Routing Dynamics of the Colony-Stimulating Factor 3 Receptor and Implications for Intracellular Signaling

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ISBN: 978-94-6169-099-9

Cover design: Martine Evenhuis
Layout: Egied Simons
Printing: Optima Grafische Communicatie

The work described in this thesis was performed at the Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands. This work was supported by the Dutch Cancer Society “Koningin Wilhelmina Fonds”.

Printing of this thesis was financially supported by the Dutch Cancer Society “Koningin Wilhelmina Fonds”, J.E. Jurriaanse Stichting and Erasmus MC University.

Routing Dynamics of the Colony-Stimulating Factor 3 Receptor and Implications for Intracellular Signaling

Dynamische routing van de CSF3 receptor
en implicaties voor intracellulaire signalering

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus Prof. dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 14 september 2011 om 11:30 uur

door
Johanna Clara Michaela Meenhuis
geboren te Almelo

PROMOTIECOMMISSIE

Promotor: Prof. dr. I.P. Touw

Overige leden: Prof. dr. H.R. Delwel
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Voor pa & ma

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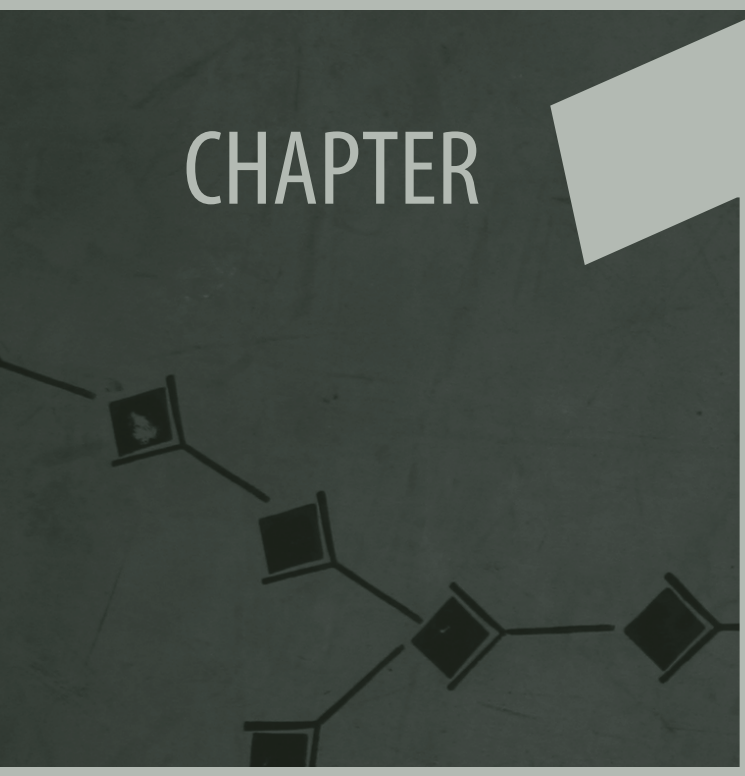
CHAPTER 1



General introduction

GENERAL INTRODUCTION

1. Hematopoiesis

Hematopoiesis is the tightly regulated formation of all blood cell types from hematopoietic stem cells (HSC), which, in adults, takes place in the bone marrow (BM). The BM of an average healthy adult produces approximately 10^{11}-10^{12} peripheral blood cells (PBCs) a day [1]. This process largely depends on hematopoietic growth factors that activate their cognate receptors, leading to proliferation, differentiation and survival of hematopoietic cells [1]. Major hematopoietic growth factors are erythropoietin (EPO), required for driving erythropoiesis; interleukins (IL); thrombopoietin (TPO), involved in megakaryocytic development; stem cell factor (SCF) and fms-related tyrosine kinase 3 ligand (FLT3L), involved in proliferation of the pluripotent stem cells and the different colony-stimulating factors (CSFs), involved in myeloid cell development [1-5].

The family of CSFs comprises multilineage colony-stimulating factor (multi-CSF also known as IL3), macrophage colony-stimulating factor (M-CSF also known as CSF1), granulocyte-macrophage colony-stimulating factor (GM-CSF also known as CSF2) and granulocyte colony-stimulating factor (G-CSF also known as CSF3) [6].

2. CSF3 and its receptor (CSF3R)

2.1 CSF3

CSF3 is a member of the cytokine class I superfamily that plays an important role in granulopoiesis by stimulating proliferation, survival and differentiation of myeloid progenitor cells [7-9]. Human CSF3 is encoded by a single gene, located on chromosome 17q11-22 [10-12], and produced by a variety of cells, of which monocytes and macrophages are the most prominent [7]. CSF3 deficient mice develop severe neutropenia with neutrophil levels ranging from 20-30% compared to wild type littermate controls [8, 13]. In addition to recruiting and activating other cells of the immune system, neutrophils play a key role in the front-line defense against invading pathogens by using three strategies to directly attack the micro-organisms: phagocytosis, release of soluble anti-microbials and generation of neutrophil extracellular traps (NETs) [14-16].

Neutrophils are the most abundant white blood cells in man, accounting for approximately 70% of all leukocytes. This number is rapidly elevated in case of a bacterial infection. CSF3 is administered to neutropenia patients in order to increase peripheral neutrophil levels, thereby reducing the risk of bacterial infection. CSF3 is also commonly used for the mobilization of HSCs into peripheral blood, to facilitate their harvest for the purpose of transplantation [17-19]. The biological activities of CSF3 are mediated by its binding to the granulocyte colony-stimulating factor receptor (CSF3R), which results in the activation of multiple intracellular signaling pathways.

2.2 CSF3R

The CSF3R is a single transmembrane protein consisting of 813 amino acids with 603 amino acids being part of an extracellular domain [20-21]. The extracellular portion consists of an Immunoglobulin-like (Ig) domain, a cytokine receptor homology domain with four cysteine residues and a WSXWS motif which is required for ligand binding (Figure 1). The cytoplasmic domain of the CSF3R comprises 3 regions, termed Box1, Box2 and Box3. The membrane proximal Box1 and Box2 are highly conserved among hematopoietin receptors and essential for transduction of proliferation signals whereas the less-conserved membrane distal Box3 region has been linked with the induction of differentiation of myeloid progenitor cells (Figure 1) [22].

Mutations in the CSF3R are present in patients with severe congenital neutropenia (SCN). Most common are nonsense mutations between amino acids 715 and 732 of the CSF3R that truncate the C-terminal cytoplasmic region of the receptor. Expression of these receptor mutants in SCN patients has been associated with a high risk to develop acute myeloid leukemia (AML) [23-25]. Expression of the d715 mutant CSF3R in mice results in hyperproliferation in response to CSF3 [26-27]. PI3K/PKB and pSTAT5 signaling via the d715 CSF3R is prolonged as compared to the wt CSF3R, due to loss of binding sites of negative inhibitory molecules [28-32]. Internalization of the d715 CSF3R is also affected because it lacks the di-leucine and tyrosine based internalization motifs present in the C-terminal region [33].

