisms to antagonize them, to ensure that signals do not exceed a critical threshold. These mechanisms of negative signaling have been shown to be crucial for normal development (Dikic and Giordano, 2003; Greenhalgh and Hilton, 2001). Negative regulation of G-CSF signaling is governed by multiple mechanisms involving protein tyrosine and inositol lipid phosphatases, suppressor of cytokine signaling (SOCS) proteins, endocytosis and lysosomal degradation of signaling complexes. The action of these mechanisms and their contribution to the down-regulation of G-CSF signaling will be briefly introduced below.

5.1. Protein tyrosine phosphatases SHP-1 and SHP-2

The Src homology 2 (SH2) domain-containing protein tyrosine phosphatase SHP-1 contains two SH2 domains and is predominantly expressed in hematopoietic cells. Mice with a loss-of-function mutation in the *shp1* gene ("moth-eaten") suffer from myeloid and lymphoid abnormalities, supporting its role in regulation of proliferation and differentiation of hematopoietic cells. SHP-1 has been shown to dephosphorylate both the activated cytokine receptors and the JAKs bound to these receptors (Valentino and Pierre, 2006) and negative regulation of signaling by SHP-1 has been demonstrated for several receptors, including the EpoR (Klingmuller et al., 1995) and G-CSFR (Ward et al., 2000). SHP-1 protein levels are up-regulated during G-CSF-mediated granulocytic differentiation of myeloid 32D cells. Enforced expression of SHP-1 in these cells resulted in inhibition of proliferation and acceleration of differentiation, while introduction of a phosphatase-dead mutant resulted in a delay of differentiation. In contrast to other cytokine receptors such as EpoR, recruitment of SHP-1 to the G-CSFR does not depend on phosphorylated tyrosines within the receptor C-terminus (Dong et al., 2001; Tapley et al., 1997; Ward et al., 2000).

Another member of SH2 domain-containing protein tyrosine phosphatase family, SHP-2, is also recruited to G-CSFR. Tyrosine 729 as well as the distal C-terminal region of receptor serves as binding motifs for this association (van de Geijn et al., 2004a). SHP-2 has been suggested to play a negative role in G-CSF signaling by dephosphorylation of STAT5 molecules (Yu et al., 2000). However SHP-2 also plays a positive role in signaling by promoting p21Ras activation (Shi et al., 2000).

5.2. SH2 domain-containing inositol phosphatase SHIP1

For several cytokine receptors including G-CSFR, SHIP1 has been suggested to negatively regulate proliferative signaling (Chacko et al., 1996; Damen et al., 1996; Ono et al., 1996). SHIP1 is an 5′-phosphatidylinositol (PtdIns) phosphatase that dephosphorylates PtdIns(3,4,5) P3, the active PI-3kinase metabolite and results in production of PtdIns(4,5)P2 lipids in the cell. PIP3 then initiates a cascade of downstream signaling pathways by interacting with pleckstrin homology (PH) domain-containing proteins, such as protein kinase B. Therefore, SHIP1 and PI-3kinase control PtdIns(3,4,5)P3 levels in the cells.

The distal C-terminal region of the G-CSFR mediates phosphorylation of SHIP1 and the formation of Shc/SHIP complexes (Hunter and Avalos, 1998). Mutation of tyrosine 764 to phenylalanine of the G-CSFR (Y764 G-CSFR) showed requirement of this tyrosine for in vivo phosphorylation of SHIP and SHIP/Shc complex formation (Dong et al., 1998; Hunter et al., 2004).

Although expression of SHIP1 is restricted to cells of hematopoietic origin, it appears that its function in myeloid linage differs from its role in lymphoid cells (Gloire et al., 2007). Whereas *SHIP1 -/-* mice are lymphopenic, they show an increased number of neutrophils

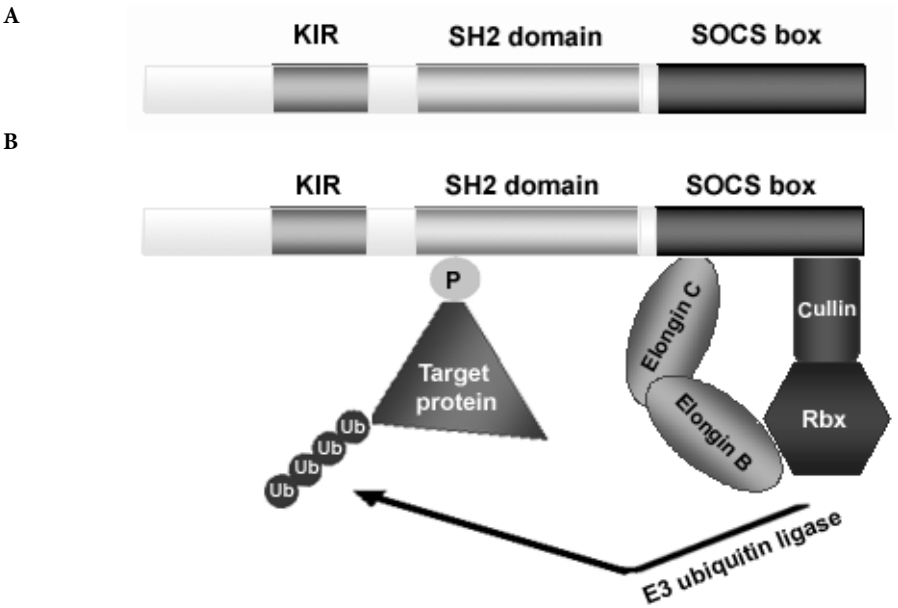

Figure 3. Schematic structure and E3 ubiquitin ligase activity of SOCS3

(A) Domain structure of SOCS3, a member of SOCS protein family. KIR: kinase inhibitory region. (B) Interaction of SH2 domain of SOCS proteins with phosphorylated tyrosine of target protein recruits members of ECS complex. This complex then functions as an E3 ubiquitin ligase in a concerted action with E1 and E2 enzymes to ubiquitinate the target protein; Ub: ubiquitin molecule.

and monocytes/macrophages in the bone marrow (Helgason et al., 1998; Liu et al., 1999; Nakamura et al., 2004). Furthermore, these mice show deficiency in controlling aggregation of platelets and in hemostasis (Giuriato et al., 2003; Severin et al., 2007).

5.3. The family of SOCS (suppressor of cytokine signaling) box-containing proteins

The SOCS protein family comprises of eight members, termed cytokine inducible SH2-containing protein (CIS) and SOCS 1 to 7 (Hilton et al., 1998; Starr et al., 1997; Yoshimura et al., 2005). All SOCS proteins contain a central SH2 domain which binds to a phosphorylated tyrosine-containing motif of target protein and a C-terminal conserved SOCS box domain consisting of approximately 40 amino acids (Figure 3A). SOCS1 and SOCS3 have an extra domain in common, the kinase inhibitory region (KIR), which downregulates JAK activity (Fujimoto and Naka, 2003; Kile et al., 2002). SOCS proteins are characterized by a variable N-terminal domain, an SH2 phosphotyrosine-interacting domain and the C-terminal SOCS box motif. Subsequent screening of DNA and protein databases revealed that at least 40 additional proteins contain a SOCS box. Instead of an SH2 domain, most of these proteins posses an alternative protein interaction domain such as WD40 repeats (Wsb proteins), ankyrin-repeats (ASB proteins) or a SPRY domain (SSB proteins) (Hilton et al., 1998; Kile et al., 2002). There are several different proteins with divergent SOCS boxes such as MUF1, elongin A and the von Hippel-Lindau (VHL) tumor suppressor protein (Aso et al., 1995; Kamura et al., 2001; Kibel et al., 1995).

The N-terminal domain of the SOCS box motif is constitutively bound to the Elongin B/C complex (Kamura et al., 1998), whereas the C-terminal region recruits members of the Cullin family (Cullin2 or Cullin5) and RING finger proteins Rbx1 or Rbx2 (Kamizono et al., 2001; Kamura et al., 2004; Maine et al., 2007; Ungureanu et al., 2002). The ECS (Elongin-Cullin-SOCS box) complex functions as an E3 ubiquitin ligase and in a concerted action with E1 (ubiquitin activating) and E2 (ubiquitin conjugating) enzymes, add ubiquitin molecules to target proteins. The ubiquitinated target is subsequently degraded in lysosomes or proteasomes (Figure 3B).

Among the various SOCS deficient mouse strains that have been generated, SOCS1 and SOCS3 deficient mice showed the most striking phenotypes. SOCS1 deficient mice are healthy at birth but die before weaning due to fatty degeneration of the liver, monocytic infiltration of organs and significant loss of lymphocytes (Naka et al., 1998; Starr et al., 1998). These mice exhibit excessive responses induced especially by interferon gamma (Alexander et al., 1999). SOCS3 deficient mice are embryonic lethal due to deregulated leukemia inhibitory factor (LIF) and cardiotrophin-1 signaling (Robb et al., 2005; Roberts et al., 2001; Takahashi et al., 2003). However there is a discrepancy between this study and an earlier study done by Marine et al. who suggested that deletion of *socs3* results in lethality due to massive erythrocytosis (Marine et al., 1999).

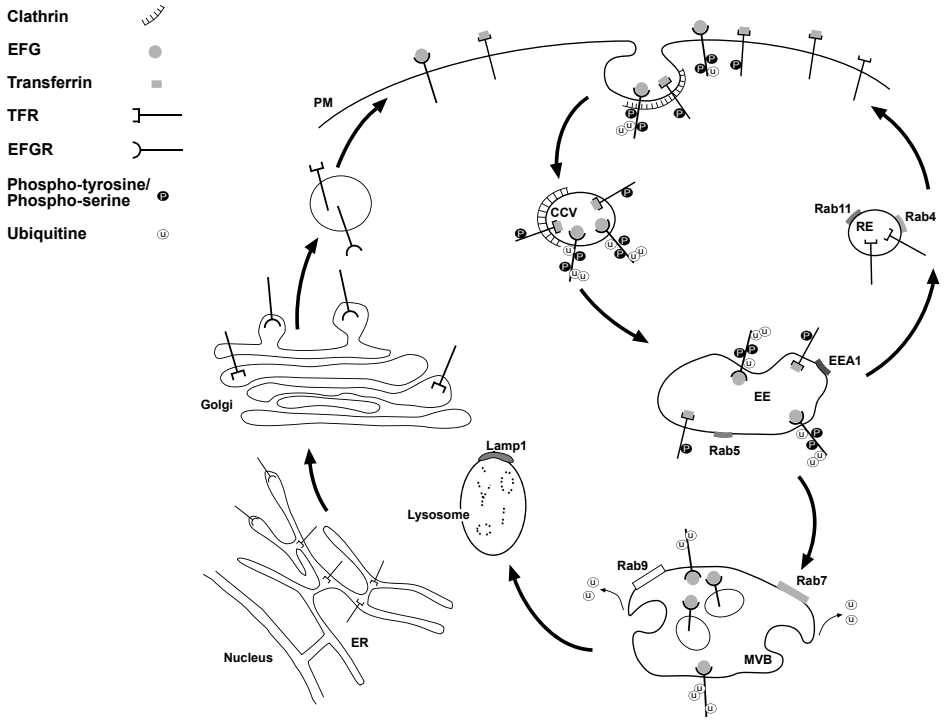

Figure 4. A schematic representation of receptor endocytosis

EGFR is representative of a receptor which is ubiquitinated and routs to lysosomes, whereas TFR is not ubiquitinated and recycles to plasma membrane. See main text for a detailed explanation. PM: plasma membrane; CCV: clathrin-coated vesicle; EE: early endosome; MVB: multivesicular body; RE: recycling endosome; EGFR: epidermal growth factor receptor; TFR: transferrin receptor.

5.3.1. SOCS proteins and their role in G-CSF signaling

Expression of SOCS mRNAs induced in response to cytokine stimulation is under transcriptional control of STATs. Both SOCS1 and SOCS3 are able to play a role in attenuation of G-CSF signaling, however SOCS3 is the most prominent physiological inhibitor. Expression of SOCS3 is robustly up-regulated in myeloid cells by G-CSF, while expression of SOCS1 is induced at much lower levels (Hortner et al., 2002; Starr et al., 1997; van de Geijn et al., 2004b). SOCS3 expression is severely reduced in STAT3 deficient mice treated with G-CSF, supporting the notion that SOCS3 is the major STAT3 target gene for negative regulation of G-CSF signaling (Lee et al., 2002).

Several mechanisms have been postulated to explain the inhibitory effects of SOCS proteins on cytokine signaling. The first mechanism is binding of SOCS SH2 domain to phosphorylated tyrosine of a cytokine receptor, and therefore competes with STAT recruitment or other positively acting signaling molecules for receptor tyrosine docking (Ram and Waxman,

2000; Yoshimura et al., 1995). The second mechanism is inhibition of JAK kinase activity via KIR; the mechanism only used by SOCS1 and SOCS3 proteins (Giordanetto and Kroemer, 2003; Nicholson et al., 1999; Yasukawa et al., 2000). Whereas the SH2 domain of SOCS1 has high affinity for JAK proteins, the SH2 domain of SOCS3 has a lower affinity for JAKs but a high affinity for phosphorylated tyrosines in receptor (Y729 in the case of G-CSFR) (Nicholson et al., 2000). The third, already discussed mechanism of inhibition of signaling by SOCS proteins involves the SOCS box, required for the formation of an ECS complex that drives degradation of target proteins. The SOCS box of SOCS1 and SOCS3 is important for G-CSF-induced inhibition of STAT5 activation and to a lesser extent for inhibition of STAT3 activation (van de Geijn et al., 2004b), highlighting a dominant inhibitory mechanism by targeting for ubiquitination rather than abrogation of JAK activity. Interestingly, it was shown recently that mice expressing a truncated SOCS3 protein lacking the C-terminal SOCS box had normal steady state hematopoiesis; however these mice are hypersensitive to G-CSF treatment leading to significantly elevated neutrophil levels when compared with wild type littermates. These findings underscore the role of the SOCS3 SOCS box for G-CSF-induced emergency granulopoiesis (Boyle et al., 2007).

5.4. Endocytosis and intracellular trafficking to degradative compartments

Intracellular routing of activated receptors plays a major role in signal attenuation. In the past decade major progress has been made in understanding the molecular mechanisms of endocytotic membrane trafficking and the influence of endocytosis on receptor signaling and vise versa (von Zastrow and Sorkin, 2007). Receptor endocytosis classically involves internalization from the plasma membrane, trafficking to early endosomes, late endosomes, multivesicular bodies (MVBs) and finally to lysosomes (Figure 4).

5.4.1. Endocytosis

Receptor endocytosis may take place by two distinct routes. Clathrin-mediated internalization involving formation of vesicles coated with clathrin is currently the best characterized mechanism and has been shown for several receptors such as G-CSFR, growth hormone receptor (GHR) and interleukin-2 receptor (IL-2R). The basic components for this type of internalization include the hetero-tetrameric adaptor complex (AP2) (Kirchhausen et al., 1997), endophilin (Guichet et al., 2002; Schmidt et al., 1999), dynamin (Marks et al., 2001; McNiven, 1998; Wang et al., 1995) and EPS15 (Fallon et al., 2006; Torrisi et al., 1999) which in turn can bind other proteins such as Epsin and Numb (Horvath et al., 2007; Iannolo et al., 1997; Santolini et al., 2000).

The second internalization route involves caveolae. These flask-like structures are enriched in cholesterol and glycosphingolipid and bud from the plasma membrane. Interestingly, in case of the transforming growth factor (TGF)-β receptor both clathrin- and caveolae-dependent receptor endocytosis was demonstrated and it was suggested that caveolae-mediated

internalization is required for receptor turnover,while clathrin-mediated endocytosis promotes TGF-β receptor signaling (Di Guglielmo et al., 2003).

Three basic classes of internalization codes have been described for receptors: tyrosine-based codes including NPXY (X representing any amino acid) and YXXφ (where φ is a hydrophobic amino acid) (Guarnieri et al., 1993; Heilker et al., 1999), dileucine motifs and lysine residues. Beside these internalization-sorting signals, conjugated ubiquitin and ubiquitination can also function as a signal for internalization as was shown for EGF receptor and IL-2R alpha chain (Haglund et al., 2003a; Hicke, 2001; Nakatsu et al., 2000; Shih et al., 2000).

Receptor internalization can either occur in response to ligand stimulation or constitutively. Whereas the internalization kinetics of ligand-stimulated receptor is rapid (within minutes), constitutive internalization occurs at a slow rate (within hours). Both ligand-induced and constitutive internalization of the G-CSFR depend on an amino acid stretch in the COOH terminus (a.a. 749-769), that contains a dileucine motif. The presence of a phosphorylated Ser749 at position -4 of the dileucine motif and a tryptophane residue critical for binding and activation of JAKs (Trp650) are essential for ligand-activated but not constitutive internalization of the G-CSFR (Aarts et al., 2004).

5.4.2. Post endocytotic trafficking into early endosomes

Following internalization, clathrin coated vesicles embracing receptors disassociate from the plasma membrane and fuse with early endosomes. One essential protein that has been implicated in fusion of endocytotic vesicles with early endosomes is the small GTPase Rab5 (Zerial and McBride, 2001) which in turn recruits early endosomal antigen-1 (EEA-1) (Christoforidis et al., 1999; Mills et al., 1998; Simonsen et al., 1998). EEA-1 localizes to the cytoplasmic face of the early endosome membrane by its cysteine-rich motif, termed the FYVE domain, which interacts with phosphatidylinositol-3-phosphate (Patki et al., 1997; Weissman, 2001). Notably, Rab5 and EEA1 proteins are frequently used as markers for early endosomes in confocal microscopy studies, including those reported in this thesis.

Early endosomes are considered as sorting endosomes, where decisions for destination of the receptor are being made. Ubiquitination of cytoplasmic lysines of receptors serves as a sorting signal at this stage for targeting them to late endosomes/ lysosomes or proteasomes, therefore ubiquitination of lysine residues plays a pivotal role in receptor turnover. Conversely, recycling back to the plasma membrane is correlated with the absence of receptor ubiquitination and loss of ubiquitination enhances receptor recycling (Levkowitz et al., 1998). Several members of the cytokine receptor family, like growth hormone receptor (GHR) (van Kerkhof and Strous, 2001), leptin receptor (LR) (Uotani et al., 1999) and gp130 (Blanchard et al., 2000; Dittrich et al., 1996) follow the endosomal/lysosomal pathway for degradation after ubiquitination. On the other hand, the thrombopoietin receptor (TpoR) does not route to lysosomes

and is recycled to the plasma membrane after endocytosis (Dahlen et al., 2003). For the EpoR, it has been shown that most of its intracellular domain is degraded after ubiquitination by the proteasomal pathway before receptor internalization, whereas the remaining part of the EpoR is internalized and routed to lysosomes for degradation (Walrafen et al., 2005).

The cytoplasmic domain of the human G-CSFR contains five conserved lysines, located at positions 681, 682 and 762. These lysines are candidates of ubiquitination and may play a potential role in intracellular trafficking of the G-CSFR.

How activated G-CSFR complexes are degraded following endocytosis and which motifs in the cytoplasmic domain of the receptor are involved in this process has not been investigated. In Chapter 2 we studied intracellular routing of the activated G-CSFR and its effect on G-CSF-induced signaling following receptor internalization. To examine the differential role of cytoplasmic lysines of the G-CSFR in sorting to degradation compartments, we made use of single lysine and lysine add back mutants of G-CSFR.

5.4.3. Different forms of ubiquitination and their role in receptor routing and degradation

Ubiquitin (Ub) is an 8.6 kDa polypeptide that is highly conserved among different species and which forms a covalent attachment to a lysine residue of the substrate by its C-terminal glycine. Conjugation of a single ubiquitin to one lysine (K) of a target substrate is referred to as monoubiquitination. Multiubiquitination is the result of attachment of single ubiquitin molecules to multiple lysine residues of the substrate. Many studies have shown that endocytosis and MVB sorting of plasma membrane receptors involves either mono-or muli ubiquitination. Ubiquitin contains seven lysines residues, which in turn can bind to ubiquitin, giving rise to polyubiquitination (Pickart, 2001). Although it was initially thought that polyubiquitination exclusively targets proteins for degradation in proteasomes, more recent studies indicate that this holds true only for polyubiquitin chains formed via K48 of ubiquitin. In contrast, polyubiquitin chains made through K29 or K63 appear to be involved in lysosomal targeting (Chastagner et al., 2006; Haglund et al., 2003b; Pickart, 2001; Weissman, 2001). However there appear to be some exceptions to this rule. For instance, the transcriptional regulator of myogenesis Pax3 is monoubiquitinated but its degradation occurs in proteasomes (Boutet et al., 2007; Boutet and Rando, 2008).

5.4.4 Function of E3 ligases

Ubiquitination is a sequential action of three enzymes, Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes (Hershko and Ciechanover, 1998). E1 mediates ATP-dependent activation of the glycine of ubiquitin for conjugation and transferring to E2

enzyme. Although some E2s can ubiquitinate substrates directly, in most cases E3 ubiquitin ligases are required to interact with E2 and transfer ubiquitin to the target substrate.

There are three major types of E3 ligases: HECT (Homologous to E6-AP Carboxyl Terminus) domain, RING (Really Interesting New Gene)-finger domain and a modified RING motif U-box (UFD2-homology domain) (Ardley and Robinson, 2005). While HECT-type E3 ligases such as Nedd4 have a direct role in ubiquitination, RING-type and U-box E3 ligases promote ubiquitination by positioning the activated E2 in close proximity to the substrate. RING-finger E3 ligase family is divided in two classes; they can be single proteins (e.g. c-Cbl) or protein complexes. The latter include the SCF (Skp1/ Cullin/ F box) and ECS (Elongin BC/ Cullin/ SOCS box) complexes (Petroski and Deshaies, 2005; Raiborg et al., 2002; Zheng et al., 2002).

The SOCS box of SOCS proteins is involved in the formation of the ECS complex that functions as an E3 ligase. Previous studies had demonstrated that SOCS3 is a negative regulator of G-CSF signalling and that the SOCS box is critical for this inhibitory effect. Because there are five conserved lysines in the cytoplasmic domain of the G-CSFR, we asked whether SOCS3 could mediate ubiquitination of the G-CSFR and whether this ubiquitination results in sorting of the receptor to a particular compartment and in attenuation of signalling. These questions have been addressed in Chapter 2, a.o. by employing ubiquitination assays and microscopic imaging in presence or absence of SOCS3 siRNA.

Despite intensive investigations on the role and mechanisms of ubiquitin conjugation within the last decade, the specificity and the selection process of the lysine residues targeted for ubiquitination is still poorly understood. Studies in chimeric models, including fusion of ubiquitin to the C-terminal region of receptors, indicate that ubiquitination and especially monoubiquitination is sufficient to promote endocytosis and MVB sorting. This model suggests that the position of ubiquitin conjugation is not a significant indicator in endocytosis. However, it has become clear that specific lysine residues are ubiquitinated in some proteins and that the precise ubiquitination site(s) can be functionally important (Mukhopadhyay and Riezman, 2007). For example, exclusive mutation of lysine residues present in the kinase domain of the EGFR is sufficient to attenuate lysosomal degradation of the receptor (Huang et al. 2006).

Furthermore, it is still unclear what determines the specificity of an E3 ligase for a given substrate. One explanation is the presence of unique determinants in the target substrate, for instance specific motifs in the cytoplasmic domain of a receptor that lead to preferential

lysine selection by an E3 ligase. Such a mechanism has been shown for β-transduction repeat-containing protein (β-Trcp), a member of RING-finger type of E3 ligase family, which can form an SCF complex via its F box. β-Trcp is involved in endocytosis and degradation of several receptors like EpoR (Meyer et al., 2007), interferon alpha receptor1 (IFNAR1) (Kumar et al., 2007) and prolactin receptor (Li et al., 2004). Recognition of a unique motif in the substrate by β-Trcp results in selection of lysine residues located 9-13 amino acids upstream of this destruction motif to be the target of ubiquitination. However, existence of a distinct motif is not always required for binding of an E3 ligase. For example, Cbl another member of RING-finger type of E3 ligase family has been shown to be involved in downregulation of receptors including hepatocyte growth factor receptor (HGFR or Met), EGFR and stem cell factor receptor (SCFR or c-Kit). Direct binding of Cbl to phosphorylated tyrosine of activated receptor is sufficient for its function (Carter et al., 2004; Masson et al., 2006; Thien and Langdon, 2005)

In Chapter 2 of this thesis, it is demonstrated that recruitment of SOCS3 to the phosphorylated tyrosine of the G-CSFR results in ubiquitination and lysosomal sorting of the activated receptor complexes. This process is strictly controlled by ubiquitination of the juxtamembrane lysine of the G-CSFR on position 632. Why this lysine is exclusively involved in G-CSFR trafficking is unknown. We explored the following possibilities: (I) K632 is part of a specific motif needed to recruit the ubiquitin machinery, (II) Positioning of K632 in proximity of the plasma membrane is essential for interaction with the molecular determinants directing lysosomal sorting. These questions have been addressed in Chapter 3.

5.4.5. Intracellular trafficking into late endosomes

Fusion of early endosomes results in the formation of late endosomes, which tend to be in close proximity of the nucleus. This localization is distinct from early endosomes, which are usually found in the cell periphery (Marmor and Yarden, 2004). Two members of Rab GTPase family, Rab7 and Rab9 have been implicated in trafficking through late endosomes (Gonzalez-Gaitan and Stenmark, 2003). Late endosomes may contain internal vesicles produced by invagination of the limiting membrane and budding into the lumen, and are then referred to as multivesicular bodies (MVBs).

Conjugation of ubiquitin to a particular target not only serves as a signal for prevention of recycling to plasma membrane but also plays an important role in efficient transport into the MVB pathway (Gruenberg and Stenmark, 2004; Katzmann et al., 2001; Reggiori and Pelham, 2002; Shaw et al., 2003). The main machinery that recognizes ubiquitinated cargo is formed by the ESCRT (the endosomal sorting complexes required for transport) proteins (Katzmann et al., 2003). There are three complexes of ESCRT proteins: ESCRT-I, ESCRT-II

and ESCRT-III, which are sequentially recruited to the endosomal membrane (Saksena et al., 2007; Slagsvold et al., 2006; Teo et al., 2006).

Interactions of proteins of the sorting machinery with ubiquitinated cargo involves one or more ubiquitin binding domains (UBDs) in these proteins (Haglund and Dikic, 2005; Haglund and Stenmark, 2006). Several UBDs have been identified, among which are UIM (Ub-interacting motif), VHS (vps27, Hrs, STAM) and GAT (GGA and TOM1) domains. Although different UBDs have different structural folds, they all contact the hydrophobic Ile44 of Ub with slight variation in the interaction (Hicke et al., 2005; Hurley et al., 2006). A comprehensive and continuosly updated overview of these motifs can be found on www.pawsonlab.mshri.on.ca.

One of the well-established UBDs is UIM, present in HRS (hepatocyte growth factor regulated tyrosine kinase substrate) and STAM (signal transducing adaptor molecule). The HRS/STAM complex is also being referred to ESCRT-0 because of its involvement in recognition of ubiquitinated cargo by their UIM prior to the ESCRT-I complex (Bache et al., 2003; Bilodeau et al., 2002; Shih et al., 2002; Slagsvold et al., 2006). HRS binds to phosphatidylinositol 3-phosphate (PIP3) domains of early endosomes via interaction of its FYVE domain (Stahelin et al., 2002). Additionally, it recruits a member of ESCRT-I complex, Tsg101 (tumor suppressor gene 101) (Bache et al., 2003; Bilodeau et al., 2002; Katzmann et al., 2003). Consequently cargo transports to ESCRT-I complex and MVB sorting continues by recruitment and function of downstream complexes, ESCRT-II and ESCRT-III. The next step is activation of deubiquitinating enzyme to remove ubiquitin tags from ubiquitinated cargo (Amerik et al., 2000). Finally ESCRT complexes dissociate from the endosomal membrane to function for sorting of other ubiquitinated cargos (Babst et al., 1997; Babst et al., 1998). Following sorting of the cargo to MVBs, these vesicles fuse with lysosomes and deliver their contents into the lumen of the lysosome. Lysosomes are characterized by expression of glycoproteins like lysosomal associated membrane (Lamp) and presence of lysosomal enzymes. Acidic pH (4.8) and digestive enzymes within lysosomes are the major factors responsible for degradation of the delivered cargo.

6. FORWARD ROUTING AND STEADY STATE DISTRIBUTION OF PLASMA MEMBRANE RECEPTORS

Within the endoplasmic reticulum, newly synthesized proteins that are properly folded package as vesicles carrying cargo and pass through a series of membrane-enclosed organelles to reach the trans-Golgi network (TGN) (Figure 4). This pathway is taken by plasma membrane-localized receptors, including cytokine receptors, which during their migration undergo post-translational modifications (e.g. glycosylation) to attain their mature status. Mature receptors then move from the TGN to the plasma membrane, their functional destination

(Duvernay et al., 2005; Mellman and Warren, 2000). Misfolded or unassembled proteins are disposed by a quality control system, endoplasmic reticulum-associated degradation (ERAD) (Meusser et al., 2005).

The density of receptors on the plasma membrane has an impact on signaling output. Therefore, regulation of steady state distribution of receptors on the cell surface plays an important role in signaling events. Cell surface localization and constitutive trafficking of several receptors have been shown to be controlled by JAK proteins; however the underlying mechanisms are heterogeneous for different receptors. Whereas members of Janus kinases enhance stability of mature forms of GH receptor and Tpo receptor and preclude their lysosomal and proteasomal degradation, respectively (He et al., 2005; Royer et al., 2005), in the case of IFNAR1 binding of TYK2 is found to attenuate receptor degradation by blocking a di-leucine internalization motif (Ragimbeau et al., 2003; Royer et al., 2005). Taken together these studies indicate that enhanced surface expression of cytokine receptors mediated by JAKs is receptor-specific and results from different mechanisms including increased receptor stability or biogenesis, altered forward routing, decreased endocytosis or combination of these processes.

The Janus kinases JAK1, JAK2 and TYK2 are activated by G-CSFR. In Chapter 4 we studied the mechanisms regulating steady state distribution of the G-CSFR and examined if JAK proteins play a role in this regulation. Furthermore, we studied if JAKs interfere with receptor endocytosis and lysosomal routing linked to dileucine-based motif and differential cytoplasmic lysines, respectively. As model systems, we used Ba/F3 cells overexpressing JAK1, JAK2 or TYK2 and human fibrosarcoma cells selectively deficient for these JAK kinases.

Besides the role of ubiquitin in receptor endocytosis and sorting to multivesicular bodies, ubiquitin can direct proteins to endosomes from the TGN (Figure 4). It has been shown that the Golgi-localized gamma-ear-containing Arf-binding (GGA) proteins, which are mainly associated with the TGN, can bind ubiquitin via their GAT domain. These proteins facilitate sorting at TGN and degradation of ubiquitinated target is hastened by sorting to endosomes. Accordingly, the appearance of ubiquitinated molecule on the cell surface gets prevented. Thus GGAs may function as ubiquitin sorting receptors in a manner similar to ESCRT-0 complex at TGN as suggested for EGF receptor and a member of human GGA family, GGA3 (Puertollano and Bonifacino, 2004; Scott et al., 2004).

Low levels of the G-CSFR on the cell surface compared to a large intracellular pool indicate that there is a tightly controlled balance between sorting to the plasma membrane and degradation of the newly synthesized receptors. Indeed, it has been shown that WT G-CSFR is

predominantly associated with Golgi apparatus and cytoplasmic structures of cell positive for lysosomal marker (Aarts et al., 2004). Therefore, ubiquitination of lysine residues of receptor plays an imperative role in maintaining the balance in constitutive distribution of G-CSFR. Although ligand-induced trafficking and signal downregulation has been largely investigated, very little is known about mechanisms controlling constitutive expression of cytokine receptors and their intracellular routing in ligand-independent pathways.

Preliminary studies in our laboratory using yeast-two hybrid assays suggested that Wsb2 (WD40 repeat and SOCS-box containing protein 2) may bind to the COOH terminus of the G-CSFR. Given that Wsb2 can form an ECS complex through its SOCS box, this protein was a potential candidate E3 ligase in ubiquitination of cytoplasmic lysines of the G-CSFR. Studies presented in Chapter 5 were performed to establish the interaction of Wsb2 with the G-CSFR using the mammalian protein-protein interaction trap (MAPPIT) system. Regulation of forward routing of G-CSFR by Wsb2 and its involvement in appropriate balance between proliferation and differentiation signals were then investigated in further detail.

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CHAPTER 2

Suppressor of cytokine signaling 3-controlled routing of G-CSF receptor

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ABSTRACT

The hematopoietic system provides an attractive model for studying growth factor-controlled expansion and differentiation of cells in relation to receptor routing and its consequences for signal transduction. Suppressor of cytokine signaling (SOCS) proteins regulate receptor signaling partly via their ubiquitin ligase (E3)-recruiting SOCS box domain. Whether SOCS proteins affect signaling through modulating intracellular trafficking of receptors is unknown. Here we show that a juxtamembrane lysine residue (K632) of the granulocyte colony-stimulating factor receptor (G-CSFR) plays a key role in receptor routing and demonstrate that the effects of SOCS3 on G-CSF signaling to a major extent depend on this lysine. Mutation of K632 causes accumulation of G-CSFR in early endosomes and leads to sustained activation of signal transducer and activator of transcription 5 and ERK, but not protein kinase B. Myeloid progenitors expressing G-CSFR mutants lacking K632 show a perturbed proliferation/differentiation balance in response to G-CSF. This is the first demonstration of SOCS-mediated ubiquitination and routing of a cytokine receptor and its impact on maintaining an appropriate signaling output.

INTRODUCTION

In the past 5 years, a major role has been demonstrated for protein ubiquitination in the control of endocytosis and intracellular routing of receptor tyrosine kinases, particularly of the epidermal growth factor (EGF) receptor (reviewed in (Haglund et al., 2003; Marmor and Yarden, 2004; Miaczynska et al., 2004; Polo and Di Fiore, 2006). For instance, upon ubiquitination by the E3 ligase Cbl, EGF receptor associates with multiple proteins containing an ubiquitin interaction motif (UIM), which guides EGF receptors into the different compartments of the endocytotic pathway. One such UIM-containing protein is hepatocyte growth factor receptor substrate (Hrs), implicated in the sorting of ubiquitinated cargo from early endosomes towards late endosomes, the lumen of multivesicular bodies and finally to lysosomes (Haglund et al., 2003). Receptor endocytosis is no longer considered as a mechanism solely involved in termination of signaling. Rather, it became clear that signaling processes proceed in endosomes and that temporal and spatial dissociation as well as integration of signals may occur during endocytosis (Miaczynska et al., 2004; Polo and Di Fiore, 2006). This form of cell signaling orchestration is believed to contribute significantly to cellular processes such as cell fate determination, migration and differentiation (Polo and Di Fiore, 2006). However, owing to a paucity of appropriate model systems, insights into the impact of receptor routing on fine-tuning of cellular responses in response to growth factors are still scarce.

Granulocyte colony-stimulating factor (G-CSF) is the main cytokine involved in the production of neutrophilic granulocytes (Avalos et al., 1990). Activation of the G-CSF receptor (G-CSFR), a member of the class I cytokine receptor superfamily, supports the proliferation, survival and differentiation of neutrophilic progenitor cells (van de Geijn et al., 2003). Similar to most cytokine receptors, G-CSFR activates multiple signaling pathways fuelled by activation of JAK kinases. For instance, JAK-phosphorylated tyrosine-based motifs become docking sites for SH2-containing signaling molecules, such as signal transducer and activator of transcription (STAT) 3 (Shao et al., 2006; Ward et al., 1999a), components of the $p21^{Ras}$/Raf/MAPK pathway (de Koning et al., 1998; Rausch and Marshall, 1997) and suppressor of cytokine signaling 3 (SOCS3) (Hermans et al., 2003; Hortner et al., 2002). In addition, the membrane-proximal cytoplasmic region of the G-CSFR is involved in the activation of protein kinase B (PKB), a serine/threonine kinase implicated in cell survival, and in the activation of STAT5 (Dong and Larner, 2000).

G-CSF stimulates proliferation and survival of myeloid progenitor cells, followed by a cell cycle arrest and differentiation towards neutrophils. Probably, this complex response requires attenuation of each of the signaling functions of G-CSFR during receptor endocytosis and intracellular routing. In support of this notion, perturbed signaling by G-CSFR truncation mutants, found in patients with acute myeloid leukemia preceded by severe chronic neutro-

penia, is to a large extent due to their inability to undergo endocytosis (Hunter and Avalos, 1999; Ward et al., 1999c).

Routing of class I cytokine receptors has been studied only to a limited extent and appears markedly heterogeneous. Most members studied thus far, for example, the growth hormone receptor (GHR) (van Kerkhof and Strous, 2001), leptin receptor (LR) (Uotani et al., 1999), prolactin receptor (PRLR) (Kelly et al., 1991), interleukin (IL)-9 receptor (Yen et al., 2000), gp130 (Blanchard et al., 2000; Dittrich et al., 1996), and the IL-2 receptor beta chain (Yen et al., 2000) follow the endosomal/lysosomal pathway. Endocytosis of LR depends on ubiquitination of two lysine residues in its cytoplasmic tail (Belouzard and Rouille, 2006). The ubiquitin-conjugating system is also required for endocytosis of GHR, but this does not depend on the cytoplasmic lysines of GHR (Alves dos Santos et al., 2001; Govers et al., 1999). In contrast, the Ub-conjugating system is not essential for internalization of PRLR (Lu et al., 2005). The thrombopoietin receptor (TPOR) forms a noteworthy exception, as it does not route to late endosomes and lysosomes but is rapidly recycled to the plasma membrane (Dahlen et al., 2003; Royer et al., 2005). This diversity in control of endocytosis and lysosomal routing emphasizes the importance of intracellular trafficking for the specific regulatory functions of the different receptors of this superfamily.

Human G-CSFR contains five cytoplasmic lysines (K632, K672, K681, K682 and K762) that are candidates for Ubiquitination and all are conserved between humans and mice. We recently reported that cytoplasmic lysines in the G-CSFR play a key role in its steady-state membrane expression and protein stability and provided evidence that WD40 and SOCS box-containing protein (Wsb) negatively controls forward routing of G-CSFR depending on these lysines (Erkeland et al., 2006). However, whether ubiquitination of one or more of these lysine residues is crucial for ligand-induced G-CSFR endocytosis, trafficking to early and late endosomes, which Ub-ligases might be involved and how this influences G-CSF-induced proliferation and differentiation signaling remained unknown.

Studies in conditional knockout mice have established that SOCS3 is a major negative regulator of G-CSF-induced neutrophil production *in vivo* (Croker et al., 2004; Kimura et al., 2004). SOCS proteins can inhibit cytokine signaling via multiple mechanisms: i.e., by competition for SH2-containing signaling substrates in receptor binding, by inhibition of JAK kinase activity through their kinase inhibitory region, or by recruitment of elongins B and C to the SOCS box to form an E3 Ub-ligase complex for degradation of signaling substrates (Kile et al., 2002). In an earlier report, we have shown that the SOCS box has a major role in SOCS3-mediated inhibition of G-CSF signaling (van de Geijn et al., 2004b), but how this is achieved remained unclear.

In this study, we show that a G-CSFR mutant lacking all five conserved cytoplasmic lysine residues (K5R) internalizes normally but fails to route to lysosomes. K5R is retained in early endosome antigen 1 (EEA1) and Hrs-positive endocytotic vesicles, where it con-

tinues to activate STAT5, but not PKB. Subsequent mutational analysis demonstrated that membrane-proximal lysine K632 is most crucial for lysosomal routing of the G-CSFR and for attenuation of G-CSF-induced STAT activity, cell proliferation and for the induction of terminal neutrophilic differentiation of myeloid 32D cells. We then show that ubiquitination of G-CSFR-K632 and lysosomal routing of the G-CSFR both depend on the integrity of the SOCS3 recruitment site (Y729) of G-CSFR. Finally, we demonstrate that inhibition of STAT5 activation by SOCS3 fully depends on the cytoplasmic lysines of G-CSF-R, in particular K632. These results reveal a major role of SOCS3 in G-CSFR routing from early (Hrs-positive) to late (Rab7-positive) endosomes and lysosomes and highlight the importance of receptor routing for an appropriate balance of proliferation and differentiation of myeloid progenitors in response to G-CSF.

MATERIAL AND METHODS

Antibodies

Mouse-anti-human CD114 (G-CSF receptor) and MoAb anti-EEA-1 were purchased from Becton-Dickinson/PharMingen (San Diego, CA). Rat anti-Lamp-1 (clone 1D4B) was obtained from Research Diagnostics (Flanders, NJ). Mouse anti-HA MoAb, goat anti EEA-1, rabbit anti- Rab7 and rabbit anti-STAT5a and anti-STAT5b were purchased from Santa Cruz, Biotechnology, Inc (Santa Cruz, CA). MoAb anti-pSTAT5 (STAT5A-pY694, STAT5B-pY699) was from Upstate Biotechnology, Inc (Charlottesville, VA) and anti-pSTAT3 (pY705) from Cell Signaling Technology, Inc (Danvers, MA). Texas Red-conjugated streptavidin (Caltag Laboratories (Burlingame, CA) was used for detection of biotinylated G-CSF (bio-G). MoAbs against pPKB, pERK and total ERK were from Santa Cruz. FLAG M2 MoAb was purchased from Sigma, St Louis, MO. Rabbit anti-Hrs was a gift from Dr. H. Stenmark (Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital and The University of Oslo, Oslo, Norway). Dye-coupled immunoglobulin (Ig) antibodies used in CLSM were purchased from Molecular Probes (Invitrogen, Breda, The Netherlands): donkey anti-mouse Alexa Fluor 488 (for G-CSFR), goat anti-rabbit Alexa Fluor 546 (for Rab7 and Hrs), rabbit anti-goat Alexa Fluor-546 (for EEA1), and goat anti-rat Alexa Fluor488 (Lamp-1). Goat anti-mouse Ig coupled to phycoerythrin (GAM-PE) used in flow cytometry was from Dako (Dako BV, Heverlee, Belgium).

Expression constructs

The pBabe expression constructs of wt G-CSFR, d749-769 and Y729F have been described previously (Aarts et al., 2004; Ward et al., 1999b). The K632R, K672R, K681R, K682R, K762R and K5R G-CSFR constructs were made in pLNCX containing wt G-CSFR using the Stratagene QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA). The single lysine add-back mutants were made in a similar way using K5R as template. C-terminal double HA-tagged versions of wt and mutant G-CSFR were created by PCR using a 5' oligonucleotide spanning a region in the cDNA corresponding to amino acids 590-595 (GRFR7) and a 3' oligonucleotide containing a segment spanning the amino acids immediately upstream of the stop codon of the G-CSFR, a double HA-tag, and a restriction site. All mutant

constructs were verified by sequencing. Expression vectors for SOCS1, SOCS2, SOCS3 and SOCS3Δbox and reporter constructs have been described previously (van de Geijn et al., 2004b). FLAG-tagged Ub in pCDNA3 was a gift from Dr. B. van der Reijden (Nijmegen, The Netherlands).