3. Forward routing and receptor membrane expression

To be able to bind its ligand CSF3, CSF3R has to be expressed at the plasma membrane. Newly synthesized and properly folded receptors are transported from the endoplasmic reticulum (ER) in vesicles that sequentially pass the ER-Golgi intermediate compartment (ERGIC), the Golgi and the Trans-Golgi Network (TGN) in order to reach the plasma membrane (Figure 2). During routing from the ER to the plasma membrane, the receptors undergo maturation by means of post-translational modifications (e.g. glycosylation) [34-35]. Misfolded proteins are degraded by the ER-associated degradation (ERAD) machinery [36]. Maintenance of a steady state level of CSF3Rs on the plasma membrane is important because increased or decreased levels of receptors on the membrane could lead to misbalanced signaling and deregulation of neutrophil numbers in blood. Previously, our group identified the WD40 and suppressor of cytokine signaling (SOCS) box protein (WSB)-2 to bind to the CSF3R C-terminal domain. Removal of this region resulted in increased membrane expression of CSF3R suggesting an important role for ubiquitination of the CSF3R by WSB2 in forward routing (Figure 2) [37].

4. Signal transduction pathways of the CSF3R

4.1 JAK kinases

The CSF3R does not have intrinsic tyrosine kinase activity and relies predominantly on the Janus kinase (JAK) family to transmit signals intracellularly. The JAK family consists of four related cytoplasmic tyrosine kinases, JAK1, JAK2, JAK3 and TYK2 [38]. These proteins contain a Band 4.1, Ezrin, Radixin, Moesin (FERM) domain, a Src homology 2-like domain, a pseudokinase domain and a functional tyrosine kinase domain [39]. Sequences within the FERM domain are involved in the interaction of JAK proteins with cytokine receptors [40]. The pseudokinase domain provides an auto-inhibitory function on the kinase activity of the protein as deletion or mutations in this domain leads to increased kinase activity [41].

JAK1, JAK2 and TYK2 are associated with the CSF3R [42-44]. A tryptophan residue at position 650, localized between Box1 and Box2 domain in the membrane proximal region of the CSF3R, is essential for JAK activation and proliferation signals [45]. Following activation, the CSF3R dimerizes and the JAK proteins become activated via trans-phosphorylation. Subsequently, JAKs phosphorylate the four tyrosine (Y) residues, located at positions 704, 729, 744 and 769, present in the cytoplasmic domain of the CSF3R. These phosphorylated tyrosines are docking sites for Src Homology (SH)-2 containing proteins, including members of the signal transducer and activator of transcription (STAT) family [43, 46-48].

JAKs have also been implicated in the regulation of cell-surface expression of various cytokine receptors. For example, 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 [49]. Likewise, binding of JAK2 or TYK2 to the thrombopoietin receptor (TPOR) increased membrane expression and stability of its mature form [50]. TYK2 binding to the interferon receptor-α1 (IFNAR1) attenuates receptor degradation and increases cell-surface expression by masking dileucine- and tyrosine-based internalization motifs present in the JAK-binding domain of the receptor [51-52]. In contrast, an alternative mechanism was demonstrated for the erythropoietin receptor (EPOR), where JAK2 binding 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 thereby leading to higher receptor expression on the plasma membrane [53]. Whether and how JAK1, JAK2 or TYK2 control CSF3R membrane expression was not known.

Are JAK1, JAK2 and TYK2 influencing CSF3R plasma membrane expression? In CHAPTER 2, we addressed this question using as model systems, Ba/F3 cells overexpressing JAK1, JAK2 or TYK2 and human fibrosarcoma cell lines deficient for either of the JAK kinases.

4.2 Signal transducer and activator of transcription (STAT)

STAT proteins are phosphorylated on specific tyrosine residues by JAKs [54]. This leads to dimerization and nuclear translocalization of the transcription factors which then results in transcription of STAT-responsive genes [55]. Suppressor of cytokine signaling (SOCS) genes are robust targets of STATs [80-81].

Among the different members of the STAT family, STAT1, STAT3 and STAT5 are prominently activated by CSF3 stimulation, whereafter they form homodimers or heterodimers of STAT1/3 and STAT3/5 [28]. Activation of STAT1 and STAT5 requires the membrane proximal region of the CSF3R [28, 56]. STAT1 deficient mice have shown that STAT1 is important for immune responses; however the contribution of STAT1 in CSF3 mediated signaling remains unclear [57]. Activation of STAT5 in response to CSF3 results in proliferation and survival signaling [28]. STAT5 knockout mice show moderately reduced colony capacity formation in response to CSF3 [58]. Mice lacking STAT3 in their hematopoietic progenitors are neutrophilic due to the lack of upregulation of *SOCS3* and cyclin-dependent kinase inhibitor *(CDKN)-1B* [59-60]. STAT3 binds to phospho-Y704 and phospho-Y744 of the CSF3R (Figure1) but can also bind independent of these tyrosines at high CSF3 concentrations [61-62]. Introduction of dominant negative STAT3 blocks differentiation and growth arrest of myeloid cells [60, 63-64] implicating a role in both cell proliferation and differentiation. Mice expressing the D715F CSF3R truncation mutant in which the remaining STAT3 binding site is mutated, which attenuates STAT5 but abolishes STAT3 activation, are neutropenic due to block in differentiation of myeloid progenitors [64].

Finally, like other hematopoietin receptors, CSF3R activates the p21Ras/Raf/MAPK and the phosphatidylinositol 3 (PI3)-kinase/protein kinase B (PKB) signaling pathways which contribute to cell proliferation and survival (Figure 1) [65-66].

5. Downmodulation of CSF3R signaling by receptor endocytosis and lysosomal routing

To avoid aberrant signaling via receptors, it is necessary that signaling pathways are correctly down regulated. One of the most important mechanisms to attenuate this signaling is by removal of the activated receptors from the membrane followed by degradation of the receptor [67]. This is a multifaceted process, encompassing different receptor entry routes and multiple adaptor proteins involved to direct trafficking of receptors to different compartments. An internalized receptor can be recycled back to cell surface or be targeted towards late endosomes for subsequent lysosomal degradation. Moreover, receptor-mediated endocytosis is also actively implicated in signal transduction from the cell membrane to the nucleus as random diffusion is too slow [68]. Scaffolding proteins, molecular motors and travelling phosphoprotein waves appear to be crucial for signal propagation to different cellular locations, thereby determining cell fate [69]. Ubiquitination of signaling receptors or their adaptors has been established as a post-translational modification that mediates internalization and/or postinternalization receptor trafficking to the lysosomes [70].

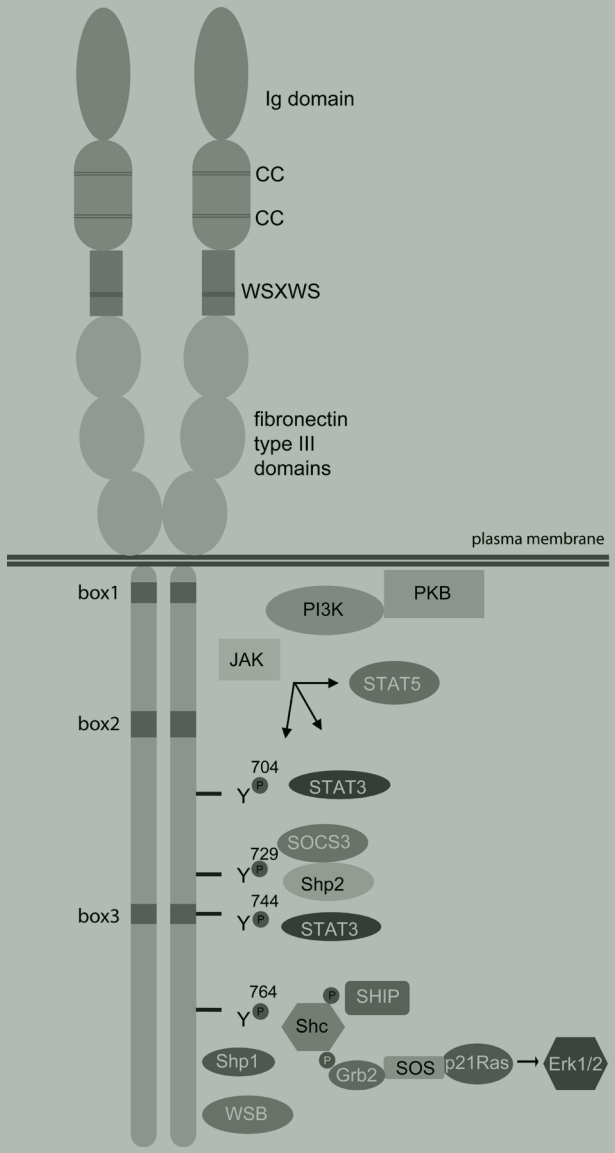


Figure 1: Schematic representation of CSF3R signaling pathways. Upon activation of the CSF3R by its ligand CSF3, JAK proteins are transphosphorylated and phosphorylate the four tyrosine residues present in the cytoplasmic part of the receptor. These phosphotyrosines are docking sites for different SH2 containing proteins. The balance between the different pathways activated and negative and positive CSF3R signaling regulation determines signaling output and cell fate.

5.1 Ubiquitination

The conjugation of ubiquitin (Ub) to an ε-NH2 group of a lysine (K) residue in the substrate protein is usually achieved by the sequential actions of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [71].

Three types of target modification with Ub can occur: monoubiquitination which is the attachment of a single Ub to one lysine residue of the target protein, multiubiquitination which comprises the attachment of single Ub moieties to multiple lysine residues of the target protein or polyubiquitination when a chain of several Ub via one or more of its seven internal lysines is affixed to the target protein. In addition linear poly-Ub chains can be formed between the C-terminal glycine residue of Ub and the N-terminal methionine residue of the next Ub molecule [72]. Polyubiquitination using lysine 48 (K48) and lysine 63 (K63) linkages of Ub have been best studied. K48 linked chains target proteins for degradation via the 26S proteasome, whereas K63 linked chains are implicated in the formation of protein complexes involved in DNA repair, transcriptional regulation, activation of protein kinases and in targeting proteins in endocytosis for lysosomal degradation [73].

5.2 Ubiquitination of the CSF3R via SOCS3

SOCS proteins are major negative regulators of cytokine receptors signaling [74-75]. The SOCS family comprises 8 members SOCS 1-7 and cytokine inducible SH2-containing protein (CIS) [76]. All SOCS proteins contain an SH2 domain and a conserved C-terminal SOCS box region. SOCS1 and SOCS3 have an extra functional domain called kinase inhibitory region (KIR) that interferes with JAK kinase activity [77-79].

SOCS3 is robustly upregulated by CSF3 stimulation in a STAT3 dependent manner [82-83]. SOCS3 is recruited to the CSF3R via its SH2 domain that interacts with phospho-Y729 [82]. The SOCS box forms an ubiquitin ligase complex with elongin B/C, RING finger proteins Rbx1 or 2 and the Cullin family members Cul2 or Cul5 [84-85].

Ligand-induced lysosomal routing of the CSF3R depends on the presence of the juxtamembrane lysine residue at position 632 (K632), although there are in total five conserved lysines present in the cytoplasmic domain of the CSF3R that can be ubiquitinated. When this lysine or the SOCS3 recruitment site is mutated, the receptor remains in early endosomes where prolonged signaling occurs [32, 86]. It is unknown why K632 is crucial for CSF3R routing from early to late endosomes.

In CHAPTER 3, we described how positioning of K632 determines the dynamics of ubiquitination, lysosomal routing and signal attenuation of the CSF3R. We investigated whether there is a specific motif or whether location of the lysine relative to the plasma membrane is crucial. To this end we 1) shifted the K632 via inserting alanines around its position and 2) reallocated the motif to the C-terminus, by fusion of ubiquitin to the C-terminus.

5.3 Role of deubiquitinating enzymes

Deubiquitinating enzymes (DUBs) are a large group of proteases that regulate ubiquitin-dependent pathways by opposing E3 ligases. Five families of DUBs have been described of which four are cysteine proteases, including the ubiquitin carboxyl hydrolases (UCH), the ubiquitin-specific proteases (USP), the Machado-Joseph disease (MJD) and the ovarian tumor proteases (OTU). The Jab1/MPN/MOV43-domain-containing (JAMM) is the fifth group and contains zinc metalloproteases [87].

Two of these DUBs, associated molecule with the SH3 domain of STAM (AMSH) and ubiquitin isopeptidase Y (UBPY), are linked to the ESCRT machinery and implicated in routing of activated receptors at the late endosomal stage [88-89]. AMSH is a member of the JAMM family, whereas UBPY is a member of the USP family of deubiquitinating enzymes family [90]. Both enzymes bind to endosomal sorting complex required for transport (ESCRT)-0 constituent signal transducing adaptor molecule (STAM) via their SH3 domain and to the ESCRT-III complex with their Microtubule Interacting and Transport (MIT) domains [91-94].

A murine DUB family comprising 4 highly homologous USP proteins (DUB1, DUB1A, DUB2 and DUB2A) was initially identified in hematopoietic cells [95-97]. DUBs are rapidly and transiently induced in response to different cytokines [95-98]. For example, DUB1 expression is induced by IL3, IL5 and CSF2, cytokines that activate receptors with a β common subunit, resulting in a G1 phase cell cycle arrest [97, 99]. A critical role for JAK2 and the Ras/Raf/MAPK pathway was shown for IL3-induced upregulation of DUB1 [100]. These pathways appeared necessary but not sufficient, because e.g. JAK2 and MAPK activation by the EPOR did not result in induction of *DUB1* expression [100-101]. DUB1A is IL3-inducible in B-lymphocytes, whereas DUB2 is induced by IL2 in T-cells [98]. DUB1 controls deubiquitination of the dynein heavy chain, which is part of the complex that regulates the movement of cargo along microtubules [102]. These data suggest that DUBs may alter endosomal trafficking and processing of cytokine receptors at distinct intracellular levels and at different time points after receptor activation.

Are different DUBs such as the murine DUB family members DUB1, DUB1A, DUB2, and DUB2A or the ESCRT associated DUBs AMSH and UBPY involved in the ubiquitination status of the CSF3R and thereby in control of signaling and routing of the receptor? These questions have been addressed in CHAPTER 4.

5.4 Protein complexes involved in intracellular trafficking and signaling from CSF3R at early endosomes

Ligand induced endocytosis of receptors involves internalization from the plasma membrane, trafficking to early endosomes, multivesicular bodies (MVB)/late endosomes, and then to lysosomes where ligand-receptor complex is degraded by proteases (Figure 2) [67].