Cell culture, transfection, retroviral transduction and introduction of siRNA

Ba/F3 cells and 32D.cl8.6 cells, a sub-line of the IL-3-dependent murine myeloid 32Dcl3 cell line that lacks endogenous G-CSFR (de Koning et al., 1998), were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and murine IL-3 (10 ng/ml) at 37°C and 5% CO_2. To obtain Ba/F3 cells expressing wt or mutant G-CSFR, cells were electroporated as described (de Koning et al., 1996). Subsequently, cells were diluted and expanded in 96 wells plates in IL-3-containing medium supplemented with puromycin (1µg/ml) to obtain individual clones. 32D.cl8.6 clones expressing different G-CSFR constructs were obtained by retroviral transduction as described (Aarts et al., 2004). HeLa and Phoenix E cells were cultured in Dulbecco Modified Eagle's medium (DMEM, glucose 4.5g/l) supplemented with 10% FCS and 100 IU/ml penicillin, 100 µg/ml streptomycin in a humidified atmosphere at 37°C and 7% CO_2. HeLa cells were plated on glass coverslips and transfected with G-CSFR constructs using lipofectamin (Invitrogen). Phoenix E cells were transfected using the calcium phosphate precipitation method. To reduce SOCS3 expression, HeLa cells (2×10^5) were transfected using an siRNA transfection kit (Mirus Bio Corporation, Madison WI, USA) according to the manufacturer's instructions. The siRNA's used were SOCS3 human ON-TARGET plus siRNA SMARTpool (Dunn et al., 2005), siCONTROL non-targeting siRNA #1 and siGLO cyclophylin B siRNA, all obtained from Dharmacon, Chicago IL, USA.

Immunoprecipitation and Western blotting

Phoenix E cells (G. Nolan, Stanford, CA) were transfected with HA-tagged wt or mutant-G-CSFR and FLAG-tagged Ub using the calcium-phosphate precipitation method. After 48 hrs, cells were stimulated with G-CSF (100 ng/ml) for 30 min or left untreated, washed with cold PBS and resuspended in lysis buffer containing 20 mM Tris HCl pH8.0, 13 7mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP40, 10% glycerol, 2 mM Na_3VO_4 and 1 mM Pefablock SC, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 50 µg/ml bacitracin, and 50 µg/ml iodoacetamide. Immunoprecipitation was performed using anti HA-antibodies and protein G-Sepharose beads. Alternatively, G-CSFR pull down was performed with biotinylated G-CSF (bio-G), prepared using a biotin labeling kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Cells were incubated with bio-G (10 µg/ml) for 15 min at 16°C and for another 30 min at 37°C. Subsequently, cells were washed with cold PBS, resuspended in lysis buffer and incubated with streptavidin-conjugated magnetic beads (Dynabeads, In vitrogen) for 1 h at 4°C. Beads were washed and resuspended in 1x Laemlli buffer (pH 11) and supernatants subjected to Western blotting as described (Ward et al., 1999b).

Immunocytochemistry and confocal microscopy

To visualize postendocytic receptor trafficking *in situ*, cells were incubated with G-CSFR antibodies (2.5 µg/ml) and G-CSF (100 ng/ml) for 20 min at 16°C, washed (PBS) and transferred to 37°C. Subsequently, cells were permeabilized by treatment for 5 min with 0.05% saponin, fixed with 3% paraformaldehyde and stained for fluorescence microscopy (Raiborg et al., 2002). To visualize receptor internalization in 32D cells, cells were incubated with bio-G for 1 h at 4°C, washed (PBS) and transferred to 37°C to induce

internalization. After washing (PBS), cells were spun onto glass slides, fixed (4% paraformaldehyde and 0.05% gluteraldehyde) and stained for CLSM (Aarts et al., 2004). Cover slips were examined in the multitrack detection mode of a Zeiss LSM 510 instrument equipped with argon/HeNe lasers and a x 63 Planapochromat oil immersion objective.

Cell proliferation assay and cytology

To determine the proliferation and differentiation characteristics of the 32D.cl8.6 cells transduced with various G-CSFR constructs in response to G-CSF, cells were washed three times in Hanks Balanced Salt Solution (HBSS) and seeded at a density of 2 x 10^5 cells/ml in RPMI 1640 medium, supplemented with 10% FCS and 100 ng/ml human G-CSF. Viable cells were counted daily using a CASY1 electronic cell counter (Schärfe-System, Reutlingen, Germany) and readjusted to 2 x 10^5 cells/ml. To analyze granulocytic differentiation, cells were spun onto glass slides and examined by light microscopy after May-Grünwald-Giemsa staining.

Bone marrow cells

Hematopoietic progenitor cells were collected from the femurs and tibiae of 8 to 12 week-old *Csf3r-/-* mice as described (Hermans et al., 2003). Cells were washed twice in HBSS/5% FCS/0.5% bovine serum albumin (BSA) and prestimulated for 2 days at a final concentration of 5×10^5 cells/ml in Cell Gro (SCGM BE SP047; Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany), supplemented with a cytokine cocktail composed of murine IL-3 (10 ng/ml), human Flt3-ligand, human thrombopoietin, murine stem cell factor (100 ng/ml) and granulocyte macrophage-colony-stimulating factor (2 U/ml). Retroviral infection was performed with viral supernatant of Phoenix E cells as described (Erkeland et al., 2003).

Colony assay

Bone marrow cells were plated in triplicate at a density of 5×10^4 cells in 1 ml of methylcellulose containing medium supplemented with 30% fetal bovine serum (FBS), 1% BSA, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, and G-CSF (20 U/ml) as described (Hermans et al., 2003). Colonies consisting of more than 50 cells were counted on day 7 of culture.

Flow cytometric analysis of G-CSFR

G-CSFR-expressing 32D.cl8.6 transfectants ($0.5x10^6$ cells) were incubated at 4°C for 1 hr with G-CSFR antibody in PSA, consisting of PBS supplemented with 1% FCS, and 0.02% NaN_3 to block endocytosis. After washing, cells were incubated with GAM-PE for 1 hour at 4°C, washed and analyzed on a FACS Calibur flow cytometer (Becton Dickinson). For internalization experiments, 32D cells ($0.5x10^6$ cells per time point) were incubated with anti-G-CSFR antibodies in the presence or absence of 100 ng/ml G-CSF for 1 hr at 4°C and subsequently incubated for 0, 15, 30 or 60 min. at 37°C. Subsequently, cells were washed in PSA, and incubated for 1 hr at 4°C with GAM-PE. To measure G-CSFR rerouting, cells incubated with G-CSF for 60 min were washed with PSA, incubated without G-CSF at 37°C for indicated times in the presence of cycloheximide (10 µg/ml) incubated with GAM-PE for 1 h at 4°C and analyzed after a final wash in PSA analyzed. Peak channel values of FITC fluorescence histograms were taken as a measure of average G-CSFR densities.

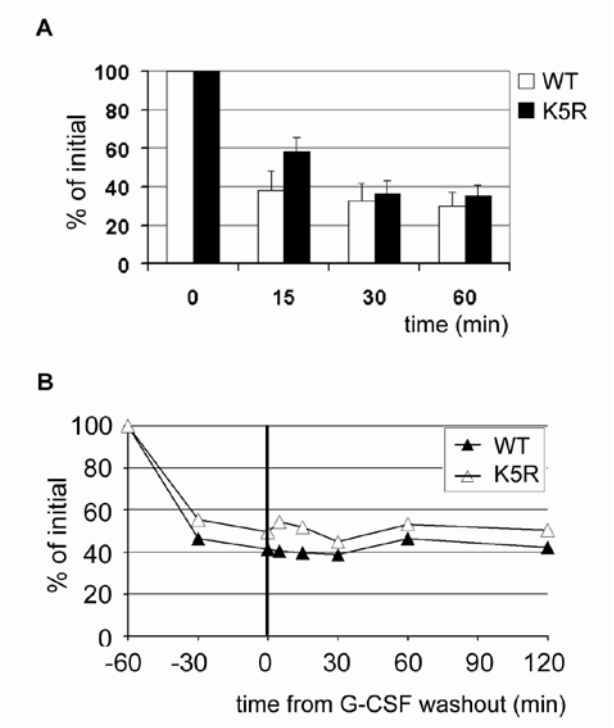


Figure 1. Cytoplasmic lysines of G-CSFR are not involved in ligand-induced receptor internalization
(A) Flow cytometric analysis of internalization kinetics of G-CSFR in 32D clones expressing wt G-CSFR (open bars) or mutant K5R (filled bars). G-CSFR cell surface expression was assessed at various time points after addition of G-CSF. Data represent the mean percentages of fluorescence compared to the fluorescence at t=0 min from three different clones. (B) G-CSFR wt and K5R are not rapidly recycled to the plasma membrane. G-CSF was added at t= -60 min and washed out at t= 0. Cells were then resuspended in the presence of cycloheximide and analyzed for cell surface expression of G-CSFR as outlined under in A. The mean fluorescence at t=-60 min was set at 100%. This result is representative of three experiments with three independent 32D clones.

Electrophoretic mobility shift assay (EMSA)

32D cells were washed in HBSS and deprived of serum and factors for 4 h at 37°C in RPMI at a density of $1x10^6$ cells/ml. Subsequently, cells were stimulated with G-CSF (100 ng/ml) for 10 min, washed twice with HBSS and incubated for 30, 60 or 120 min in RPMI in the absence of G-CSF. Preparation of nuclear extracts and STAT5 EMSA were carried out as described (de Koning et al., 2000).

Luciferase assay

Luciferase assays to assess the effects of increasing levels of SOCS proteins on STAT5 activity induced by wt and mutant G-CSFR were performed as described (van de Geijn et al., 2004a; van de Geijn et al., 2004b). In brief, HEK293 cells were transfected with the indicated G-CSFR constructs, increasing amounts of SOCS constructs and a STAT5-responsive luciferase construct. After 2 days, cells were stimulated with G-CSF for 6 hrs and assayed for luciferase activity. G-CSF-induced luciferase reporter

activity without transfected SOCS constructs was set at 100%. All experiments were performed in triplicate and data presented are representative of three independent experiments.

RESULTS

Cytoplasmic lysines are not essential for ligand-induced endocytosis of G-CSFR

Previously, we reported that internalization of the G-CSFR is guided by two adjacent internalization motifs, one of them containing a serine-type dileucine motif (Aarts et al., 2004; Ward et al., 1999c). To determine a possible role of cytoplasmic lysine residues in endocytosis and routing, we first compared internalization kinetics of wild type (wt) G-CSFR and mutant K5R, in which cytoplasmic lysines had been replaced by arginines, in 32D cells. As shown in Figure 1A, the rates of G-CSF-induced internalization of wt and K5R G-CSFR were similar. In

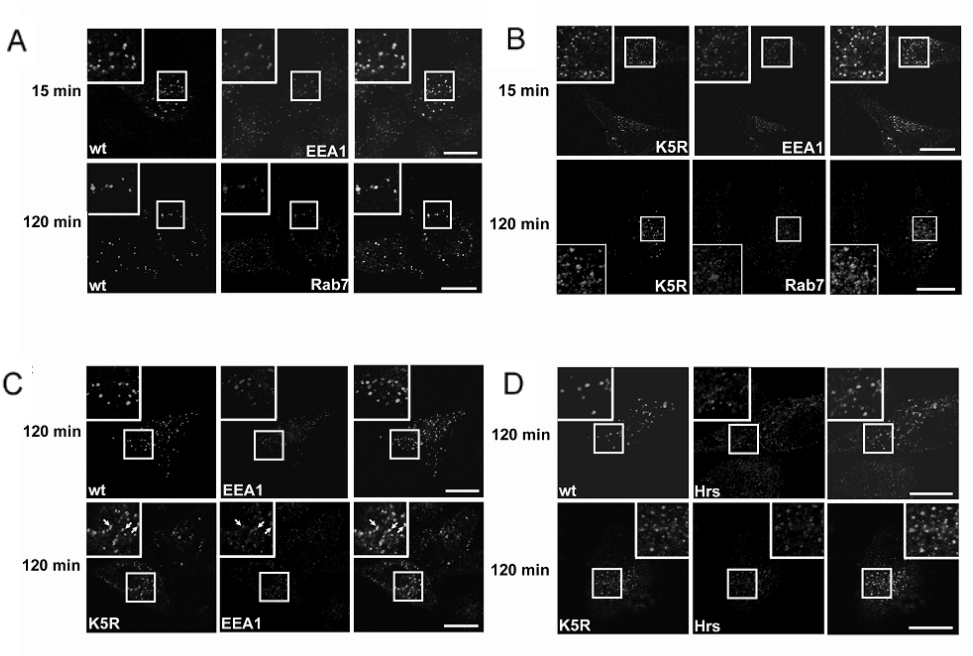
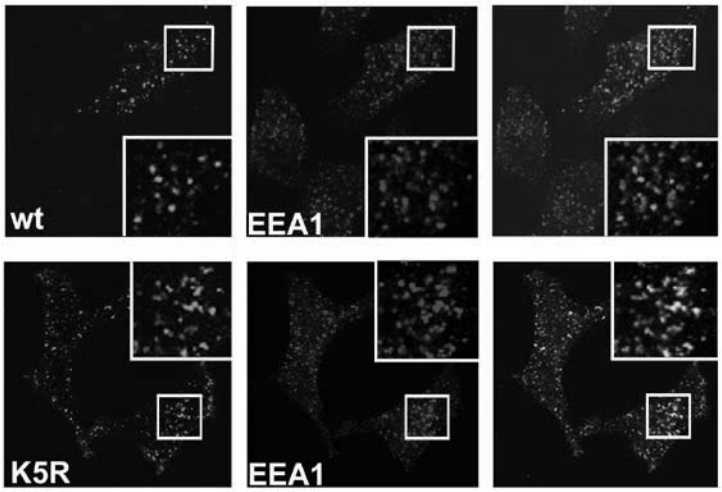


Figure 2. Role of G-CSFR lysines in lysosomal routing

HeLa cells ectopically expressing wt or K5R G-CSFR were incubated with G-CSFR antibodies and G-CSF for 20 min at 16°C, washed and transferred to 37°C for 15 or 120 min as indicated. Subsequently, cells were fixed and stained for internalized G-CSFR (green, left panels) and organelle markers EEA1, Rab7 or Hrs (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels, indicating co-localization in yellow. Insets show enlargements of the boxed areas. (A) Presence of wt G-CSFR in EEA1-positive early endosomes at 15 min and in Rab7- positive late endosomes/lysosomes at 120 min after internalization. (B) K5R is present in EEA1-positive endosomes at 15 min, but is not detectable at Rab7-positive endosomes/lysosomes at 120 min after internalization. (C) Presence of K5R (arrows) but not wt G-CSFR in EEA1-positive endosomes at 120 min after internalization. (D) Presence of K5R but not wt G-CSFR in Hrs-positive endosomes 120 min after internalization. Scale bar, 10μm



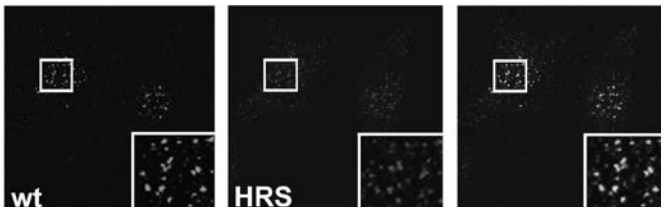
Supplementary Figure 1. Retention of K5R G-CSFR in early endosomes 4hrs after internalization
Hela cells were transfected with wt or K5R G-CSFR and stained for localization in EEA1 positive endosomes as detailed under Figure 2.

addition, the amount of cell surface receptors did not substantially increase within 2 hrs after G-CSF withdrawal (Figure 1B). Moreover, no co-localization was found between internalized anti-G-CSFR antibodies and a marker for recycling endosomes (TRITC-conjugated transferrin) from 30 min post-internalization onwards (data not shown). These results establish that cytoplasmic lysines does not contribute significantly to ligand-induced G-CSFR endocytosis and that internalized wt or K5R G-CSFR do not massively recycle to the plasma membrane.

Lysosomal routing of G-CSFR depends on cytoplasmic lysines

To examine the post-endocytic fate of the internalized G-CSFR, nonactivating antibodies directed against an extracellular epitope of G-CSFR were used to track receptor localization by confocal laser scanning microscopy (CLSM) (Aarts et al., 2004). HeLa cells transfected with wt G-CSFR were allowed to bind G-CSFR antibodies and G-CSF for 20 min at 16°C, washed and then transferred to 37°C to permit internalization. Fifteen minutes after transfer, the internalized G-CSFR resided predominantly in EEA1-positive early endosomes (Figure 2A, upper panel). From 30 min onward, the G-CSFR accumulated in Rab7 positive late endosome/lysosomes, which was complete after 2 h (Figure 2A, lower panel). These results show that the G-CSFR follows the endosomal-lysosomal degradation route.

We then asked whether replacement of the cytoplasmic lysines affected intracellular trafficking of the G-CSFR. Similar to the wt G-CSFR, mutant K5R accumulated in EEA1 positive early endosomes 15 min after induction of internalization (Figure 2B, upper panel). However, contrary to the wt G-CSFR, no transfer of K5R to Rab7 positive late endosomes had occurred after 2 h (Figure 2B, lower panel). Instead, a fraction of K5R was retained in EEA1- positive



Supplementary Figure 2. Co-localization of wt G-CSFR with Hrs, 30min after internalization
Hela cells were transfected with wt G-CSFR and stained for localization in HRS positive endosomes as detailed under Figure 2.

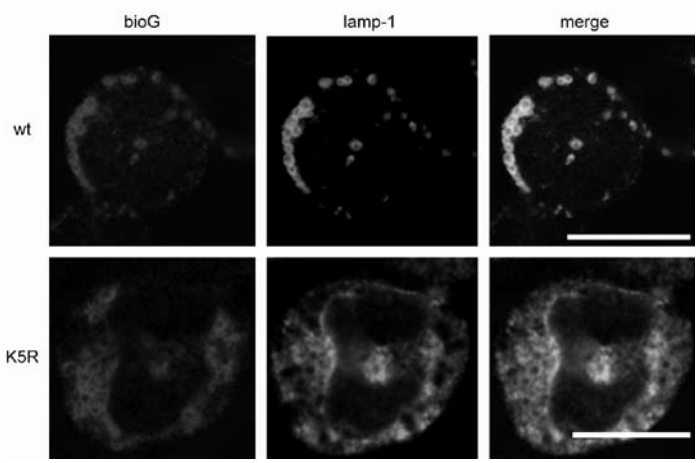


Figure 3. Intracellular localization of K5R G-CSFR in myeloid 32D cells
32D clones expressing wt or K5R G-CSFR were allowed to bind biotinylated G-CSF (bio-G) for 60 min at 4°C, washed and transferred to 37°C for an additional 60 min. Subsequently, cells were spun on glass slides, fixed, immunostained for bio-G and Lamp-1 and analyzed by CLSM. Scale bar: 10 μm

early endosomes (Figure 2C; lower panel, arrows), which was not the case for wt G-CSFR (Figure 2C, upper panel). Even 4 hr after internalization, mutant K5R was still found in early endosomes, indicating a block or severe delay in lysosomal routing (supplementary Figure 1). A large fraction of K5R co-localized with Hrs (Figure 2D, lower panel), a marker for prelysosomal endosomes. In contrast, wt G-CSFR co-localized with Hrs at 30 min (Supplementary Figure 2), but not at 2 h post-internalization (Figure 2D, upper panel). Together, these data show that internalized G-CSFR route to an Hrs-positive endosomal compartment via a lysine independent mechanism. Subsequently, one or more lysines in the cytoplasmic tail are required for routing to lysosomes. Co-localization of the wt G-CSFR or mutant K5R with caveolin 1 was not observed (data not shown), indicating that G-CSFR internalization via the raft/caveolar endocytotic pathway does not occur under these conditions.

As cell context may potentially influence routing mechanisms, we validated the results obtained in HeLa cells in myeloid 32D cells. Because Rab7 immunoreactivity is low in these cells, antibodies against lysosome-associated membrane protein-1 (Lamp-1) were used instead to mark the lysosomal compartment (Granger et al., 1990). Kinetics of lysosomal routing of G-CSFR in 32D cells were similar to those in HeLa cells: 1 hr after receptor activation, most wt G-CSFR accumulated in lysosomes (Figure 3, upper panel), whereas K5R did not (Figure 3, lower panel).

Differential involvement of internalization motifs and cytoplasmic lysines in attenuation of G-CSF signaling

To compare the effects of receptor internalization domain and lysosomal routing on G-CSF signaling, we studied the kinetics of signaling off-switch from wt G-CSFR, mutant K5R and a mutant lacking the internalization domain (d749-769). 32D and Ba/F3 clones with comparable expression levels of wt, d749-769 or K5R G-CSFR were used (Figure 4A and data not shown). First, we assessed the kinetics of downregulation of G-CSF-induced STAT5 activity in 32D cells by electrophoretic mobility shift assay (EMSA) following G-CSF washout after 10 min of stimulation (Figure 4B). Compared with wt G-CSFR, both d749-769 and K5R G-CSFR showed a significant delay in the attenuation of STAT5 activity (Figure 4B). Western blot analysis with phosphospecific antibodies against signaling substrates showed that phosphorylation of STAT3, STAT5 and ERK induced by mutants K5R and d749-769 is prolonged compared with wt G-CSFR after G-CSF washout (Figure 4C). In contrast, PKB activitation was prolonged after activation of d749-769, but normally attenuated after activation of mutant K5R (Figure 4C). These results indicate that PKB activation is abrogated immediately upon receptor internalization, whereas activation of STAT3, STAT5 and Erk continues upon routing of the G-CSFR to early endosomes. The latter experiments were performed in Ba/F3 cells instead of 32D cells, because 32D cells were less optimal for Western blot analysis, probably due to higher levels of protease activity. A quantification of these data is presented in supplementary Table 1.

Subsequently, we assessed the consequences of removal of the internalization domain and substitution of the lysine residues for G-CSF-induced proliferation and differentiation. As demonstrated previously, 32D cells expressing wt G-CSFR proliferated 6 to 8 days and differentiated into neutrophilic granulocytes from day 6 onwards (Figure 4D and E) (Ward et al., 1999a). In contrast, 32D cells expressing d749-769 or K5R continued with proliferation in response to G-CSF at the expense of terminal granulocytic differentiation (Figure 4D and E). Colony assays with G-CSFR-deficient (*Csf3r-/-*) bone marrow cells, retrovirally transduced with wt, d749-769 or K5R G-CSFR and cultured in G-CSF-supplemented semi-solid medium were then performed. Both the numbers and size of G-CSF-induced colonies formed by d749-769 or K5R G-CSFR-expressing bone marrow cells exceeded those of wt G-CSFR transduced cells (Figure 4F), indicating that both defective internalization and lysosomal routing result

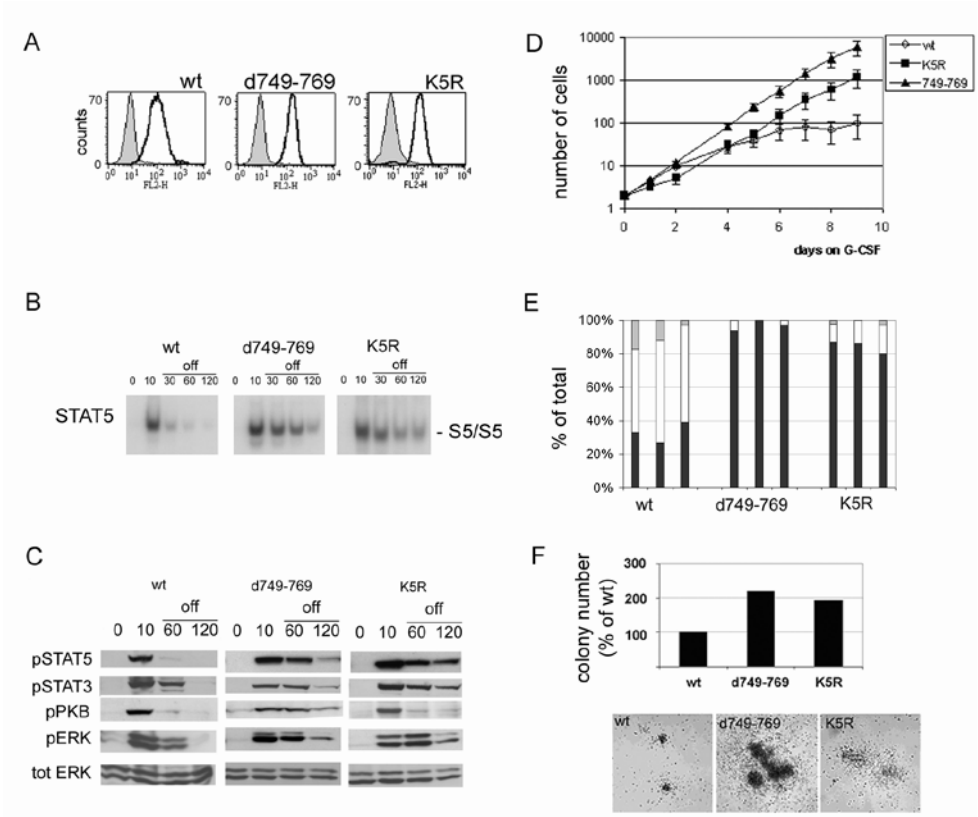


Figure 4. Role of internalization motif and cytoplasmic lysines in G-CSF signal attenuation and myeloid differentiation

(A) Flow cytometric analysis of membrane expression of wt, d749-769 or K5R G-CSFR in 32D cells. Bold histograms: cells stained with anti-G-CSFR and GAM-PE; shaded histograms: cells stained with GAM-PE only. (B) 32D cells expressing wt or mutant G-CSFR were deprived of IL-3 and serum for 4 hrs, incubated with G-CSF for 10 min at 37°C, washed and kept at 37°C in medium without factors. Nuclear extracts were assayed at the indicated time points by EMSA for activated STAT5 using β-casein oligonucleotides (C) Ba/F3 cells expressing wt or mutant G-CSFR were treated as described under B and lysates immunoblotted and stained with anti-pSTAT5, anti-pSTAT3, anti-pPKB, anti-pERK and restained with anti-ERK to verify equal loading. Results are representative of three independent experiments in different Ba/F3 clones. (D) Proliferation of 32D.cl8.6 cells expressing wt or mutant G-CSFR during 9 days of culture in the presence of G-CSF. Numbers of viable cells were determined at the indicated times. Data represent mean values +/- SEM of 3 clones per construct. (E) Differential counts of cells taken from cultures shown in panel D. On day 8, three clones per construct were analyzed and scored for percentages of blast cells (black bars), band forms (gray bars) or mature neutrophils (white bars). (F) G-CSF-induced colony formation by *Csfr3-/-* primary bone marrow cells after retroviral transduction of wt or mutant G-CSFR constructs. Upper panel: mean colony numbers per 25.000 infected bone marrow cells from triplicate colony assays. Lower panel: photomicrograph of representative colonies showing differences in colony size. Data are representative of two independent experiments. No G-CSF-induced colonies were observed with *Csfr3-/-* bone marrow cells transduced with empty vector.

Supplementary Table 1. Quantification of Figure 4C*

phosphoSTAT5

G-CSF stimulation		after G-CSF washout		
WT	3%	100%	1%	1%
D749-69	9.6%	100%	86%	32.6%
K5R	7.5%	100%	91%	67%

phosphoSTAT3

G-CSF stimulation		after G-CSF washout		
G-CSFR type	0 min	10 min	60 min	120 min
WT	7.3%	100%	11.5%	1%
D749-69	4%	100%	95.5%	35.4%
K5R	1%	100%	87%	60%

phosphoPKB

G-CSF stimulation		after G-CSF washout		
G-CSFR type	0 min	10 min	60 min	120 min
Wt	7.3%	100%	11.5%	1%
D749-69	33,2%	100%	92%	50%
K5R	10%	100%	26%	15%

phosphoERK

G-CSF stimulation		after G-CSF washout		
G-CSFR type	0 min	10 min	60 min	120 min
WT	43.6%	100%	86%	3.5%
D749-69	15%	100%	99%	49.5%
K5R	6%	100%	98%	55%

Data were quantified using ImageQuant 5.2 software (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). Data are expressed as signal intensities relative to signals at 10 min after G-CSF stimulation, which were set at 100%.

in a hyperproliferative response of primary hematopoietic progenitors to G-CSF. Notably, the internalization defect had a more pronounced impact on proliferation signaling than the lysosomal routing defect, which might relate to the fact that G-CSF-induced PKB activation by the internalization defective mutant is sustained, whereas it is downregulated with normal kinetics after activation of mutant K5R (Figure 4C and supplementary Table 1) (Dong and Larner, 2000).

G-CSFR-K632 is the most critical lysine for lysosomal routing and attenuation of G-CSF signaling

To determine which of the five cytoplasmic lysines of G-CSFR are involved in lysosomal routing and termination of signaling, we constructed single lysine substitution mutants. Mutant K632R failed to accumulate in Rab7-positive lysosomes 2 hr after internalization, whereas a significant proportion of mutants K672R, K681R, K682R and K762R co-localized

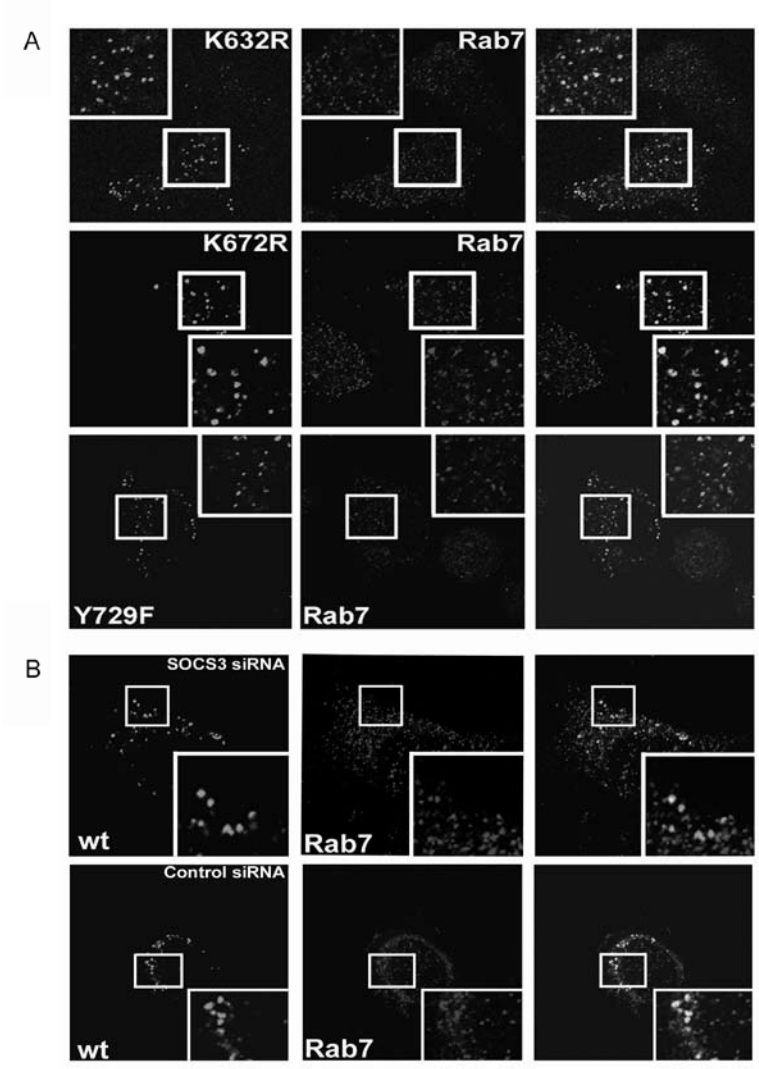
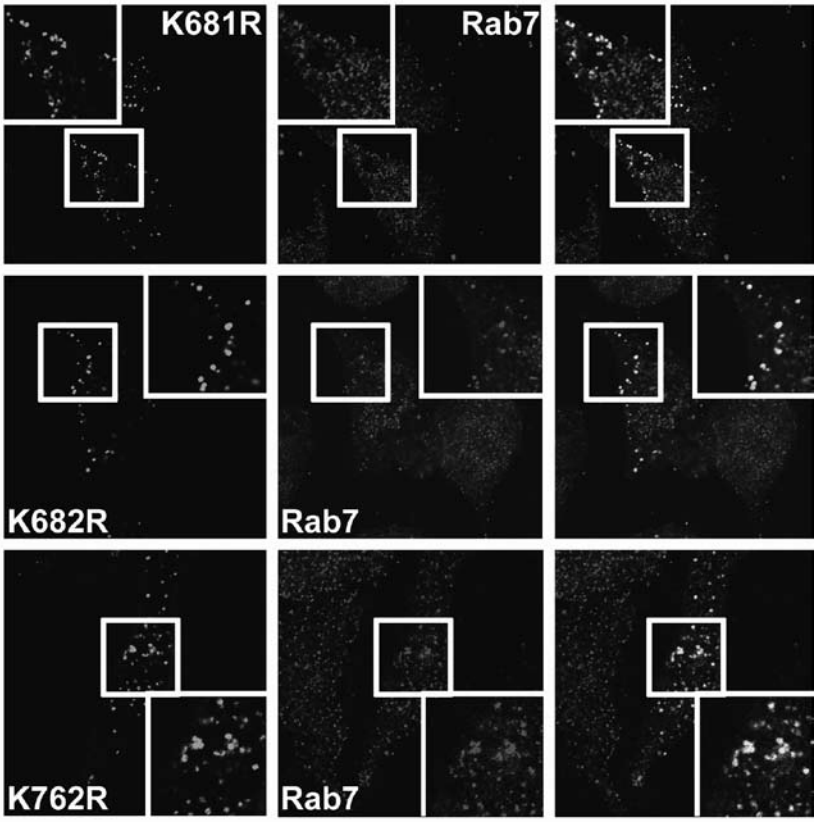


Figure 5. Intracellular localization of G-CSFR mutants

(A) HeLa cells were transfected with G-CSFR mutants K632R, K672R and Y729F and examined for localization in Rab7-positive endosomes or lysosomes after 120 min as detailed under Figure 2. (B) HeLa cells were co-transfected with wt G-CSFR and 30 nM SOCS3 siRNA (upper panel) or 30 nM control siRNA (lower panel) and analyzed as outlined above.



Supplementary Figure 3. Intracellular localization of G-CSFR mutants
HeLa cells were transfected with G-CSFR mutants K6812R, K682R and K762R and examined for localization in Rab7 positive endosomes or lysosomes as detailed under Figure 2.

with Rab7 at this time point (Figure 5A and supplementary Figure 3). EMSA's on stable 32D clones expressing either one of these mutants showed that only mutation of G-CSFR-K632 resulted in prolonged STAT activation following ligand washout (Figure 6A upper panel). To further substantiate this result, we made G-CSFR constructs with single lysine residues added back into K5R (mKA to mKE). Only the add-back of K632 (mKA) restored the attenuation of STAT activation at a rate comparable to wt G-CSFR, whereas adding back each of the other lysines had little or no effect (Figure 6A, lower panel). In G-CSF-containing medium, 32D/K632R cells showed sustained and enhanced proliferation in response to G-CSF, whereas clones expressing the other lysine substitution mutants were arrested in proliferation and differentiated comparably to 32D/wt cells (Figure 6B). Together, these results establish that G-CSFR-K632 plays a key role in lysosomal receptor degradation, termination of G-CSF-induced STAT activation and inhibition of proliferation signaling.

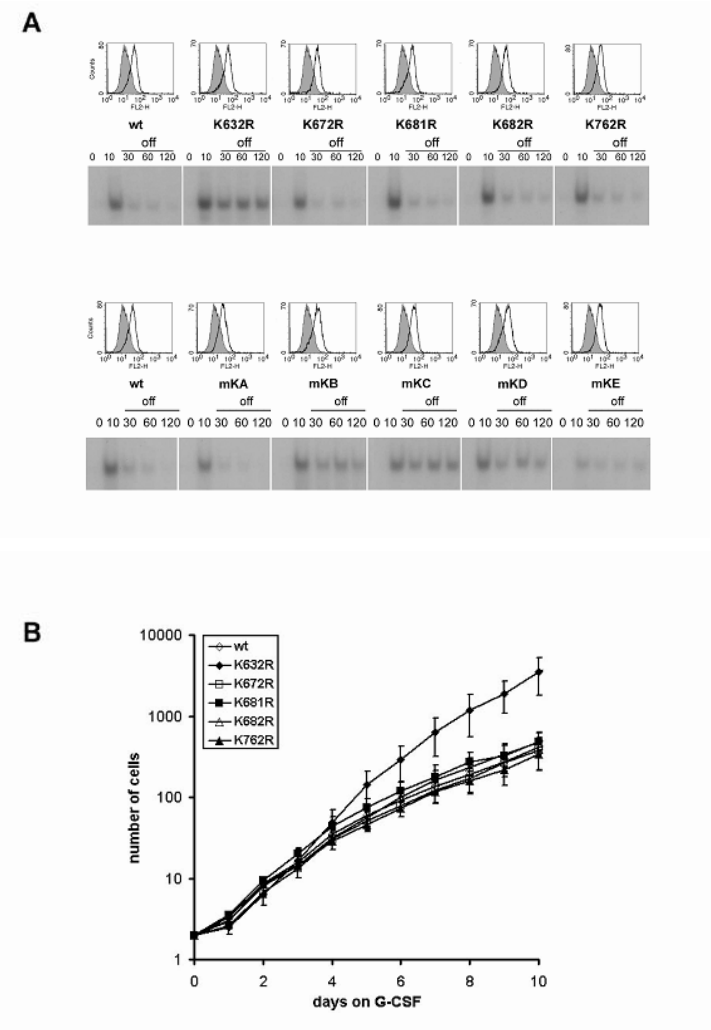


Figure 6. Involvement of G-CSFR K632 in attenuation of G-CSF-induced STAT5 activation and cell proliferation

(A) STAT5 EMSA on 32D cells expressing wt G-CSFR or single K to R substitution mutants (upper panel) or single add-back mutants (lower panel). Cells were deprived of IL-3 and serum, incubated for 10 min at 37°C with 100 ng/ml G-CSF, washed to remove G-CSF and subjected to EMSA. FACS histograms indicate expression levels of different mutants, see Figure 4B for explanation. (B) Proliferation of 32D cells expressing wt or single K to R substitution mutants cultured in the presence of G-CSF. Numbers of viable cells were determined at the indicated time points. Data represent mean values +/- s.e.m of three independent clones.

G-CSFR is ubiquitinated on K632

To investigate whether G-CSFR lysines are acceptors for ubiquitination, we transfected cells with haemagglutinin (HA)-tagged G-CSFR constructs and FLAG-tagged Ub and subjected these to HA immunoprecipitation and FLAG Western blot analysis. G-CSFR immunopre-

cipitates showed FLAG-immunoreactive bands with estimated molecular sizes between 140 and 170 kDa (Figure 7A). In contrast, no significant FLAG-immunoreactivity was detected in immunoprecipitates from cells transfected with K5R, indicating that ubiquitination is receptor lysine specific. Mutant K5R was significantly more abundant than G-CSFR wt in cell lysates, as was expected based on its reduced lysosomal routing. However, because only a minor fraction (approximately 5%) of the total G-CSFR protein is present on the plasma membrane (Aarts et al., 2004; Harada et al., 2005), these results may reflect predominantly intracellular G-CSFR. To examine specifically ubiquitination of G-CSFR expressed on the cell membrane, cells were incubated with biotinylated G-CSF (bio-G) and ligand-bound G-CSFR was precipitated using streptavidin-coated magnetic beads. FLAG-immunoreactivity was observed with wt G-CSFR (figure 7B) but not with K5R. In addition, substantial FLAG immunoreactivity of mKA was seen, indicating that G-CSFR-K632 is subject to ubiquitination. Notably, substitution mutant K632R is still robustly ubiquitinated, indicating that other lysines in G-CSFR also serve as substrates for E3 ligases (supplementary Figure 4). Additional experiments with single add-back mutants indicated that K672, K681, K682 and to a lesser

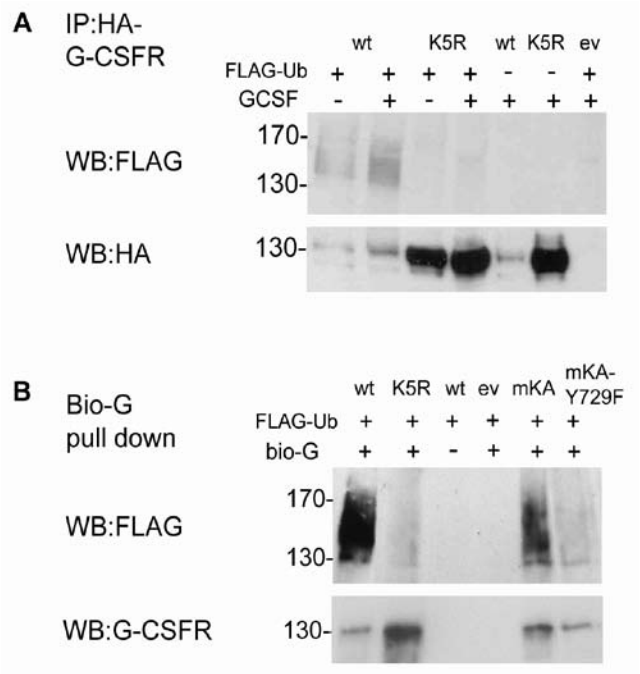


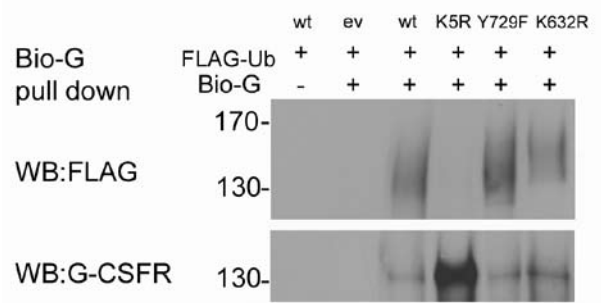
Figure 7. Ubiquitination of G-CSFR

(A) Phoenix E cells cotransfected with HA-tagged wt or K5R G-CSFR and FLAG-tagged Ub were incubated with (+) or without (-) G-CSF for 30 min, and HA-immunoprecipitates analyzed by Western blotting using anti FLAG (upper panel) or anti HA-antibodies (lower panel). (B) A similar set up as in (A), but instead of HA immunoprecipitation, cells were incubated with bio-G for 20 min at 16°C and for 30 min at 37°C and subsequently G-CSFR was pulled down with streptavidin-coated magnetic beads

extent K762 are ubiquitinated (data not shown). Having established that these lysines are not critical for lysosomal routing in the endocytotic pathway, they may possibly be involved in other functions, such as forward routing and protein stability, as recently proposed (Erkeland et al., 2006). The E3 ligase involved in ubiquitination of these lysines is not SOCS3, as is evident from the observation that G-CSFR mutant Y729F (lacking the SOCS3 docking site) is ubiquitinated (supplementary Figure 4).