Activated receptors generally internalize either via clathrin-coated pits or via clathrin independent structures, such as caveolae [67]. Internalization of the CSF3R can occur ligand dependent or ligand-independant; which is much slower compared to activated receptor internalization [33]. For both processes a dileucine motif present between amino acids 749-769 in the C-terminal part of the CSF3R is important [33]. Internalized vesicles containing the CSF3R fuse with early endosomes, that comprise the GTPase Rab5 which also recruits the early endosomal antigen-1 (EEA1) [103-105].

It is largely unknown which proteins drive endocytic routing and which proteins are involved in signaling of the activated CSF3R from the early endosomes. To address these questions, we performed Bio-CSF3 pull down experiments in combination with MALDI-TOF proteomics analyses to identify candidate proteins involved these processes. These studies are presented in CHAPTER 5.

Late endosomes are formed from the early endosome network and these vesicles undergo conversion by losing Rab5 and recruiting Rab7 [106]. Late endosomes have more luminal vesicles than early endosomes and are often described as multivesicular bodies (MVBs) [107]. The luminal vesicles are generated by invagination of the limiting endosomal membrane and are enriched in either lysobisphosphatidic acid or phosphatidylinositol 3-phosphate (PtdIns3P) [108]. The formation of the luminal vesicles and the sorting of ubiquitinated proteins into these vesicles requires the sequential recruitment and assembly of ESCRT-0, -I, -II and-III on to the endosome-limiting membrane [109-110]. The final step of delivery of endocytosed receptor to lysosomal proteases requires direct fusion events between the late endosomes and lysosomes [111].

5.5. Role of an ubiquitin binding protein Sequestosome-1

Sequestosome 1 (SQSTM1) was identified in the activated CSF3R complex by mass-spectometry (Chapter 5). SQSTM1 contains a ubiquitin-associated (UBA) domain that preferentially binds to K63-polyubiquitinated substrates [112]. Depletion of SQSTM1 results in an inhibition of ubiquitin proteasome-mediated degradation and an accumulation of ubiquitinated proteins [112]. SQSTM1 has been implicated as a regulator of autophagy-mediated protein degradation [113] and binds simultaneously ubiquitin and the autophagasome protein LC3 providing a molecular link between ubiquitination and autophagy [114]. SQSTM1 colocalizes with the epidermal growth factor receptor (EGFR) and the late endosomal markers Rab7 and LAMP1, implicating functions of SQSTM1 predominantly in late endosomes and lysosomes [115]. Moreover SQSTM1 was found to be downregulated by miRNAs with an AAAGUGC seed. When these miRNA are expressed in hematopoietic cells CSF3 induced expansion was enhanced significantly.

Does SQSTM1 influence CSF3R signaling and trafficking, thereby enhancing cell proliferation? This question was addressed in CHAPTER 6.

The studies presented in this thesis were aimed at understanding the role of ubiquitination of the CSF3R and associated complexes and the underlying mechanisms in CSF3R trafficking and signaling. The implications of these results for normal neutrophil development and myeloid diseases such as acute myeloid leukemia and severe congenital neutropenia will be discussed in Chapter 7.

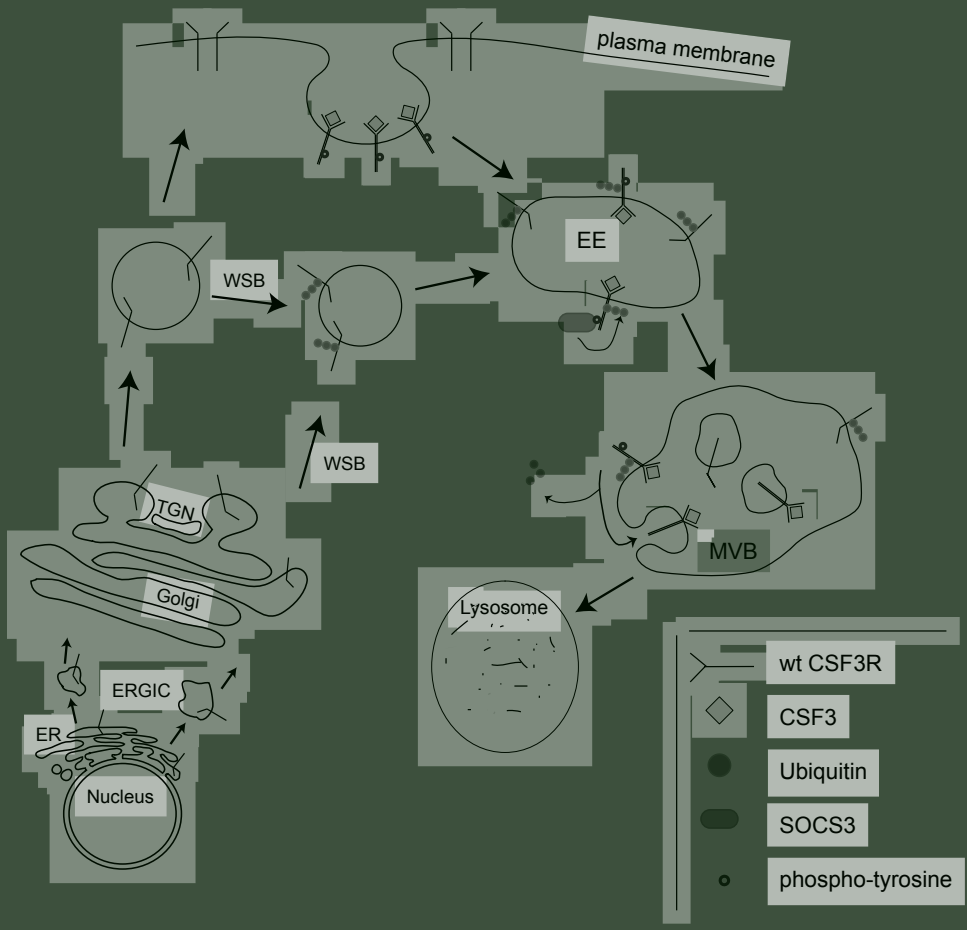


Figure 2: Schematic representation of CSF3R intracellular trafficking. Ubiquitination is an important regulator of CSF3R forward routing, controlled by the E3 ligase WSB and lysosomal routing, controlled by the E3 ligase SOCS3. EE: early endosome, ER: endoplasmic reticulum, ERGIC: ER-Golgi intermediate compartment, MVB: multivesicular body, TGN: Trans-Golgi Network.