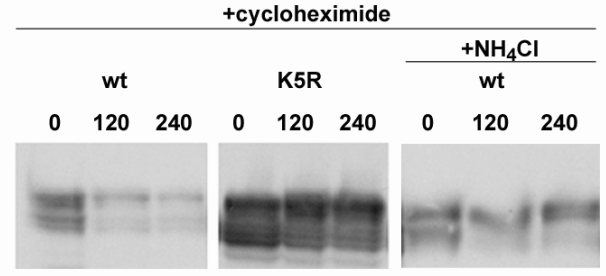
Involvement of SOCS3 in ubiquitination and lysosomal routing of G-CSFR

Previously, it was shown that the inhibitory action of suppressor of cytokine signaling 3 (SOCS3) in G-CSF-induced STAT5 activation to a major extent depends on the SOCS-box (van de Geijn et al., 2004b). This raises the question whether SOCS3 may be directly involved in ubiquitination of G-CSFR, specifically on K632. We addressed this possibility using a G-CSFR mutant that lacks the SOCS3 recruitment site Y729 (Hortner et al., 2002; van de Geijn et al., 2004a). Substitution of Y729 by phenylalanine resulted in a significantly reduced



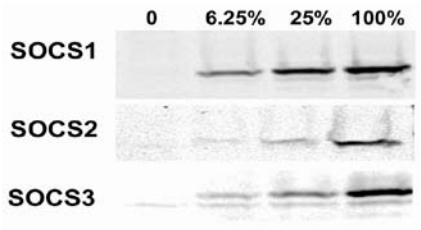
Supplementary Figure 4. Ubiquitination of G-CSFR mutants

Phoenix E cells were cotransfected with FLAG-tagged Ub and different mutants of G-CSFR. G-CSFR pull down was performed with bio-G and SA-conjugated magnetic beads.



Supplementary Figure 5. Lysosomal degradation of G-CSFR depending on receptor lysines

Phoenix E cells were transfected with wt or K5R G-CSFR and cultured for 120 min and 240 min in the presence of cycloheximide (5 µg/ml) to block de novo protein synthesis and for wt receptor also with NH_4Cl to inhibit lysosomal acidification. Western blot stained for G-CSFR shows that mutant K5R is not degraded and that NH_4Cl inhibits degradation of wt G-CSFR.



Supplementary Figure 6. Expression of SOCS proteins in luciferase experiments
Western blot analysis with Myc antibodies showing expression levels of Myc-Tagged SOCS1, SOCS2 and SOCS3 in luciferase experiments shown in Figure 8. Experimental details can be found in (van de Geijn et al., 2004)

ubiquitination of G-CSFR-K632 (compare mKA to mKA/Y729F, Figure 7B). This led us to examine the possible involvement of SOCS3 in lysosomal routing of G-CSFR. Two hours after internalization, no substantial co-localization of G-CSFR mutant Y729F with Rab7 was seen (Figure 5A), indicating that loss of SOCS3 recruitment to Y729 prevents lysosomal routing of G-CSFR. To further substantiate the role of SOCS3 in the lysosomal routing of G-CSFR, we inhibited its expression using siRNA in wt G-CSFR-expressing cells. In these experiments, the efficacy of transfection as detected by siGlo Cyclophilin B siRNA exceeded 90% (data not shown). As expected, SOCS3-siRNA but not control siRNA significantly reduced co-localization of G-CSFR with Rab7 2 h after G-CSF-induced internalization (Figure 5B). Protein stability assays in which lysosomal activity is inhibited by NH_4CL-mediated de-acidification confirmed that lysosomes play a major role in G-CSFR degradation (supplementary Figure 5).

G-CSFR-K632 is the most critical lysine for the inhibitory effects of SOCS3 on G-CSF-induced STAT5 activation

SOCS3 causes a dose-dependent reduction of G-CSF-induced STAT5 activity from the wt G-CSFR in luciferase reporter assays (van de Geijn et al., 2004b). Using this assay, we asked whether SOCS3-mediated inhibition of G-CSF signaling directly depends on the cytoplasmic lysines of the G-CSFR. As demonstrated previously, inhibition of STAT5 activation was lost completely after mutation of Y729 of the G-CSFR (mutant Y729F) (Figure 8A). Strikingly, mutant K5R was also hardly inhibited by SOCS3, indicating that lysines of the G-CSFR are crucial for SOCS3-controlled attenuation of STAT5 activation (Figure 8A). In contrast, adding back K632 (mKA) fully restored sensitivity to SOCS3 (Figure 8A). These inhibitory effects of SOCS3 on wt and mKA G-CSFR were lost upon removal of the SOCS box, consistent with the role of the SOCS box domain in recruiting ubiquitin ligase activity (Figure 8B). Individual add- backs of the lysines K672 (mKB), K681 (mKC) or K682 (mKD), restored SOCS3 responses only at the highest concentrations, whereas adding back K762 (mKE) had even less effect (Figure 8C). These results identify the juxtamembrane lysine K632 as the most critical target within the G-CSFR for SOCS3-mediated termination of STAT5 activation. In contrast

to SOCS3, the inhibitory effect of SOCS1 on STAT5 activation was not abrogated by mutation of Y729 of G-CSFR, supporting the notion that recruitment to G-CSFR Y729 is required for SOCS3, but not SOCS1 to inhibit STAT5 activation (Figure 8D). This result complies with a study showing that phosphorylated Y729-containing peptides have high affinity for the SH2 domain of SOCS3, but not SOCS1 (Hortner et al., 2002), but is in apparent conflict with a study showing some Y729 dependent inhibitory effects of SOCS1 on STAT5 activation (Zhuang et al., 2005). However, also in this latter study, the authors failed to demonstrate a physical interaction between G-CSFR-Y729 and SOCS1. Notably, the inhibitory effects of SOCS1 were not affected by mutation of K632 or other lysines in the G-CSFR cytoplasmic domain (Figure 8D), further arguing against a direct effect of SOCS1 on G-CSFR routing.

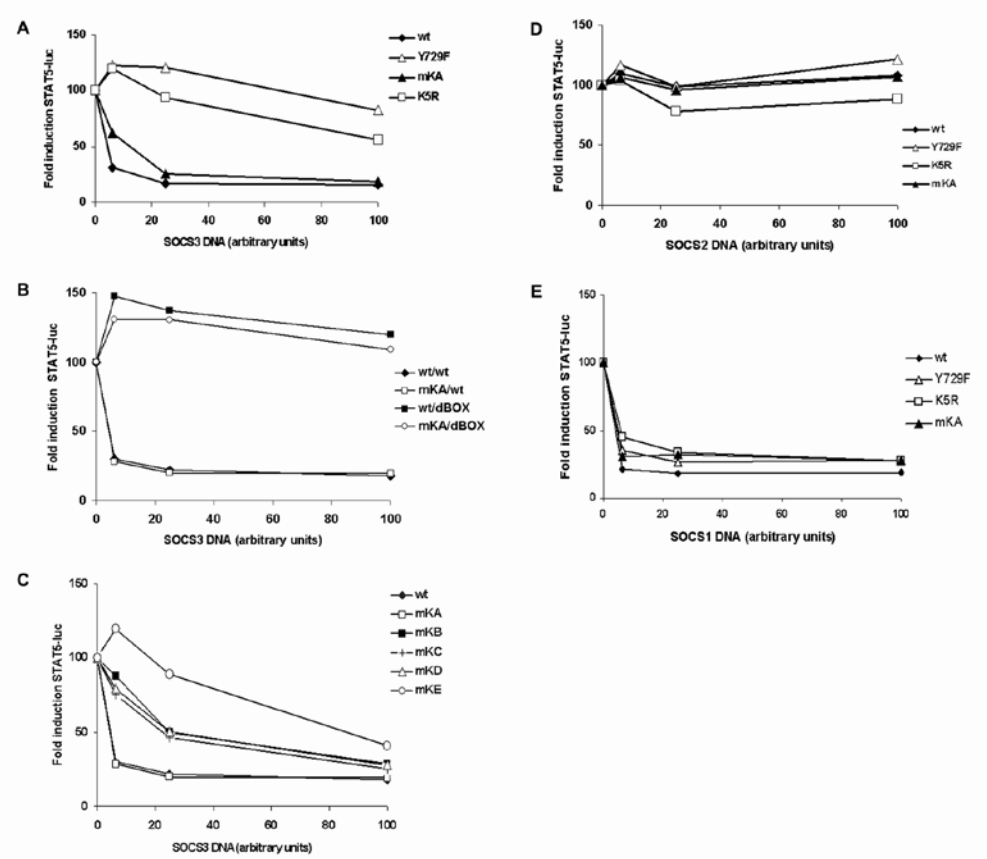


Figure 8. Role of lysines of G-CSFR in SOCS3-mediated inhibition of STAT5 activation

HEK293 cells were transfected with the indicated G-CSFR constructs, increasing amounts of SOCS3 (panel A and C), SOCS3 and SOCS3dBox (panel B), SOCS 2 (panel D) and SOCS1 (panel E) and a STAT5-responsive luciferase construct. SOCS protein levels were determined by Western blotting, of which a representative analysis is shown in supplementary Figure 6. For each G-CSFR construct, G-CSF-induced luciferase reporter activity in the absence of SOCS was set at 100%. Data are representative of 3 independent experiments each performed in triplicate.

Two recent studies have shown that SOCS2 interacts with and degrades SOCS3, thereby playing a unique role in cytokine signaling (Piessevaux et al., 2006; Tannahill et al., 2005). We therefore investigated the effects of SOCS2 on STAT5 activation by wt G-CSFR and mutants, but found that SOCS2 had no effect on G-CSF-induced STAT5 activation (Figure 8E) and did not neutralize the inhibitory effects of ectopically expressed SOCS3 (data not shown).

DISCUSSION

Role of receptor lysine K632 in G-CSFR routing and control of G-CSF signaling

In this study, we have shown that G-CSFR routing from early endosomes to lysosomes depends on the integrity of a single lysine, G-CSFR-K632, in the juxtamembrane cytoplasmic region and that maintaining an appropriate balance of G-CSF-induced signals largely depends on this mechanism. In contrast, G-CSFR internalization was not affected by substitution of any of the five conserved cytoplasmic lysines for arginines. This result differs from a recent study on the related LR, which showed that two lysines in the cytoplasmic domain are critical for internalization (Belouzard and Rouille, 2006). The reason for this discrepancy might relate to the fact that the LR lacks tyrosine or dileucine-based endocytosis motifs found in other class I cytokine receptor chains such as G-CSFR and gp130, and instead carries a Ub-based novel internalization motif (Belouzard and Rouille, 2006). However, it remains possible that the Ub-conjugation system controls G-CSFR endocytosis via an indirect mechanism, as has been demonstrated for GHR (Govers et al., 1999).

An important question is which effector protein(s) linked to K632 is responsible for lysosomal routing of G-CSFR. We considered the UIM-containing protein Hrs as the most plausible candidate, as it has been shown to form a multivalent complex, including other UIM-containing proteins such as STAM1/STAM2 and Eps15 that guides endocytosed membrane proteins to lysosomes (Bache et al., 2003). However, we were unable to demonstrate association of Hrs to G-CSFR in immunoprecipitation assays. Furthermore, siRNA-mediated knockdown of Hrs did not alter off-rate kinetics of STAT5 activation as seen with G-CSFR mutants lacking K632 (data not shown). It thus remains to be determined which proteins or protein complexes are crucial for ligand-induced lysosomal routing of G-CSFR and attenuation of STAT5 activation.

Consequences of G-CSFR routing for G-CSF signaling

Another major objective was to determine how intracellular trafficking impacts on G-CSF signaling. From the off-rate experiments with G-CSFR mutant K5R, which internalizes normally but fails to route to late endosomes and lysosomes, it can be deduced that activation of STAT5 and ERK continues in early endosomes. In contrast, PKB activation by K5R was terminated following G-CSF washout with kinetics similar to wt G-CSFR, whereas it was

sustained with internalization defective G-CSFR mutant d749-769 (Figure 4C). These findings suggest that distinct mechanisms of signal attenuation are involved in the termination of PKB versus STAT5 and ERK activation, depending on the intracellular localization of G-CSFR. Although this needs further study, several potential mechanisms can be envisaged. For instance, as demonstrated for the insulin receptor and the tyrosine phosphatase PTP1B (Romsicki et al., 2004), an endosome-associated phosphatase activity might be involved that affects PKB but not STAT5 and ERK. Alternatively, divergence in intracellular routing of signaling intermediates, for example, through caveolin-mediated internalization, may cause dissociation of the PI-3K/PKB pathway from the G-CSFR complex in early clathrin-coated endosome, as was recently shown for the abrogation of PI-3K/PKB signaling after internalization of integrin receptors (del Pozo et al., 2005).

SOCS3 as a key regulator of G-CSFR routing and STAT5 activation

Together with previous reports, our present data support a model in which SOCS3, recruited to the phosphorylated Y729 of G-CSFR following receptor activation, inhibits G-CSF signaling by two mechanisms (Figure 9). The first mechanism engages the kinase inhibitory region (KIR) of SOCS3 to inhibit JAK kinase activity and has been documented for a number of receptors (Kile and Alexander, 2001). The second, new mechanism supported by our data involves ubiquitination of the G-CSFR protein on a membrane proximal lysine, which triggers routing of the G-CSFR from an Hrs positive compartment to lysosomes. Although functionally distinct, these mechanisms do not necessarily have to act independently. For

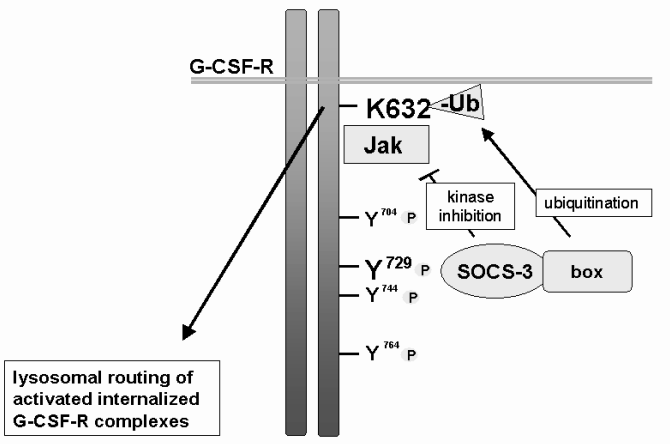


Figure 9. Model of SOCS3-controlled termination of G-CSF signaling

Following activation by G-CSF, G-CSFR recruits SOCS3 via phosphorylated tyrosine residue Y729. SOCS3 inhibits JAK activity via its kinase inhibitory region to inactivation of G-CSFR. This may also be achieved in part by direct recruitment of SOCS3 to JAK itself (Kile and Alexander, 2001). A second and novel mechanism involves recruitment of E3 ligase activity, leading to ubiquitination of K632 of G-CSFR. This triggers G-CSFR transition from Hrs-positive endosomes to lysosomes, attenuating activation of STAT5.

instance, the unidentified protein(s) recruited to Ub-K632 and involved in lysosomal routing may be negatively controlled by JAK activity, which would imply that the KIR of SOCS3 indirectly also controls lysosomal routing of G-CSFR. Conversely, the SOCS box of SOCS3 may also be involved in degradation of JAK proteins (Kile and Alexander, 2001), which in turn could have an effect on routing of G-CSFR.

The K632 of G-CSFR resides at position –5 relative to the box 1 domain (PGIPSP). Intriguingly, this lysine is conserved among several other class I cytokine receptors, for example, leptin receptor, gp130, IL-4R and IL-7R. This might be suggestive of a general role of this conserved juxtamembrane lysine and merits investigations aimed at determining whether control of receptor routing by members of the SOCS protein family may be a more common theme in cytokine receptor signaling.

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CHAPTER 3

Site-specific ubiquitination determines lysosomal sorting and signal attenuation of the G-CSF receptor

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(Manuscript submitted)

ABSTRACT

Ubiquitination of cytokine receptors controls intracellular receptor routing and signal duration but the underlying molecular determinants are unclear. The suppressor of cytokine signaling protein SOCS3 drives lysosomal degradation of the granulocyte colony-stimulating factor receptor (G-CSFR), depending on SOCS3-mediated ubiquitination of a specific lysine located in a conserved juxtamembrane motif. Here, we show that despite ubiquitination of other lysines positioning of a lysine within the membrane-proximal region is indispensable for this process. Neither reallocation of the motif nor fusion of ubiquitin to the C-terminus of the G-CSFR could drive lysosomal routing. However, within this region, the lysine could be shifted 12 amino acids towards the C-terminus without losing its function, arguing against the existence of a linear sorting motif and demonstrating that positioning of the lysine relative to the SOCS3 docking site is flexible. G-CSFR ubiquitination peaked after endocytosis, was inhibited by methyl-β-cyclodextrin and severely reduced in internalization defective G-CSFR mutants, indicating that ubiquitination mainly occurs at endosomes. Apart from elucidating structural and spatio-temporal aspects of SOCS3-mediated ubiquitination, these findings have implications for the abnormal signaling function of G-CSFR mutants found in severe congenital neutropenia, a hematopoietic disorder with a high leukemia risk.

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is the major cytokine involved in the production of neutrophilic granulocytes (Akbarzadeh and Layton, 2001; Avalos et al., 1990). G-CSF induces the proliferation and survival of myeloid progenitor cells, followed by a cell cycle arrest and neutrophilic differentiation (Touw and van de Geijn, 2007). This response depends on the balanced activation and subsequent attenuation of signaling pathways linked to the G-CSF receptor (G-CSFR), a member of the class I cytokine receptor superfamily (Touw and van de Geijn, 2007). Signal attenuation of G-CSFR is severely compromised by mutations causing truncations in the cytoplasmic domain of the G-CSFR, which are found in patients with a severe form of neutropenia and a predisposition to acute myeloid leukemia (Germeshausen et al., 2007). Two major mechanisms implicated in the perturbed signaling functions of these truncated G-CSFR mutants are the loss of a dileucine-based internalization motif(Hunter and Avalos, 1999; Ward et al., 1999) and the deletion of Y729, the tyrosine residue involved in the recruitment of the suppressor of cytokine signaling protein SOCS3 (Hermans et al., 2003; Hortner et al., 2002).

Covalent attachment of ubiquitin (Ub) to cytoplasmic lysine residues of activated membrane receptors is one of the major mechanisms involved in downregulation of signaling (Mukhopadhyay and Riezman, 2007). The process of ubiquitination requires Ub-activating (E1), -conjugating (E2) and -ligase (E3) activities. Substrate specificity is mediated by the E3 ligase, by recognizing and interacting with the target protein. The role of ubiquitination in internalization and intracellular degradation of membrane receptors has been best studied for receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) (Marmor and Yarden, 2004). Upon ligand binding, the E3 ligase Cbl is recruited to the tyrosine-phosphorylated receptor and induces ubiquitination of lysine residues located within the kinase domain (Huang et al., 2006). While ubiquitination of the EGFR itself is dispensable for receptor endocytosis, it is crucial for lysosomal routing and degradation of the EGFR (Huang et al., 2007; Marmor and Yarden, 2004). However, recent studies on other receptor systems, for instance the leptin receptor OB-Ra and the interferon α receptor type 1 (IFNAR1), have revealed that ubiquitination may also play a major role in receptor internalization (Belouzard and Rouille, 2006; Kumar et al., 2007), indicating that depending on the receptor type and cell context, E3 ligases control multiple steps in receptor trafficking and lysosomal degradation.

We recently reported that lysosomal routing and degradation of the granulocyte colony-stimulating factor receptor (G-CSFR) depends on recruitment of suppressor of cytokine-signaling 3 (SOCS3) to the activated receptor (Irandoust et al., 2007). Besides inhibiting signaling by interfering with JAK kinase activity through its kinase inhibitory region (KIR) (Yoshimura et al., 2007), SOCS3 forms a cullin-based E3 ligase complex by recruitment of

Elongin C and B as well as Cullin 2 via its SOCS box (a so called ECS E3 ligase) (Kamura et al., 2004). SOCS3-directed lysosomal routing and signaling downregulation of the G-CSFR was dependent on ubiquitination of lysine residues of the G-CSFR as well as on the presence of the SOCS box in SOCS3 (Irandoust et al., 2007). Importantly, *in vivo* evidence for a specific role of the SOCS box in G-CSFR negative signaling recently emerged from studies in knock-in mice expressing a truncated SOCS3 protein lacking the C-terminal SOCS box (Boyle et al., 2007).

A key finding of our previous study was that SOCS3-induced downregulation of STAT5 activity and lysosomal routing of the G-CSFR to a major extent depended on a juxtamembrane lysine residue at position 632 (K632), even though there are in total five conserved lysines present in the cytoplasmic domain of the G-CSFR (Irandoust et al., 2007). This result differed from studies showing that endocytosis and lysosomal routing could be triggered by a linear fusion of Ub to the cytoplasmic tail of a membrane receptor (Haglund et al., 2003; Hicke and Dunn, 2003; Raiborg et al., 2003; Sigismund et al., 2004). On the other hand, more recent studies have suggested that specific positioning of lysines within target proteins may be important for certain ubiquitination-dependent mechanisms because some E3 ligases preferentially select lysines next to their recruitment site within the target protein (Hunter, 2007). For example, this applies to the F-box protein β-Transducin repeat-containing protein (β-Trcp) (Laney and Hochstrasser, 1999), which together with the linker Skp1 and Cul1 forms a multiprotein SCF (Skp1-Cul1-F-box) E3 ligase complex. β-Trcp ubiquitinates lysine residues at position 9-13 upstream of its binding motif (Fuchs et al., 2004). Targets of β-Trcp include some cytokine receptors, like interferon α receptor 1 (IFNAR1) (Kumar et al., 2004; Kumar et al., 2003), prolactin receptor (Li et al., 2004) and the erythropoietin receptor (EPOR) (Meyer et al., 2007), but not the G-CSFR. Although ECS and SCF belong to the same cullin-RING ubiquitin ligase superfamily and share considerable structure similarity, nothing is known about preferential lysine selection by ECS E3 ligases, such as SOCS3. In addition to being important for accessibility to E3 complexes, positioning of a lysine residue within a target protein may also be critical for binding with a downstream interacting protein, as has recently been implicated to be of major importance to regulate IFNAR1 internalization (Kumar et al., 2007).

In the present study, we investigated how positioning of the critical lysine residue determines the dynamics of ubiquitination, lysosomal routing and signal attenuation of the G-CSFR. We show that lysosomal sorting activity of a ubiquitinated lysine is confined to the membrane-proximal region despite ubiquitination of other lysines. However, within this domain and relative to the SOCS3 binding site (Y729), positioning of the crucial lysine is to some extent flexible. We further show that G-CSFR ubiquitination predominantly takes place after receptor internalization. Finally, we demonstrate that a G-CSFR deletion mutant (d715) found in

patients with neutropenia is severely hampered in ubiquitination, which corroborates the importance of ubiquitination for proper G-CSFR function and provides a mechanism for the dysfunction of this receptor mutant associated with leukemic progression of this disease.

MATERIAL AND METHODS

Expression constructs

The constructs of human G-CSFR wt, K5R, K632R, mKA, mKB, mKC, mKD, mKE, d715 and d749-769 in pBabe and pLNCX2 vector have been described previously (Aarts et al., 2004; Irandoust et al., 2007). Domain-fusion G-CSFR constructs were generated by PCR using GRFR7 as a forward primer (Aarts et al., 2004) and a reverse primer spanning the last 21 nucleotides of the G-CSFR sequence excluding the stop codon, the 36 nucleotides corresponding to 12 amino acids of the juxtamembrane domain and box-1 region (R631 to P642), a stop codon and a *Bgl*II restriction site. G-CSFR wt, K5R and K632R were used as templates. PCR fragments were then digested with *Hpa*I and *Bgl*II (Aarts et al., 2004) and ligated in pBabe or pLNCX2 containing wt G-CSFR, which had also been digested with *Hpa*I and *Bgl*II. Other single amino acid changes and multiple amino acids insertions were created using oligonucleotides containing the desired mutations and Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The authenticity of all constructs was verified by restriction enzyme analysis and DNA sequencing.

To create the K5R G-CSFR-Ub fusion construct, a first PCR was performed using GRFR7 as a forward primer (Aarts et al., 2004) and a 3' chimeric oligonucleotide (G-CSFR/Ub), spanning a 3' segment of the G-CSFR and a 5' segment of Ub, as the reverse primer to amplify a C-terminal lysine-less G-CSFR fragment using K5R as template. Secondly, to amplify the Ub cDNA, a 5' chimeric oligonucleotide (Ub/G-CSFR), spanning the 5' segment of Ub and the 3' segment of the G-CSFR, and a 3' oligonucleotide spanning the 3' segment of Ub plus containing a *Bgl*II restriction site (Ub/*Bgl*II) was used with Flag-tagged ubiquitin in pCDNA3 (see below) as a template. Finally, the products of theses two PCR were isolated, mixed 1:1, and used as templates for a PCR using GRFR7 and Ub/*Bgl*II as primers. The resulting fragment was digested with *Hpa*I and *Bgl*II and ligated in pBABE or pLNCX2 containing wt G-CSFR, from which the wt *Hpa*I/*Bgl*II fragment has been removed. The G-CSFR-d715-Ub fusion construct was made in a similar way using GRFR7 and Ub/*Bgl*II together with the appropriate chimeric primers spanning the 5' segment of Ub and the 3' segment of d715.

Flag-tagged Ub in pCDNA3 was a gift from Dr. B. van der Reijden (Nijmegen, The Netherlands). SOCS3 expression constructs and reporter constructs for luciferase assays have been reported previously (van de Geijn et al., 2004).

Antibodies

Flag M2 monoclonal antibody (mAb) was purchased from Sigma (St Louis, MO). mAb anti-human CD114 (G-CSFR) and anti-CD114 coupled to phycoerythrin (PE) were from Becton-Dickinson/PharMingen (San Diego, CA). Mouse anti-ubiquitin (P4D1), rabbit anti-Rab7, mAb anti-HA and rabbit polyclonal anti-STAT5 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary goat anti-rabbit Alexa Fluor 546 and goat anti-mouse Alexa Fluor 488 were purchased from Molecular

Probes (Invitrogen, Breda, The Netherlands). For western blot analysis secondary goat anti-mouse and goat anti-rabbit either coupled to IRDyeTM680 or IR-DyeTM800CW were obtained from LI-COR Biosciences (Nebraska, USA). mAb anti-pSTAT5 (STAT5A-pY694, STAT5B-pY699) was from Upstate Biotechnology Inc. (Charlottesville, VA).

Cell culture, transfection and retroviral transduction

Phoenix E, HEK293 and HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM, glucose 4.5 g/l) supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin in humidified atmosphere at 37°C and 5% CO_2. HeLa cells were plated on glass coverslips and transiently transfected using lipofectamine (Invitrogen). Transfection of Phoenix E cells and HEK cells was done by the calcium phosphate precipitation method.

Murine myeloid 32D.cl8.6 and Ba/F3 cells, which both lack endogenous G-CSFR expression (de Koning et al., 1998), were cultured in RPMI-1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml murine IL-3 at 37°C and 5% CO_2. 32D.cl8.6 clones and Ba/F3 cells expressing different G-CSFR constructs were obtained by retroviral transduction as described (Aarts et al., 2004). Levels of G-CSFR expression were assessed by FACS analysis and clones with comparable G-CSFR expression were used in further experiments.

Bio-G-CSF pull downs

G-CSFR pull-down was performed with biotinylated G-CSF (bio-G) as described previously (Irandoust et al., 2007). Briefly, after transfection with G-CSFR constructs and Flag-tagged Ub, Phoenix E cells were incubated with bio-G (10 μg/ml; biotin labeling kit, Roche Molecular Biochemicals, Mannhein, Germany) for 15 min at RT and subsequently at 37°C for 30 min. Subsequently, cells were harvested, washed twice with ice-cold PBS and lysed in lysis buffer (20 mM Tris HCl pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP40, 10% glycerol, 2 mM Na_3VO_4 and 1 mM Pefablock SC, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml bacitracin, and 50 μg/ml iodoacetamide as protease inhibitors). After incubating the lysates with streptavidin-coated magnetic beads (Dynabeads, Invitrogen) for 1hr at 4°C, beads were washed, resuspended in Laemlli buffer (pH 11) and boiled for 5 min. Subsequent SDS-polyacrylamide gel electrophoresis and Western blotting was performed as described (Ward et al., 1999). Detection of proteins was done using fluorescently labeled secondary antibodies and the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA). Quantification of ubiquitination levels in Ba/F3 cells was done using the Odyssey Infrared Imaging System software according to the manufacturer's instructions.

For bio-G pull-down experiments with Ba/F3 cells stably expressing indicated G-CSFR mutants, cells (1×10^7) were washed in HBSS and resuspended in RPMI containing 250 nM bafilomycin (Calbiochem, Invitrogen). In experiments determining the role of endocytosis in G-CSFR ubiquitination, methyl-β-cyclodextrin (5 mM, Sigma-Aldrich, Zwijndrecht. The Netherlands) was added concomitantly with bafilomycin. After preincubation for 30 min at 37°C, bio-G was added for 1 hr. Subsequent procedures were done as described for Phoenix E cells.

Cell proliferation assay and G-CSF-induced STAT5 phosphorylation

To investigate the proliferation characteristics of 32D8.6cl clones transduced with various G-CSF constructs, cells were washed twice in Hank's balanced salt solution (HBSS) and seeded at a density of 1

x 10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS and 10 ng/ml human G-CSF. Viable cells were counted and readjusted to 1 x 10^5 cells/ml every second day.

To determine STAT5 phosphorylation off-rate kinetics after G-CSFR stimulation, Ba/F3 cells stably expressing the indicated G-CSFR mutants were washed twice in HBSS and serum and cytokine starved for 4 hours in RPMI-1640. After stimulation for 10 min with G-CSF (100 ng/ml), cells were washed twice with HBSS to remove G-CSF and monitored for residual STAT5 phosphorylation 60 and 120 min after stimulation. Cell lysates (2 x 10^6 cells) were prepared at the indicated time points and subjected to western blot analysis as described above.

Luciferase assay

STAT5 luciferase assays were performed as described (Hermans et al., 2003). Briefly, HEK293 cells were transfected by calcium phosphate precipitation with the indicated G-CSFR constructs, with or without a SOCS3 construct (G-CSFR DNA to SOCS3 DNA ratio 16:1) and a STAT5-responsive luciferase construct. After overnight starvation in DMEM + 1% BSA, cells were stimulated with 250 ng/ml G-CSF on the second day after transfection for 6 hrs and assayed for luciferase activity. All experiments were done in triplicate.

Immunocytochemistry and confocal microscopy

Visualization of lysosomal routing of membrane G-CSFR after receptor activation was done using confocal laser scanning microscopy (CLSM) as described (Irandoust et al., 2007). In brief, Hela cells transiently expressing indicated G-CSFR constructs were incubated with G-CSFR antibody and G-CSF for 15min at RT, washed twice and incubated at 37°C for 1 hr. Subsequently, cells were permeabilized with saponin, fixed with 3% paraformaldehyde and stained for internalized receptors (DaM-488) and Rab7 (GaR-546) as marker of lysosomes (Raiborg et al., 2002).

Statistics

Statistical significance was determined by a one-way ANOVA analysis followed by a two-sided Dunnett post-hoc test, unless otherwise stated. p-values <0.05 were considered significant.

RESULTS

Ubiquitination of cytoplasmic lysine residues after G-CSFR activation

Although the cytoplasmic domain of the G-CSFR contains five highly conserved lysines, SOCS3-induced downregulation of G-CSFR signaling via lysosomal routing and degradation criticallly depended on the presence of the juxtamembrane K632 (Irandoust et al., 2007). To test whether this relates to differential ubiquitination, we expressed the GCSFR mutant K5R, lacking all cytoplasmic lysine residues, or single lysine add-back mutants (mutants mKA to mKE) together with Flag-tagged Ub in phoenix E cells. After activation with biotinylated G-CSF (bio-G), ligand-bound receptors were pulled down using streptavidin-coated beads. Flag-immunoreactivity was observed with constructs mKA, mKB, mKC, and mKD and to a lesser extent with mKE, indicating robust ubiquitination of lysine residues K632, K672,

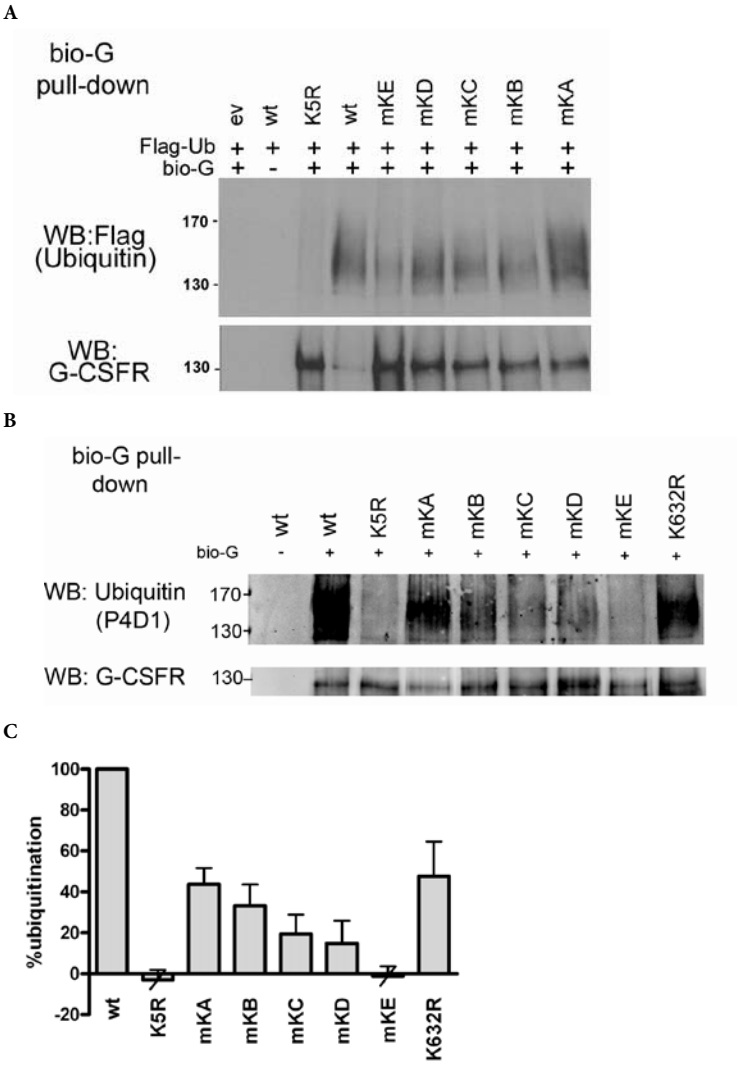


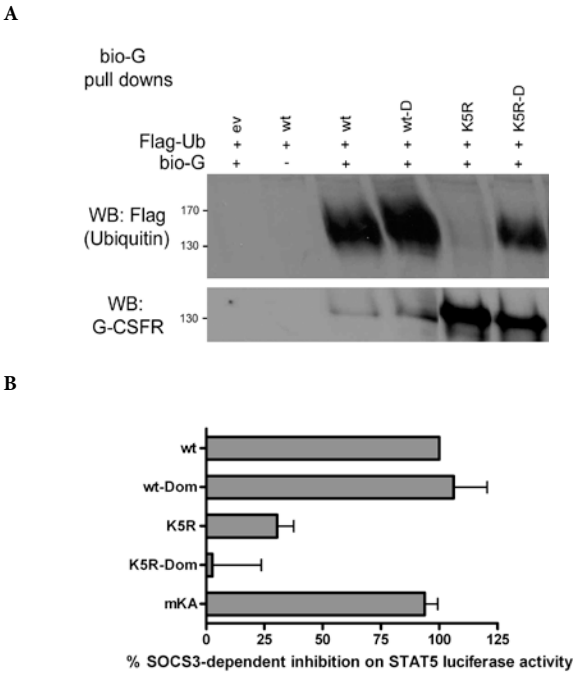
Figure 1. Ubiquitination of cytoplasmic lysine residues after G-CSFR activation

(A) Phoenix E cells co-transfected with the indicated G-CSFR constructs and Flag-Ub were incubated with biotinylated G-CSF (bio-G) for 15 min at 16°C and for 30min at 37°C. Ligand-bound receptors were precipitated using streptavidin-coated beads and analyzed by Western blotting using anti-Flag (upper panel) or anti-G-CSFR antibodies (lower panel). (B) Ba/F3 cells stably expressing the indicated G-CSFR mutants were preincubated with bafilomycin (250nM) for 30 min to inhibit acidification of late endosomes and then stimulated with biotinylated G-CSF for 60 min at 37°C. Ligand-bound receptors were precipitated using streptavidin-coated beads and analyzed by Western blotting using anti-Ub (upper panel) or anti-G-CSFR antibodies (lower panel). (C) The mean amounts of Ub in the G-CSFR immunoprecipitates were normalized to total receptor levels from four experiments performed as in B and expressed as a percentage (± SD) of the ubiquitination of WT G-CSFR. Receptor levels were lower for WT and mKA because the lysine on position 632 also regulates steady-state membrane expression of the G-CSFR (Meenhuis et al., Manuscript accepted)

K681, and K682 after receptor activation (see Figure 1A). To rule out that overexpression of Ub caused physiologically irrelevant ubiquitination on these lysines (Ea et al., 2006), we generated Ba/F3 cells stably expressing wt G-CSFR and lysine add-back mutants at levels comparable to primary bone marrow cells, without introducing ectopic Flag-Ub. Ubiquitination of G-CSFR could barely be detected with the Ub antibody P4D1, due to rapid lysosomal degradation (data not shown). Upon inhibition of lysosomal degradation using the specific inhibitor bafilomycin (Tanaka et al., 2007) we could readily detect a Ub smear with mutants mKA, mKB, mKC and mKD but not with mKE (Figure 1B). We thus confirmed that ubiquitination of K632, K672, K681 and K682 also occurred at physiological Ub levels. The amount of ubiquitination gradually declined from the membrane-proximal K632 to the more distal K682 (Figure 1C). Notably, substitution mutant K632R, which fails to route to lysosomes (Irandoust et al., 2007) is as robustly ubiquitinated as mKA, indicating that the overall level of ubiquitination is not the determinant for lysosomal targeting (Figure 1B and 1c, compare lanes 4 and 9).

K632 is not part of a motif that retains its function upon relocation

Because the unique involvement of K632 in lysosomal routing of G-CSFR could not be assigned to differences in ubiquitination levels, we wondered whether K632 is part of a motif, e.g., specifically recognized by proteins involved in lysosomal routing. To this end, we generated mutant K5R-D, in which amino acid stretch R631 to P642, was fused to the C-terminus



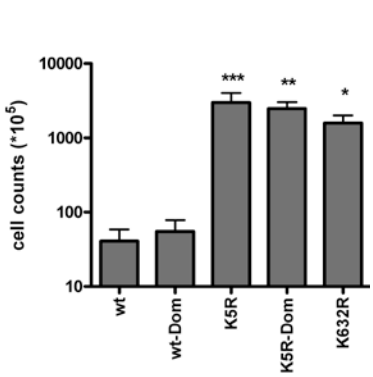


Figure 2. Ubiquitination and function of the K5R-D fusion G-CSFR mutant

(A) Phoenix E cells were co-transfected with the indicated constructs and incubated with bio-G as described in Figure 1a. Ligand-bound receptors were precipitated and analyzed by Western blotting using anti-Flag (upper panel) or anti-G-CSFR antibodies (lower panel). (B) HEK293 cells were transfected with the indicated G-CSFR constructs, a STAT5-responsive luciferase construct and with or without SOCS3. SOCS3-dependent inhibition of STAT5 luciferase activity observed by WT-G-CSFR was set at 100%. The mean (± SE) of three independent experiments done in triplicate is shown. (C) Proliferation of 32D cells expressing the indicated G-CSFR constructs in response to G-CSF (10 ng/ml). Mean cell numbers (± SE) from three to four independent cell clones per construct are shown. ** $p<0.01$, *** $p<0.001$, relative to wt G-CSFR.

of mutant K5R (see also scheme in Figure 4A). We first tested whether the relocated K632 in K5R-D is still ubiquitinated. Indeed, ubiquitination of K5R-D was clearly observed in Flag-Ub overexpressing Phoenix E cells (Figure 2A).

We then studied the functionality of the relocated motif using a STAT5 reporter assay (Irandoust et al., 2007). Whereas SOCS3 strongly inhibited STAT5 activation by wt and mKA G-CSFR, these inhibitory effects were not seen with mutant K5R-D (Figure 2B). In addition, stable expression of mutants K5R-D in 32D cells conferred a hyperproliferative response to G-CSF similar to K5R, indicating that, also in a myeloid cellular context, the lysine present in the domain fused to the C-terminus could not substitute for a properly positioned K632 to attenuate G-CSF signaling (Figure 2C).

Fusion of Ub to the G-CSFR C-terminus does not restore lysosomal sorting and signal downregulation

Fusion of Ub to either the C-terminus of a degradation-defective EGFR (Mosesson et al., 2003) or to an EGFR mutant lacking the whole cytoplasmic domain (Haglund et al., 2003) is sufficient to restore ligand-dependent internalization and lysosomal sorting of these receptor chimeras. Because ubiquitination of the K5R-D mutant varied with experimental conditions, we tested whether fusion of Ub directly to the C-terminus restored lysosomal sorting of K5R. However, contrary to wt G-CSFR and the Ub-tagged EGFR, K5R-Ub did not route to rab7

positive lysosomes, but accumulated in Hrs-positive early endosomes (Figure 3A), as was seen with K5R (Irandoust et al., 2007). Accordingly, prolonged STAT5 phosphorylation in both K5R as well as K5R-Ub expressing Ba/F3 cells was observed after removal of G-CSF (Figure 3B). These results indicate that fusion of Ub to the C-terminus of K5R is not sufficient to restore lysosomal sorting and signal downregulation in a lysine-deficient G-CSFR, corroborating our finding that site-specific ubiquitination of K632 is a prerequisite for lysosomal degradation of the G-CSFR.

Mutational analysis within the juxtamembrane domain and the box1 region reveals some flexibility in positioning of K632

The lysine within the juxtamembrane motif is highly conserved in the G-CSFR of different species as well as in related cytokine receptor chains such as gp130 and leptin receptor (Figure 4A). To further delineate how imperative the exact positioning of K632 is for its function, we inserted five alanines immediately upstream or downstream of K632 using mutagenesis PCR (mutants 5A-mKA and mKA-5A, respectively; see scheme Figure 4B). In these and the following mutants the remaining four lysines were replaced by arginines, to assure that the observed effects could be uniquely assigned to repositioning of the juxtamembrane lysine (Figure 4B). Insertion of 5 alanines either N- or C-terminal of K632 did not affect

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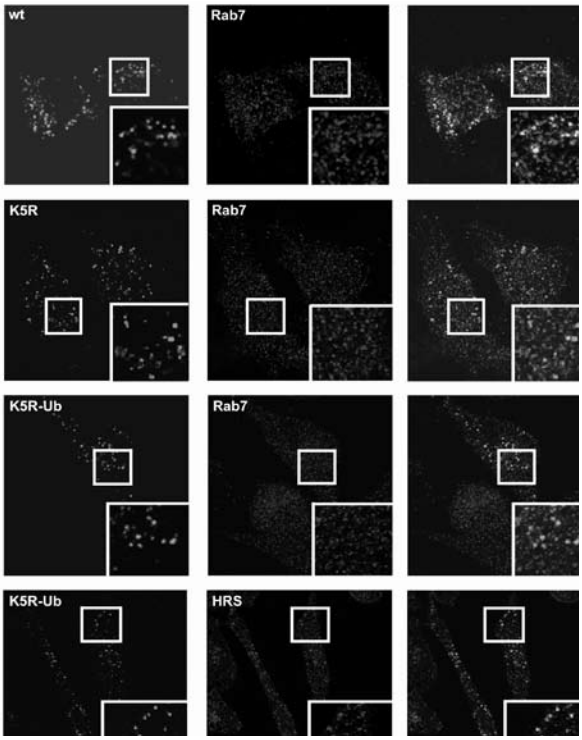

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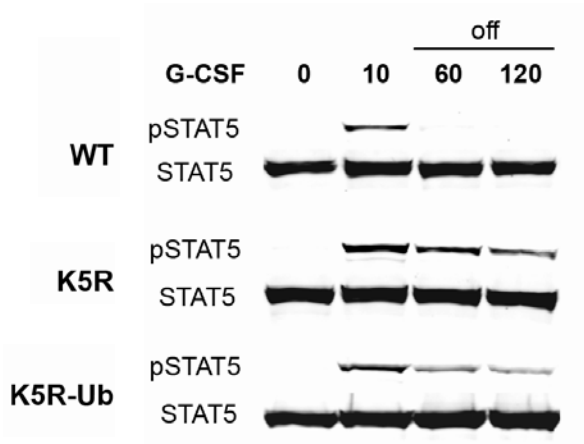

Figure 3. Fusion of ubiquitin to the C-terminus does not restore lysosomal sorting and downregulation STAT5 activation

(A) HeLa cells ectopically expressing WT, K5R or K5R-monUb G-CSFR were incubated with anti-G-CSFR antibodies and G-CSF for 20 min at 16°C, washed and transferred to 37°C for 60 min. Subsequently, cells were fixed and stained for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels indicating colocalization in yellow. Insets show enlargements of boxed areas. (B) Ba/F3 cells expressing WT or mutant G-CSFR were deprived of IL-3 and serum for 4h, incubated with G-CSF for 10 min at 37°C, washed and kept at 37°C in medium without factors for the indicated time periods. Cell lysates were then immunoblotted and stained with anti-pSTAT5 and anti-STAT5. Results of one out of four experiments are shown.

SOCS3-dependent inhibition of STAT5 luciferase activity in HEK cells stimulated with G-CSF (see Supplementary Figure 1A). In addition, 32D cells expressing these mutants showed proliferation characteristics comparable to cells expressing mKA (see Supplementary Figure 1B). These results show that neither a stringent proximity of a lysine to the cell membrane, nor a strict positioning relative to the SOCS3 binding site are required for downregulation of G-CSFR signaling.

To more precisely define the region within which the lysine retained its function, we 'walked' lysines through the juxtamembrane domain and the adjacent box 1-region of mutant K5R by changing single amino acids at the indicated positions into lysines (Figure 4B). Whereas a lysine residue on position 631, 633, 634, 638 and 644 was ubiquitinated comparably to K632 (Figure 4C), the mutants 628K and 630K were hardly ubiquitinated after G-CSF stimulation, suggesting that lysines at these positions are not accessible for SOCS3. Indeed, a lysine at position 628 or 630 failed to support SOCS3-mediated inhibition of G-CSF-induced STAT5 activation (Figure 5A), affected lysosomal routing (see Supplementary Figure 2), and led to prolonged STAT5 activity upon G-CSF removal (Figure 5B). In addition, 32D cell clones expressing 628K or 630K exhibited hyperproliferation in response to G-CSF comparable to

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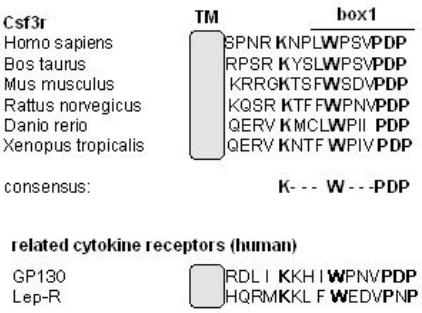

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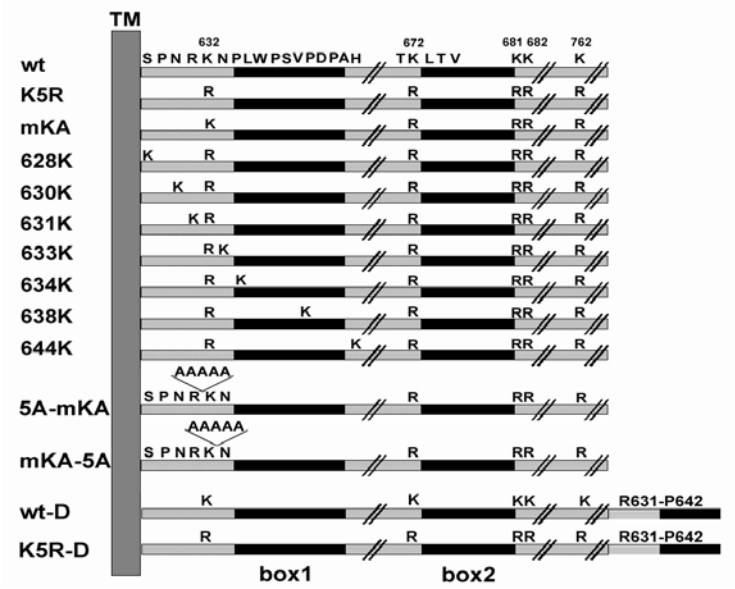

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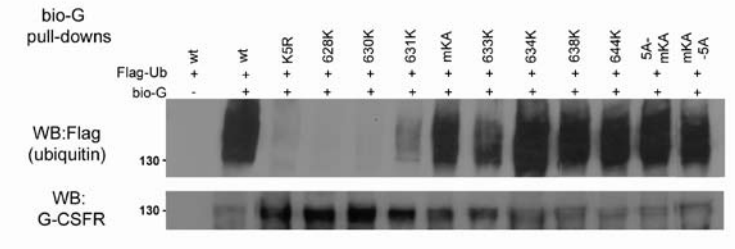

Figure 4. Ubiquitination of juxtamembrane alanine-insertion and 'walking' lysine G-CSFR mutants

(A) Schematic representation showing conservation of juxtamembrane motif in G-CSFR (CSF3R) and related cytokine receptors gp130 and leptin receptor. TM: transmembrane domain (B) Schematic representation of G-CSFR mutants used in this study. (C) Phoenix E cells cotransfected with the indicated G-CSFR constructs and Flag-Ub were incubated with bio-G as described in Figure 1a. Ligand-bound receptors were precipitated using streptavidin-coated beads and analyzed by Western blotting using anti-Flag (upper panel) or anti-G-CSFR antibodies (lower panel).

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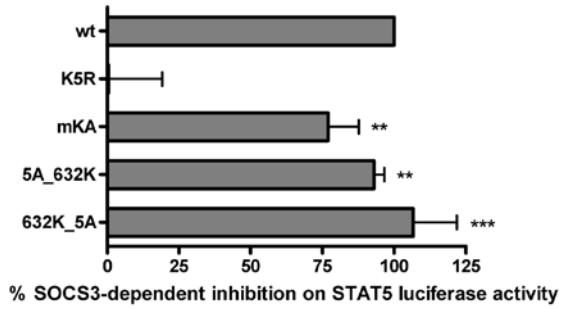

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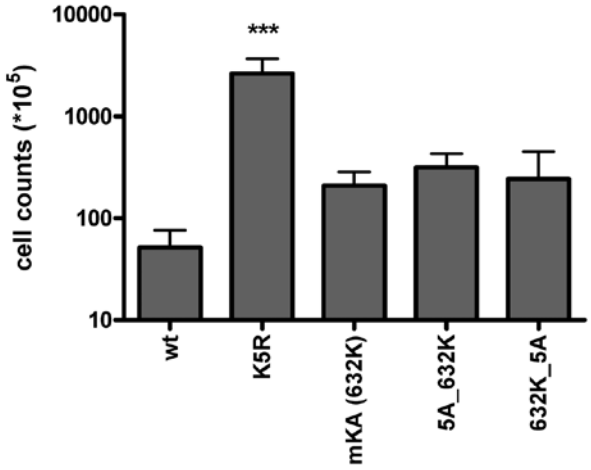


Supplementary figure 1. Insertion of 5 alanines either up- or downstream of K632 does not affect K632-mediated G-CSFR signal downregulation

(A) STAT5-responsive luciferase assays with or without SOCS3 were done as described in Figure 2c. The mean (± SE) of three independent experiments done in triplicate is shown. (B) Proliferation in 32D cells expressing the indicated G-CSFR constructs. Numbers (± SE) of cells of three to four independent cell clones per constructs after 10 days of G-CSF treatment are given.

clones expressing K5R (Figure 5C). In contrast, lysines on position 631, 633, 634, 638 and 644 were ubiquitinated (Figure 4B) and these G-CSFR mutants showed normal lysosomal routing (see Supplementary Figure 2), SOCS3 dependent downregulation of G-CSF-induced STAT5 luciferase activity and attenuation of STAT5 phosphorylation (Figure 5A and 5B). G-CSF-induced growth characteristics of 32D cells expressing G-CSFR mutants with a lysine at position 631, 633 and 634 were indistinguishable from mKA, but cells expressing mutants 638K and 644K displayed an intermediate phenotype in terms of cell proliferation (Figure 5C). This suggests a gradual decline of functionality of lysines moved towards box-1. Finally, moving the lysine downstream to position 672 resulted in a complete loss of functionality (Figure 5A

and 5B). Taken together, these results indicate that, within the membrane-proximal region, repositioning of the lysine towards the TM region is not tolerated, whereas there is some flexibility towards the C-terminus. However, moving the lysine upstream of position 631 results in an abrupt loss of ubiquitination and function, while positioning towards box-1 and further downstream leads to a decreased functional activity eventually resulting in a complete loss of function within the box-2 region.

G-CSFR ubiquitination increases during endocytosis

Because ubiquitination of receptor lysines is not the prime determinant for G-CSFR internalization (Irandoust et al., 2007), we sought to determine when and in which intracellular compartment(s) ubiquitination of activated G-CSFR takes place. In Ba/F3 cells ubiquitination of the wt G-CSFR steadily increased over time and was maximal 30 min after receptor activation (Figure 6A). Because G-CSFR endocytosis is completed within minutes (Aarts et al., 2004), these data imply that G-CSFR ubiquitination mainly occurs at early endosomes. This is further substantiated by our finding that ubiquitination of wt G-CSFR was significantly inhibited by methyl-ß-cyclodextrin (mßCD), a drug which impairs clathrin-mediated endocytosis of the G-CSFR (Figure 6A). In addition, an internalization defective G-CSFR mutant (d749-769), which lacks the critical dileucine-based endocytosis motif (Aarts et al., 2004), showed a markedly delayed and reduced ubiquitination compared to wt G-CSFR, despite much higher membrane expression levels (Supplementary Figure 3). These results suggest that ubiquitination of G-CSFR increases progressively during endocytosis, possibly due to a more efficient recruitment of the ubiquitination machinery to early endosomes than to the plasma membrane.

Fusion of ubiquitin to G-CSFR-d715 restores internalization but not lysosomal sorting

Somatically acquired nonsense mutations in the CSF3R gene are found in patients with severe congenital neutropenia (SCN) who develop acute myeloid leukemia (AML)(Dong et al., 1995; Germeshausen et al., 2007; Link et al., 2007). The truncated G-CSFR forms caused by these mutations, e.g. G-CSFR-d715, lack both the internalization motif and the binding site for SOCS3 (Y729). As a consequence, activated G-CSFR-d715 is hardly ubiquitinated (Figure 6B) and is not routing to late endosomes/lysosomes (Figure 6C), thus resulting in prolonged STAT5 phosphorylation after G-CSF washout (Figure 6D). However, while internalization of G-CSFR-d715 is clearly inhibited compared to wt G-CSFR (compare Figure 7A, middle and left panel), some endocytotic vesicles containing internalized G-CSFR-d715 can be detected 30 min. after G-CSF stimulation (Figure 7A, middle panel).

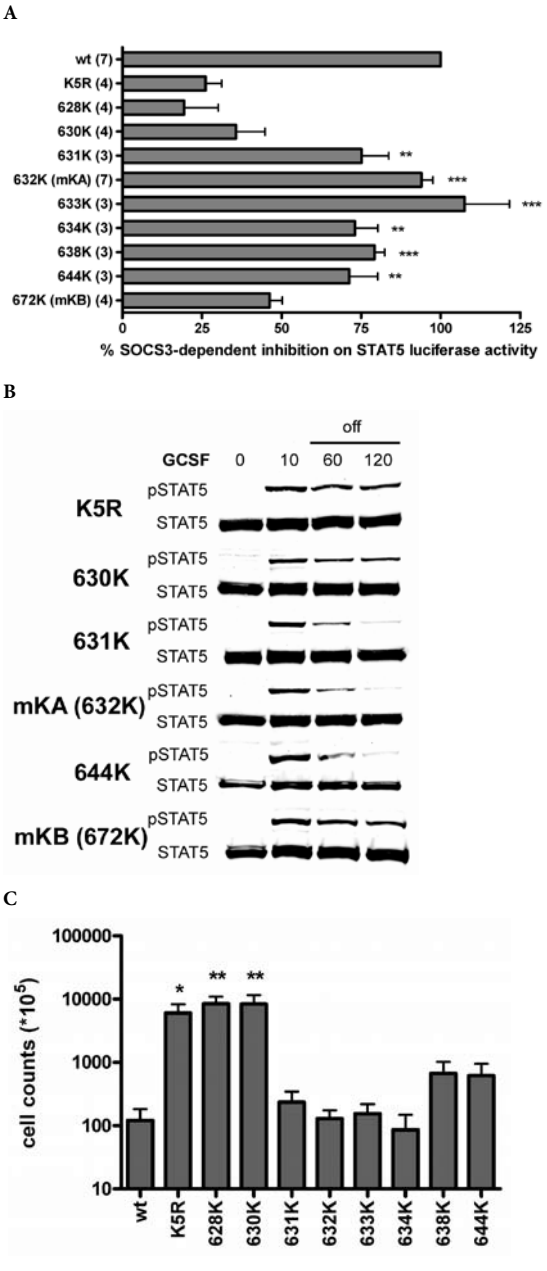
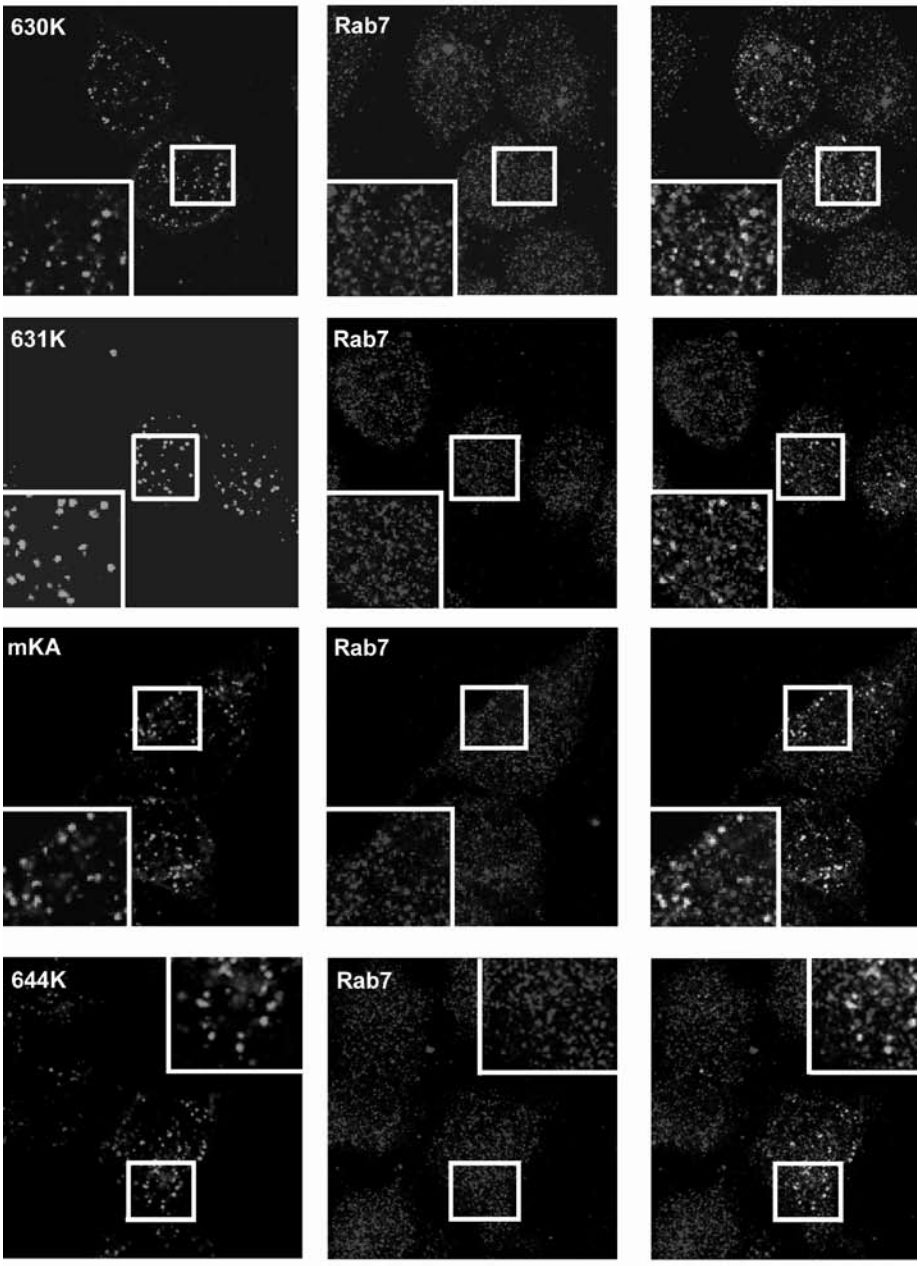


Figure 5. Position of a lysine within the juxtamembrane and box-1 region determines its role in G-CSFR signal downregulation

(A) STAT5-responsive luciferase assays with or without SOCS3 were done as described in Figure 2C. Mean values ± SE of at least three independent experiments (the total number of experiments for each construct is shown in parenthesis) each done in triplicate are shown. ** $p<0.01$, *** $p<0.001$ (B) STAT5 phosphory-lation patterns in Ba/F3 cells expressing indicated mutant G-CSFR as described in detail in Figure 3B. (C) G-CSF-induced proliferation in 32D cells expressing the indicated G-CSFR constructs as described in detail in Figure 2C. * $p<0.05$, ** $p<0.01$



Supplementary figure 2. Lysosomal routing of selected 'walking' lysine G-CSFR mutants

HeLa cells ectopically expressing indicated G-CSFR mutants were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow. Insets show enlargements of boxed areas

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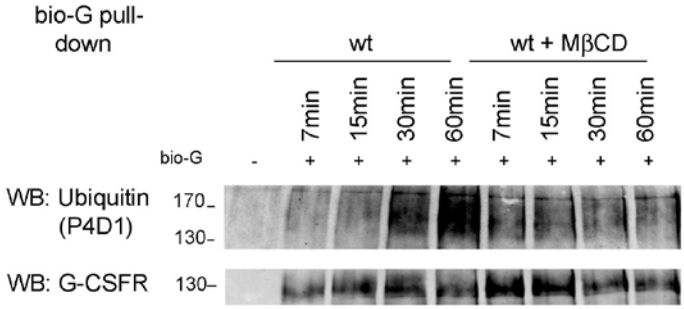
bio-G pull-down
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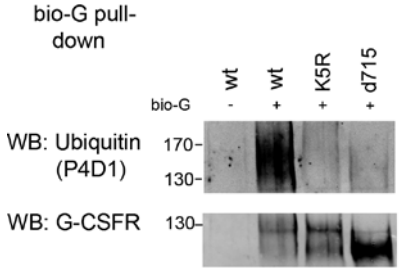
bio-G pull-down
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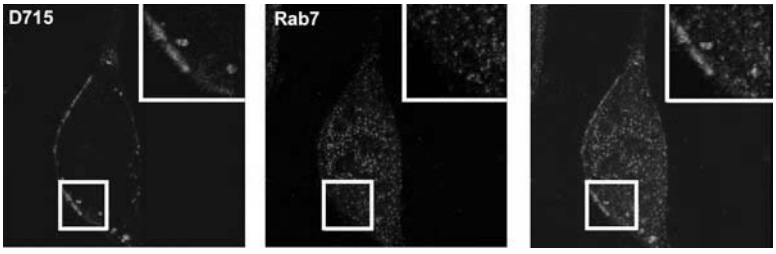
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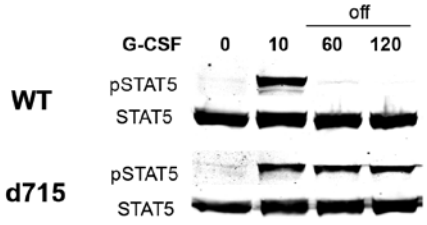
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Figure 6. G-CSFR ubiquitination is inhibited by methyl-β-cyclodextrin (mßCD) and a disease-associated G-CSFR mutant (d715) lacks ubiquitination completely

(A) and (B) Ba/F3 cells stably expressing the indicated G-CSFR mutants were preincubated with bafilomycin ± mßCD and stimulated with bio-G either for the indicated time periods (A) or 60 min (B) as described in Figure 1b. Ligand-bound receptors were precipitated using streptavidin-coated beads and analyzed by Western blotting using anti-ubiquitin (upper panel) or anti-G-CSFR antibodies (lower panel). (C) HeLa cells ectopically expressing G-CSFR_d715 mutants were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized G-CSFR (green, left panels) and the late endosomal-lysosomal marker rab7 (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow. (D) STAT5 phosphorylation patterns in Ba/F3 cells expres-sing WT or G-CSFR-d715 as described in detail in Figure 3B.

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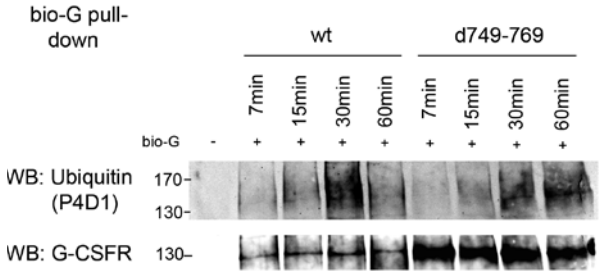

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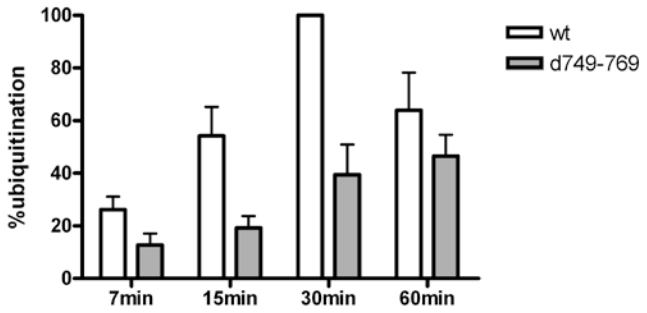

Supplementary Figure 3. A G-CSFR mutant lacking the di-leucine-based internalization motif (d749-69) is hampered and delayed in ubiquitination

Ba/F3 cells stably expressing the indicated G-CSFR mutants were preincubated with bafilomycin and stimulated with bio-G either for the indicated time periods as described in Figure 1b. (A) Ligand-bound receptors were pre-cipitated using streptavidin-coated beads and analyzed by Western blotting using anti-Ub (upper panel) or anti-G-CSFR antibodies (lower panel). (B) Quantification of data as shown in A. The mean amounts of Ub in the G-CSFR immunoprecipitates were normalized to total receptor levels from three experiments and expressed as a percentage (± SD) of the ubiquitination of WT G-CSFR.

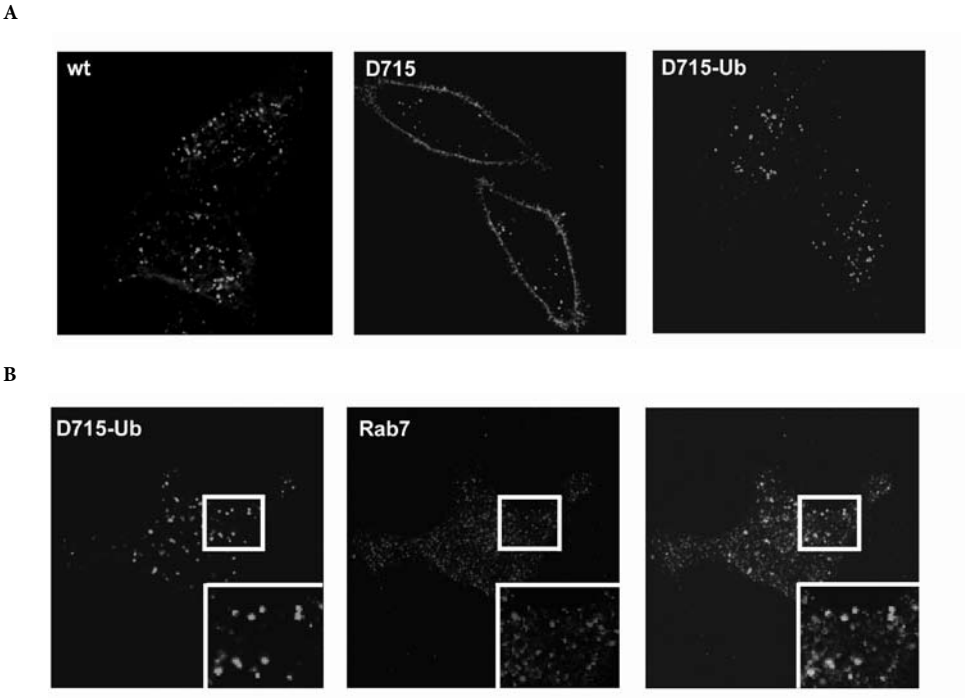

Figure 7. Fusion of ubiquitin to the C-terminus of G-CSFR_d715 restores internalization but not lysosomal sorting

(A) HeLa cells ectopically expressing wt, d715 or d715-Ub G-CSFR were labeled with anti-GCSFR antibody prior to incubation with G-CSF for 30min. After washing cells were permeabilized, stained with a secondary goat anti-mouse Alexa Fluor 488 and analyzed by CLSM. (B) HeLa cells ectopically expressing G-CSFR_d715-Ub were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow.

In view of the reported effects of Ub on internalization of the OB-Ra and IFNAR1 receptors (Belouzard and Rouille, 2006; Kumar et al., 2007), we wondered whether fusion of Ub to the C-terminus of G-CSFR-d715 could restore endocytosis and then promote lysosomal routing, given that K632 is preserved in this mutant. Indeed, we observed that attachment of a Ub moiety resulted in the rapid formation of intracellular vesicles after ligand binding, comparable to wt G-CSFR (Figure 7A, right panel). However, in contrast to wt G-CSFR, but similar to K5R-Ub (Figure 3A), d715-Ub failed to enter rab7 positive late endosomes/lysosomes (Figure 7B). These results establish that fusion of Ub to the C-terminus of d715 can overcome its internalization defect as was recently also reported for a leptin receptor isoform lacking endocytotic motifs (Belouzard and Rouille, 2006), but cannot restore lysosomal sorting. This emphasizes the marked distinction between Ub-mediated endocytosis, for which positioning of the Ub appears quite irrelevant for its function, and lysosomal targeting, for which site-specific ubiquitination in the juxtamembrane region is crucial. The data also indi-

cate that even when G-CSFR-d715 is artificially forced to route to early endosomes, this does not suffice to trigger lysosomal routing, implicating that the loss of SOCS3 activity recruited via Y729 of G-CSFR is not being compensated for by other E3 complexes once the receptor reaches the early endosome compartment.

DISCUSSION

In this study, we provided direct evidence that site-specific ubiquitination of a juxtamembrane lysine controls lysosomal sorting of the G-CSFR and that disturbances thereof result in perturbation of G-CSF signaling. In addition, our data suggest that, instead of preferential lysine selection by the ubiquitination machinery, selectivity of the lysine may stem from the interaction of the specifically ubiquitinated cargo protein with downstream sorting proteins. Finally, the observation that G-CSFR ubiquitination peaks around 30 min after activation and that an internalization defective G-CSFR mutants showed delayed and reduced ubiquitination indicates that ubiquitination predominantly takes place in early endosomes.

We observed robust ubiquitination of four of the five lysines within the cytoplasmic domain of G-CSFR. The detection of Ub smears ranging from 135 to 170 kDa rather than discrete single ubiquitinated forms of the G-CSFR add-back mutants is indicative of polyubiquitination of these lysines. This is in line with the emerging concept that activated membrane receptors predominantly undergo poly- rather than mono-ubiquitination, which then provokes sorting of receptors from early to late endosomes/lysosomes (Huang et al., 2006; Traub and Lukacs, 2007; Umebayashi et al., 2008). The finding that a single lysine (K632) is not only sufficient but also exclusive to direct lysosomal sorting and signal downregulation of the G-CSFR argues against an earlier assumption, derived from studies on Ub-dependent proteasomal degradation, that multiple lysines can act as redundant Ub acceptor sites (King et al., 1996). It also challenges the current view that the amount of poly-Ub attached to a receptor, rather than the specific site of ubiquitination, is important for lysosomal sorting (Huang et al., 2006; Mukhopadhyay and Riezman, 2007; Umebayashi et al., 2008). This latter notion is supported by the observation that although ubiquitination levels of mutants mKA and K632R were comparable, their intracellular behaviour after activation was entirely different: mKA is sorted to lysosomes with normal signal downregulation, whereas K632R accumulates in early endosomes and displays prolonged signaling (Irandoust et al., 2007). However, in recent years it has been increasingly recognized that for functions other than protein degradation specific lysine residues are ubiquitinated and that the position of these lysines can be functionally important. For instance, mutation of 5 lysines within the kinase domain of the EGFR to arginine is already sufficient to inhibit its degradation, although there are in total 28 lysines present in the cytoplasmic domain (Huang et al., 2006). Similarly,

lysosomal degradation of the IFNAR1 is dependent on three out of seven cytoplasmic lysines (Kumar et al., 2003). Together with our data presented here, these findings imply that site-specific ubiquitination, rather than a certain amount of (poly-)Ub, is essential for directing lysosomal sorting of these membrane receptors.

Transposition of the entire juxtamembrane domain surrounding the functional lysine to the C-terminus of a lysine-less G-CSFR did not restore lysosomal routing and signal downregulation. On the other hand, the position of the lysine within the membrane-proximal region of the G-CSFR was to a limited extent flexible. These findings have several implications: First, the flexible position within the membrane-proximal region argues against the idea that K632 is part of a strictly conserved spatial motif, in which positioning of the lysine relative to other structurally important residues is crucial for interaction with proteins involved in lysosomal sorting. However, our findings do not definitely rule out that there is a so far unknown linear motif within the G-CSFR in proximity to the functional lysine, which may cooperate with the ubiquitinated lysine in regulating lysosomal sorting. Cooperation between site-specific ubiquitination and a linear endocytic motif nearby has recently been implicated in ligand-induced IFNAR1 internalization by regulating interaction of the receptor with the AP2 adaptin complex (Kumar et al., 2007). Second, the prerequisite for a membrane-proximal positioning of the lysine suggests an imperative role of the cell membrane in the interaction of the ubiquitinated G-CSFR with certain components of the lysosomal sorting machinery. Indeed, several sorting proteins, like Hrs, epsins, and some members of ESCRT complexes contain binding sites for membrane phosphoinositides in addition to their Ub binding domains (Slagsvold et al., 2006). For instance, EAP45/Vps36, a component of the ESCRT-II complex that is responsible for sorting cargo proteins to the lumenal vesicles of multi-vesicular bodies/late endosomes, interacts with Ub and membrane posphoinositides with its GLUE domain (Slagsvold et al., 2005; Teo et al., 2006). Crystal structures of the EAP45 GLUE domain/Ub complex revealed that their binding sites are linked physically indicating cooperative binding of Ub and phosphoinositides (Alam et al., 2006). It is tempting to speculate that interaction of the G-CSFR with proteins involved in this sorting step, like EAP45, relies on ubiquitination of a properly positioned juxtamembrane lysine of the G-CSFR.

In a recent study employing a temperature sensitive E1 expressing cell line (ts20), it was demonstrated that inhibition of E1 activity interferes with internalization of the G-CSFR (Kindwall-Keller et al., 2008). These results suggest that internalization of the activated G-CSFR requires intact ubiquitination machinery. This might be interpreted as contradictory to our results, showing that lysine-less G-CSFR mutant K5R internalizes normally. However, as has been demonstrated for the growth hormone receptor, this may involve ubiquitination of intermediate proteins that link a nonlysine-containing ubiquitin-dependent endocytosis motif within the receptor to the clathrin-mediated endocytosis machinery (Govers et al., 1999).

Fusion of Ub to the internalization-deficient G-CSFR-d715 restored internalization implicating that Ub acts as an endocytotic signal independent of its positioning. Accordingly, fusion of Ub to the C-terminus of MHC I molecules (Duncan et al.,2006), CD33 (Walter et al., 2008), and leptin receptor (Belouzard and Rouille, 2006) resulted in ligand-induced and/or constitutive internalization of these fusion proteins. These studies commonly establish that Ub can act as an endocytotic signal independent of its positioning. This emphasizes the marked distinction between Ub-mediated endocytosis, for which positioning of Ub appears nonessential, and lysosomal sorting, for which site-specific ubiquitination in the juxtamembrane region is crucial.

Although our observations are limited so far to the G-CSFR, site-specific ubiquitination of a juxtamembrane lysine may also be implicated in regulating lysosomal sorting of other receptors. Intriguingly, a lysine exactly corresponding to the crucial K632 in the G-CSFR (position -5 to box-1) is found to be highly conserved in many cytokine receptors, like gp130, LR, IL-4R and IL-7R. In addition, given the flexible position of the lysine within this region, other cytokine receptors harbor a conserved lysine within their juxtamembrane domain in close proximity to the position of K632 in the G-CSFR, e.g. the EPOR at position -4 to box-1. Finally, the finding that the SCN/AML derived mutant G-CSFR-d715 is not only grossly delayed in internalization but is also completely blocked in its lysosomal degradation provides an additional mechanism for its ability to induce prolonged STAT5 activation, a feature that was recently linked to clonal dominance of hematopoietic stem cells expressing such truncated G-CSFR forms and to its predicted role in the leukemic evolution of the disease (Liu et al., 2008).

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CHAPTER 4

Janus kinases promote cell surface expression and provoke autonomous signaling from routing defective G-CSF receptors

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ABSTRACT

The G-CSF receptor (CSF3R) controls survival, proliferation and differentiation of myeloid progenitor cells via activation of multiple Janus kinases (JAKs). In addition to their role in phosphorylation of receptor tyrosines and downstream signaling substrates, JAKs have recently been implicated in controlling expression of cytokine receptors, predominantly by masking critical motifs involved in endocytosis and lysosomal targeting. Here we show that increasing the levels of JAK1, JAK2 and TYK2 elevated steady state CSF3R cell surface expression and enhanced CSF3R protein stability in hematopoietic cells. This effect was not due to inhibition of endocytotic routing, since JAKs did not functionally interfere with the dileucine-based internalization motif nor lysine-mediated lysosomal degradation of CSF3R. Rather, JAKs appeared to act on CSF3R in the biosynthetic pathway at the level of the ER. Strikingly, increased JAK levels synergized with internalization or lysosomal routing defective CSF3R mutants to confer growth factor independent STAT3 activation and cell survival, providing a model for how increased JAK expression and disturbed intracellular routing of CSF3R synergize in the transformation of hematopoietic cells.

INTRODUCTION

The granulocyte colony-stimulating factor receptor (G-CSFR, now referred to as CSF3R) is the major hematopoietic growth factor receptor involved in neutrophil development (Avalos et al., 1990). The CSF3R contains four conserved tyrosine residues in the cytoplasmic domain, which upon phosphorylation by Janus tyrosine kinases (JAKs) become docking sites for Src homology 2 (SH2)-containing signaling proteins such as signal transducer and activator of transcription (STAT)-3, the adapter proteins SHC and GRB2, and suppressor of cytokine signaling (SOCS)-3 (Touw and Bontenbal, 2007). The cytoplasmic domain of CSF3R further comprises a dileucine-based motif crucial for internalization (Aarts et al., 2004), and five conserved lysine (K) residues. We have recently shown that one of these lysines, the juxtamembrane K632, is the major determinant for ligand-induced lysosomal routing of the activated CSF3R, a process that involves SOCS3-mediated ubiquitination of K632 (Irandoust et al., 2007).

Ligand-induced CSF3R internalization and lysosomal sorting are both crucial for a balanced signaling output and disruption of either of these processes results in increased proliferation signaling at the expense of G-CSF-induced neutrophilic differentiation (Aarts et al., 2004; Irandoust et al., 2007). Perturbed signaling from CSF3R, owing to mutations in the *CSF3R* gene or overexpression of signaling defective splice variants, has been implicated in the development of acute myeloid leukemia (AML) (Touw and van de Geijn, 2007). Importantly, C-terminal truncation mutants of CSF3R found in patients with severe congenital neutropenia that are at high risk of developing AML lack the dileucine-based internalization motif as well as the recruitment site Y729, essential for SOCS3-induced ubiquitination of K632 (Irandoust et al., 2007; Ward et al., 1999b). While signal attenuation involving ligand-induced endocytosis and lysosomal degradation has been studied in considerable detail for different receptor systems, less attention has been paid to mechanisms regulating ligand independent distribution and stability of cytokine receptors and their consequences for signal intensity and duration.

Recently, JAKs have been implicated in the regulation of cell surface expression of various cytokine receptors. For instance, JAK1 binding to the oncostatin M receptor (OSMR) has been suggested to mask a signal within the juxtamembrane part of the receptor that inhibits expression of the mature form of the OSMR at the plasma membrane (Radtke et al., 2006). Similarly, binding of JAK2 or TYK2 to the thrombopoietin receptor (TpoR) augmented membrane expression and stability of its mature (EndoH resistant) form (Royer et al., 2005; Tong et al., 2006). Studies on the interferon receptor-α1 (IFNAR1) revealed that binding of TYK2 attenuates degradation and increases cell surface expression by masking dileucine- and tyrosine-based internalization motifs present in the JAK binding domain of the receptor (Kumar et al., 2008; Ragimbeau et al., 2003). Taken together, these findings fit into a model in which JAKs interfere with the function of motifs involved in receptor endocytosis and

lysosomal degradation, thereby increasing receptor expression on the cell surface. On the other hand, an alternative mechanism was demonstrated for erythropoietin receptor (EpoR), where JAK2 binding does not affect receptor endocytosis, but enhances the appropriate folding of the EpoR protein in the ER, leading to more efficient Golgi processing and enhanced forward routing of mature protein to the cell surface (Huang et al., 2001).

Increased JAK expression has been implicated in several hematopoietic malignancies, including AML, and amplification of the *JAK2* gene has been reported in Hodgkin's lymphoma (Joos et al., 2000; Rucker et al., 2006). In the present study, we examined whether JAKs control the expression of CSF3R and how this might affect signaling in the absence of ligand-induced receptor activation. We show that JAKs elevate the steady state CSF3R cell surface expression but that this occurs independent of the major determinants of receptor internalization (dileucine motif) or lysosomal routing (conserved lysine residues). Strikingly, increased JAK levels resulted in growth factor independent cell survival and spontaneous STAT3 activation in cells expressing internalization or lysosomal routing defective CSF3R mutants.

MATERIAL AND METHODS

Antibodies

Mouse anti-human CSF3R (un- or PE- conjugated) were purchased from Becton-Dickinson/PharMingen (San Diego, CA). Monoclonal rabbit anti-JAK2, polyclonal rabbit anti-JAK1, rabbit anti-TYK2, rabbit anti-pSTAT3 and the secondary goat anti-rabbit coupled to horseradish peroxidase (GAM-HRP) were obtained from Cell Signaling Technology (Danvers, MA). Mouse anti-pSTAT5a/b was obtained from Upstate Biotechnology (New York, USA). Goat anti-actin and mouse anti-ubiquitin (P4D1) were purchased form Santa Cruz Biotechnology inc. (Santa Cruz, CA). Anti-mouse CD2 coupled to fluorescein isothiocyanate (FITC) and anti-human CD4-FITC were purchased from Becton Dickinson Biosciences (BD Biosciences, Erembodegem, Belgium). Secondary goat anti-mouse, goat anti-rabbit and donkey anti-goat either coupled to IRDyeTM680 or IR-DyeTM800CW were obtained from LI-COR Biosciences (Nebraska, USA). Goat anti-mouse Ig coupled to phycoerythrin (GAM-PE) was obtained from Dako (DakoBV, Heverlee, Belgium). Donkey anti-mouse Alexa fluor 488 and 7-AAD were purchased from Molecular Probes (Invitrogen, Breda, The Netherlands).

Expression constructs

The pLNCX and pBabe retroviral plasmids containing wild type (wt) CSF3R, the single lysine mutants K632R, K672R, K681R, K682R, K762R , the lysine null mutant K5R, and the single lysine add-back mutants mKA, mKB, mKC, mKD and mKE were described previously (Irandoust et al., 2007), as were the W650R and the d715 receptor mutants (Barge et al., 1996; Dong et al., 1993). The pSG513 vector with wt JAK2 and the bicistronic vectors pRex TYK2-IRES-CD2, pRex JAK1-IRES-CD2 were previously reported (Royer et al., 2005; van den Akker et al., 2004).

Cell culture, transfection and retroviral transduction

Murine myeloid 32D.cl8.6, a subline of the interleukin 3 (IL-3) dependent murine myeloid 32Dcl3 cell line that lacks endogenous CSF3R expression (Greenberger et al., 1983) and Ba/F3 cells, which are mouse pro-B cells that lack endogenous CSF3R (Greenberger et al., 1983; Palacios and Steinmetz, 1985) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 ng/ml murine IL-3, 100 IU/ml penicillin and 100μg/ml streptomycin at 37°C and 5% CO2. Ba/F3 cells expressing murine JAK1, murine JAK2 or human TYK2 have been described previously (Royer et al., 2005). Phoenix E, HeLa and the parental human fibrosarcoma cells 2C4 (Watling et al., 1993) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO2. JAK1-deficient U4C (Muller et al., 1993) and JAK2-deficient γ2A (Watling et al., 1993) human fibrosarcoma cell lines were cultured in DMEM containing 400 μg/ml geneticin (G418). The TYK2-deficient human fibrosarcoma cell line 11.1 (Velazquez et al., 1992) was cultured in DMEM in the presence of 400 μg/ml hygromycin. Ba/F3 cells and 32D cells were retrovirally transduced with virus from the Phoenix E packaging cell line (G. Nolan, Stanford, CA) as previously described (Aarts et al., 2004).

Constitutive and ligand-induced internalization determined with flow cytometry

For constitutive internalization 32D cells expressing wt or K5R CSF3R were incubated with mouse anti-human CSF3R in PSA (PBS supplemented with 1% v/v FCS and 0.05% w/v NaN3) for 1 hr at 4°C. After washing, cells were incubated at 37°C for 0, 30, 60, 120 and 240 min. Next, cells were stained with secondary antibody goat anti-mouse-PE in PSA for 1 hr at 4°C and, after a final wash, analyzed by flow cytometry using a FACS Calibur (Becton-Dickinson Biosciences). Mean fluorescence intensity of histograms was taken as a measure of the average CSF3R expression.

To study ligand-induced internalization of the CSF3R along with JAK overexpression, Ba/F3 cells were incubated with 10 ng/ml human G-CSF for 30 min. at 4°C. Thereafter the cells were incubated at 37°C for 0, 15, 30 and 60 min. Cell surface expression of the G-CSF receptor was determined by labeling the cells with PE-conjugated mouse anti-human CSF3R antibody for 1 hr at 4°C followed by flow cytometric analysis.