REFERENCES

1. Lotem, J. and L. Sachs, *Cytokine control of developmental programs in normal hematopoiesis and leukemia.* Oncogene, 2002. **21**(21): p. 3284-94.
2. D'Andrea, A.D., et al., *The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains.* Mol Cell Biol, 1991. **11**(4): p. 1980-7.
3. Ihle, J.N., et al., *Interleukin 3: possible roles in the regulation of lymphocyte differentiation and growth.* Immunol Rev, 1982. **63**: p. 5-32.
4. Lyman, S.D., et al., *Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells.* Cell, 1993. **75**(6): p. 1157-67.
5. Witte, O.N., *Steel locus defines new multipotent growth factor.* Cell, 1990. **63**(1): p. 5-6.
6. Clark, S.C. and R. Kamen, *The human hematopoietic colony-stimulating factors.* Science, 1987. **236**(4806): p. 1229-37.
7. Demetri, G.D. and J.D. Griffin, *Granulocyte colony-stimulating factor and its receptor.* Blood, 1991. **78**(11): p. 2791-808.
8. Lieschke, G.J., et al., *Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization.* Blood, 1994. **84**(6): p. 1737-46.
9. Seymour, J.F., et al., *Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity, perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival.* Blood, 1997. **90**(8): p. 3037-49.
10. Nagata, S., et al., *Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor.* Nature, 1986. **319**(6052): p. 415-8.
11. Nagata, S., et al., *The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor.* Embo J, 1986. **5**(3): p. 575-81.
12. Simmers, R.N., et al., *Localization of the G-CSF gene on chromosome 17 proximal to the breakpoint in the t(15;17) in acute promyelocytic leukemia.* Blood, 1987. **70**(1): p. 330-2.
13. Liu, F., et al., *Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice.* Immunity, 1996. **5**(5): p. 491-501.
14. Clark, S.R., et al., *Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood.* Nat Med, 2007. **13**(4): p. 463-9.
15. Nauseef, W.M., *How human neutrophils kill and degrade microbes: an integrated view.* Immunol Rev, 2007. **219**: p. 88-102.
16. Hickey, M.J. and P. Kubes, *Intravascular immunity: the host-pathogen encounter in blood vessels.* Nat Rev Immunol, 2009. **9**(5): p. 364-75.
17. Dale, D.C., A.A. Bolyard, and W.P. Hammond, *Cyclic neutropenia: natural history and effects of long-term treatment with recombinant human granulocyte colony-stimulating factor.* Cancer Invest, 1993. **11**(2): p. 219-23.
18. Welte, K., C. Zeidler, and D.C. Dale, *Severe congenital neutropenia.* Semin Hematol, 2006. **43**(3): p. 189-95.
19. Welte, K., et al., *Differential effects of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in children with severe congenital neutropenia.* Blood, 1990. **75**(5): p. 1056-63.

20. Anaguchi, H., et al., *Ligand binding characteristics of the carboxyl-terminal domain of the cytokine receptor homologous region of the granulocyte colony-stimulating factor receptor.* J Biol Chem, 1995. **270**(46): p. 27845-51.
21. Fukunaga, R., et al., *Functional domains of the granulocyte colony-stimulating factor receptor.* Embo J, 1991. **10**(10): p. 2855-65.
22. Dong, F., et al., *Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation.* Mol Cell Biol, 1993. **13**(12): p. 7774-81.
23. Dong, F., et al., *Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia.* N Engl J Med, 1995. **333**(8): p. 487-93.
24. Dong, F., et al., *Mutations in the granulocyte colony-stimulating factor receptor gene in patients with severe congenital neutropenia.* Leukemia, 1997. **11**(1): p. 120-5.
25. Freedman, M.H. and B.P. Alter, *Risk of myelodysplastic syndrome and acute myeloid leukemia in congenital neutropenias.* Semin Hematol, 2002. **39**(2): p. 128-33.
26. Hermans, M.H., et al., *Perturbed granulopoiesis in mice with a targeted mutation in the granulocyte colony-stimulating factor receptor gene associated with severe chronic neutropenia.* Blood, 1998. **92**(1): p. 32-9.
27. McLemore, M.L., J. Poursine-Laurent, and D.C. Link, *Increased granulocyte colony-stimulating factor responsiveness but normal resting granulopoiesis in mice carrying a targeted granulocyte colony-stimulating factor receptor mutation derived from a patient with severe congenital neutropenia.* J Clin Invest, 1998. **102**(3): p. 483-92.
28. Dong, F., et al., *Stimulation of Stat5 by granulocyte colony-stimulating factor (G-CSF) is modulated by two distinct cytoplasmic regions of the G-CSF receptor.* J Immunol, 1998. **161**(12): p. 6503-9.
29. Dong, F., et al., *The carboxyl terminus of the granulocyte colony-stimulating factor receptor, truncated in patients with severe congenital neutropenia/acute myeloid leukemia, is required for SH2-containing phosphatase-1 suppression of Stat activation.* J Immunol, 2001. **167**(11): p. 6447-52.
30. Hunter, M.G., et al., *Loss of SHIP and CIS recruitment to the granulocyte colony-stimulating factor receptor contribute to hyperproliferative responses in severe congenital neutropenia/acute myelogenous leukemia.* J Immunol, 2004. **173**(8): p. 5036-45.
31. Ward, A.C., et al., *The SH2 domain-containing protein tyrosine phosphatase SHP-1 is induced by granulocyte colony-stimulating factor (G-CSF) and modulates signaling from the G-CSF receptor.* Leukemia, 2000. **14**(7): p. 1284-91.
32. Irandoust, M.I., et al., *Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor.* Embo J, 2007. **26**(7): p. 1782-93.
33. Aarts, L.H., et al., *Receptor activation and 2 distinct COOH-terminal motifs control G-CSF receptor distribution and internalization kinetics.* Blood, 2004. **103**(2): p. 571-9.
34. Duvernay, M.T., C.M. Filipeanu, and G. Wu, *The regulatory mechanisms of export trafficking of G protein-coupled receptors.* Cell Signal, 2005. **17**(12): p. 1457-65.
35. Mellman, I. and G. Warren, *The road taken: past and future foundations of membrane traffic.* Cell, 2000. **100**(1): p. 99-112.
36. Meusser, B., et al., *ERAD: the long road to destruction.* Nat Cell Biol, 2005. **7**(8): p. 766-72.

37. Erkeland, S.J., et al., *Novel role of WD40 and SOCS box protein-2 in steady-state distribution of granulocyte colony-stimulating factor receptor and G-CSF-controlled proliferation and differentiation signaling.* Oncogene, 2007. **26**(14): p. 1985-94.
38. Haan, C., et al., *Jaks and cytokine receptors--an intimate relationship.* Biochem Pharmacol, 2006. **72**(11): p. 1538-46.
39. Yamaoka, K., et al., *The Janus kinases (Jaks).* Genome Biol, 2004. **5**(12): p. 253.
40. Zhou, Y.J., et al., *Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases.* Mol Cell, 2001. **8**(5): p. 959-69.
41. Saharinen, P. and O. Silvennoinen, *The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction.* J Biol Chem, 2002. **277**(49): p. 47954-63.
42. Shimoda, K., et al., *G-CSF induces tyrosine phosphorylation of the JAK2 protein in the human myeloid G-CSF responsive and proliferative cells, but not in mature neutrophils.* Biochem Biophys Res Commun, 1994. **203**(2): p. 922-8.
43. Nicholson, S.E., et al., *Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation.* Proc Natl Acad Sci U S A, 1994. **91**(8): p. 2985-8.
44. Marino, V.J. and L.P. Roguin, *The granulocyte colony stimulating factor (G-CSF) activates Jak/STAT and MAPK pathways in a trophoblastic cell line.* J Cell Biochem, 2008. **103**(5): p. 1512-23.
45. Barge, R.M., et al., *Tryptophan 650 of human granulocyte colony-stimulating factor (G-CSF) receptor, implicated in the activation of JAK2, is also required for G-CSF-mediated activation of signaling complexes of the p21ras route.* Blood, 1996. **87**(6): p. 2148-53.
46. Ihle, J.N., et al., *Jaks and Stats in cytokine signaling.* Stem Cells, 1997. **15 Suppl 1**: p. 105-11; discussion 112.
47. Shimoda, K., et al., *Jak1 plays an essential role for receptor phosphorylation and Stat activation in response to granulocyte colony-stimulating factor.* Blood, 1997. **90**(2): p. 597-604.
48. Rane, S.G. and E.P. Reddy, *Janus kinases: components of multiple signaling pathways.* Oncogene, 2000. **19**(49): p. 5662-79.
49. Radtke, S., et al., *Three dileucine-like motifs within the interbox1/2 region of the human oncostatin M receptor prevent efficient surface expression in the absence of an associated Janus kinase.* J Biol Chem, 2006. **281**(7): p. 4024-34.
50. Tong, W., et al., *The membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms.* J Biol Chem, 2006. **281**(50): p. 38930-40.
51. Kumar, K.G., et al., *Basal ubiquitin-independent internalization of interferon alpha receptor is prevented by Tyk2-mediated masking of a linear endocytic motif.* J Biol Chem, 2008. **283**(27): p. 18566-72.
52. Ragimbeau, J., et al., *The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression.* Embo J, 2003. **22**(3): p. 537-47.
53. Huang, L.J., S.N. Constantinescu, and H.F. Lodish, *The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor.* Mol Cell, 2001. **8**(6): p. 1327-38.
54. Flores-Morales, A., et al., *In vitro interaction between STAT 5 and JAK 2; dependence upon phosphorylation status of STAT 5 and JAK 2.* Mol Cell Endocrinol, 1998. **138**(1-2): p. 1-10.
55. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark, *Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.* Science, 1994. **264**(5164): p. 1415-21.

56. de Koning, J.P., et al., *The membrane-distal cytoplasmic region of human granulocyte colony-stimulating factor receptor is required for STAT3 but not STAT1 homodimer formation.* Blood, 1996. **87**(4): p. 1335-42.

57. Durbin, J.E., et al., *Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease.* Cell, 1996. **84**(3): p. 443-50.

58. Teglund, S., et al., *Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses.* Cell, 1998. **93**(5): p. 841-50.

59. Lee, C.K., et al., *STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation.* Immunity, 2002. **17**(1): p. 63-72.

60. de Koning, J.P., et al., *STAT3-mediated differentiation and survival and of myeloid cells in response to granulocyte colony-stimulating factor: role for the cyclin-dependent kinase inhibitor p27(Kip1).* Oncogene, 2000. **19**(29): p. 3290-8.

61. Ward, A.C., et al., *Tyrosine-dependent and -independent mechanisms of STAT3 activation by the human granulocyte colony-stimulating factor (G-CSF) receptor are differentially utilized depending on G-CSF concentration.* Blood, 1999. **93**(1): p. 113-24.

62. Chakraborty, A., et al., *Identification of a novel Stat3 recruitment and activation motif within the granulocyte colony-stimulating factor receptor.* Blood, 1999. **93**(1): p. 15-24.

63. Shimozaki, K., et al., *Involvement of STAT3 in the granulocyte colony-stimulating factor-induced differentiation of myeloid cells.* J Biol Chem, 1997. **272**(40): p. 25184-9.

64. McLemore, M.L., et al., *STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation.* Immunity, 2001. **14**(2): p. 193-204.

65. Dong, F. and A.C. Larner, *Activation of Akt kinase by granulocyte colony-stimulating factor (G-CSF): evidence for the role of a tyrosine kinase activity distinct from the Janus kinases.* Blood, 2000. **95**(5): p. 1656-62.

66. de Koning, J.P., et al., *Proliferation signaling and activation of Shc, p21Ras, and Myc via tyrosine 764 of human granulocyte colony-stimulating factor receptor.* Blood, 1998. **91**(6): p. 1924-33.

67. Zwang, Y. and Y. Yarden, *Systems biology of growth factor-induced receptor endocytosis.* Traffic, 2009. **10**(4): p. 349-63.

68. Howe, C.L., *Modeling the signaling endosome hypothesis: why a drive to the nucleus is better than a (random) walk.* Theor Biol Med Model, 2005. **2**: p. 43.

69. Birtwistle, M.R. and B.N. Kholodenko, *Endocytosis and signalling: a meeting with mathematics.* Mol Oncol, 2009. **3**(4): p. 308-20.

70. Acconcia, F., S. Sigismund, and S. Polo, *Ubiquitin in trafficking: the network at work.* Exp Cell Res, 2009. **315**(9): p. 1610-8.

71. Hershko, A., et al., *Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.* J Biol Chem, 1983. **258**(13): p. 8206-14.

72. Kirisako, T., et al., *A ubiquitin ligase complex assembles linear polyubiquitin chains.* Embo J, 2006. **25**(20): p. 4877-87.

73. Woelk, T., et al., *The ubiquitination code: a signalling problem.* Cell Div, 2007. **2**: p. 11.

74. Krebs, D.L. and D.J. Hilton, *SOCS proteins: negative regulators of cytokine signaling.* Stem Cells, 2001. **19**(5): p. 378-87.

75. Starr, R. and D.J. Hilton, *SOCS: suppressors of cytokine signalling.* Int J Biochem Cell Biol, 1998. **30**(10): p. 1081-5.

76. Hilton, D.J., et al., *Twenty proteins containing a C-terminal SOCS box form five structural classes.* Proc Natl Acad Sci U S A, 1998. **95**(1): p. 114-9.

77. Kile, B.T., et al., *The SOCS box: a tale of destruction and degradation.* Trends Biochem Sci, 2002. **27**(5): p. 235-41.

78. Nicola, N.A. and C.J. Greenhalgh, *The suppressors of cytokine signaling (SOCS) proteins: important feedback inhibitors of cytokine action.* Exp Hematol, 2000. **28**(10): p. 1105-12.

79. Fujimoto, M. and T. Naka, *Regulation of cytokine signaling by SOCS family molecules.* Trends Immunol, 2003. **24**(12): p. 659-66.

80. Auernhammer, C.J., C. Bousquet, and S. Melmed, *Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter.* Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6964-9.

81. Davey, H.W., et al., *STAT5b mediates the GH-induced expression of SOCS-2 and SOCS-3 mRNA in the liver.* Mol Cell Endocrinol, 1999. **158**(1-2): p. 111-6.

82. Hortner, M., et al., *Suppressor of cytokine signaling-3 is recruited to the activated granulocyte-colony stimulating factor receptor and modulates its signal transduction.* J Immunol, 2002. **169**(3): p. 1219-27.

83. van de Geijn, G.J., et al., *G-CSF receptor truncations found in SCN/AML relieve SOCS3-controlled inhibition of STAT5 but leave suppression of STAT3 intact.* Blood, 2004. **104**(3): p. 667-74.

84. Kamura, T., et al., *The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families.* Genes Dev, 1998. **12**(24): p. 3872-81.

85. Kamura, T., et al., *VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases.* Genes Dev, 2004. **18**(24): p. 3055-65.

86. van de Geijn, G.J., J. Gits, and I.P. Touw, *Distinct activities of suppressor of cytokine signaling (SOCS) proteins and involvement of the SOCS box in controlling G-CSF signaling.* J Leukoc Biol, 2004. **76**(1): p. 237-44.

87. Nijman, S.M., et al., *A genomic and functional inventory of deubiquitinating enzymes.* Cell, 2005. **123**(5): p. 773-86.