Receptor protein stability assay

Phoenix E cells were transiently transfected with CSF3R constructs in pBabe with or without co-transfecting murine JAK2 using calcium phosphate precipitation. Two days after transfection, cells were incubated for 3 hrs with 50 μg/ml cycloheximide (CHX) to block protein synthesis or left untreated. Ba/F3 cells stably transduced with CSF3R and JAK vectors were treated with 50 μg/ml CHX for 5 hrs. Cells were washed with cold PBS and resuspended in lysis buffer containing 20 mM Tris-HCl pH8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% v/v NP-40, 10% v/v Glycerol, 50 μg/ml aprotinin, 1 mM pefablock and 2 mM Na3VO4. Lysates were put on ice for 30 min, centrifuged for 15 min. at 14,000 rpm to remove insoluble proteins. CSF3R was pulled down from lysates by adding magnetic protein G-beads (Dynabeads, Invitrogen) pre-incubated with CSF3R o/n at 4°C. Beads were incubated in Laemmli buffer for 5 min. at 95°C and then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting as described previously (Ward et al., 1999a). Proteins were detected by fluorescently

labeled secondary antibodies followed by detection using Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

Ubiquitination of CSF3R

Ba/F3 cells were incubated with 500 nM bafilomycin A1 (Calbiochem, Darmstadt, Germany) for 30 min. Cells were stimulated with biotinylated G-CSF (Bio-G) prepared using a biotin labeling kit according to manufacturers protocol (Roche Molecular Biochemicals, Mannheim, Germany) for 1 hr at 37°C. Cells were washed twice with cold PBS and lysed. CSF3R was pulled down using streptavidin coated magnetic beads (Dynabeads, Invitrogen). Beads were washed and resuspended in Laemmli buffer, incubated for 5 min. at 95°C and subjected to Western blotting as described (Ward et al., 1999a).

Confocal microscopy

HeLa cells were seeded on glass cover slips and transfected with wt or K5R CSF3R in pLNCX using lipofectamin (Invitrogen). Cells were prepared and analyzed by confocal microscopy using a LSM510 microscope equipped with argon and He/Ne lasers as described (Irandoust et al., 2007).

Statistical analysis

For comparing two groups Student *t*-tests were performed. When comparing multiple groups One-Way ANOVA was performed followed by a post-hoc test.

Endoglycosidase H (EndoH) treatment

Phoenix E cells were transiently transfected with wt CSF3R. For membrane receptor pull-down, cells were incubated with 1:100 BioG for 30 min. at 37°C. Cells were then washed twice with cold PBS and resuspended in lysis buffer. For total CSF3R levels cells were first lysed and subsequently 1:50 BioG was added o/n at 4°C. CSF3R was pulled down using streptavidin-coated magnetic beads. For EndoH treatment (New England Biolabs, Ipswich, United States), beads were washed and resuspended in 50µl 1x GD denaturation buffer and boiled for 10 min. at 100°C. After adding 10x G5 reaction buffer, the supernatant was divided into two portions, to one of which EndoH was added and to the other H2O as a control. The samples were incubated for 3 hrs at 37°C and thereafter resuspended in Laemmli buffer, boiled for 5 min. at 95°C and subjected to Western blotting.

Proliferation and survival assay

JAK2 overexpressing Ba/F3 cells stably expressing wt, K5R or d715 were washed and transferred to RPMI medium without growth factors. Cells were counted and cell viability was assessed by flow cytometry using 7-AAD on a daily basis. For the inhibitor study, 10 µM LY 294002, 10 µM JAK inhibitor I, 10 µM SB 203580, 10 µM U0126, 0.2 µM Akt inhibitor IV or 10 µM PP2 (Calbiochem, Darmstadt, Germany) all dissolved in DMSO, or DMSO as solvent control was added to the medium. Half of the medium was replaced daily by fresh medium with fresh inhibitors.

RESULTS

JAKs enhance CSF3R cell surface expression

To determine the effects of elevated JAK1, JAK2 or TYK2 expression on CSF3R cell membrane levels, we used the previously described Ba/F3 cell system co-expressing CD2 or CD4 from an IRES-containing expression (Royer et al., 2005). First, we confirmed that the amount of CD2 or CD4 on the cell surface, determined by flow cytometry, correlated with the expression of the different JAKs, determined by Western blot analysis (Figure 1A and 1B). Parental Ba/F3 cells and the JAK-overexpressing cells were then retrovirally transduced to express wt CSF3R and cell membrane expression was determined by flow cytometry. JAK1, JAK2 or TYK2 transduced cells showed a 2- to 4-fold higher level of CSF3R expression on the cell surface compared to parental control cells (Figure 1C). Complementary to the experiments in the Ba/F3 transfectants, we studied the consequences of the absence of each of these JAK proteins on CSF3R expression using JAK-deficient fibrosarcoma cell lines (Figure 1D). All cell lines showed comparable cell surface expression of CSF3R, implicating that JAK1, JAK2 and TYK2 are redundant in controlling steady-state cell CSF3R expression (Figure 1E). These results raised the additional question whether CSF3R cell surface expression would be significantly hampered in the absence of any JAK binding. To address this, we introduced the CSF3R mutant W650R, which fails to activate JAKs (Barge et al., 1996), into Ba/F3 cells. CSF3R-W650R was severely hampered in both JAK2 binding and activation (Figure 2A and 2B). In agreement with its inability to bind JAKs, no significant increase in cell surface expression of CSF3R-W650R was seen in the JAK overexpressing Ba/F3 cells relative to parental controls (Figure 2C). Notably, membrane expression of CSF3R-W650R in parental Ba/F3 cells was equal to wt CSF3R, suggesting that JAK binding is not required for CSF3R expression as such (Figure 2C).

Role of receptor lysines in steady state cell surface expression and stability of CSF3R

One of the mechanisms implicated in the JAK-mediated effects on cell surface expression of cytokine receptors is that motifs involved in receptor endocytosis and lysosomal degradation are masked by steric interference (Kumar et al., 2008; Radtke et al., 2006; Ragimbeau et al., 2003). We previously demonstrated that ubiquitination of lysines in the intracellular domain of CSF3R mediated by G-CSF-induced expression of the E3-ligase SOCS3 is important for ligand-induced receptor endocytosis (Irandoust et al., 2007). However, we did not analyze whether this ubiquitination of lysine residues affects ligand-independent receptor endocytosis. We first studied membrane expression of a receptor mutant in which all 5 cytoplasmic

lysines were replaced by arginines (mutant K5R). Myeloid 32D cells stably expressing K5R showed higher membrane expression levels compared to wt CSF3R (Figure 3A), a result that was confirmed by confocal microscopy in 32D cells (Figure 3B, upper panel) and in

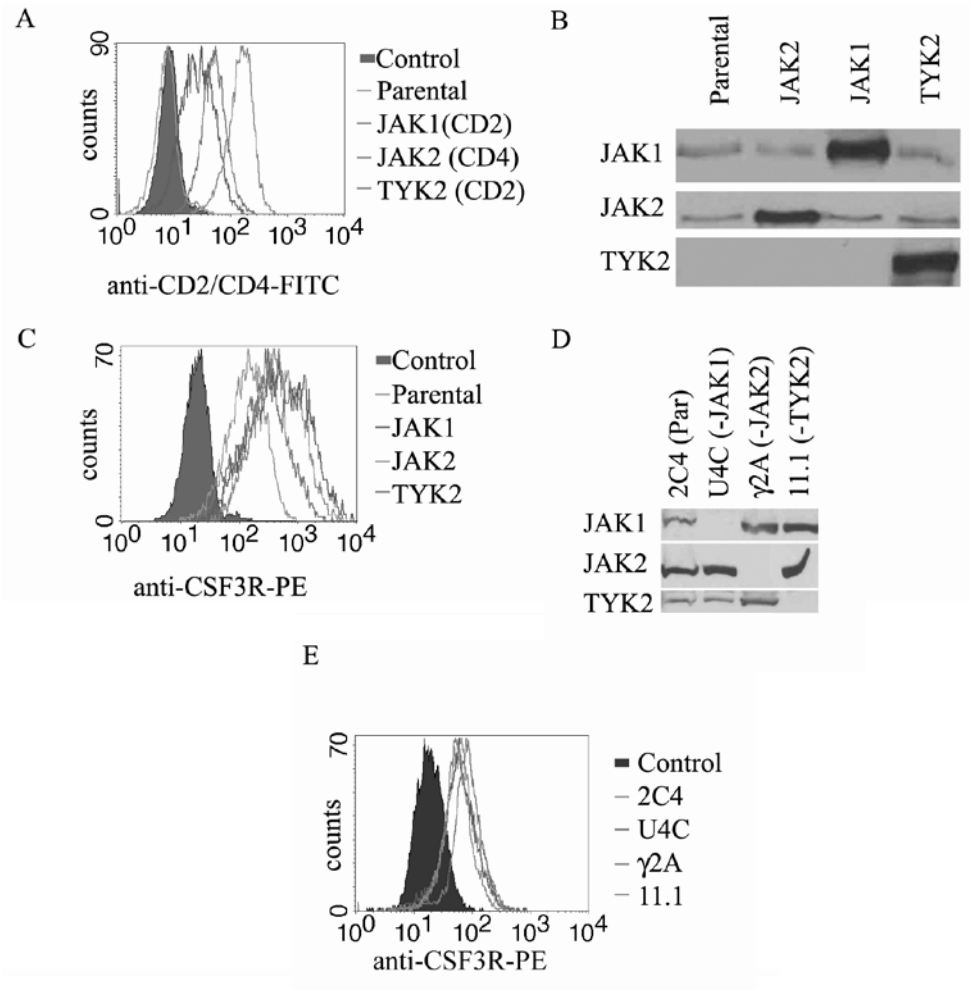


Figure 1. JAK1, JAK2 and TYK2 augment cell surface expression of CSF3R

(A) JAK overexpression in Ba/F3 cells assessed by flow cytometry using CD2- or CD4-FITC antibodies. Control is Ba/F3 cells without antibody staining. (B) Western blot analysis showing JAK levels in parental Ba/F3 cells and JAK transfectants. (C) Expression of wt CSF3R in stably transduced Ba/F3 cell lines determined by flow cytometry using CSF3R-PE antibody. Control is Ba/F3 parental cells without antibody staining. (D) Immunoblot of total cell lysates from human parental fibrosarcoma cells (2C4) and derivatives deficient for either JAK1, JAK2 or TYK2 (U4C, γ2A and 11.1, respectively). Cells were stained for anti-JAK1, anti-JAK2 and anti-TYK2 to confirm JAK-deficiencies. (E) Flow cytometric analysis of CSF3R cell surface levels in the human fibrosarcoma cell lines stably transduced with wt CSF3R. Control is 2C4 cells without antibody staining.

HeLa cells (Figure 3B, lower panel). However, kinetics of ligand independent internalization of wt and K5R CSF3R were similar, indicating that lysines are not critical in this process (Figure 4A). Taken together, these results suggest that lysines within the CSF3R cytoplasmic

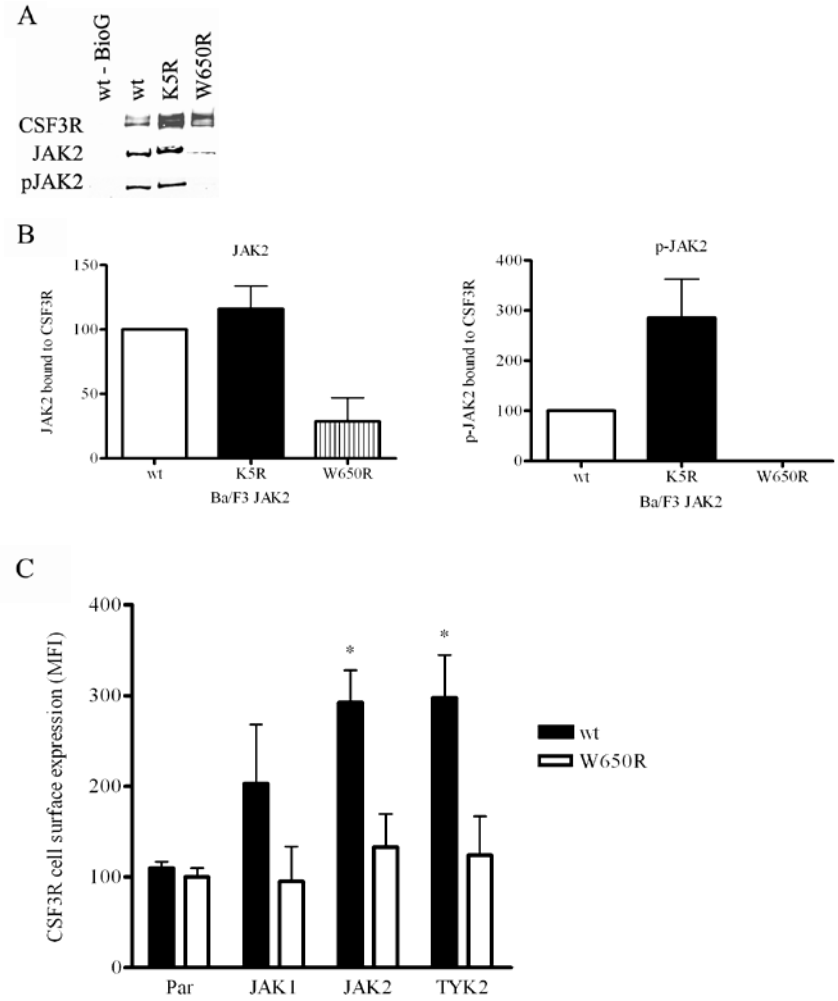


Figure 2. JAK-induced surface expression of CSF3R depends on a tryptophan residue (W650) that is critical for JAK binding

(A) JAK2 overexpressing Ba/F3 cells with the indicated CSF3R variants were stimulated with biotinylated G-CSF (Bio-G) for 30 min. Receptor complexes were isolated using streptavidin pull down and analyzed by Western blotting for the presence of CSF3R, JAK2 or phospho-JAK2. A representative blot of three independent experiments is shown. (B) Quantification of total JAK2 (left panel) and phospho-JAK2 (right panel) binding to wt CSF3R, mutant K5R and W650R. Data are expressed as mean ± SD of three experiments. (C) Expression of mutant W650R relative to wt CSF3R in Ba/F3 parental and JAK overexpressing cells. Cell surface expression was determined by flow cytometry. Data are expressed as mean fluorescence intensities (MFI) ± SEM. *P< 0.05 compared to parental Ba/F3 cells expressing wt CSF3R.

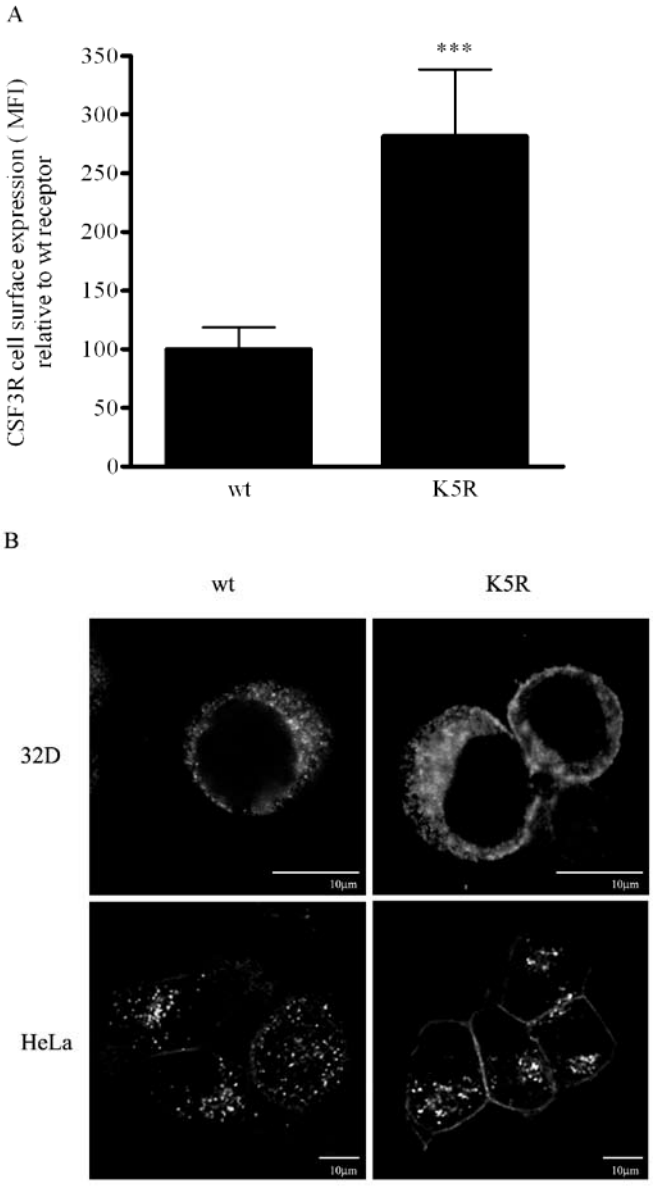


Figure 3. Role of receptor lysines in CSF3R cell surface expression

(A) Sublines of 32D cells were generated with wt or K5R CSF3R. Membrane expression was determined by flow cytometry using CSF3R-PE antibody. Results are shown as MFI ± SEM of 12 clones relative to wt receptor. ***P< 0.001 compared to wt receptor. (B) Confocal microscopy images of 32D clones stably expressing wt or K5R CSF3R (upper panel) and HeLa cells transiently transfected with wt or K5R CSF3R constructs (lower panel).

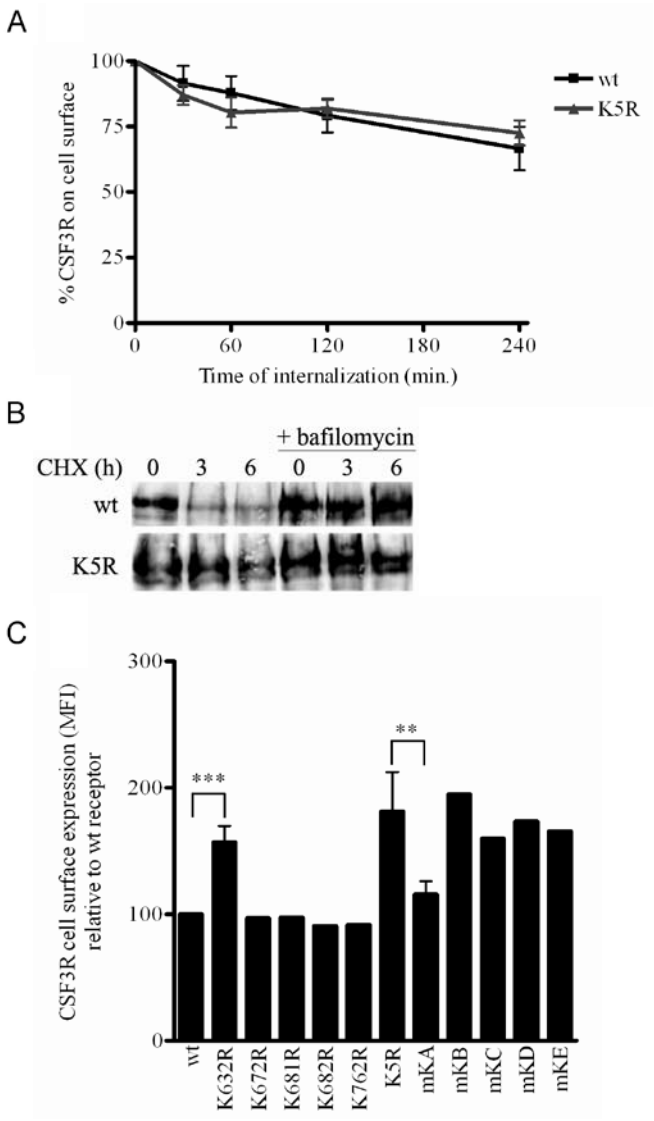
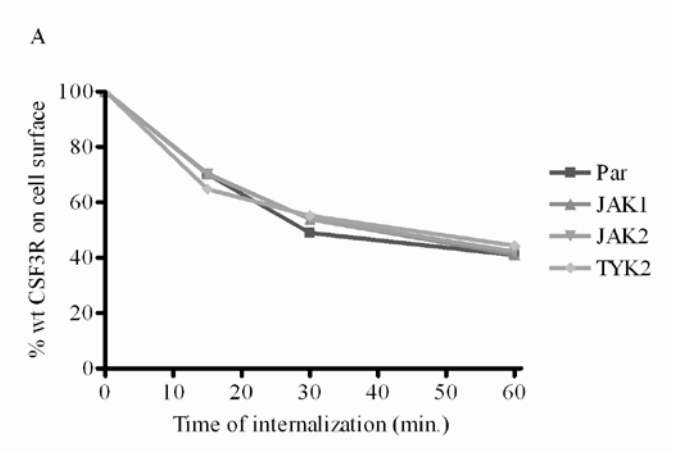
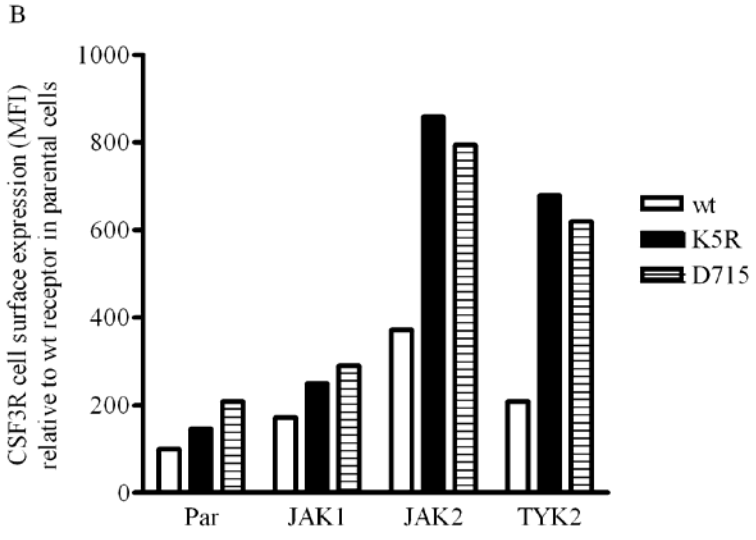


Figure 4: Lysines are important for steady state CSF3R cell surface expression but are not involved in constitutive internalization

(A) 32D cells stably expressing wt or K5R CSF3R were labeled with CSF3R antibody and were allowed to internalize during 4 hrs in the absence of ligand. CSF3Rs remaining at the cell surface were labeled with goat anti-mouse-PE and analyzed by flow cytometry. Data from three independent experiments are expressed as MFI ± SEM relative to t=0, which was set at 100%. (B) Protein synthesis in Ba/F3 cells stably expressing wt or K5R was blocked by CHX added to the medium for 0, 3 and 6 hrs. The amount of CSF3R in total cells lysates was visualized on Western blot using CSF3R specific antibody (lanes 1-3). Addition of the lysosomal inhibitor bafilomycin shows that CSF3R is constitutively degraded via the lysosomal pathway (lanes 4-6). (C) CSF3R cell surface expression on Ba/F3 parental cells transduced with wt CSF3R, lysine substitution (K632R, K672R, K681R, K682R and K762R) or add-back mutants (mKA, mKB, mKC, mKD and mKE). Data are expressed as in A. ***P<0.001 K632R compared to wt CSF3R. **P<0.01 K5R compared to mKA CSF3R.

region are involved in control of membrane expression through the biosynthetic pathway. To confirm this, we studied the role of the receptor cytoplasmic lysines in CSF3R stability. To this end, Ba/F3 cells stably expressing wt or K5R CSF3R were incubated for 3-6 hrs with cycloheximide (CHX) to inhibit de novo protein synthesis. While degradation of wt CSF3R was observed after inhibition of protein synthesis, K5R CSF3R was found to be stable (Figure 4B). The lysosome inhibitor bafilomycin prevented degradation of wt CSF3R, suggesting that steady state receptor levels are regulated via lysosomal degradation and that lysines are important for this mechanism.





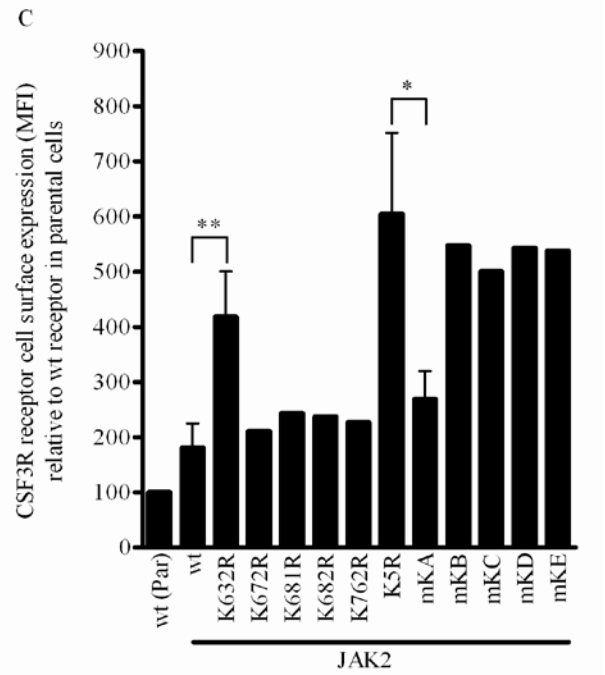


Figure 5: Enhanced CSF3R cell surface expression by JAKs is not due to masking of internalization or lysosomal sorting motifs

(A) Ba/F3 parental and JAK overexpressing cells stably expressing wt CSF3R were incubated with G-CSF (100 ng/ml) and at indicated time points stained with anti-CSF3R-PE followed by flow cytometric analysis. Data are expressed as mean fluorescence intensities relative to t=0, which was set at 100%. (B) Cell surface expression of internalization defective CSF3R-d715 and lysosomal routing defective K5R is strongly increased by JAK2 or TYK2 and to a lesser extent JAK1 overexpression. Receptor membrane expression was determined as in A and data are expressed as MFI relative to parental Ba/F3 cells expressing wt CSF3R (set at 100%). (C) CSF3R cell surface expression on JAK2 overexpressing Ba/F3 cells transduced with wt CSF3R, lysine substitution (K632R, K672R, K681R, K682R and K762R) or add-back mutants (mKA, mKB, mKC, mKD and mKE) and parental Ba/F3 cells transduced with wt receptor (set at 100%). Data are expressed as in 4C. **P<0.01 K632R compared to wt CSF3R. *P<0.05 K5R compared to mKA CSF3R.

The lysines in the cytoplasmic domain of CSF3R are fully conserved between multiple species. Using a panel of lysine substitution (K→R) and single lysine add-back (mKA-mKE) mutants, we investigated which of these lysines are involved in the negative control of steady state CSF3R membrane expression. Among the 5 conserved lysines, K632 is the major inhibitory determinant for constitutive CSF3R cell surface expression (Figure 4C).

JAKs do not mask motifs involved in CSF3R endocytosis or lysosomal routing

Next we examined the kinetics of CSF3R internalization in parental and JAK transfected Ba/F3 cells. As shown in Figure 5A, none of the JAKs had a significant influence on the kinetics and magnitude of CSF3R endocytosis. Studying the effects of JAKs on the expres-

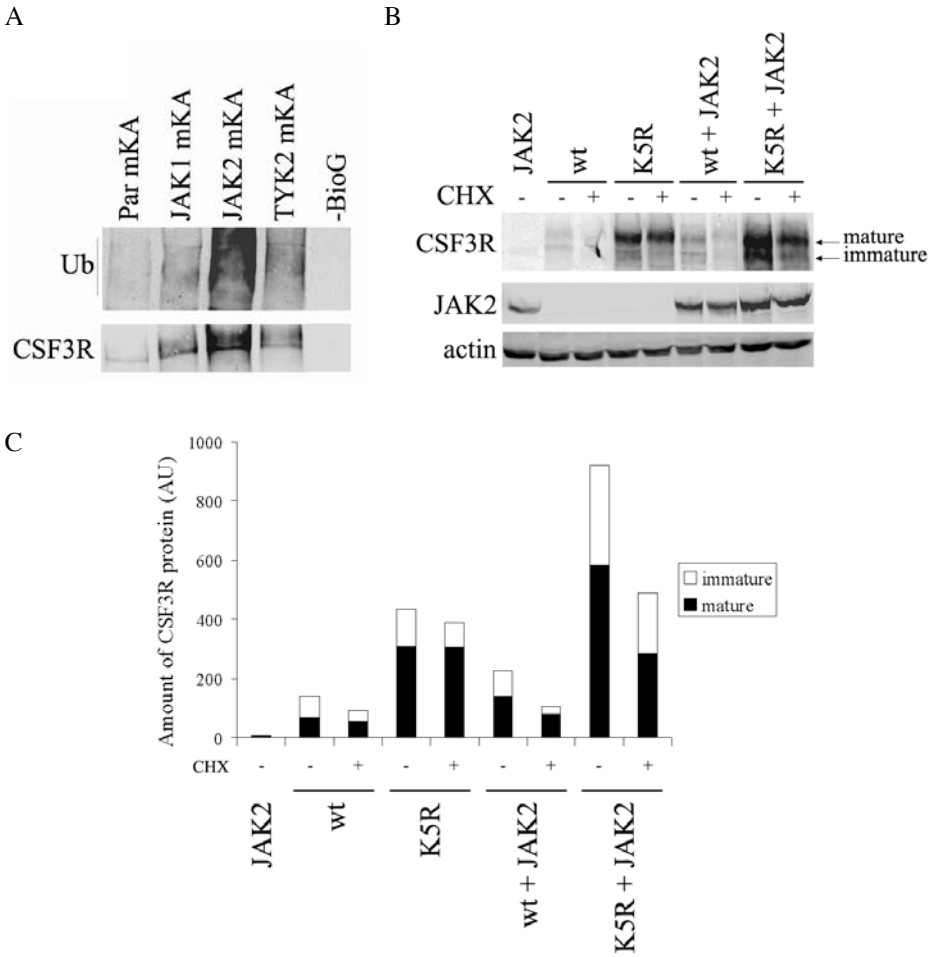


Figure 6. JAKs control CSF3R expression in the biosynthetic pathway irrespective of receptor ubiquitination
(A) Ubiquitination status of the lysine at position 632 of membrane CSF3R determined in Ba/F3 parental and JAK overexpressing cells. Cells were stimulated with Bio-G for 30 min. in the presence of bafilomycin, where after CSF3R was pulled down with streptavidin-coated magnetic beads, eluted and subjected to Western blot analysis using anti-ubiquitin antibody P4D1. Vertical line (left) indicates size variation of receptor-ubiquitin complexes. Blots were restained with anti-CSF3R to determine CSF3R loading. As an input control, an immunoblot with whole cell lysate was stained for actin. (B) Stability of wt and K5R CSF3R transiently expressed with or without JAK2 in Phoenix E cells. Cells were left untreated or treated with CHX for 3 hrs to block protein synthesis. CSF3R was immunoprecipitated using CSF3R antibodies and subjected to western blotting using the same antibodies. Quantification of mature and immature forms of CSF3R is shown in part C; protein levels are expressed in arbitrary units (A.U.). Total cell lysates were also stained for JAK2 content and for actin (loading control). Endogenous JAK2 is below detection level on this blot. Results shown are representative of three independent experiments.

sion of internalization defective mutant d715 (lacking the dileucine motif) and the lysosomal routing defective mutant K5R we observed that JAK2 and TYK2, and to a lesser extent JAK1, significantly enhanced surface expression of CSF3R-d715 and K5R (Figure 5B). This indicates that JAKs do not elevate CSF3R expression by blocking internalization or lysine-based lysosomal sorting motifs (which are lacking in these mutants). Also in the presence of excess JAKs, K632 remains the most prominent lysine inhibiting CSF3R surface expression (Figure 5C, compare to Figure 4C).

JAKs do not affect ubiquitination of K632 in ligand-activated CSF3R

From the above studies, K632 emerges as a major negative determinant in constitutive CSF3R expression, not functionally affected by JAKs. Upon ligand activation of the CSF3R-JAK complex, K632 is ubiquitinated (a process involving SOCS3), which is essential for G-CSF-induced lysosomal routing of CSF3R (Irandoust et al., 2007). Because JAKs are activated in this setting, we wondered whether increased JAK levels may interfere with ubiquitination of K632 to attenuate ligand-induced lysosomal degradation. As shown in Figure 6A, ubiquitination of K632, the only cytoplasmic lysine retained in mKA, increased proportionally with elevated CSF3R levels in JAK1, JAK2 or TYK2-overexpressing cells relative to parental cell controls. Together, these data indicate that neither in forward nor retrograde routing of CSF3R JAKs interfere with the function of K632.

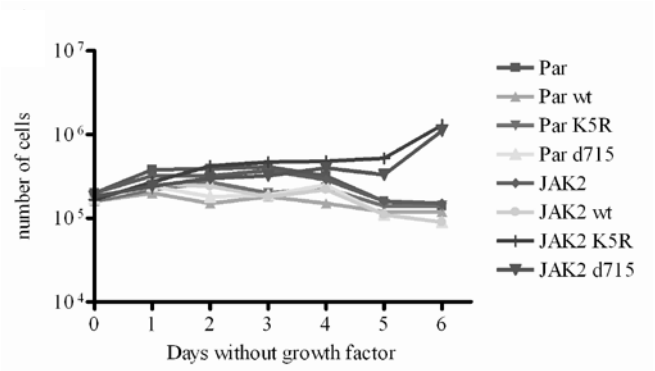
JAKs enhance protein stability of CSF3R

To determine if JAKs influence CSF3R protein stability, as e.g., shown for the TpoR (Royer et al., 2005), Ba/F3 parental cells and JAK transfectants expressing wt CSF3R were cultured in the presence or absence of CHX for 5 hrs to block protein synthesis. Immunoblots revealed two CSF3R species, the higher band representing the mature form and the lower band the immature protein that was sensitive to Endo H treatment (data not shown). Overexpression of JAK1, JAK2 and TYK2 predominantly enhanced the stability of the mature form of the wt CSF3R (Figure 6B and data not shown). In contrast, both the mature and immature forms of K5R were stabilized (Figure 6B). This was seen in three independent experiments and also observed when overexpressing JAK1 and TYK2 (data not shown). These data indicate that JAKs protect both the mature and immature form of CSF3R, provided that lysine-mediated degradation is prevented.

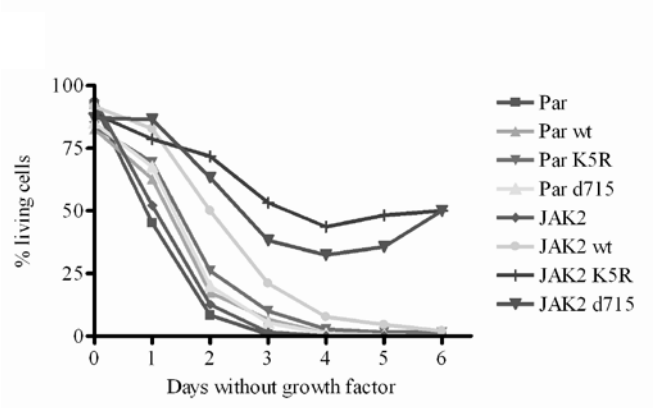
Increased JAK levels confer ligand independent signaling to routing defective K5R and internalization defective d715 mutants

Having established that JAKs increase the stability of CSF3R by a mechanism that is distinct from delayed internalization or lysosomal sorting, we wondered whether increased JAK levels can cooperate with mutant CSF3R defective in receptor downregulation to enhance

A


number of cells
Days without growth factor
Par
Par wt
Par K5R
Par d715
JAK2
JAK2 wt
JAK2 K5R
JAK2 d715

B


% living cells
Days without growth factor
Par
Par wt
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Par d715
JAK2
JAK2 wt
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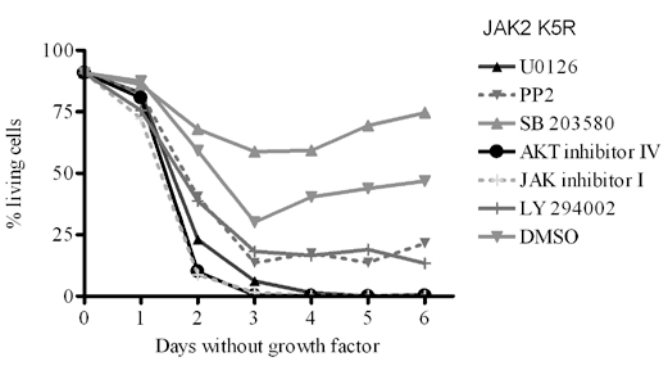
JAK2 d715

C


JAK2 K5R
% living cells
Days without growth factor
U0126
PP2
SB 203580
AKT inhibitor IV
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LY 294002
DMSO

D

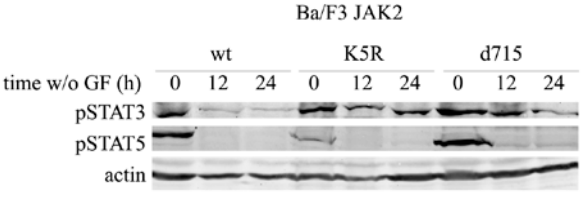
Ba/F3 JAK2
wt
K5R
d715
time w/o GF (h)
0 12 24 0 12 24 0 12 24
pSTAT3
pSTAT5
actin

Figure 7. JAK2 overexpression provokes ligand-independent pro-survival signalling by internalization defective and lysosomal routing deficient CSF3R mutants

Parental and JAK2 overexpressing Ba/F3 transfectants were cultured in medium with IL3 and transferred to medium without growth-factors. (A) total cell numbers and (B) the percentage of living cells based on 7-AAD staining were assessed at the indicated time points. Representative graphs of three independent experiments are shown. (C) Effects of various kinase inhibitors on cell survival. See main text for details on the specificity of different compounds. DMSO: solvent control. Representative graphs of three independent experiments are shown. (D) Cell lysates were made after removal of growth factor (GF) at the indicated time points (hrs) and subjected to Western blot analysis with antibodies against pSTAT3, pSTAT5 and actin for loading control.

downstream signaling. As reported previously (Lu et al., 2005), both parental and JAK2-overexpressing Ba/F3 cells failed to proliferate or survive in the absence of growth factor (IL-3) (Figure 7A). Expression of wt CSF3R in these cells did not alter this growth behavior. In contrast, JAK2-overexpressing Ba/F3 cells transduced with either the lysosomal routing defective mutant K5R or the internalization defective mutant d715 showed a moderately enhanced proliferation and a pronounced increase in cell survival (Figure 7B). To determine which pathways are responsible for the growth factor independent cell survival of the JAK2 overexpressing Ba/F3 cells stably expressing K5R CSF3R, cells were cultured in the presence of a variety of inhibitors (Figure 7C). JAK inhibitor I, a potent inhibitor of JAK2 (IC50 = 1nM), MEK inhibitor U0126 and Akt inhibitor IV completely abolished cell survival. Blocking of PI-3 kinase, acting upstream of PKB/Akt, by LY294002 also severely diminished survival as did inhibition of Src activity by tyrosine kinase inhibitor PP2. This result indicates that multiple pathways contribute to the growth-factor independent cell survival of the routing defective K5R CSF3R mutant. In contrast, inhibition of the stress-associated p38 MAPK [25] by SB203580, resulted in significantly increased survival. Because both STAT3 and STAT5 have been implicated as important signalling molecules for G-CSF-induced proliferation and survival [26, 27], we investigated whether these STATs are constitutively activated in JAK2-overexpressing Ba/F3 cells harbouring CSF3R K5R or d715. A prominent ligand independent activation of STAT3, but not STAT5, was observed in JAK2-overexpressing Ba/F3 cells transduced with mutants K5R or d715, but not with wt CSF3R (Figure 7D).

DISCUSSION

We investigated the role of JAK proteins in controlling CSF3R expression and intracellular routing. Specifically, we addressed to what extent JAKs interfere with the internalization and lysosomal routing machinery, linked to the dileucine-based internalization and conserved cytoplasmic lysines, respectively. Major observations were: (1) increased JAKs significantly elevated CSF3R membrane expression and protein levels, (2) JAKs did not exert this effect by masking motifs essential for internalization, as previously demonstrated for IFNAR1, or by reducing the ubiquitination of the lysosomal routing determinant K632 of CSF3R and

(3) simultaneous perturbation of internalization or lysosomal routing and JAK-mediated increase in forward routing resulted in ligand-independent activation of CSF3R complexes.

Enhanced cell surface expression after JAK overexpression has been reported for a number of cytokine receptors (Huang et al., 2001; Radtke et al., 2002; Radtke et al., 2006; Ragimbeau et al., 2003; Royer et al., 2005), but whether endogenous JAKs control receptor routing has been a matter of controversy. For example, a variety of EpoR mutants that failed to bind JAK2 showed only moderately reduced cell surface expression compared to wt EpoR and EpoR was still expressed on the cell surface of JAK2-deficient embryonic fibroblasts (Pelletier et al., 2006). Because EpoR exclusively binds JAK2, these results implied that cell surface expression of EpoR does not require JAKs. On the other hand, a clear relationship between loss of TYK2 and reduced membrane expression was reported for IFNAR1, owing to TYK2-mediated masking of endocytosis motifs (Kumar et al., 2008; Ragimbeau et al., 2003) and similarly JAK1 was found to inhibit internalization of OSMR (Radtke et al., 2006). In this respect, CSF3R resembles the EpoR, as increased JAK levels did not affect endocytosis and because cell surface expression of CSF3R mutant W650R, which fails to activate JAKs and is hampered in JAK binding, is approximately similar to that of wt CSF3R. However, it remains possible that the residual 10-20% JAK binding observed with CSF3R-W650R might still suffice to promote cell surface routing.

Among the 5 conserved lysines in the cytoplasmic tail of CSF3R, the juxtamembrane residue K632, in addition to being a major determinant for ligand-induced lysosomal routing (Irandoust et al., 2007), also appeared to be uniquely involved in the control of CSF3R cell surface expression. Although lysines have been reported to regulate constitutive internalization of the short isoform of the leptin receptor (Belouzard and Rouille, 2006), we could not detect any role of lysines in constitutive internalization of the CSF3R. Hence, loss of K632 rather leads to enhanced cell surface expression by redirection of receptors from the endoplasmic reticulum-lysosomal degradative pathway towards the plasma membrane. Major players involved in intracellular trafficking of surface membrane proteins are the endosomal sorting required for transport (ESCRT) and Golgi-localized γ-ear containing (GGA) protein complexes, respectively (Hurley et al., 2006). Because key proteins within these complexes, such as Hrs and GGA, interact with ubiquitinated lysines in cargo proteins but simultaneously require interaction with phosphoinositides to increase affinity (Hurley, 2008; Wang et al., 2007), this might explain why membrane proximal positioning of lysine K632 is imperative for lysosomal routing of CSF3R. This is supported by recent experiments showing that relocation of K632 to a more membrane distant position disrupted its role in routing despite the fact that ubiquitination was unaffected (Wölfler et al., submitted for publication).

How JAKs control basal cell surface expression of CSF3R remains to be resolved. Because JAKs bind with their N-terminal FERM domain to the juxtamembrane domain of CSF3R that encompasses K632, a feasible explanation was that JAK overexpression would mask the function of this lysine, e.g., by preventing its ubiquitination or by hindering the binding of

effector proteins. This hypothesis can now be discarded because increased JAK levels did not prevent ubiquitination of K632 and elevated cell surface levels of mutants K632R and K5R at as efficiently as the wt CSF3R. Most likely, JAKs associate with the CSF3R already early in the biosynthetic pathway and help to protect CSF3R from misfolding and degradation, similar to what has been proposed for the EpoR (Huang et al., 2001).

Abnormal responses to hematopoietic growth factors, including G-CSF, have long been suspected to be involved in leukemic cell growth in AML (Lowenberg and Touw, 1993). The discovery of acquired mutations in *CSF3R* in patients with severe congenital neutropenia that are strongly associated with progression to AML have strongly supported this idea (Freedman et al., 2000). Although *CSF3R* mutations are rarely detected in *de novo* AML, a scenario can be envisaged in which increased levels of JAK confer spontaneous pro-survival signaling from lysosomal routing defective CSF3R. Such a combinatorial mechanism might, e.g., involve deubiquitinating enzymes that attenuated lysosomal routing by deubiquitination of critical lysines in CSF3R or proteins of the ESCRT machinery. Notably, this is not without precedent, since it was demonstrated earlier that expression of the deubiquitinating enzyme DUB-2 increases survival of haematopoietic cells following cytokine withdrawal (Migone et al., 2001).

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CHAPTER 5

Novel role of Wsb2 in steady state distribution of G-CSF receptor and G-CSF-controlled proliferation and differentiation signaling

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ABSTRACT

Signals induced by granulocyte colony-stimulating factor (G-CSF), the major cytokine involved in neutrophil development, are tightly controlled by ligand-induced receptor internalization. Truncated G-CSF receptors that fail to internalize show sustained proliferation and defective differentiation signaling. Steady state forward routing also determines cell surface levels of cytokine receptors, but mechanisms controlling this are poorly understood. Here, we show that Wsb-2, a SOCS box-containing WD40 protein with currently unknown function, binds to the COOH-terminal region of G-CSF receptor. Removal of this region did not affect internalization, yet resulted in increased membrane expression of G-CSF receptor and enhanced proliferation signaling at the expense of differentiation induction. Conversely, Wsb-2 binding to the G-CSF receptor reduced its cell surface expression and inhibited proliferation signaling. These effects depended on the SOCS box involved in ubiquitylation and on cytosolic lysines of G-CSF receptor and imply a major role for ubiquitylation through the G-CSFR C-terminus in forward routing of the receptor. Importantly, the *Wsb-2* gene is commonly disrupted by virus integrations in mouse leukemia. We conclude that control of forward routing of G-CSF receptor is essential for a balanced response of myeloid progenitors to G-CSF and suggest that disturbance of this balance may contribute to myeloid leukemia.

INTRODUCTION

The granulocyte colony-stimulating factor receptor (G-CSFR) is the major cytokine receptor involved in neutrophil development (Avalos, 1996; Demetri and Griffin, 1991; van de Geijn et al., 2003). G-CSFR activates multiple signaling molecules that control the proliferation and survival of myeloid precursors. In myeloid cell line models such as 32D, G-CSFR induces proliferation in the first 4-6 days of culture, followed by growth arrest and terminal granulocytic differentiation (Dong et al., 1993; Fukunaga et al., 1993). How this balance is maintained is largely unknown. Truncated G-CSFR resulting from nonsense mutations in *CSFR3* are found in ~20% of patients with severe congenital neutropenia (SCN). These mutations are linked with progression of SCN to acute myeloid leukemia (AML) (van de Geijn et al., 2003). The truncated G-CSFRs confer a hyperproliferative response to G-CSF in knock-in mouse models and are unable to transduce differentiation signals in 32D cells (Aarts et al., 2004; Dong et al., 1998; Hermans et al., 1999; McLemore et al., 1998; Ward et al., 1999b). Importantly, these mutants are defective in internalization, supporting the importance of a tight control of G-CSFR membrane expression for a balanced signaling output (Aarts et al., 2004; Hunter and Avalos, 1999; Ward et al., 1999b). While ligand-induced receptor endocytosis is a major mechanism for signal attenuation, less is known of mechanisms that control steady state expression of cytokine receptors on the plasma membrane and their contribution to signaling.

We recently identified the gene encoding WD40-repeat and suppressor of cytokine signaling (SOCS) box-containing protein 2 (*Wsb-2*) in a retroviral screen for leukemia genes (Erkeland et al., 2004). Wsb-1 and Wsb-2 proteins share 46% homology and belong to a protein family characterized by a SOCS box (Hilton et al., 1998). Together with elongin B/C and cullin 2 or 5 these proteins form Elongin-Cullin-SOCS protein (ECS) complexes, which function as E3 ubiquitin (Ub) ligases and in which the SOCS-box proteins are thought to bind the substrates that are targeted for ubiquitylation (Kamura et al., 2004; Kamura et al., 1998). This was recently demonstrated for ASB-2, a SOCS box protein involved in myeloid differentiation and for WSB-1, implicated in thyroid hormone activation (Dentice et al., 2005; Heuze et al., 2005). Substrate specificities of ECS complexes are still largely unknown. Moreover, it is unclear whether SOCS box proteins predominantly participate in polyubiquitylation and proteasomal degradation or also in lysosomal routing involving mono-or multi-ubiquitylation of substrates (Schnell and Hicke, 2003).

Here, we show that Wsb-2 (and the homologous Wsb-1) binds to the distal 13 amino acids of G-CSFR. In myeloid 32D cells, mutants lacking this region confer a hyperproliferative response, associated with reduced granulocytic differentiation. Wsb affects cell surface expression of G-CSFR via a mechanism depending on the conserved lysine residues in the G-CSFR and on the Wsb SOCS box, implicated in ubiquitylation of target substrates. All

integrations in *Wsb-2* identified in the retroviral screen disrupted the gene structure, suggesting that *Wsb-2* may act as a tumor suppressor or as a haplo-insufficient gene. *WSB-2* expression was also reduced in a subclass of AML patients. Based on these findings, we propose a model in which Wsb-2 induces ubiquitylation of the G-CSFR, thereby controlling steady state surface expression levels. This control appears essential for the appropriate balance of G-CSF-induced proliferation and differentiation, and may be lost in cases of murine and human acute myeloid leukemia.

MATERIAL AND METHODS

PCR primers

Primers are listed in table 1.

Table 1. PCR primers

primers	sequence
wsb2a	5'-GGGACCTGAATAAGCACG-3'
wsb2b	5'-CTCCTGTGATTTCTCTCCTG-3'
wsb2c	5'-AGACAACACTGCTTTGGTG-3'
wsb2d	5'-CTGTCATCTGC-CACCGTAG -3'
wsb2e	5'-AGATCCAGGTGTTATCCG-3
wsb2f	5'-ACCAG TGTGATTATGTGGGA-3'
wsb2g	5'-GGATTAGTGTGTAGGACCG-3'
wsb2h	5'-AGATACAAGCC TTCAGGTGAG-3'
L1	5'-TGCAAGATGGCGTTACTGTAGCTAG-3'
L1N	5'-AGCCTTATGGTGGGGTCTTTC-3'
L2	5'-CCAGGTTGCCCCAAAGACCTG-3'
L2N	5'-AAAGACCTGAAACGACCTTGC-3'
WSB F6	5'-CTTCTTACGATACCAATGTGA-3'
WSB R6	5'-TACAAGCCTTCTGGAGAGAA-3'
Y2HGCSFRF1	5'-CCGAATTCTCCTATGAGAACCTCTGGTTCCAG-3'
Y2HGCSFRR1	5'-AACTGCAGTTTTAGTCATGGGCTTATGGACC-3'
Y2HGCSFRR2	5'-AACTGCAGCTACAGGAGGGGGAAGTTGAG-3'
WSB1fw	5'-AAGAATTCAAGCCAGCTTTCCCCCGAG-3'
WSB1r1	5'-AACTCGAGTTAGCCCCTGTAGGAGAGAAACGCCAATA-3'
WSB2fw	5'-AAGAATTCAAGAGGCCGGAGAGGAGCC-3'

Yeast-two-hybrid (Y2H) analysis

Y2H was performed using yeast strain PJ69-4A (Dr. Philip James, University of Wisconsin, Madison) as described (James et al., 1996; van Zon et al., 2002). As prey library, a mouse 17-day embryo cDNA library (Matchmaker, Clontech laboratories, Palo Alto, CA) was used. For a second screen, cDNA library plasmids were co-transformed with the pGBT9-GCSFR762-813 or pGBT9-GCSFR762-790 bait. Prey inserts from this second selection were nucleotide sequenced (ABI 3100, Applied Biosystems).

Constructs generated for yeast two hybrid analysis

Bait constructs. The DNA encoding the 51 C-terminal 51 amino acids of G-CSFR (G-CSFR762-813) was obtained with primers Y2HGCSFRF1 and Y2HGCSFRR1. A fragment encoding G-CSFR amino acids 762 to 790 (G-CSFR762-790, lacking the 23 most C-terminal amino acids) was amplified with primers Y2HGCSFRF1 and Y2HGCSFRR2 using pBabe-G-CSFR (Hermans et al., 2003) as template. EcoRI and PstI fragments were cloned in-frame with the DNA binding domain (BD) in pGBT9 vector (Clontech), resulting in pACT-BD-GCSFR762-813 and pACT-BD-GCSFR762-790.

Prey constructs. Wsb-1 wild type and Wsb-1 without SOCS box (Wsb-1-ΔSB) sequences were amplified with primers WSB1fw and WSB1r1 using pMG2-WSB-1 and pMG2-WSB-1-ΔSB (see MAPPIT) as templates. Similarly, Wsb-2 and Wsb-2-ΔSB were amplified with primers WSB2fw and WSB2r1 using

primers	sequence
WSB2r1	5'-AACTCGAGCTAGAAAGTCCTGTATGTGAGGAACTC-3'
GCSFRHA	5'-GGAAGATCTCTACGCGTAATCTGGAACATCGTATGGGTACGCG TAATCTGGAACATCGTATGG-GTAGAAGCTCCCCAGCGCCTC-3'
pMGEcoMutF	5'-GGCGGTTCGAGAATTCGAAGCA-3'
pMGEcoMutR	5'-TGCTTCGAATTCTCGAACCGCC-3'
WSBMYCf	5'-AAGAATTCAAGCCAGCTTTCCC-3'
Mappit11_749-769F	5'-GGCGAGCTCATCCACCTAGCCCCTCTTG-3'
1250RV	5'ATCTAGATTAGTCATGGGCTTATGGA-3'
Mappit5	5'-GGCGAGCTCAAGGAAGAATCCCCTCTGG-3'
PMG2-SHCf	5'-AAGAATTCAACAAGCTGAGTGGAGGCGGC-3'
PMG2-SHCr	5'-AACTCGAGTCACAG TTTCCGCTCCACAGGTT-3'
WSB1stopf	5'-TGGGCCACTCCAAGGTAAGTCCCTAGCCTT-3'
WSB1stopr	5'AAGGCTAGGGACTTACCTTGGAGTGGCCCA-3'
WSB2stopf	5'-TCCCCGGGTCTAGTCCTCAC-3'
WSB2stopr	5'-GTGAGGACTAGACCCGGGGA-3'
Fw7	5'-GTCCTCACCCTGATGACC-3'
769WSB1fusionR	5'-AAAGCTGGCTGATCCTCC*GAACCAGAGGTTCTCATA*-3'
769WSB1fusionF	5'-*CTCTGGTTC*GGAGGATCAGCCAGCTTTCCCCCGAGG-3'
769WSB2fusionR	5'-TCCGGCCTCTGATCCTCC*GAACCAGAGGTTCTCATA*-3'
769WSB2fusionF	5'-*CTCTGGTTC*GGAGGATCAGAGGCCGGAGAGGAGCCGCT-3'

pMG2-WSB-2 and pMG2-WSB-2-ΔSB as templates. The PCR fragments were EcoRI-XhoI digested and ligated into the pACT prey vector (Clontech).

Immunoprecipitation and Western blotting

COS cells were transfected with pSG5-HAHA-G-CSFR together with pMG2-WSB-1, pMG2-WSB-2, pMG2-FzR-WD40, or pMG2-SVT (Eyckerman et al., 2002; Eyckerman et al., 2001). After 48 h, cell lysates were incubated with anti-FLAG (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) or anti-HA (Santa Cruz Biotechnology, CA) antibodies. Immune-complexes were visualized by Western blotting as described (Dong et al., 2001). HEK293 cells were transfected with the different G-CSFR mutants. After 48 h, cell lysates were either incubated with anti-Jak-2 antibodies (Bio Connect, Veenendaal, The Netherlands) or analyzed by Western blotting using phosphorylation specific antibodies against Stat5 (Becton Dickinson, Alphen a/d Rijn, The Netherlands), Stat3 (Westburg, Leusden, The Netherlands), P38 (Westburg) and Erk (Tebu-Bio, Heerhugowaard, The Netherlands) and against total Erk for loading control (Campro, Veenendaal, The Netherlands). Phosphorylated Jak-2 and total Jak-2 input were visualized by Western blotting using anti-phosphotyrosine (PY99) and anti-Jak2 antibodies (Tebu-bio) respectively.

Constructs generated for co-immunoprecipitation

PSG5-HAHA-G-CSFR. A G-CSFR fragment was amplified using primers GCSFRHA and Fw7 creating a double HA-tag at the G-CSFR C-terminus. The HpaI-BglII digested PCR fragment replaces the HpaI-BglII fragment of pBabe-G-CSFR. The EcoRI-SalI fragment of pBabe-HA-G-CSFR was ligated into pSG5 expression vector resulting in pSG5-HAHA-G-CSFR.

Ligation of pACT-prey insert into pMG2. The pMG2 vector was mutated with QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA), using primers pMGEcoMutF and pMGEcoMutR resulting in pMG2-mut vector. The EcoRI-XhoI fragment from pGBT9-Fzr-WD was cloned in the EcoRI and XhoI sites of the pMG2-mut vector resulting in pMG2-Fzr-WD.

Immunofluorescence microscopy

HEK293 cells were transfected with EGFP-fusion constructs of WT G-CSFR, G-CSFRΔ715, G-CSFRΔ769 or G-CSFRΔ749-769 in pBabe (Aarts et al., 2004) together with pMT2-MYC-WSB. Cells were fixed and stained using mouse anti-MYC monoclonal antibodies (9E10, Santa Cruz Biotechnology, CA) and TRITC-conjugated Goat-anti-mouse secondary antibodies as described (Aarts et al., 2004). To examine subcellular localization of the fluorescent proteins, confocal laser scanning microscopy (CLSM) was performed on a Zeiss LSM510 (Carl Zeiss BV, Sliedrecht, The Netherlands).

Constructs generated for CSLM

pMT2-MYC-WSB-1. WSB was amplified with WSBMYCf and WSB1r1 using pMG2-WSB as template and cloned in TA cloning vector. The EcoRI fragment was cloned in the EcoRI site of pMT2SM-MYC vector (Aarts et al., 2004; van Dijk et al., 2000)

MAPPIT

MAPPIT was performed as described (Eyckerman et al., 2002). In summary, HEK293-T cells ($2x10^5$) were transfected with bait and prey constructs in presence of luciferase reporter gene (pXP2d2-rPAP-Luci). 48h after transfection, cells were treated bait receptors were activated with Epo (0,5 U/ml) for 24 h. Cultures without Epo stimulation were run in parallel. Luciferase activity from a STAT3 luciferase reporter was determined in triplicate using the Steady-Glo luciferase assay system (Promega, Leiden, The Netherlands). Activities are expressed as fold induction by dividing luciferase activity of the Epo-stimulated samples over non-stimulated values. To determine expression levels of the FLAG-tagged prey proteins, Western blotting using anti-FLAG M2 monoclonal antibodies (Sigma-Aldrich Chemie, Steinheim, Germany) was performed as described (Ward et al., 1999a).

Bait constructs. The C-terminal 65 amino acids fragment of human G-CSFR was generated with primers Mappit11_749-769F and 1250RV with pLNCX-G-CSFR (Ward et al., 1999a) as template. The entire intracellular domains of G-CSFR (G-CSFR630-813) and G-CSFR630-813-Y (intracellular domain lacking all tyrosines) were amplified with primers Mappit5 and 1250RV using pLNCX-G-CSFR and pLNCX-mutant "null" as templates (Ward et al., 1999a). The fragments were cloned in TA cloning vector, sequence checked, SacI and XbaI digested and ligated into pCEL(2L) vector (Eyckerman et al., 2002; Tavernier et al., 2002), resulting in pCEL(2L)-G-CSFR748-813, -G-CSFR630-813 and -G-CSFR630-813-Y. Deletion mutants of the G-CSFR748-813 bait were made by introducing stop codons with the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

Prey constructs. Human SHC was amplified using primers PMG2-SHCf and PMG2-SHCr using pHMT-SHC as template (Gotoh et al., 1996). Murine Wsb-1 and Wsb-2 were amplified from 32D cDNA with primers WSB1f and WSB1r1 (Y2H constructs), WSB2f and WSB2r2 (Y2H constructs). PCR products were cloned in TA vector, sequence checked and EcoRI-XhoI digested allowing ligation into pMG2 prey vector (Eyckerman et al., 2002), thus resulting in SHC-, WSB-1- and WSB-2- Flag-tagged-gp130 fusions. The WSB-1-ΔSB and WSB-2-ΔSB mutants were made by site-directed mutagenesis (QuickChange) using primers WSB1stopf, WSB1stopr and primers WSB2stopf, WSB2stopr, respectively.

Retroviral gene transfer in 32D cells

IL-3-dependent murine myeloid 32D cells were infected with the different Babe-based retroviral vectors and selected on 1 μg/ml puromycin (Sigma, Zwijndrecht, The Netherlands) as described (Aarts et al., 2004).

Production of retroviral vectors

The G-CSFR part of the G-CSFRΔ769-Wsb-1 fusion was amplified using primers Fw7 and 769WSB1fusionR, in which italics denote G-CSFR sequence, underlined a flexible linker sequence encoding for two glycines and a serine (GGS) (Eyckerman et al., 2002) and regular fond Wsb sequence using pBabe-G-CSFR (Aarts et al., 2004) as template. The Wsb part was amplified using primers 769WSB1fusionF and WSB1r1 (Y2H construct), and pMG2-WSB-1 and pMG2-WSB-1-SBD as template. Both amplification products were used as template and the annealed fragments amplified using primers Fw7 and WSB1r1. The G-CSFR part of G-CSFRΔ769-Wsb2 fusion was amplified using primer 769WSB2fusionR and Fw7. The Wsb-2 part was amplified using primers 769WSB2fusionF and WSB2r1 (Y2H construct), with pMG2-WSB-2 and pMG2-WSB-2-SBD as input. These fragments were used as PCR template and

amplified with primers Fw7 and WSB2r1. Fusion products were cloned in TA vector and sequenced. pBabe-G-CSFR was digested with HpaI and BglII, blunted (DNA polymerase I large fragment, Invitrogen) and dephosphorylated (shrimp alkaline phosphatase, Roche, Mannheim, Germany). Fusion products were digested with HpaI and XhoI, blunted, ligated in pBabe-G-CSFR and checked by restriction analysis and nucleotide sequencing. G-CSFR mutant K5RΔ769-Wsb-2, in which cytoplasmic lysine residues K632, K672, K681, K682 and K762 are replaced by arginines was created using QuickChange site-directed mutagenesis (Stratagene) using G-CSFRΔ769-Wsb-2 as a template. 32D cells expressing G-CSFRΔ800 and G-CSFRΔ715 have been described previously (Aarts et al., 2004).

Flow cytometric analysis of G-CSFR expression and internalization in transduced 32D cells

To determine G-CSFR expression, transduced 32D cells were labeled with biotinylated antihuman antibody recognizing an N-terminal extracellular epitope of the G-CSFR (LMM741; Pharmingen, San Diego, CA), subsequently incubated with phycoerythrin-conjugated streptavidin (SA-PE; DAKO Diagnostics, Glostrup, Denmark) and analysed by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA) as described (Aarts et al., 2004). Ligand induced internalization was assessed at several time points after G-CSF treatment as described (Aarts et al., 2004).

STAT3 and STAT5 electrophoretic mobility assay (EMSA)

EMSA to detect activated STAT3 and STAT5 complexes in nuclear extracts of 32D cell transfectants were performed as described (de Koning et al., 2000).

G-CSFR ubiquitination

Phoenix E cells (G. Nolan, Stanford, CA) were transfected with HA-tagged wt or mutant-G-CSFR and flag-tagged Ub using the calcium-phosphate precipitation method. After 48 h, cells were stimulated with G-CSF (100 ng/ml) for 30 minutes or left untreated, washed with cold PBS and resuspended in lysis buffer. (Lysis buffer: 20mM Tris HCl pH8.0, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% NP40, 10% glycerol, 2mM Na_3VO_4 and 1mM Pefablock SC, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml bacitracin, and 50 μg/ml iodoacetamide). Immunoprecipitations using protein G-sepharose beads and subsequent Western blotting were performed using anti-Flag and anti-HA and anti polyUb (FK2, Affiniti Research Products, Exeter, UK) antibodies. Sepharose beads were washed and resuspended in 1x laemlli buffer (pH 11) before gel electrophoresis and Western blotting.

Detection of virus integrations in *Wsb-2*

To determine orientation and localization of Gr-1.4 MuLV integrations in *Wsb-2*, a nested PCR was performed on genomic DNA from primary tumors as described (Erkeland et al., 2004) (Figure 1A). For the first PCR, *Wsb-2* specific primers a, b, c, and d were used in combination with Gr-1.4 LTR specific primers L1 and L2. For the nested PCR, Wsb-2 specific primers e, f, g and h were used in combination with Gr-1.4 LTR specific primers L1N and L2N. PCR fragments were analyzed as described (Erkeland et al., 2004).

WSB-2 expression in human AML

Real-time PCR was performed on complementary DNA from 260 AML patients using an Applied Biosystems 7900 instrument (Applied Biosystems, Weiterstadt Germany) and SYBR Green PCR Master Mix (Applied Biosystems) (Erkeland et al., 2003; Valk et al., 2004). Primers were WSB-2F6 and WSB-2R6. The porphobilinogen deaminase (PBGD) gene was taken as reference (Erkeland et al., 2003).

RESULTS

Wsb binds to the COOH terminus of G-CSFR in Y2H and co-immunoprecipitation assays and colocalizes with G-CSFR in intact cells

Previous studies indicated that the C-terminal part of the G-CSFR is crucial for G-CSF-induced granulocytic differentiation (Dong et al., 1993; Fukunaga et al., 1993). Yeast two hybrid analysis was performed to identify proteins that interact with this region. Using G-CSFR762-813 as bait, candidate interacting proteins were identified, including the WD40 repeat domain proteins Fizzy-related 1 (FzR1), Wsb-1 and Wsb-2 (Figure 1 upper panel). Deletion of the SOCS box domain (ΔSB) did not affect Wsb-1/2 binding to the G-CSFR bait (Figure 1, upper panel). Wsb proteins failed to interact with G-CSFR762-790, indicating that the distal 23 amino acids of G-CSFR are crucial for binding (Figure 1, lower panel). In COS cells, FLAG-tagged Wsb-1 and Wsb-2 co-immunoprecipitated with G-CSFR in HA-IP's, as did the WD40 domain of FzR1 (Figure 2A). No interactions were seen with an irrelevant control protein SV40 large T (SVT) or with empty vector controls (Figure 2A). Similar results were obtained in the reverse FLAG IP/HA Western experiments (Figure 2B). No interactions were observed between G-CSFRΔ715 and Wsb-1, Wsb-2, or FzR-WD40 (Figure 2C,D). Confocal laser scanning microscopy (CLSM) showed that Wsb-2 co-localized with WT-G-CSFR in transfected HEK293-T cells (Figure 3A), but not with G-CSFRΔ715 or G-CSFRΔ769, mutants that lack the WD40-interacting distal 23 amino acids (Figure 3B,C). Conversely, G-CSFRΔ749-769, a mutant defective in internalization but retaining its distal region, again co-localized with Wsb in intracellular vesicles (Figure 3D).

G-CSFR/Wsb interactions in mammalian protein-protein interaction trap (MAPPIT) assay

To establish that Wsb and G-CSFR proteins interact in living mammalian cells in the correct subcellular context, we performed MAPPIT, a cytokine receptor based two-hybrid assay (Eyckerman et al., 2001). Intracellular fragments of G-CSFR were coupled to the C-terminus of the EPO/Leptin receptor hybrid bait (Figure 4A) (Tavernier et al., 2002). First, we validated the MAPPIT system for G-CSFR-containing baits using preys containing full length Shc or Grb2, proteins that bind directly to phosphorylated Y764 of G-CSFR (Ward et al., 1999a). As

predicted, Shc and Grb2 interacted efficiently with bait C65, but not with C65-Y764F (Figure 4B). Wsb-1 and Wsb-2 strongly interacted with C65, while no binding with the control bait (EPO-R) was observed (Eyckerman et al., 2002) (Figure 4C). These results show that Wsb specifically binds to the G-CSFR and not to the leptin receptor backbone of the bait. FzR1

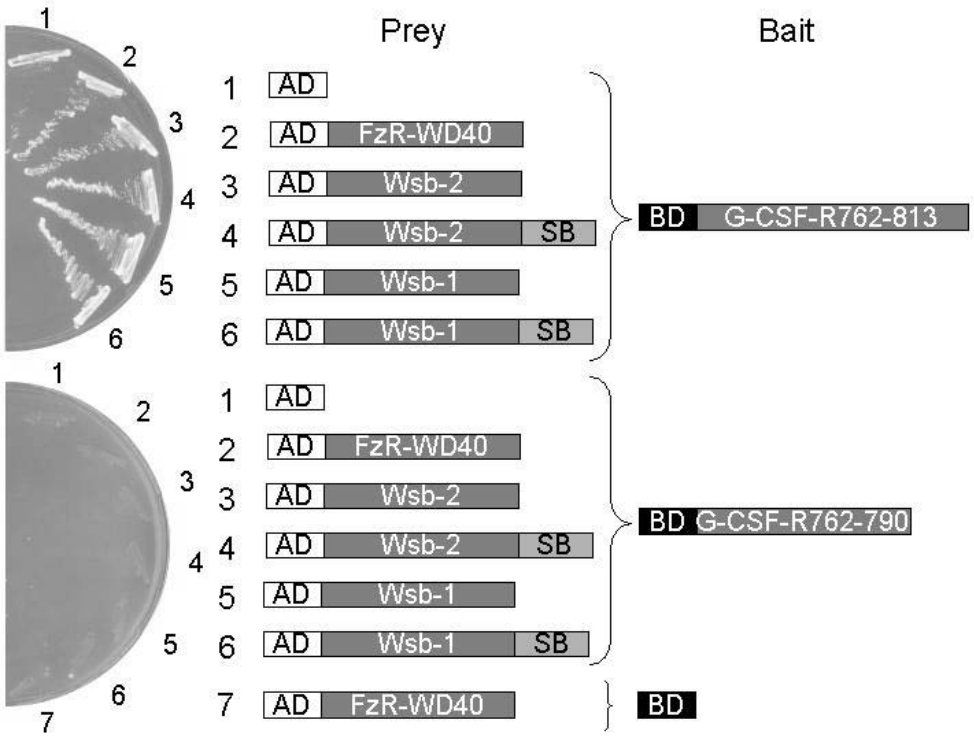

Figure 1. Yeast-two-hybrid analysis

Yeast strain PJ69-4A was transformed according to the LiAc/SS-DNA/PEG procedure with either pGBT9-GCSFR762-813, containing amino acids 762 to 813 of G-CSFR (upper section) or pGBT9-G-CSFR762-790, containing amino acids 762 to 790 of G-CSFR (lower section) in combination with Wsb-1, Wsb-1 without SOCS Box (SB), Wsb-2, Wsb-2 without SB, Fzr-WD40 or empty prey vector expressing the ADdomain only.

did not bind to the G-CSFR bait (data not shown), indicating that Wsb, but not FzR1 may control G-CSFR function. Removal of the SOCS box did not affect Wsb binding to C65. Wsb binding to C65Δ803 was strongly reduced and no longer detected with C65Δ792 (Figure 4D). These results corroborate the Y2H, immunoprecipitation and CLSM experiments and establish that Wsb binding specifically occurs to the carboxyl terminal 10 to 20 amino acids of the G-CSFR.

The Wsb-binding region of G-CSFR influences steady state expression levels and controls the balance between proliferation and differentiation signals

To study the role of Wsb-binding amino acids in G-CSFR distribution and function, we generated multiple independent clones of 32Dcl10 cells expressing a C-terminal deletion

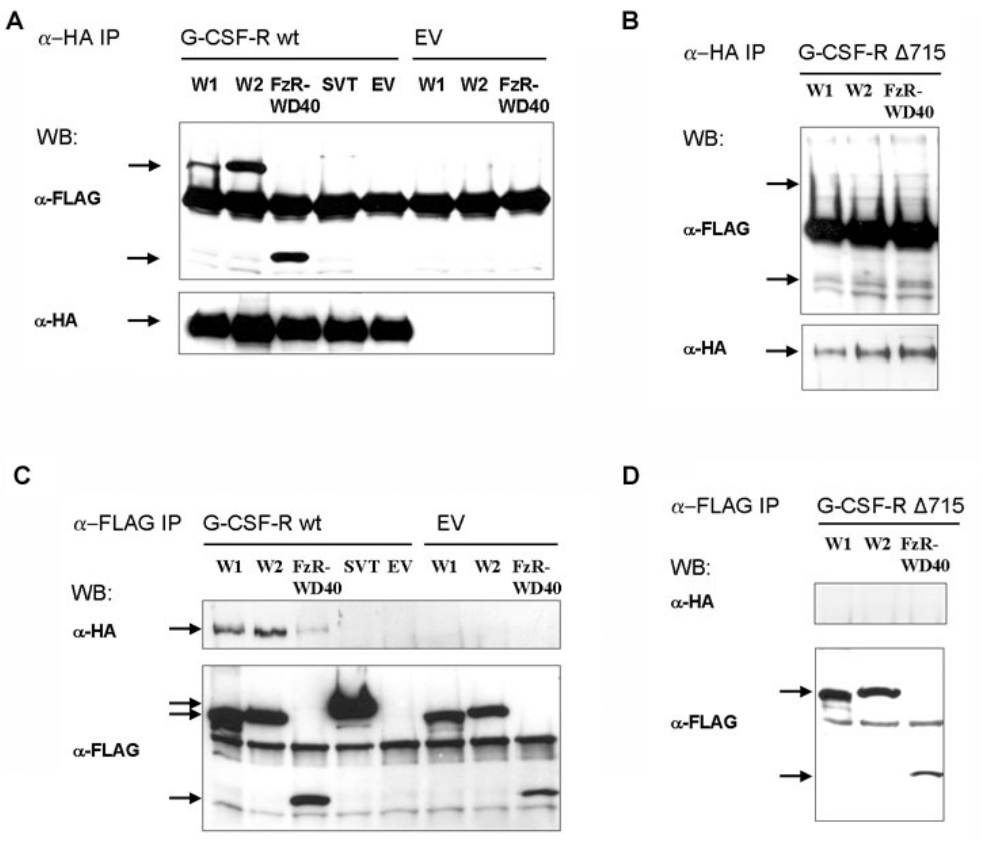

Figure 2. Co-immunoprecipitation of Wsb-1 and Wsb-2 with G-CSFR

COS cells were transfected with pSG5-HA-G-CSFR, pSG5-HA-Δ715 or pSG5-empty vector in combination with pMT2-FLAG-Wsb1, pMT2-FLAG-Wsb-2, pMT2-FLAG-FzRWD40 (positive control) or pMT2-FLAG-SVT (negative control). After 48 hours, cells were lysed and proteins immunoprecipitated with antibodies against HA-tagged G-CSFR (A, C) or FLAG-tagged Wsb and control proteins (B, D). Blots were restained with anti-FLAG and anti-HA (Santa Cruz Biotechnology, CA) as indicated. Arrows mark positions of co-precipitated products (upper panels) and input of precipitates (lower panels). W1,W2: Wsb1,Wsb2; EV: empty vector; SVT: SV40 large T.

mutant lacking the 13 carboxyl-terminal amino acids (G-CSFRΔ800). Steady state membrane expression of G-CSFRΔ800 was consistently increased compared to WT G-CSFR (Figure 5A). Contrary to G-CSFRΔ715, ligand-induced internalization of G-CSFRΔ800 in 32D cells

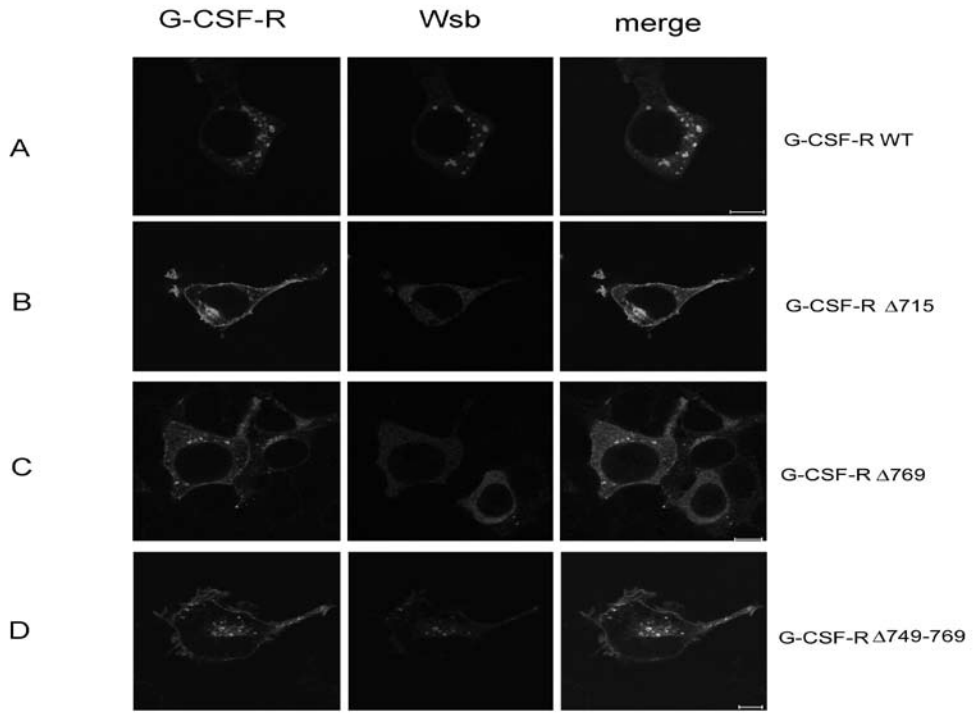

Figure 3. Co-localization of Wsb and G-CSFR in intracellular vesicles
CLSM analysis of HEK293-T cells transfected with G-CSFR wt (A), Δ715 (B), Δ749-769 (C) or Δ769 (D) together with myc-tagged Wsb-2. Cells growing on glass slides were fixed 2 days after transfection and immunostained for G-CSFR (left panels; green) or myc-Wsb (middle panels). After merging left and middle panels, co-localization appears as yellow (right panels). Size bar indicates 10μM.

was normal and occurred with similar kinetics as internalization of WT G-CSFR (Figure 5B), indicating that increased expression levels were not due to defective endocytosis. The rate of proliferation of 32D/G-CSFRΔ800 cells in G-CSF-containing cultures was significantly increased compared to 32D/G-CSFR-WT and matched growth rates of 32D/G-CSFRΔ715 in the first days of culture (Figure 5C). This was accompanied by a lack of terminal neutrophilic differentiation (Figure 5D), indicating a major role of the C-distal 13 amino acids of G-CSFR in controlling the proliferation/differentiation balance in response to G-CSF.

Wsb fusion reduces steady-state G-CSFR expression on the plasma membrane
Next, we determined the effects of Wsb on G-CSFR distribution. Unfortunately, we could not generate 32D clones stably expressing Wsb-1 or Wsb-2, possibly because WD40 repeats bind to substrates that are crucial for cell growth (Neer and Smith, 2000; Smith et al., 1999). To overcome this problem, we generated constructs with Wsb-1 or Wsb-2 fused to G-CSFR mutant Δ769 via a flexible linker (GGS) (Figure 6A). While membrane expression of G-CSFRΔ769 was

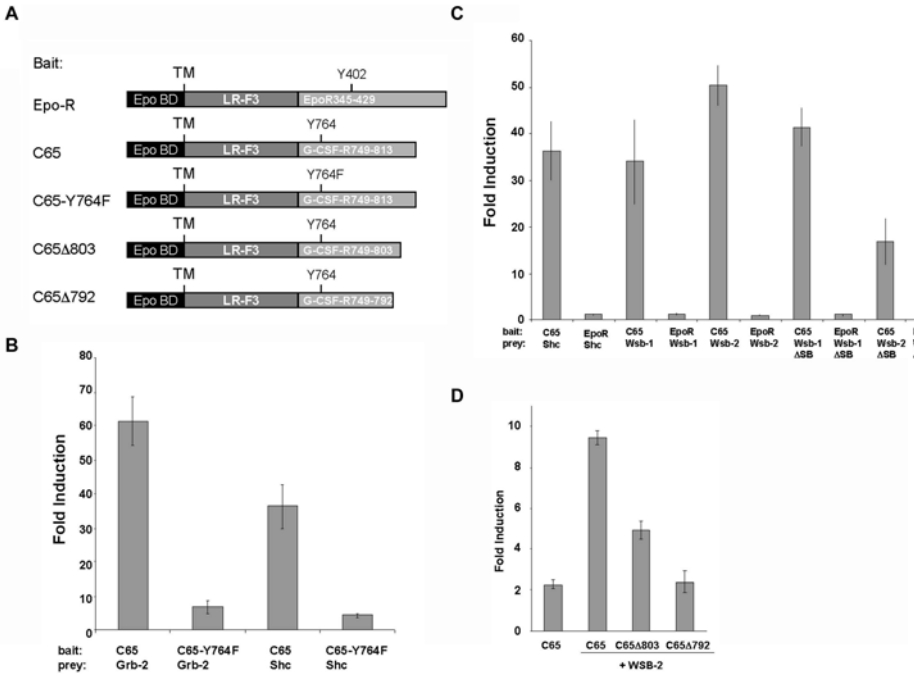

Figure 4. Interactions of Wsb, Shc and Grb2 to G-CSFR detected by MAPPIT assay

HEK293-T cells were transfected with Epo/LeptinR-G-CSFR bait or Epo/LeptinR-EpoR (control) bait constructs and different gp130-based prey constructs as indicated together with a STAT3-driven luciferase reporter (pXP2d2-rPAP-luci). Transfected cells were cultured with or without Epo and luciferase activity was measured 24 hours after stimulation. Fold induction was calculated by dividing luciferase values of Epo-treated over non Epo-treated cells. Empty prey vector was used as negative contol. (A) schematic view of the different MAPPIT bait constructs, with Epo-R and G-CSFR bait sequences shown in light grey. EpoBD: extracellular Epo binding domain, TM: transmembrane domain, LR-F3: leptin receptor intracellular domain devoid of STAT3 binding receptor tyrosines; (B) specific binding of Shc and Grb-2 to Y764 of G-CSFR, indicating correct configuration of the G-CSFR C-terminus in MAPPIT bait constructs; (C) binding of Wsb-1, Wsb-2 and Wsb1/2-ΔSB to the G-CSFR C-terminus; C65/Shc and EpoR/Shc bait/prey combinations were included as positive and negative controls, respectively; (D) progressive truncation of G-CSFR C-terminus showing Wsb-2 binding to distal 10-21 amino acids of G-CSFR.

consistently higher than that of WT G-CSFR, fusion of Wsb-2 to G-CSFRΔ769 significantly reduced membrane expression levels, an effect that depended on the SOCS box (Figure 6B). Similar results were obtained with G-CSFRΔ769-Wsb1 fusions (data not shown). Replacement of the 5 conserved lysines in the G-CSFR moiety of G-CSFRΔ769-Wsb2 by arginines (K5R-G-CSFRΔ769-Wsb2) reverted membrane expression to normal levels, suggesting a role of ubiquitylation of receptor lysines in steady state distribution of G-CSFR (Figure 6C). Indeed, we could demonstrate ubiquitylation of lysines in WT-G-CSFR (Figure 6D upper panel). The size distribution of Ub-G-CSFR species was suggestive of mono- or multi- instead of polyubiquitylation, which is in agreement with the lack of reactivity with FK2, a mAb with

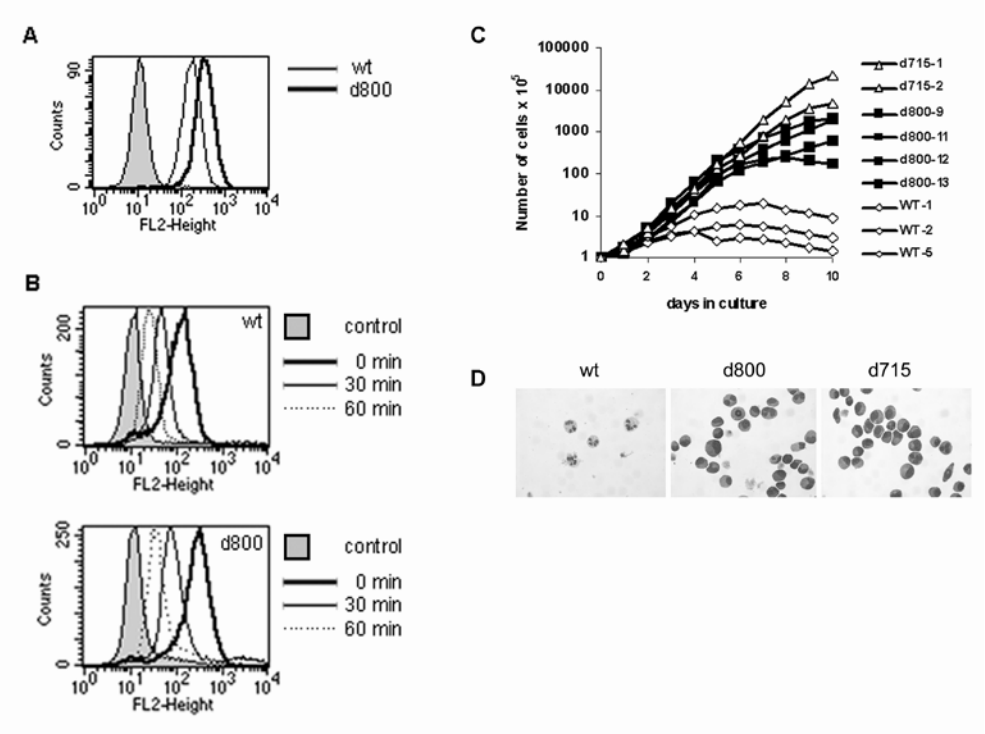

Figure 5. Role of C-terminal region of G-CSFR in steady-state membrane expression, receptor internalization and proliferation/differentiation signaling

(A) FACS histograms showing steady-state expression of G-CSFR-WT (thin line) and G-CSFRΔ800 (thick line) in 32D cell clones. Results are representative for 5 independent clones; (B) FACS histograms showing time kinetics of G-CSF-induced internalization of G-CSFR-WT (upper panel) and G-CSFRΔ800 (lower panel). Results are representative of 5 independent clones; (C) Proliferation of independent 32D cell clones stably expressing G-CSFRΔ800, G-CSFRΔ715 or G-CSFR WT in G-CSF-containing suspension culture; (D) Light micrographs of May Grünwald-Giemsa stained cells on day 10 of culture (x 690).

high affinity for polyUb (Figure 6D, middle panel) (Haglund et al., 2003). Staining for G-CSFR showed significantly higher protein levels of K5R compared with WT G-CSFR (Figure 6D, lower panel), suggesting that the receptor lysines are crucial for protein degradation. G-CSFRΔ769-Wsb2 predominantly accumulated in the ER and intracellular vesicles, while G-CSFRΔ769-Wsb2ΔSB and K5R-G-CSFRΔ769-Wsb2 were both mainly found on the cell membrane (Figure 6E). In agreement with this, G-CSF-induced phosphorylation of STAT3, STAT5, Erk, $p38^{MAPK}$ (Figure 7A) and JAK2 (Figure 7B) in G-CSFRΔ769-Wsb1/2 expressing cells was strongly diminished compared to G-CSFRΔ769. This inhibitory effect of Wsb again depended on the presence of the SOCS box. EMSA analysis of activated STAT3 and STAT5 as well as long-term suspension cultures gave similar results and confirmed that the lysines in G-CSFR are crucial for the inhibitory effects of Wsb (Figure 7C,D).