88. McCullough, J., M.J. Clague, and S. Urbe, *AMSH is an endosome-associated ubiquitin isopeptidase.* J Cell Biol, 2004. **166**(4): p. 487-92.

89. Kato, M., K. Miyazawa, and N. Kitamura, *A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP.* J Biol Chem, 2000. **275**(48): p. 37481-7.

90. Clague, M.J. and S. Urbe, *Endocytosis: the DUB version.* Trends Cell Biol, 2006. **16**(11): p. 551-9.

91. Agromayor, M. and J. Martin-Serrano, *Interaction of AMSH with ESCRT-III and deubiquitination of endosomal cargo.* J Biol Chem, 2006. **281**(32): p. 23083-91.

92. Kaneko, T., et al., *Structural insight into modest binding of a non-PXXP ligand to the signal transducing adaptor molecule-2 Src homology 3 domain.* J Biol Chem, 2003. **278**(48): p. 48162-8.

93. Row, P.E., et al., *The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation.* J Biol Chem, 2007. **282**(42): p. 30929-37.

94. Tanaka, N., et al., *Possible involvement of a novel STAM-associated molecule "AMSH" in intracellular signal transduction mediated by cytokines.* J Biol Chem, 1999. **274**(27): p. 19129-35.

95. Baek, K.H., et al., *DUB-2A, a new member of the DUB subfamily of hematopoietic deubiquitinating enzymes.* Blood, 2001. **98**(3): p. 636-42.

96. Zhu, Y., et al., *DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes.* J Biol Chem, 1997. **272**(1): p. 51-7.

97. Zhu, Y., et al., *The murine DUB-1 gene is specifically induced by the betac subunit of interleukin-3 receptor.* Mol Cell Biol, 1996. **16**(9): p. 4808-17.

98. Baek, K.H., et al., *DUB-1A, a novel deubiquitinating enzyme subfamily member, is polyubiquitinated and cytokine-inducible in B-lymphocytes.* J Biol Chem, 2004. **279**(4): p. 2368-76.

99. Zhu, Y., et al., *DUB-1, a deubiquitinating enzyme with growth-suppressing activity.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3275-9.

100. Jaster, R., et al., *JAK2 is required for induction of the murine DUB-1 gene.* Mol Cell Biol, 1997. **17**(6): p. 3364-72.

101. Jaster, R., K.H. Baek, and A.D. D'Andrea, *Analysis of cis-acting sequences and trans-acting factors regulating the interleukin-3 response element of the DUB-1 gene.* Biochim Biophys Acta, 1999. **1446**(3): p. 308-16.

102. Lee, M.Y., et al., *DUB-1, a fate determinant of dynein heavy chain in B-lymphocytes, is regulated by the ubiquitin-proteasome pathway.* J Cell Biochem, 2008. **105**(6): p. 1420-9.

103. Christoforidis, S., et al., *The Rab5 effector EEA1 is a core component of endosome docking.* Nature, 1999. **397**(6720): p. 621-5.

104. Zerial, M. and H. McBride, *Rab proteins as membrane organizers.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 107-17.

105. Mills, I.G., A.T. Jones, and M.J. Clague, *Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes.* Curr Biol, 1998. **8**(15): p. 881-4.

106. Rink, J., et al., *Rab conversion as a mechanism of progression from early to late endosomes.* Cell, 2005. **122**(5): p. 735-49.

107. Woodman, P.G. and C.E. Futter, *Multivesicular bodies: co-ordinated progression to maturity.* Curr Opin Cell Biol, 2008. **20**(4): p. 408-14.

108. Gillooly, D.J., et al., *Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells.* Embo J, 2000. **19**(17): p. 4577-88.

109. Piper, R.C. and D.J. Katzmann, *Biogenesis and function of multivesicular bodies.* Annu Rev Cell Dev Biol, 2007. **23**: p. 519-47.

110. Shields, S.B., et al., *ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting.* J Cell Biol, 2009. **185**(2): p. 213-24.

111. Luzio, J.P., P.R. Pryor, and N.A. Bright, *Lysosomes: fusion and function.* Nat Rev Mol Cell Biol, 2007. **8**(8): p. 622-32.

112. Seibenhener, M.L., et al., *Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation.* Mol Cell Biol, 2004. **24**(18): p. 8055-68.

113. Kirkin, V., et al., *A role for ubiquitin in selective autophagy.* Mol Cell, 2009. **34**(3): p. 259-69.

114. Pankiv, S., et al., *p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy.* J Biol Chem, 2007. **282**(33): p. 24131-45.

115. Sanchez, P., et al., *Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62.* Mol Cell Biol, 1998. **18**(5): p. 3069-80.


CHAPTER
2

JANUS KINASES PROMOTE CELL-SURFACE EXPRESSION AND PROVOKE AUTONOMOUS SIGNALING FROM ROUTING DEFECTIVE CSF3 RECEPTORS

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Biochem. J. (2009) 417, 737-746

ABSTRACT

CSF3R [CSF3 (granulocyte colony-stimulating factor) receptor] 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 tyrosine residues and downstream signalling substrates, JAKs have recently been implicated in controlling expression of cytokine receptors, predominantly by masking critical motifs involved in endocytosis and lysosomal targeting. In the present study, we show that increasing the levels of JAK1, JAK2 and TYK2 (tyrosine kinase 2) elevated steady-state CSF3R cell-surface expression and enhanced CSF3R protein stability in haematopoietic cells. This effect was not due to inhibition of endocytotic routing, since JAKs did not functionally interfere with the dileucine-based internalization motif or lysine-mediated lysosomal degradation of CSF3R. Rather, JAKs appeared to act on CSF3R in the biosynthetic pathway at the level of the ER (endoplasmic reticulum). Strikingly, increased JAK levels synergized with internalization- or lysosomal-routing-defective CSF3R mutants to confer growth factor independent STAT (signal transducer and activator of transcription) -3 activation and cell survival, providing a model for how increased JAK expression and disturbed intracellular routing of CSF3R synergize in the transformation of haematopoietic cells.

INTRODUCTION

The CSF3R is the major haematopoietic growth factor receptor involved in neutrophil development [1]. The CSF3R contains four conserved tyrosine residues in the cytoplasmic domain, which upon phosphorylation by JAKs (Janus tyrosine kinases) become docking sites for SH2 (Src homology 2)-containing signalling proteins such as STAT (signal transducer and activator of transcription) 3, the adapter proteins Shc (Src homology and collagen homology) and Grb2 (growth-factor-receptor-bound protein 2), and SOCS3 (suppressor of cytokine signalling 3) [2]. The cytoplasmic domain of CSF3R further comprises a dileucine-based motif crucial for internalization [3], and five conserved lysine residues. We have recently shown that one of these lysine residues, the juxtamembrane Lys^{632}, is the major determinant for ligand-induced lysosomal routing of the activated CSF3R, a process that involves SOCS3-mediated ubiquitination of Lys^{632} [4].