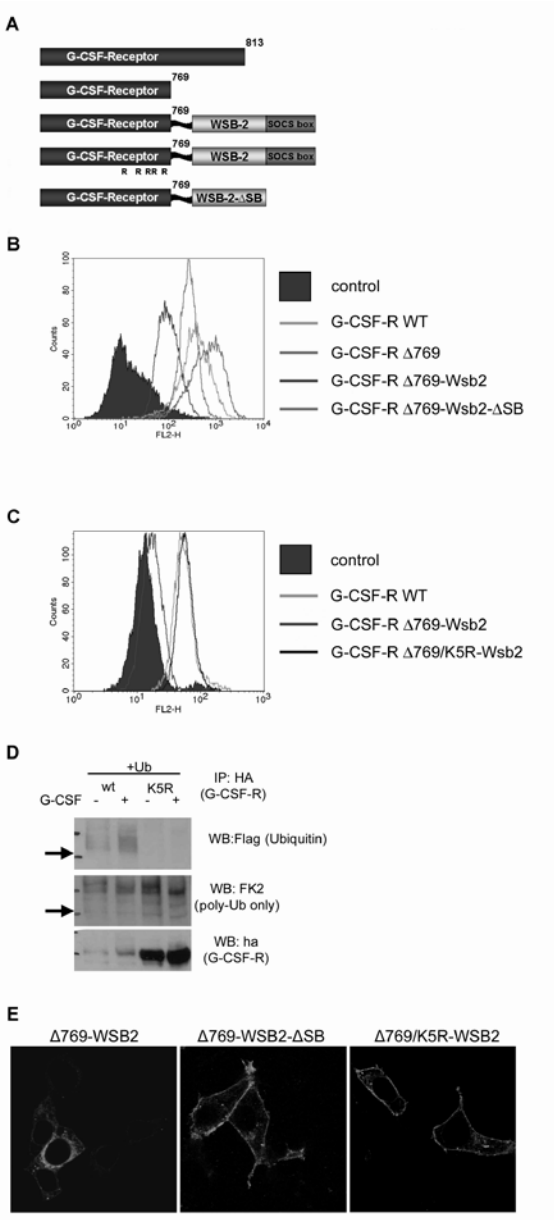

Figure 6. Steady-state distribution of G-CSFR-Wsb fusion proteins.

(A) G-CSFR constructs. Wsb moieties were fused to G-CSFRΔ769 via a flexible linker (GGS), R's indicate 5 conserved lysine residues replaced by arginines (K5R); (B) FACS histograms showing steady-state membrane expression levels of G-CSFR-WT, G-CSFRΔ769, G-CSFRΔ769-Wsb-2 and G-CSFRΔ769-Wsb-2ΔSB on 32D cells; (C) FACS histograms comparing steady state expression levels of K5R-G-CSFRΔ769-Wsb-2 and G-CSFRΔ69-Wsb-2; (D) IP/Western blot analysis showing ubiquitylation of lysine residues of G-CSFR (upper panel) and lack of reactivity with anti poly-Ub mAb FK2 (middle panel); arrows indicate position of nonubiquitylated G-CSFR; lower panel: backstain for WT and K5R G-CSFR; (E) CLSM of steady-state distribution of G-CSFRΔ769-Wsb-2 (left), G-CSFRΔ769-Wsb-2ΔSB (middle) and K5R-G-CSFRΔ769-Wsb-2 (right) on HEK293-T transfectants.

Involvement of *Wsb-2* in acute myeloid leukemia

Previously, we identified *Wsb-2* in a common virus integration site in a murine myeloid leukemia model (Erkeland et al., 2004). Locus specific PCR (Fig 8A) showed that the 5 integrations in *Wsb-2* identified in independent leukemia samples occurred between exon 4 and exon 7, leading to disruption of the gene (Figure 8B). Because of the non-clonal nature of MuLV-induced tumors (Erkeland et al., 2003; Erkeland et al., 2004), we were unable to verify the status of the other allele or to determine *Wsb-2* expression levels in single leukemic clones. As an alternative, we examined *WSB-2* expression in human AML, using gene expression profiling data set of 285 AML cases (Valk et al., 2004). Significance analysis of microarrays (SAM) (Tusher et al., 2001) revealed that *WSB-2* expression is significantly decreased (fold change = 0.45152, score = -6.33656, q-value = 0.109866108) in a subclass of patients (cluster 4), characterized by mutations in *CEBPA* (Valk et al., 2004). These data were confirmed by

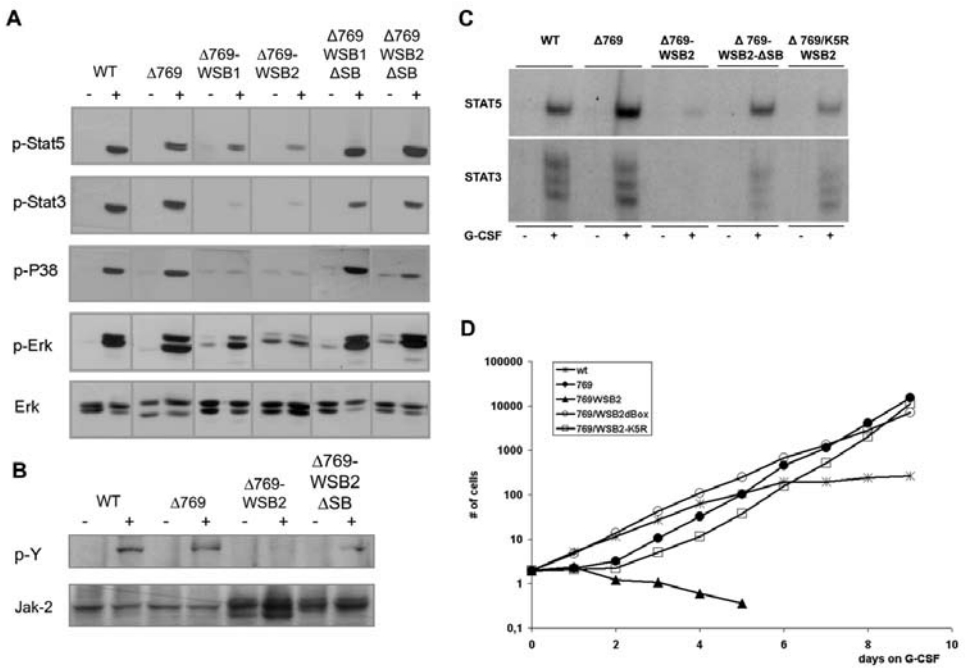

Figure 7. Effects of Wsb on G-CSF induced STAT activation and proliferation

32D clones transduced with indicated G-CSFR constructs were serum deprived for 4 hours and then incubated with (+) or without (-) 100 ng/ml G-CSF for 10 min at 37°C. (A) cell lysates analyzed by Western blotting using phosphorylation specific antibodies against Stat5, Stat3, p38, and Erk and against total Erk (loading control); (B) Anti Jak-2 immunoprecipitation; blots were stained with anti-phospho-tyrosine and anti-Jak2 as indicated; (C) STAT3 and STAT5 EMSA; nuclear extracts were prepared and assayed by EMSA using STAT5 and STAT3 specific radiolabeled probes as described in material and methods. Results of one out of three representative clones per construct are shown. (D) Proliferation of 32D clones transduced with indicated G-CSFR constructs in G-CSF-containing suspension culture.

quantitative RT-PCR (not shown) and suggest that reduced WSB-2 levels may play a role in perturbed G-CSF responses in this group of AML patients.

DISCUSSION

The key observations reported here are that (i) Wsb proteins bind to the G-CSFR C-terminus and thereby modulate plasma membrane levels of G-CSFR, (ii) removal of the Wsb-binding domain increases the proliferation signaling abilities of G-CSFR at the expense of its ability to induce differentiation and (iii) lysines in the G-CSFR and the E3 ligase-recruiting SOCS box of Wsb are both necessary for G-CSFR distribution and signaling function, suggesting that Wsb acts via ubiquitylation of the G-CSFR. Given that one Ub moiety increases

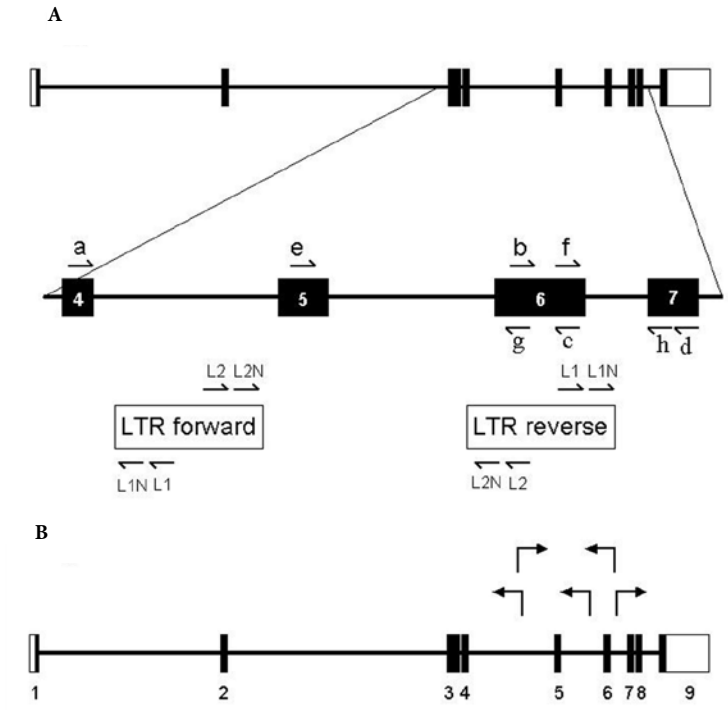

Figure 8. Gr-1.4 MuLV integrations in *Wsb-2*

(A) PCR strategy to detect position and orientation of Gr-1.4 integrations in *Wsb-2*. Translated exons of *Wsb-2* are shown as black boxes; white boxes represent untranslated regions of exons 1 and 9. LTR forward and LTR reverse indicate orientations of the integrated virus. Depending on position and orientation of the virus integration, PCR products will be formed. For example, when the virus is integrated in the forward orientation in intron 5, use of primer combination (a, L1) and nested primers (e, L1N), and/or primer combination (c, L2) and nested primers (g, L2N) will result in PCR products. Primer combinations are described in material and methods; (B) Examples of virus integration sites in *Wsb-2*. Arrows indicate positions and orientation of the integrations.

the molecular size of a protein with ~ 8 kD, it appears that G-CSFR is mainly mono- or multi-ubiquitylated. Whereas poly-ubiquitylation serves as a signal for proteasome-mediated protein degradation, mono-Ubs act as a sorting signals for delivery of proteins in the biosynthetic or endocytic pathway to multi vesicular bodies (MVB) and lysosomes (Aguilar and Wendland, 2003; Hicke and Dunn, 2003). Direct or indirect association with E3 ligases regulates cell surface expression of a number of receptors. For instance, for the degradation of the interferon α receptor 1 (IFNAR1) a mechanism was proposed involving the Homolog of Slimb (HOS) F-Box protein, which interacts with Skp1 and Cullin-1 to form the SCF^{HOS} E3 complex (Kumar et al., 2004; Kumar et al., 2003). Similar to Wsb, HOS (alternatively termed β-TrCP2 or Fbw1b) binds to substrates via its WD40 domain. Lysine residues in the cytoplasmic tail that are ubiquitylated by SCF^{HOS} are essential for IFNAR1 degradation (Kumar et al., 2003). However, a major difference is that binding of Wsb to G-CSFR does not require receptor activation. This supports the notion that Wsb proteins are dispensable for ligand-induced internalization but mainly affect the steady state membrane expression of G-CSFR by targeting excess protein for lysosomal degradation. A similar role has recently been proposed for SOCS5 in the control of the steady state distribution of the epidermal growth factor receptor (Kario et al., 2005).

To study the consequences of reduced Wsb levels on G-CSFR distribution, we have attempted to knockdown of Wsb-1 and Wsb-2 expression using small interfering (si) RNA. However, although significant reduction of Wsb transcript levels in HEK293 and 32D cells was achieved, siRNA's were found to nonspecifically reduce G-CSFR expression on the cell membrane. Recent studies have revealed that siRNA's cause the activation of Toll-like receptors and induce strong interferon responses in cells, which might explain these nonspecific effects (Reynolds et al., 2006). Because Wsb-1 and Wsb-2 (double) knockout mice are not yet available, the question whether Wsb-1 and Wsb-2 have unique or (partly) redundant functions in G-CSFR routing and signaling cannot be addressed at present.

Wsb fusion to the G-CSFR resulted in more prominent effects than the presence of the G-CSFR region involved in Wsb binding, both on receptor routing (compare Figures 6D and 3A, left panel) and on signaling (Figure 7). The reason for this is unclear but likely relates to temporal and stoichiometric aspects of the protein-protein interaction that that are overridden by the fusion. Nonetheless, the results are compatible with a role of the receptor C-terminus in recruiting E3 ligase activity that ubiquitylates one or more of the conserved lysine residues in the G-CSFR, thereby controlling its steady-state membrane expression as well as overall protein stability. Previous studies showed that SOCS3 acts in a negative feedback loop, involving G-CSF-induced activation of STAT3, STAT3-induced transcription of SOCS3 and binding of SOCS3 protein to phosphorylated Y729 of G-CSFR [Hermans, 2003 #17;Hortner, 2002 #25;van de Geijn, 2004 #26]. In contrast, Wsb-1 and Wsb2 transcript

levels were high in nonstimulated 32D cells and primary bone marrow progenitors and not altered by G-CSF (data not shown). While SOCS3 attenuates G-CSF signaling from activated receptors, Wsb appears to control steady-state lysosomal degradation as well as the levels of G-CSFR expressed on the cell membrane, thereby contributing up front to a balanced response of myeloid precursors to G-CSF. The observation that disruption of the *Wsb-2* gene (mouse leukemia) and truncation of the G-CSFR C-terminus (SCN/AML patients) are both associated with acute myeloid leukemia supports the notion that disruption of the control of steady state expression of G-CSFR leads to perturbed myeloid differentiation in vivo and may contribute to leukemic outgrowth.

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CHAPTER 6

Summary & Discussion

6.1. SUMMARY

In the past decade, considerable attention has been paid to the intracellular trafficking of growth factor receptors and the mechanisms controlling this process. These studies have revealed that ubiquitination of lysine residues within the cytoplasmic domain of receptor play a crucial role in endocytosis and sorting of the receptor complex to degradation compartments. Much of this work has been performed on model systems of intrinsic tyrosine kinase receptors, the EGF receptor in particular (Haglund et al., 2003a; Huang et al., 2007; Huang et al., 2006; McCullough et al., 2006; Pennock and Wang, 2008; Row et al., 2006; Stang et al., 2004). While these studies have provided general paradigms of how these processes are controlled, investigations of other receptor systems, for instance the cytokine receptors, have revealed a significant level of variation on the theme. For instance, this applies to the role of receptor lysines in ligand-induced internalization, the ubiquitin ligation complexes involved, specific domains that interact with these complexes and the type of ubiquitin modification (mono-, multi- and poly-ubiquitination). Experiments reported in this thesis have illustrated the mechanisms that regulate intracellular trafficking of the G-CSFR.

Chapter 1 offers an overview of current knowledge in ligand-dependent and –independent routing of cytokine receptors. Endocytosis of receptor complex is one of the mechanisms that negatively control cytokine signalling. Trafficking of G-CSFR through endocytotic pathway, including internalization, routing to early endosomes, late endosomes, multivesicular bodies and lysosomes and its effect on signal downregulation have been introduced in more detail in this chapter. Furthermore, the role of receptor ubiquitination by the function of E3 ubiquitin-ligases and involvement of effector proteins directing lysosomal sorting in both retrograde and forward routing are described.

While Chapter 2 and 3 focus on endocytosis and retrograde routing of the G-CSFR following its activation, forward routing and constitutive distribution of receptor have been discussed in Chapters 4 and 5. In *Chapter 2*, the role of G-CSFR ubiquitination and the E3 ubiquitin ligase involved in ligand-induced endocytosis and lysosomal routing was studied. Specifically, the differential involvement of cytoplasmic lysines of the G-CSFR in intracellular routing and downregulation of signalling was investigated. These studies established that the juxtamembrane lysine located at position 632 is target for ubiquitination by SOCS3 following G-CSFR activation. Interestingly, lysosomal sorting and signal downregulation of the G-CSFR exclusively relied on this lysine residue, even though there are five lysines present in the C-terminal region of the receptor that are highly conserved among different species. Mutation of K632 G-CSFR in myeloid progenitors expressing this receptor mutant leads to sustained signalling and hyperproliferation phenotype in response to G-CSF due to perturbed signalling and accumulation in early endosomes.

Findings presented in *Chapter 3* show that endocytosis is a prerequisite for receptor site-specific ubiquitination. In addition, the molecular determinants which identify the proper

functionality of the G-CSFR lysine 632 were investigated in further detail. An imperative membrane-proximal position of this lysine residue was indispensable to direct lysosomal sorting and signal downregulation of the G-CSFR, therefore specificity of K632 stems from its interaction with downstream effector proteins.

In *Chapter 4,* it is shown that two mechanisms lead to increased cell surface expression and stability of the G-CSFR in forward routing: 1) the juxtamembrane lysine residue of G-CSFR at position 632, which controls the distribution of receptor by regulation of its degradation, and 2) elevated levels of JAK proteins. The effect of JAKs is not exerted by masking motifs essential for receptor internalization or by reducing the level of ubiquitination. Furthermore, it is observed that increased JAK levels confer ligand independent signalling and cell survival to routing defective G-CSFR mutants.

In *Chapter 5*, regulation of G-CSFR expression on the cell surface by its cytoplasmic lysine residues has been addressed in further detail. It has been demonstrated that Wsb proteins bind to the G-CSFR C-terminus and function as an E3 ubiquitin ligase in steady state situations. Fusion of Wsb2 protein to the C-terminal region of G-CSFR indicates that ubiquitination of G-CSFR lysine residues by the SOCS box of Wsb modulate receptor distribution on the plasma membrane and play an important role in appropriate balance between proliferation and differentiation of myeloid cells.

6.2. DISCUSSION AND FUTURE PERSPECTIVES

6.2.1. Involvement of ubiquitination in endocytotic pathway of G-CSGR

Proper downregulation of activated G-CSFR and therefore attenuation of signalling are critical mechanisms in maintaining granulopoiesis. Retrograde endocytosis followed by sorting to intracellular compartments and post translational modifications such as phosphorylation and ubiquitination of receptor and its complex are involved in regulation of this process. In chapter 2 and 3 of this thesis, endocytosis and ubiquitination of the G-CSFR following its activation were studied. Ligand stimulation results in rapid endocytosis of G-CSFR in clathrin coated vesicles. Ubiquitin modification of cytoplasmic lysines of the G-CSFR is not required for internalization; however fusion of a single ubiquitin molecule to the C-terminal region of an internalization-deficient mutant can induce its internalization. This observation is in agreement with the findings presented for EGFR (Haglund et al., 2003a; Mosesson et al., 2003) and IL-2 receptor α chain (Nakatsu et al., 2000). However, in a recent report dealing with endocytotic routing of the fibroblast growth factor receptor (FGFR) (Haugsten et al., 2008), it was shown that fusion of an ubiquitin moiety does not always give rise to an endocytosis signal This discrepancy could relate to the type of internalization-defective receptors used for mentioned studies or to the dominant pathway governing endocytosis at the cell membrane.

Whereas ubiquitination appears dispensable for G-CSFR internalization, it plays a crucial role in intracellular trafficking of receptor from early to late endosomes/lysosomes. A schematic representation of intracellular trafficking of G-CSFR is shown in Figure 1. After ligand stimulation, SOCS3 recruits to the phosphorylated tyrosine of G-CSFR on position 729 (Y729). In addition, we observed that ligand stimulation results in ubiquitination of four out of five conserved lysines located at the cytoplasmic tail of the G-CSFR. Although we cannot exclude that SOCS3 is involved in the ubiquitination of these four lysine, , we showed that SOCS3 is responsible for ligation of ubiquitin molecules to the juxtamembrane lysine of G-CSFR at position 632 and that this results in signal attenuation, lysosomal trafficking and subsequent degradation of receptor. Other E3 ubiquitin ligases appear to be involved in ubiquitin modification of lysine residues 672, 681 and 682, since the G-CSFR lacking the SOCS3 binding site (Y729F G-CSFR) is still ubiquitinated, although to a significantly lower extent compared to wt receptor.

One candidate E3 ubiquitin-ligase is c-Cbl, which has been reported to play a negative role in G-CSF signalling (Grishin et al., 2000; Wang et al., 2002). C-Cbl is a member of RING-type ubiquitin ligase family and has been shown to be involved in downregulation of receptors including hepatocyte growth factor receptor (HGFR/ Met), EGFR and stem cell factor receptor (SCFR/ c-Kit). Direct binding of c-Cbl to phosphorylated tyrosine of activated receptor is sufficient for its function (Carter et al., 2004; Masson et al., 2006; Thien and Langdon, 2005). However, we were not able to demonstrate the association of c-Cbl with G-CSFR by employing different techniques such as co-immunoprecipitation and the MAPPIT assays as described in Chapter 5 (Eyckerman et al., 2002).

6.2.2. Ubiquitin-dependent sorting of G-CSFR in the biosynthetic pathway

Newly synthesized proteins that arrive at the *trans*-Golgi network (TGN) are sorted either to the plasma membrane or to an endosomal compartment and route to the lysosome degradation pathway (Figure 1). Although there is controversy over the type of ubiquitin modifications in this pathway, studies on yeast amino acid permeases made it evident that ubiquitin signals play an important role in regulation of forward trafficking (Beck et al., 1999; Helliwell et al., 2001; Soetens et al., 2001).

Low levels of G-CSFR expression at the cell surface compared to a large intracellular pool, implicates in a balance between routing of the receptors to the plasma membrane and their turn over. In Chapter 5, it is proposed that SOCS box-containing Wsb proteins serve as the E3 ubiquitin-ligases involved in regulation of receptor in forward routing (Figure 1). Like SOCS3, Wsb proteins are able to form an ECS complex through their SOCS box. Importantly, binding of WD40 domain of Wsb to the COOH terminus of the G-CSFR is not ligand dependent, supporting the notion that it affects constitutive routing. Controlling membrane expression of G-CSFR by Wsb2 at steady state situation depends on its E3 ligase activity and cytoplasmic lysines of the receptor, particularly the juxtamembrane lysine residue. Accord-

ingly, lysine-less G-CSFR has a high cell surface expression, which is a due to increased forward routing rather than inefficient constitutive internalization. Involvement of lysines and their ubiquitination in controlling the receptor distribution has also been recently reported for CD33, an ITIM-containing immunoreceptor (Walter et al., 2007)and gp130 (Tanaka et al., 2008).

Whether Wsb proteins are exclusively involved in the control of forward routing of G-CSFR or whether alternative E3 complexes may play a role remains to be elucidated. Interestingly, another WD40-containing protein, β-Trcp, which comprises an F box, has been identified as the E3 ligase responsible for ubiquitinatiion of several cytokine receptors, including EpoR (Meyer et al., 2007) and IFNAR1 (Kumar et al., 2004). Although for these latter receptors, it was shown that β-Trcp binds to the activated, i.e., serine phosphorylated receptor chains (Fuchs et al., 2004; Li et al., 2004), it is possible that β-Trcp binds directly to the G-CSFR C-terminus via its WD40 domain.

6.2.3. The role of E2 ubiquitin-conjugating and E3 ubiquitin-ligating enzymes in intracellular trafficking of membrane receptors

As described earlier, ubiquitin modification is a concerted action of three enzymes. E1 activates and generates a thiol ester bond with Gly76 of ubiquitin in an ATP-dependent manner and the ubiquitin molecule is then transferred to an E2 ubiquitin-conjugating enzyme (Ubc). Finally the ubiquitin ligates to a lysine residue of the substrate by the action of E3 ubiquitin-ligating enzyme. There are only one or two E1 enzymes, approximately 60 E2 enzymes and more than 400 E3 ligases in the human genome (Li et al., 2005). Interaction of E2 with E3 and recognition of limited substrates by certain E3s regulate specificity of the ubiquitin modification (Jackson et al., 2000).

All E2 ubiquitin-conjugating enzymes share a conserved catalytic domain consisting of approximately 150 amino acids. Some family members have N- or C-terminal extensions that may facilitate specific interactions (Pickart, 2001; Pickart and Eddins, 2004). Certain E2s interact with E3s from both HECT- and RING-domain families. For example, UbcH7 is able to bind to HECT domain of E6-AP (Huang et al., 1999; Kumar et al., 1997; Nuber et al., 1996) and also to several known RING E3 ubiquitin ligases like Cbl (Moynihan et al., 1999; Yokouchi et al., 1999; Zheng et al., 2000).

It has been proposed that specificity of target lysine in formation of polyubiquitin chains may be determined by the E2 (Pickart and Eddins, 2004). For example Lysine48-linked ubiquitin chains are formed by members of Ubc4, Ubc5 and Ubc7, whereas generation of polyubiquitin chains via Lysine63 is mediated by Ubc13 and some other Ubc variants (Hofmann and Pickart, 1999). In agreement with this, it was recently reported that CHIP, a U-box E3 ubiquitin ligase, is able to form lysine63-linked polyubiquitin chains with its functional partner, Ubc13 (Zhang et al., 2005). However a more recent study demonstrated that Ubc4/5

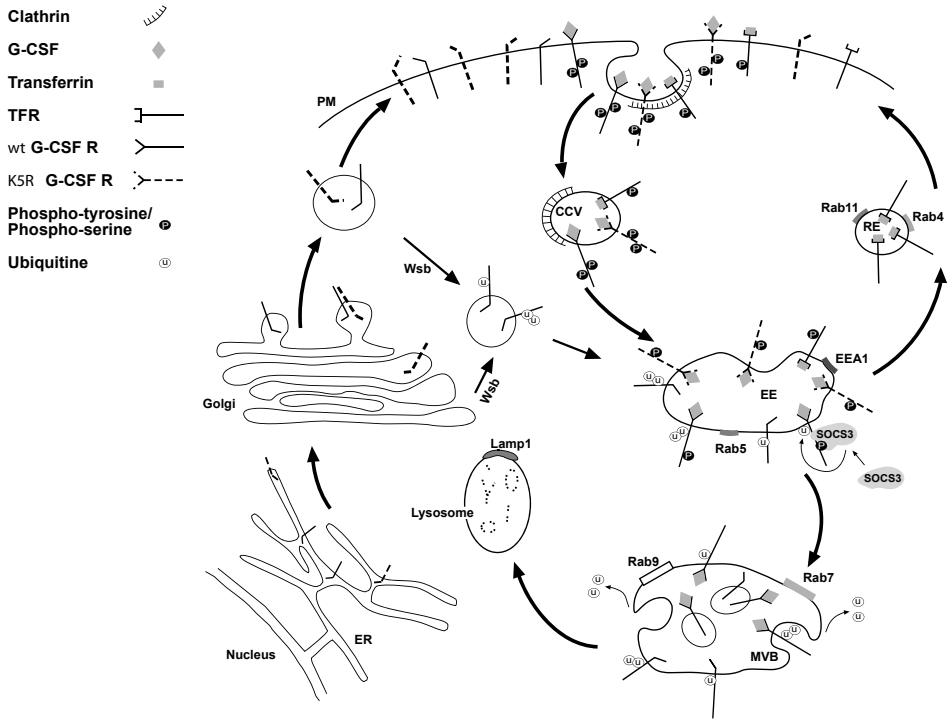

Figure1. Schematic representation of endocytosis and forward trafficking of G-CSFR
PM: plasma membrane, CCV: clathrin-coated vesicles, RE: recycling endosome, EE: early endosome, MVB: multivesicular bodies, ER: endoplasmic reticulum. See main text for detailed explanation.

family is the primary E2 for Cbl mediated EGFR polyubiquitination (Umebayashi et al., 2008), which is made by lysine63-linked ubiquitin chains (Huang et al., 2006).

The E2 ubiquitin-conjugating enzymes involved in ubiquitination of G-CSFR have not yet been identified. Screening different members of E2 family in E2-E3 interaction assays can assist in uncovering the E2 partner of SOCS3-mediated G-CSFR ubiquitination. Importantly, functional studies such as colocalization, knockdown and in vitro analyses should be conducted to confirm the protein-protein interactions, since binding assays do not always identify functional E2-E3 pairs (Christensen et al., 2007; Deffenbaugh et al., 2003). Interestingly, localization studies of E2-E3 pair can reveal where receptor ubiquitination occurs and if this proceeds after internalization in intracellular compartments such as early endosomes. Indeed, localization of Ubc4/5 as an E2 for Cbl-mediated EGFR ubiquitination in endosomal vesicles demonstrated that ubiquitinated status of EGFR is in fact maintained owing to the continuous ubiquitination (Umebayashi et al., 2008).

Despite intensive studies on ubiquitin-mediated events, very little is known concerning the specificity of an E3 ligase for a certain substrate and subsequent ubiquitin modifications. One mechanism is presence of linear motifs within the cytoplasmic tail of plasma membrane receptors which has been shown to be involved in site-specific ubiquitination and recognition of substrate by E3 ubiquitin ligases as it was shown for β-Trcp (Kumar et al., 2007; Meyer et al., 2007). More recently, a consensus pattern was identified as a recognition determinant for substrates of a RING domain E3 ubiquitin ligase, TRAF6 (Jadhav et al., 2008). In the case of G-CSFR, however, we can not definitely rule out that there is a so far unknown linear motif within the proximity of functional lysine of G-CSFR, which may cooperate with recruitment of SOCS3 and activate lysosomal sorting pathway.

On the other hand, the type of ubiquitination (mono versus polyubiquitination) and topology of ubiquitin chains generated by E3 ligases can be specific for a certain target and therefore play an important role in the fate of ubiquitinated target. Whereas monoubiquitination and lysine-63 linked polyubiquitination are involved in endocytosis and lysosomal sorting of membrane receptors, elongation of ubiquitin chains via lysine48 of ubiquitin molecule is a signal for proteasomal degradation. Some E3 ligases can catalyze both mono and polyubiquitination such as c-Cbl and β-Trcp E3 ubiquitin ligases in the case EGFR and EpoR, respectively (Haglund et al., 2003b; Huang et al., 2006; Kumar et al., 2007; Rubin et al., 2003; Soubeyran et al., 2003; Yokouchi et al., 2001). Although it is mostly reported that SOCS proteins through their SOCS box are involved in proteasomal degradation of their targets, we have shown that ligand-induced downregulation of G-CSFR can also be regulated by lysosomal sorting of receptor by SOCS3. The nature of ubiquitin chains made by SOCS3-mediated G-CSFR ubiquitination needs further investigation. However the smear of ubiquitination ranging from 135 to 170kDa rather than single ubiquitin implies oligoubiquitination or lysine-63 linked polyubiquitination. Further studies like mass spectrometry (Huang et al., 2006; Kirkpatrick et al., 2006; Kumar et al., 2007)or utilizing ubiquitin mutants lacking functional lysines (for example lysine-63) are required to determine the precise role of these modifications.

6.2.4. Possible endocytotic adaptors linked to lysine-632 responsible for G-CSFR lysosomal sorting

G-CSFR lysine-632 is located at position –5 relative to the box1 region (PGIPSP). Intriguingly, this residue is a highly conserved lysine upstream of box1 region among several other cytokine receptors such as leptin receptor, gp130 and human EpoR (table I). Furthermore, most of these receptors have a SOCS3 binding site. This might be suggestive of a general mechanism of the juxtamembrane lysine and merits investigations to demonstrate its potential role in regulation of lysosomal routing. Interestingly, TpoR possessing an arginine at this position recycles to the plasma membrane in myeloid cells (Dahlen et al., 2003). However mutation of K632 to arginine in G-CSFR (K632R G-CSFR) does not generate a recycling

receptor and results in retention of the mutant receptor in early endosomal compartment and preclude its trafficking to lysosomes.

In the experiments described in Chapter 3, it was shown that the position of the ubiquitinated lysine of the G-CSFR in an imperative proximity of the plasma membrane is crucial for its appropriate function. Therefore, selection of this lysine by ubiquitin machinery may stem from the interaction of the specifically ubiquitinated cargo protein with downstream sorting proteins linked to the plasma membrane. Indeed, it has been postulated that several sorting proteins containing ubiquitin-binding domains (UBDs) posses binding sites for membrane phosphoinositides (Slagsvold et al., 2005; Slagsvold et al., 2006; Stahelin et al., 2002). These proteins such as epsin, Hrs (a member of ESCRT-0 complex) and Eap45 (a member of ESCRT-II complex) play a critical role in sorting of the cargo to late endosomes/ multivesicular bodies. Furthermore, electron microscopic studies show localization of these proteins with clathrin coated vesicles (Stang et al., 2004; Wendland, 2002).

An interesting question is which UBD-containing protein(s) linked to K632 is involved in sorting of the G-CSFR. Several strategies, such as pull-down assays, mass spectrometry and employing siRNAs against candidate sorting proteins could assist to address this question. Thus far, we were unable to show interactions of UBD-containing proteins such as Hrs, STAM and Eap45 with G-CSFR. However, it must be noted that showing these interactions is not effortless for several reasons. First, binding of a UBD to a free ubiquitin has a low affinity (Kd=0.1-1 mM) (Fisher et al., 2003). Additionally, these proteins are target of ubiquitination themselves, which hasten their degradation (Di Fiore et al., 2003; Katz et al., 2002; Polo et al., 2002). And finally, given the fact that ubiquitination is a dynamic and reversible process, dissociation of ubiquitin molecules from the ubiquitinated cargo reduces the chance of detection of ubiquitin-UBD association. Recent developments by using siRNAs against proteins such as Hrs and STAM give the opportunity to demonstrate the role of these proteins in lysosomal trafficking. However, siRNAs against such essential components of MVB sorting may have a secondary effect which is aggregation of endosomes. Generation of enlarged/abnormal endosomes can in turn inhibit further routing of the ubiquitinated cargo to lysosomes.

6.2.5. Potential role of deubiquitinating enzymes in trafficking of G-CSFR

It has been shown that the attachment of ubiquitin molecules to the cytoplasmic lysine residues of several cytokine receptors results in sorting to lysosomal compartments, whereas the receptors such as tranferrin and low-density lipoprotein receptors that do not undergo ubiquitination, recycle back to the plasma membrane. Therefore, the process of routing to multivesicular bodies and lysosomes is regulated by ubiquitin modifications at the level of endosomes. Ubiquitination is a reversible post translational modification and ubiquitin molecules can be removed from ubiquitinated cargo by deubiquitinating enzymes (DUBs).

Table I. Alignment of receptors harbouring the conserved juxtamembrane lysine residue and involvement of SOCS3 in receptor signalling

Receptor	Species	Sequence	SOCS3 binding (Reference)
G-CSFR	mouse	KRRG**K**TSFW *SDV**P**D**P***	Hortner et al. 2002
	human	SPNP**K**NPLW *PSV**P**D**P***	van de Geijn et al. 2004
EpoR	mouse	RRTLQQKIW *PGI**P**S**P***	Sasaki et al. 2000
	human	RRAL**K**QKIW *PGI**P**S**P***	Hortner et al. 2005
GHR	mouse	QQRI**K**MLIL *PPV**P**V**P***	Ram et al.1999
	human	QQRI**K**MLIL *PPV**P**V**P***	Riddersrale et al. 2003
gp130	mouse	RDLI**K**KHIW *PNV**P**D**P***	Nicholson et al. 2000
	human	RDLI**K**KHIW *PNV**P**D**P***	Bergamin et al. 2006
IL4R	mouse	ITKI**K**KIWW *DQI**P**T**P***	Hague et al. 2000
	human	ITKI**K**KEWW*DQI**P**N**P***	Ratthe et al. 2007
IL7R	mouse	KKRI**K**PVVW *PSL**P**DH*	
	human	KKRI**K**PIVW *PSL**P**DH*	
IL9R	mouse	SPRL**K**RIFY *QNI**P**S**P***	Lejeune et al. 2001
	human	SPRV**K**RIFY *QNV**P**S**P***	
LepR	mouse	HQRM**K**KLFW *DDV**P**N**P***	Eykerman et al. 2000 Bjorbak
	human	HQRM**K**KLFW *EDV**P**N**P***	et al. 2000
TpoR	mouse	YRRLRHALW *PSL**P**DL*	-
	human	YRRLRHALW *PSL**P**DL*	
IL2 Rβ	mouse	GPWL**K**TVLK CHI***PDP***	Cohney et al.1999
	human	GPWL**K**KVLK CNT***PDP***	

There are approximately 95 putative DUBs encoded by human genome (Nijman et al., 2005). Functions of DUB and their substrate specificity are poorly studied as compared to that of the ligase enzymes.

The Src homology 3 (SH3) domain of STAM, a member of ESCRT-0 complex is a common binding site for two DUBs called UBPY (Ub-specific protease 1) and AMSH (associated molecule with the SH3 domain of STAM) (Kato et al., 2000; McCullough et al., 2006; Tanaka et al., 1999). Both UBPY and AMSH have been shown to be required for efficient degradation of EGFR (McCullough et al., 2006; Mizuno et al., 2005; Row et al., 2006). However deubiquitination of EGFR by UBPY depletion and its involvement in enhancement or inhibition of degradation is a matter of debate (Alwan and van Leeuwen, 2007; Mizuno et al., 2005; Row et al., 2006). Whereas UBPY does not discriminate between polyubiquitin chains elongated via lysine 63 or lysine 48, AMSH can only process lysine 63-linked polyubiquitin chains. Therefore, AMSH does not play a direct role in proteasomal degradation. Primary studies in overexpression of both AMSH and UBPY showed decreased ubiquitination of G-CSFR

(ongoing experiments). Controlling the level of protein ubiquitination by DUBs takes place at early stages of the endosomal sorting, indicating that DUBs act before full commitment of ubiquitinated cargo to MVBs (Clague and Urbe, 2003). Removing ubiquitin at this step may enhance receptor recycling; however wt G-CSFR does not recycle to the plasma membrane. In addition lack of ubiquitination in K5R G-CSFR mutant does not influence recycling pathway. One explanation is immediate attachment of ubiquitin molecules to the deubiquitinated G-CSFR and subsequent routing to the lysosomal pathway. Another explanation is involvement of DUBs in later stages of sorting when ubiquitinated G-CSFR is already committed to MVBs. The latter model has been proposed for AMSH in receptor tyrosine kinase sorting by competing with Vps4 for CHMP (ESCRT-III) binding after commitment of RTK to the MVB (Agromayor and Martin-Serrano, 2006; Clague and Urbe, 2006; Tsang et al., 2006). However a recent report suggests that ubiquitinating and deubiquitinating activities compete along the endocytotic pathway (Umebayashi et al., 2008). Involvement of DUBs in G-CSFR deubiquitination and their potential role in regulation of lysosomal sorting by opposing SOCS3 need to be investigated in more detail.

6.2.6. SOCS3 as a negative regulator of G-CSF signaling *in vivo*

The studies presented in this thesis and in earlier work from our laboratory (van de Geijn et al., 2004b) have elucidated the various functions of SOCS3 protein in the negative regulation of G-CSF signaling in vitro. However, the biological role of SOCS3 is best exemplified in mouse models.

As described earlier, SOCS3 knockout mice were shown to be embryonic lethal (Marine et al., 1999; Roberts et al., 2001). To investigate the role of SOCS3 in adult tissues several conditional mouse models were generated to examine the role of SOCS3 in various systems including hematopoietic system. Two models were used for deletion of SOCS3 in all hematopoietic and endothelial tissues (Croker et al., 2004; Kimura et al., 2004). In both models, SOCS3 was found to be a negative regulator of G-CSF signaling. These mice are viable and healthy; however from 17 weeks of age they demonstrate neutrophil leukocytosis and infiltration of hematopoietic cells into the liver and lungs (Croker et al., 2004). Administration of G-CSF to these mice resulted in neutrophilia, with neutrophil levels exceeding those of wild type littermates, indicating that loss of Socs3 results in hypersensitive to G-CSF stimulation in vivo (Croker et al., 2004). Recently, a mouse model lacking the SOCS box motif of SOCS3 was generated by Boyle and colleagues. These mice are viable and do not reveal any abnormalities (Boyle et al., 2007). Interestingly, they exhibited exaggerated responses to G-CSF administration. Furthermore, JAK/STAT signaling downstream of G-CSFR was enhanced in the bone marrow cells. This was in agreement of in vitro studies suggesting that SOCS box was required for negative regulation of STAT5 and, to a lesser extent, STAT3 activation following G-CSF stimulation (van de Geijn et al., 2004a). The delta SOCS box mouse model was

the first in vivo demonstration that the SOCS box of SOCS3 plays a crucial role in inhibition of G-CSF signaling.

6.2.7. Altered ubiquitin signalling and its implications for myeloid disorders

As discussed above maintenance of balance between proliferation and differentiation of myeloid progenitors to mature neutrophils is controlled by appropriate signal transduction via G-CSFR. Aberrant signalling function of G-CSFR caused by a number of mutations or rare polymorphisms in the *G-CSFR* gene (Touw and van de Geijn, 2007), results in enhanced number of blood cells and upregulated levels of signalling in myeloid disorders. Lack of lysosomal sorting of a G-CSFR mutant, due to lack of ubiquitination leads to prolonged G-CSF signalling. This receptor mutant contributes to hyperproliferation phenotype without induction of differentiation in myeloid 32D cells. Therefore, G-CSFR turnover and signal downregulation plays a pivotal role in G-CSF-controlled neutrophil development.

Analysis of AML patients did not show any mutations in the cytoplasmic lysines of the *G-CSFR* or *SOCS3*. However further investigation is required to determine abnormal activation of endocytotic adaptor proteins directing lysosomal trafficking, other E3 ligases and DUBs involved in ubiquitination or deubiquitination of G-CSFR, respectively.

Involvement of endocytotic adaptors and their alteration such as loss or overexpression have been implicated in oncogenesis (for review see Crosetta et al. 2005). An extensively-studied example is Tsg101 which has also been identified in truncated transcripts in human leukaemia and lymphoma cell lines (Hosokawa et al., 2000; Li et al., 1998)Ferrer et al. 1999). Regarding E3 ubiquitin ligases, different types of molecular alterations such as mutation or misexpression have been demonstrated that occur in haematopoietic malignancies. For instance, inactivating mutations in the Von Hippl-Lindau (VHL) E3 ligase is detected in patients with congenital polycythemia (Pastore et al., 2003a; Pastore et al., 2003b). An example of E3 ligase misexpression was presented in chapter 3. Using gene expression profiling, we showed that Wsb2 is downregulated in a subclass of patients with CEBPα mutations (Erkeland et al., 2007; Gaullier et al., 1999). Furthermore, Wsb2 has been identified in a common virus integration site in a murine myeloid leukemia, leading to disruption of the gene (Erkeland et al., 2004). Therefore misexpression of Wsb2 may contribute to myeloid leukemia through G-CSFR accumulation. And finally, deregulation of specific DUBs has also been implicated in leukemogenesis. For instance, the DUB USP42 was identified in a translocation with RUNX1 in acute myeloid leukaemia (Paulsson et al., 2006). These observations suggest that altered ubiquitin signalling pathways are important in normal haematopoiesis and characterization of such molecular alterations is critical to develop new treatments for blood cancer.

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Nederlandse samenvatting

SAMENVATTING

In het afgelopen decennium is veel onderzoek verricht aan intracellulaire "trafficking" van groeifactorreceptoren en de mechanismen die dit proces besturen. Deze studies hebben laten zien dat ubiquitinering van lysine residuen in het cytoplasmatisch domein van receptoren een cruciale rol speelt bij de endocytose en sortering van membraanreceptoren naar compartimenten waarin eiwitten worden afgebroken. Veel werk is uitgevoerd in modelsystemen van de zogenaamde intrinsieke tyrosine kinase receptoren, in het bijzonder de EGF receptor {Pennock et al MBC 2008, Huang et al. PNAS 2007, Huang et al. Mol cell 2006, Stang et al MBC 2004, Haglund et al MCB 2003, Row et al. JBC 2006, McCullough et al. Curr Biol 2006}. Hoewel deze studies de algemene principes van internalisering en endosomale sortering hebben blootgelegd, is uit onderzoek aan andere receptorsystemen, met name de cytokinereceptoren, een aanzienlijke mate van diversiteit naar voren gekomen. Dit geldt bijvoorbeeld voor de rol van receptor lysines in de ligand-geïnduceerde internalisering, de ubiquitine ligase (E3) complexen betrokken bij deze processen, specifieke domeinen die een interactie met deze complexen aangaan en de aard van de ubiquitine modificaties (mono-, multi- en poly-ubiquitinering). De experimenten beschreven in dit proefschrift hadden tot doel de mechanismen betrokken bij de intracellulaire "trafficking" van de G-CSF receptor op te helderen.

Hoofdstuk 1 geeft een overzicht van de huidige kennis van zaken over ligand-afhankelijke en –onafhankelijke intracellulaire routing van cytokine receptoren. Endocytose van het receptor complex is een van de mechanismen verantwoordelijk voor het uitschakelen van cytokine-geïnduceerde signaaltransductie. Trafficking van de G-CSFR via de endocytotische route, middels internalisering, routing via vroege endosomen naar late endosomen, multivesicular bodies (MVB) en lysosomen, en de effecten daarvan op de remming van de signaalfunctie worden in dit hoofdstuk geïntroduceerd. Tevens wordt de rol van receptor ubiquitinering door E3 ubiquitine-ligasen en de rol van effector-eiwitten betrokken bij lysosomale sortering in zowel de retrograde als de voorwaartse richting beschreven.

Hoofdstukken 2 en 3 behandelen endocytose en retrograde routing van de G-CSFR na activering door het ligand G-CSF. In *Hoofdstuk 2* wordt de rol van G-CSFR ubiquitinering en de daarbij betrokken E3 ubiquitine-ligase beschreven in relatie tot endocytose en lysosomale routing. In het bijzonder is de differentiële betrokkenheid van de cytoplasmatische lysines van de G-CSFR onderzocht. Deze studies hebben laten zien dat K632, een lysine residu gelegen in het juxtamembraan domein van de G-CSFR, afhankelijk van SOCS3 wordt geubiquitineerd na receptor activering. De lysosomale sortering en SOCS3-geinduceerde signaalremming van de G-CSFR blijken exclusief afhankelijk van dit lysine residu, ondanks het feit dat er in totaal 5 geconserveerde lysines in het cytoplasmatisch domain van de G-CSFR aanwezig zijn.

De bevindingen beschreven in *Hoofdstuk 3* laten zien dat endocytose een voorwaarde is voor de ubiquitinering van de G-CSFR. Tevens werden de moleculaire determinanten ve-

rantwoordelijk voor de functie van K632 van de G-CSFR lysine 632 verder onderzocht. De membrane-proximale positionering van het lysine residue blijkt essentieel voor de rol die deze lysine speelt bij lysosomale sortering en signaalremming, hoewel een beperkte mate van flexibiliteit in de positionering getolereerd wordt. De in dit hoofdstuk beschreven resultaten suggereren verder dat de specifieke betrokkenheid van K632 bij lysosomale routing voortkomt uit interacties met effector-eiwitten die voor hun functie afhankelijk zijn van een positie dicht bij de plasmamembraan. Bovendien blijkt uit de resultaten beschreven in dit hoofdstuk dat er geen rigide positionering van K632 ten opzichte van de SOCS3 bindingsplaats Y729 noodzakelijk is voor het ubiquitineren van K632 door SOCS3.

Voorwaartse routing en constitutieve distributie van de G-CSFR zijn beschreven in hoofdstukken 4 en 5. In *Hoofdstuk 4* worden twee determinanten verantwoordelijk voor de regulatie van membraanexpressie en stabiliteit van de G-CSFR in de context van voorwaartse routing nader bestudeerd: 1) de juxtamembraan gelokaliseerde lysine K632 van G-CSFR, betrokken bij de regulatie van degradatie, en 2) verhoogde expressie van JAK eiwitten. De effecten van JAKs blijken niet te worden veroorzaakt door maskering van motieven in de G-CSFR die essentieel zijn voor receptor internalisering en lysosomale routing of door het reduceren van de ubiquitinering van G-CSFR. Wel blijkt uit dit hoofdstuk dat verhoogde expressie van JAK eiwitten aanleiding geeft tot spontane (G-CSF onafhankelijke) signaalactiviteit van routing-defectieve G-CSFR mutanten, hetgeen leidt tot verhoogde celoverleving van groeifactor afhankelijke cellen in afwezigheid van groeifactoren.

In *Hoofdstuk 5* wordt de regulatie van G-CSFR expressie op het celoppervlak door de cytoplasmatische lysine residuen verder onderzocht. Getoond wordt dat Wsb eiwitten binden aan de C-terminus van de G-CSFR, waar zij vervolgens als E3 ubiquitine ligasen functioneren onder ligand onafhankelijke condities. Fusie van Wsb2 aan de C-terminus van G-CSFR laat zien dat ubiquitinering van de G-CSFR lysine residues via de SOCS box van Wsb G-CSFR expressie op de plasmamembraan negatief beinvloedt en op die wijze een belangrijke rol speelt bij de het in stand houden van de balans tussen proliferatie en differentiatie van myeloide voorlopercellen onder invloed van G-CSF.

List of abbreviations

LIST OF ABBREVIATIONS

AML: acute myeloid leukaemia
AMSH: associated molecule with the SH3 domain of STAM
Bio-G: biotinylated G-CSF
β-Trcp: β-transduction repeat-containing protein
CCV: clathrin-coated vesicle
CHX: cycloheximide
CLSM: confocal laser scanning microscopy
CLP: common lymphoid progenitor
CMP: common myeloid progenitor
CRH: cytokine receptor homology
CSF: colony-stimulating factors
DMEM: dulbecco modified Eagle's medium
DUB: deubiquitinating enzyme
ECS: Elongin-Cullin-SOCS box
EE: early endosome
EEA-1: early endosomal antigen-1
EMSA: electrophoretic mobility shift assay
EPO: erythropoietin
EPOR: erythropoietin receptor
EGFR: epidermal growth factor receptor
ERAD: endoplasmic reticulum-associated degradation
ESCRT: endosomal sorting complexes required for transport
FBS: fetal bovine serum
FCS: fetal calf serum
FGFR: fibroblast growth factor receptor
FITC: fluorescein isothiocyanate
FN III: fibronectin type III
FzR1: Fizzy-related 1
GAM: goat anti-mouse Ig
GAT: GGA and TOM1
G-CSF: granulocyte colony stimulating factor
G-CSFR: granulocyte colony-stimulating factor receptor
GF: growth factor
GGA: golgi-localized gamma-ear-containing Arf-binding
GHR: growth hormone receptor
GM-CSF: granulocyte-macrophage colony-stimulating factor
GMP: granulocyte macrophage progenitor

HA: haemagglutinin
HECT: homologous to E6-AP carboxyl terminus
HGFR: hepatocyte growth factor receptor
HRP: horseradish peroxidise
HSC: hematopoietic stem cell
HGF: hematopoietic growth factor
HOS: homolog of Slimb
HRS: hepatocyte growth factor regulated tyrosine kinase substrate
IFNAR1: interferon alpha receptor1
Ig: immunoglobulin
IL: interleukin
IL-2R: interleukin-2 receptor
JAK: Janus tyrosine kinase
KIR: kinase inhibitory region
Lamp-1: lysosome-associated membrane protein-1
LIF: leukemia inhibitory factor
LR: leptin receptor
mAb: monoclonal antibody
MAPPIT: mammalian protein-protein interaction trap
MEP: megakaryocyte erythroid progenitor
MFI: mean fluorescence intensity
MVB: multivesicular body
OSMR: oncostatin M receptor
PE: phycoerythrin
PH: pleckstrin homology
PI3-kinase: phosphatidylinositol 3-kinase
PIP3: phosphatidylinositol 3-phosphate
PKB: protein kinase B
PM: plasma membrane
PRLR: prolactin receptor
PtdIns: phosphatidylinositol
PY: phosphotyrosine
RE: recycling endosome
SCF: Skp1/ Cullin/ F box
SCFR: stem cell factor receptor
SCN: severe congenital neutropenia
SH2: Src homology 2
SOCS: suppressor of cytokine signalling
STAM: signal transducing adaptor molecule

STAT: signal transducer and activator of transcription
TFR: transferrin receptor
TGF: transforming growth factor
TGN: *trans*-golgi network
TOM1: target of Myb1
TPO: thrombopoietin
TPOR: thrombopoietin receptor
Tsg101: tumor suppressor gene 101
Ub: ubiquitin
UBD: ubiquitin binding domain
UBPY: Ub-specific protease
UIM: Ub-interacting motif
VHL: von Hippel-Lindau
VHS: Vps27, Hrs, STAM
Wsb: WD40 repeat and SOCS-box containing protein
WT: wild type
Y: tyrosine

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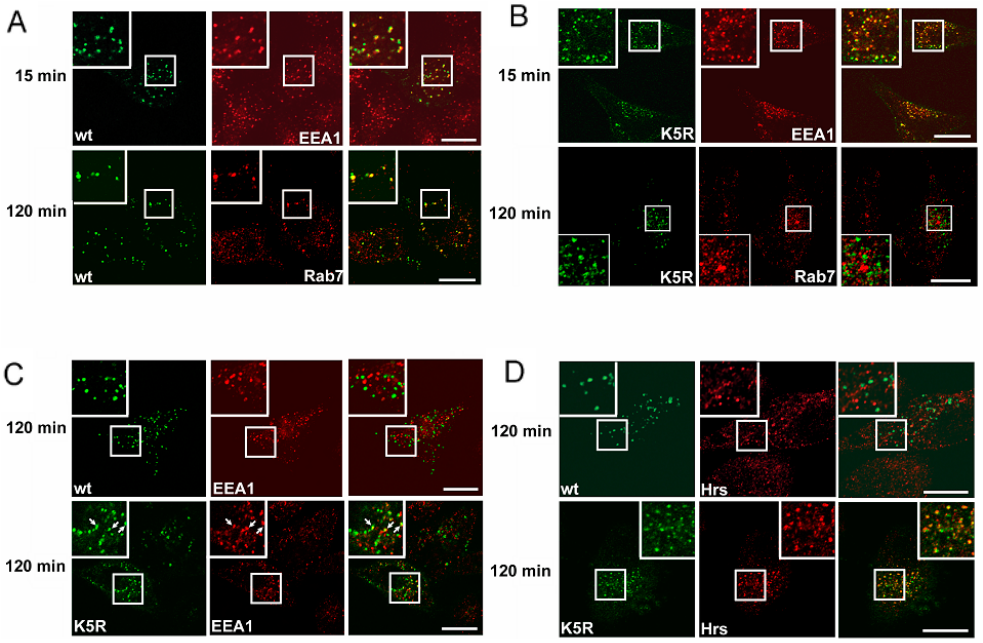
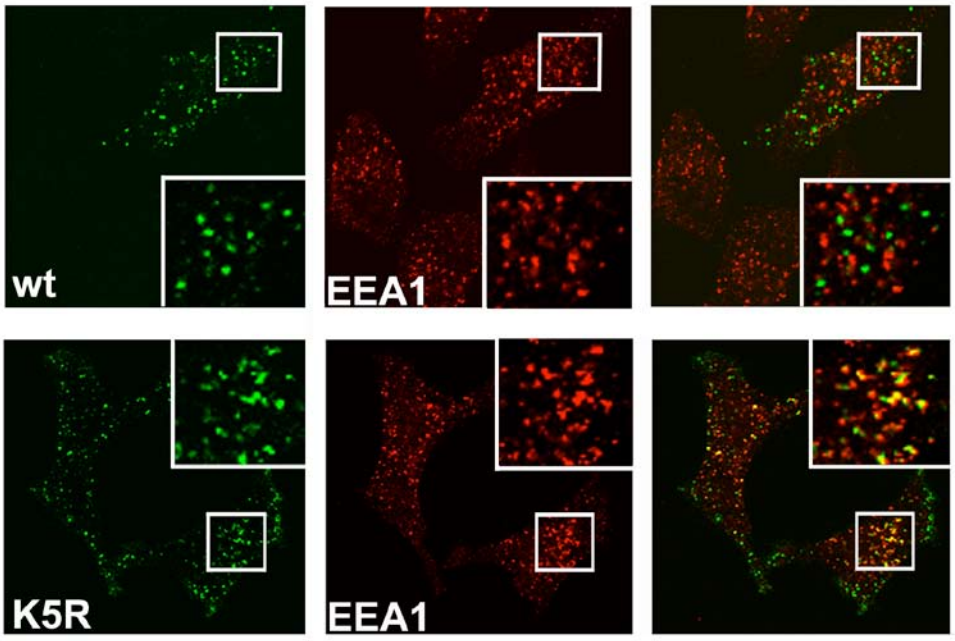


Figure 2. Role of G-CSFR lysines in lysosomal routing

HeLa cells ectopically expressing wt or K5R G-CSFR were incubated with G-CSFR antibodies and G-CSF for 20 min at 16°C, washed and transferred to 37°C for 15 or 120 min as indicated. Subsequently, cells were fixed and stained for internalized G-CSFR (green, left panels) and organelle markers EEA1, Rab7 or Hrs (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels, indicating co-localization in yellow. Insets show enlargements of the boxed areas. (A) Presence of wt G-CSFR in EEA1-positive early endosomes at 15 min and in Rab7- positive late endosomes/lysosomes at 120 min after internalization. (B) K5R is present in EEA1-positive endosomes at 15 min, but is not detectable at Rab7-positive endosomes/lysosomes at 120 min after internalization. (C) Presence of K5R (arrows) but not wt G-CSFR in EEA1-positive endosomes at 120 min after internalization. (D) Presence of K5R but not wt G-CSFR in Hrs-positive endosomes 120 min after internalization. Scale bar, 10μm



Supplementary Figure 1. Retention of K5R G-CSFR in early endosomes 4hrs after internalization
Hela cells were transfected with wt or K5R G-CSFR and stained for localization in EEA1 positive endosomes as detailed under Figure 2 (chapter 2).

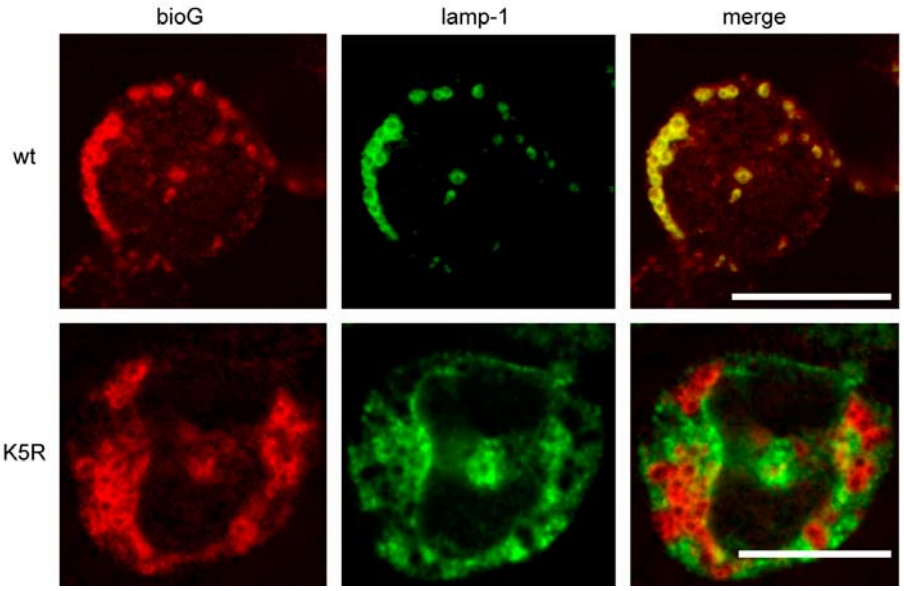
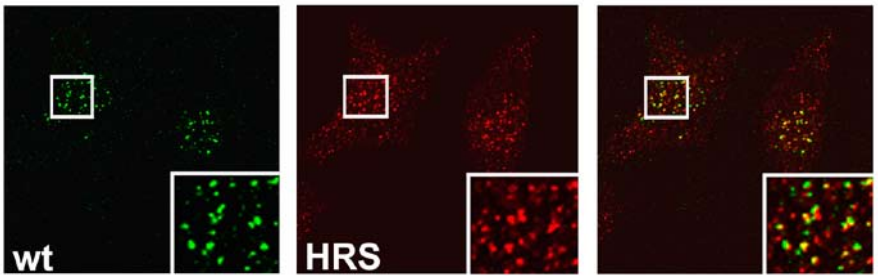


Figure 3. Intracellular localization of K5R G-CSFR in myeloid 32D cells

32D clones expressing wt or K5R G-CSFR were allowed to bind biotinylated G-CSF (bio-G) for 60 min at 4°C, washed and transferred to 37°C for an additional 60 min. Subsequently, cells were spun on glass slides, fixed, immunostained for bio-G and Lamp-1 and analyzed by CLSM. Scale bar: 10 µm



Supplementary Figure 2. Co-localization of wt G-CSFR with Hrs, 30min after internalization

Hela cells were transfected with wt G-CSFR and stained for localization in HRS positive endosomes as detailed under Figure 2 (chapter 2).

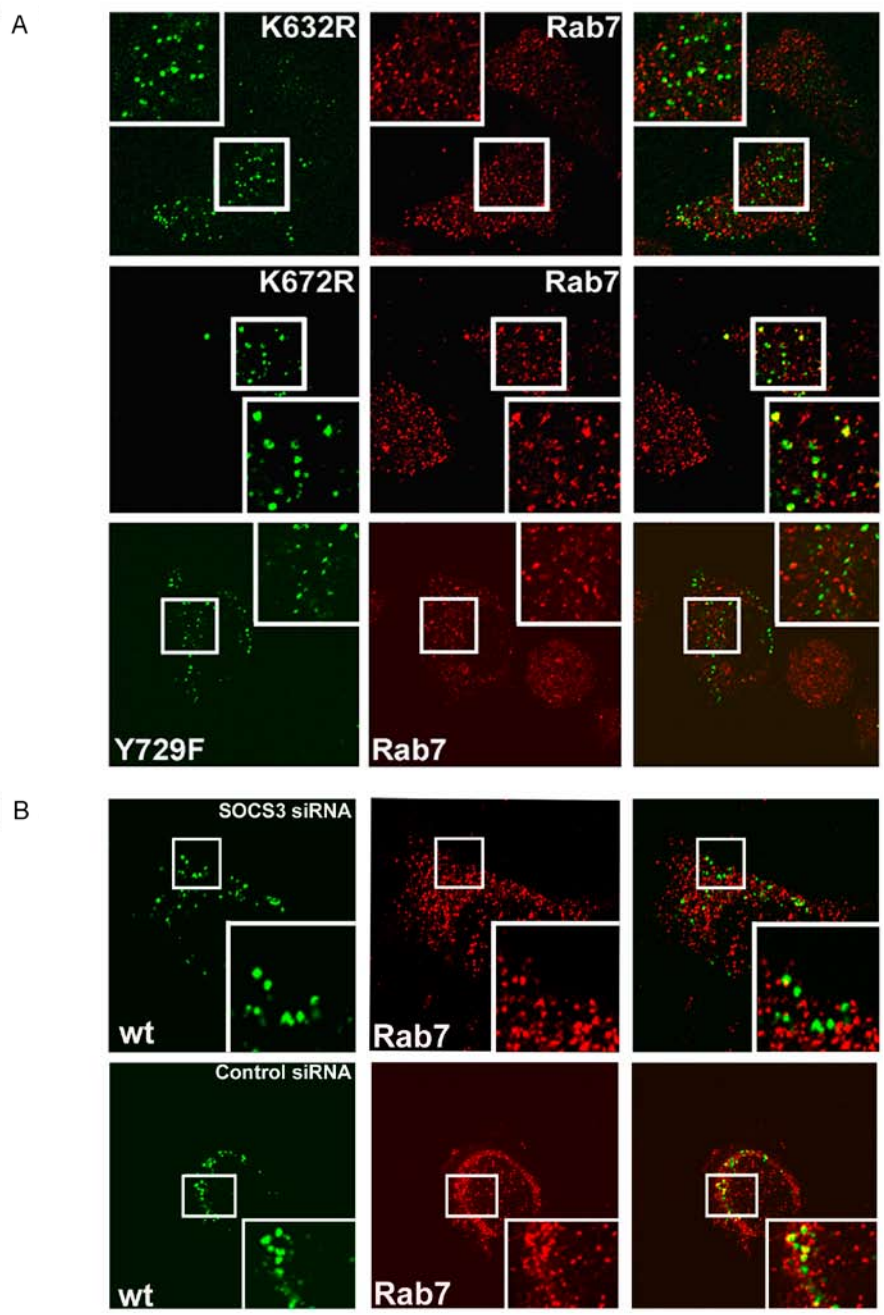
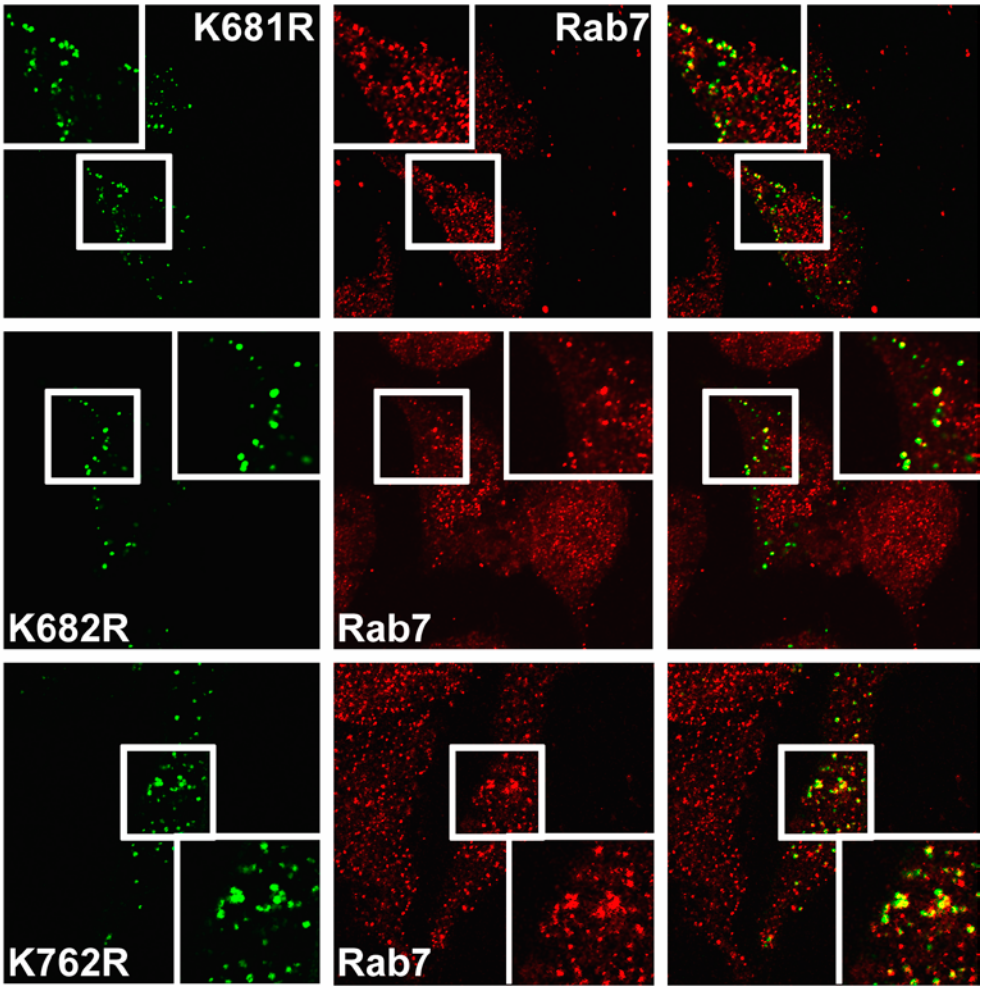


Figure 5. Intracellular localization of G-CSFR mutants

(A) HeLa cells were transfected with G-CSFR mutants K632R, K672R and Y729F and examined for localization in Rab7-positive endosomes or lysosomes after 120 min as detailed under Figure 2. (B) HeLa cells were co-transfected with wt G-CSFR and 30 nM SOCS3 siRNA (upper panel) or 30 nM control siRNA (lower panel) and analyzed as outlined above.



Supplementary Figure 3. Intracellular localization of G-CSFR mutants

HeLa cells were transfected with G-CSFR mutants K6812R, K682R and K762R and examined for localization in Rab7 positive endosomes or lysosomes as detailed under Figure 2 (chapter 3).

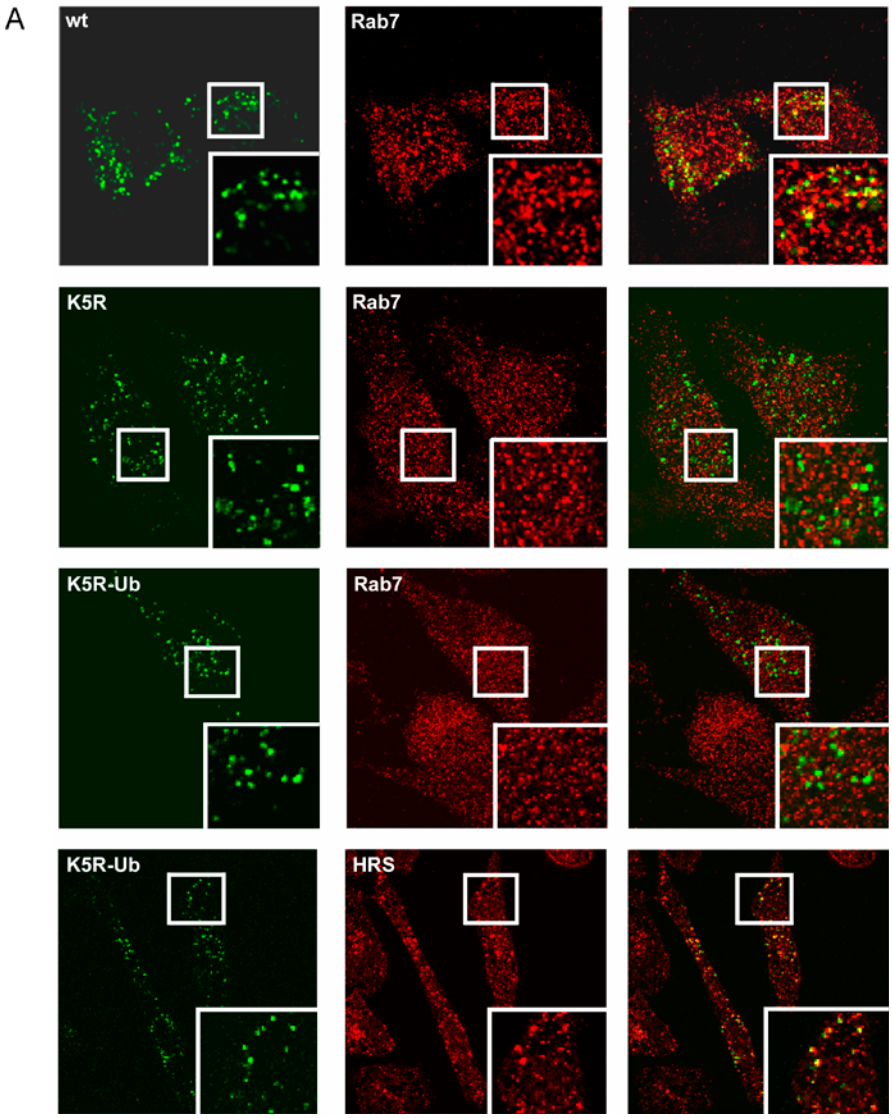
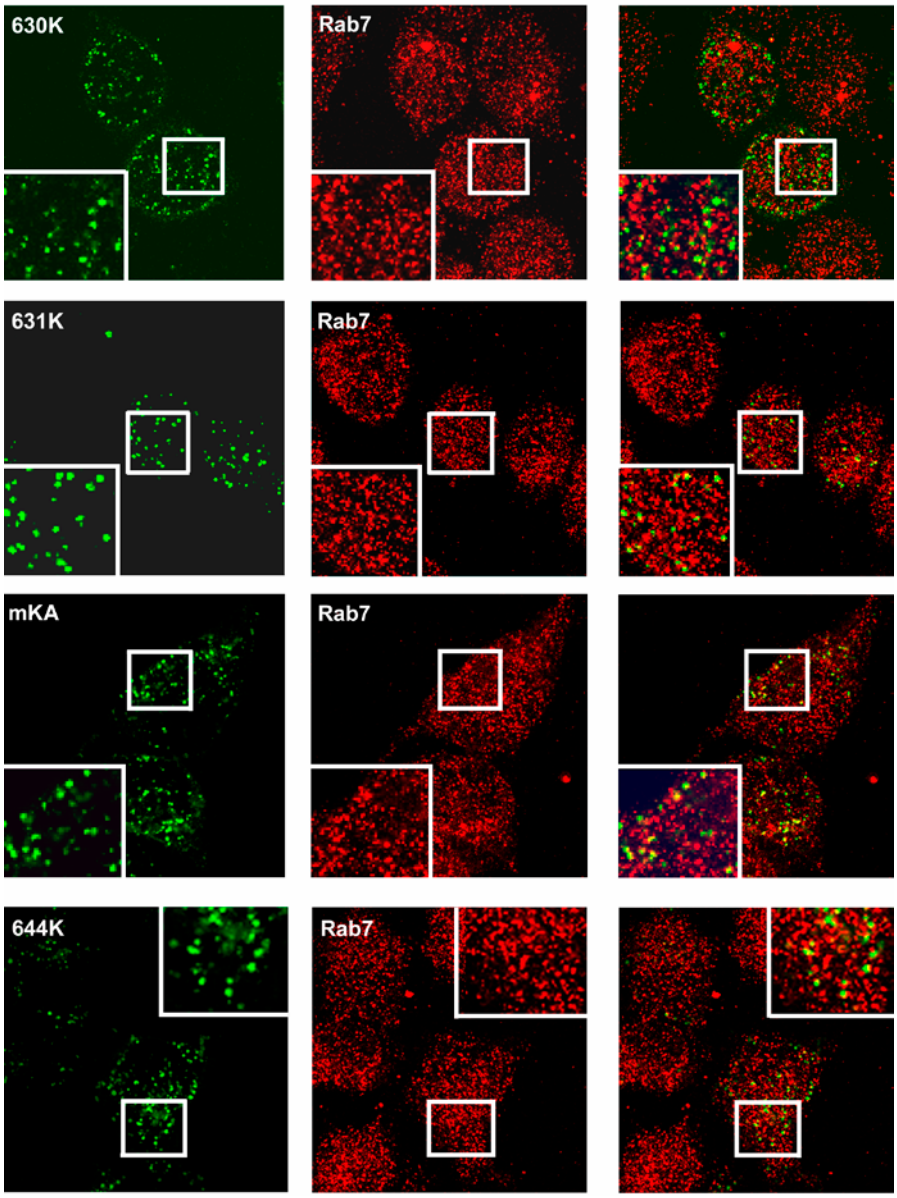


Figure 3. Fusion of ubiquitin to the C-terminus does not restore lysosomal sorting and downregulation STAT5 activation

(A) HeLa cells ectopically expressing WT, K5R or K5R-monUb G-CSFR were incubated with anti-G-CSFR antibodies and G-CSF for 20 min at 16°C, washed and transferred to 37°C for 60 min. Subsequently, cells were fixed and stained for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels indicating colocalization in yellow. Insets show enlargements of boxed areas.



Supplementary figure 2. Lysosomal routing of selected 'walking' lysine G-CSFR mutants

HeLa cells ectopically expressing indicated G-CSFR mutants were processed as described (Figure 3A) and analyzed by CLSM after staining for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow. Insets show enlargements of boxed areas

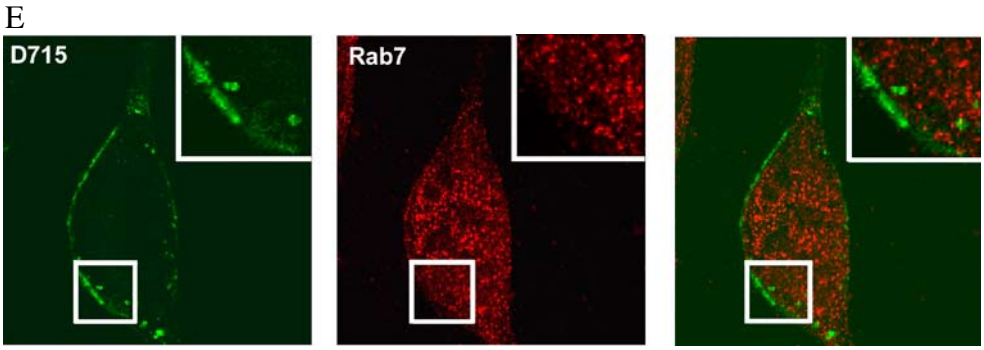


Figure 6. A G-CSFR mutant lacking the di-leucine-based internalization motif (d749-69) is hampered and delayed in ubiquitination, while a disease-associated truncated G-CSFR mutant lacks ubiquitination completely
(E) HeLa cells ectopically expressing G-CSFR_d715 mutants were processed as described (Figure 3A) and analyzed by CLSM after staining for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow.

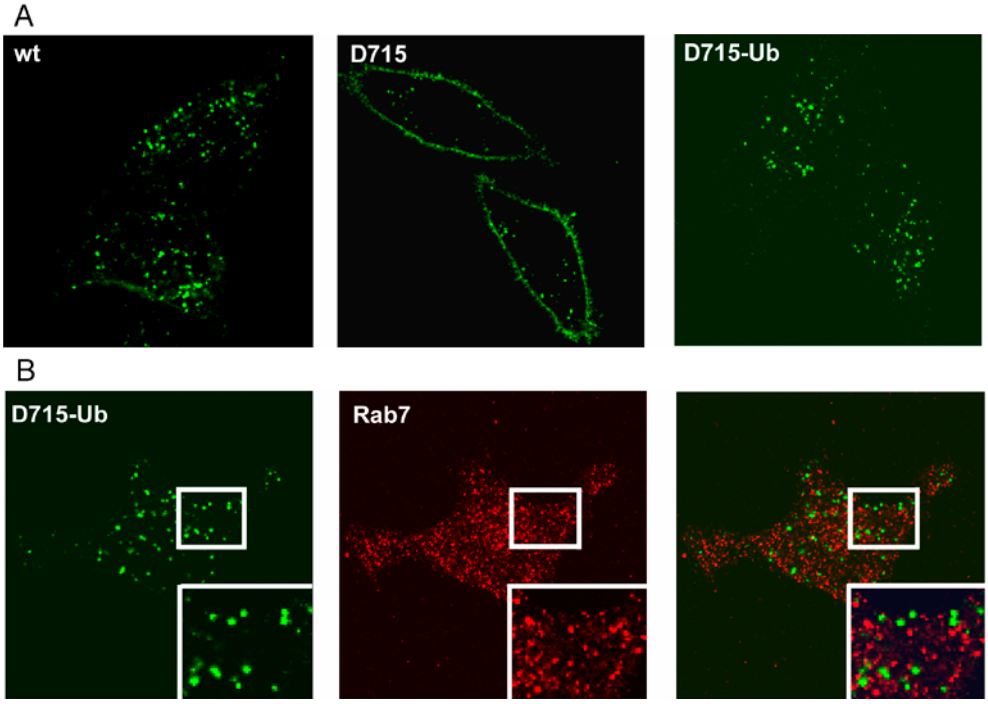


Figure 7. Fusion of ubiquitin to the C-terminus of G-CSFR_d715 restores internalization but not lysosomal sorting
(A) HeLa cells ectopically expressing wt, d715 or d715-Ub G-CSFR were labeled with anti-GCSFR antibody prior to incubation with G-CSF for 30min. After washing cells were permeabilized, stained with a secondary goat anti-mouse Alexa Fluor 488 and analyzed by CLSM. (B) HeLa cells ectopically expressing G-CSFR_d715-Ub were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized G-CSFR (green, left panels) and the late endosomal/lysosomal marker rab7 (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow.

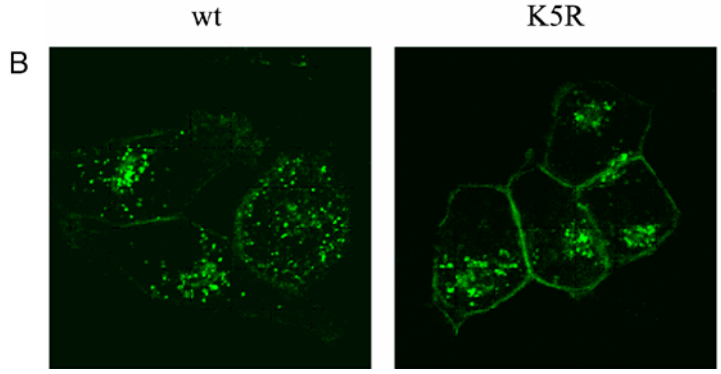


Figure 3. Role of receptor lysines in CSF3R cell surface expression

(B) Confocal microscopy images of HeLa cells transiently transfected with wt or K5R CSF3R constructs.

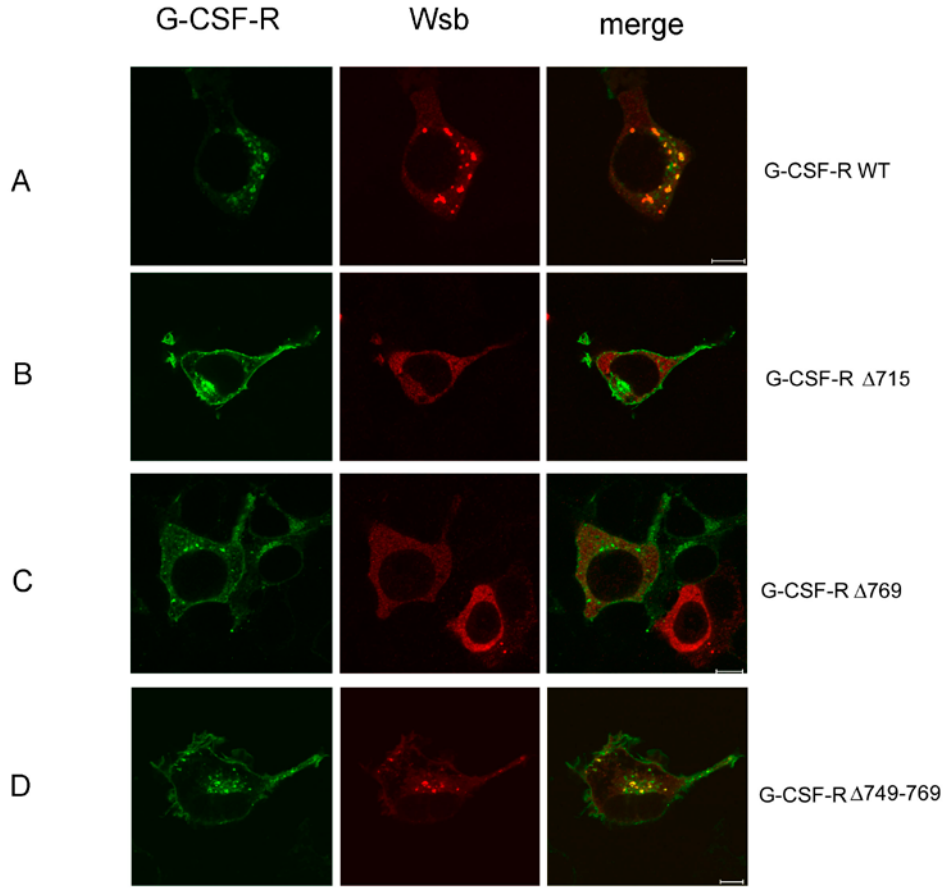


Figure 3. Co-localization of Wsb and G-CSFR in intracellular vesicles

CLSM analysis of HEK293-T cells transfected with G-CSFR wt (A), Δ715 (B), Δ749-769 (C) or Δ769 (D) together with myc-tagged Wsb-2. Cells growing on glass slides were fixed 2 days after transfection and immunostained for G-CSFR (left panels; green) or myc-Wsb (middle panels). After merging left and middle panels, co-localization appears as yellow (right panels). Size bar indicates 10μM.

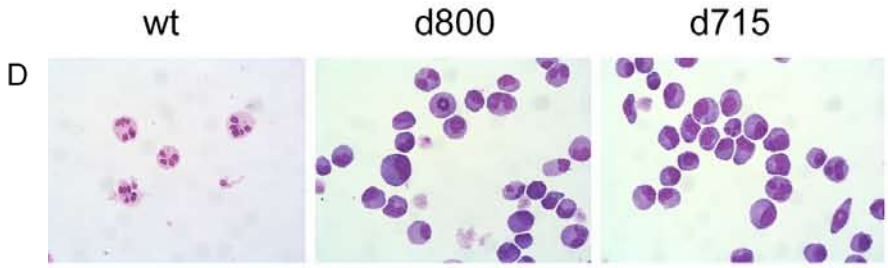


Figure 5. Role of C-terminal region of G-CSFR in steady-state membrane expression, receptor internalization and proliferation/differentiation signaling

(D) Light micrographs of May Grünwald-Giemsa stained cells on day 10 of culture (x 690).

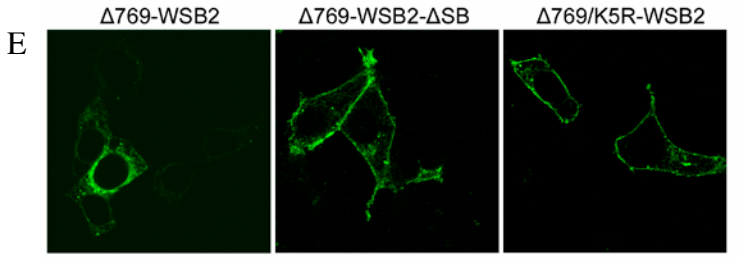


Figure 6. Steady-state distribution of G-CSFR-Wsb fusion proteins.

(E) CLSM of steady-state distribution of G-CSFRΔ769-Wsb-2 (left), G-CSFRΔ769-Wsb-2ΔSB (middle) and K5R-G-CSFRΔ769-Wsb-2 (right) on HEK293-T transfectants.