Ligand-induced CSF3R internalization and lysosomal sorting are both crucial for a balanced signalling output and disruption of either of these processes results in increased proliferation signalling at the expense of CSF3-induced neutrophilic differentiation [3, 4]. Perturbed signalling from CSF3R, owing to mutations in the *CSF3R* gene or expression of signalling defective splice variants, has been implicated in the development of AML (acute myeloid leukaemia) [5]. 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 Tyr^{729}, essential for SOCS3-induced ubiquitination of Lys^{632} [4, 6]. 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 OSMR (oncostatin M receptor) 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 [7]. Similarly, binding of JAK2 or TYK2 to the TpoR (thrombopoietin receptor) augmented membrane expression and stability of its mature [EndoH (endoglycosidase H) resistant] form [8, 9]. Studies on the IFNAR1 (interferon receptor-α1) 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 [10, 11]. 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 EpoR (erythropoietin receptor), where JAK2 binding does not affect receptor endocytosis, but

enhances the appropriate folding of the EpoR protein in the ER (endoplasmic reticulum), leading to more efficient Golgi processing and enhanced forward routing of mature protein to the cell surface [12].

Increased JAK expression has been implicated in several haematopoietic malignancies, including AML, and amplification of the *JAK2* gene has been reported in Hodgkin's lymphoma [13, 14]. In the present study, we examined whether JAKs control the expression of CSF3R and how this might affect signalling in the absence of ligand-induced receptor activation. We show that JAKs elevate the steady-state CSF3R cell-surface expression, independently 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 involving activation of multiple kinases and spontaneous STAT activation in cells expressing internalization- or lysosomal-routing-defective CSF3R mutants.

EXPERIMENTAL

Antibodies

Mouse anti-human CSF3R antibodies 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 (GAR-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 [4], as were the W650R and the d715 receptor mutants [15, 16]. The pSG513 vector with wt JAK2 and the bicistronic vectors pRex TYK2-IRES-CD2, pRex JAK1-IRES-CD2 have been previously reported [8, 17].

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 [18] and Ba/F3 cells, mouse pro-B cells that lack endogenous CSF3R [18, 19] were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 ng/ml murine IL-3, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO2. Ba/F3 cells expressing murine JAK1, murine JAK2 or human TYK2 have been described previously [8]. Phoenix E, HeLa and the parental human fibrosarcoma cells 2C4 [20] 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 [21] and JAK2-deficient γ2A [20] human fibrosarcoma cell lines were cultured in DMEM containing 400 μg/ml geneticin (G418). The TYK2-deficient human fibrosarcoma cell line 11.1 [22] 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 [3].

Constitutive and ligand-induced internalization determined by flow cytometry

To determine 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 CSF3 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 CSF3 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 (Dyna-

beads, 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 [23]. 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 CSF3 (Bio-CSF3) 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 [23].

Endoglycosidase H (EndoH) treatment

Phoenix E cells were transiently transfected with wt CSF3R. For membrane receptor pull-down, cells were incubated with 1:100 Bio-CSF3 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 Bio-CSF3 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.

Confocal microscopy

HeLa cells were seeded on glass cover slips and transfected with wt or K5R CSF3R in pLNCX using lipofectamin (Invitrogen). 32D.cl8.6 clones stably expressing wt or K5R CSFR were spun down on glass slides. Cells were prepared and analyzed by confocal microscopy using a LSM510 microscope equipped with argon and He/Ne lasers as described [4].

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.

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.

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 vector [8]. 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 with 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 CSF3R cell 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 [15], 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 as such is not required for CSF3R expression (Figure 2C).

Role of receptor lysine residues 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 [7, 10, 11].

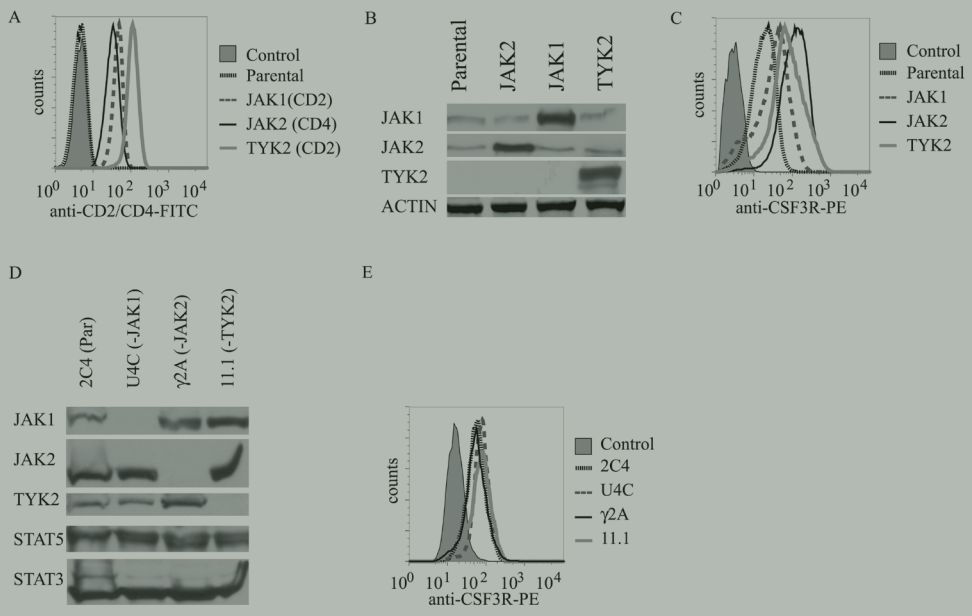

Figure 1: JAK1, JAK2 and TYK2 augment cell-surface expression of CSF3R. (**A**) JAK overexpression in Ba/F3 cells assessed by flow cytometry using CD2- or CD4-FITC antibodies. The control is Ba/F3 cells without antibody staining. (**B**) Western blot analysis showing JAK levels in parental Ba/F3 cells and JAK transfectants. To control for loading, the blot was restained for actin. (**C**) Expression of wt CSF3R in stably transduced Ba/F3 cell lines determined by flow cytometry using CSF3R-PE antibody. The control is Ba/F3 parental cells without antibody staining. (**D**) Immunoblots 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). Blots were stained for anti-JAK1, anti-JAK2 and anti-TYK2 to confirm JAK-deficiencies. Stainings with anti-STAT5 and anti-STAT3 were included for loading controls. (**E**) Flow cytometric analysis of CSF3R cell-surface levels in the human fibrosarcoma cell lines stably transduced with wt CSF3R. The control is 2C4 cells without antibody staining.

We have previously demonstrated that ubiquitination of lysine residues in the intracellular domain of CSF3R is important for ligand-induced lysosomal targeting but not for receptor internalization [4]. To determine whether receptor lysine residues are involved in ligand-independent (constitutive) endocytosis, we studied membrane expression of a receptor mutant in which all five cytoplasmic lysine residues were replaced by arginines residues (mutant K5R). Myeloid 32D cells stably expressing K5R showed higher membrane expression levels compared with wt CSF3R (Figure 3A), a result that was confirmed by confocal microscopy in 32D cells (Figure 3B, top panels) and in HeLa cells (Figure 3B, bottom panels). However, kinetic analysis of ligand-independent internalization of wt and K5R CSF3R were similar, indicating that lysine residues are not critical in this process (Figure 4A). Taken together, these results suggest that lysine residues within the CSF3R cytoplasmic region are involved in control of membrane expression through the biosynthetic pathway. To confirm this, we studied the role of the receptor cytoplasmic lysine residues in CSF3R stability. To this end,

Ba/F3 cells stably expressing wt or K5R CSF3R were incubated for 3-6 h with 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 lysine residues are important for this mechanism.

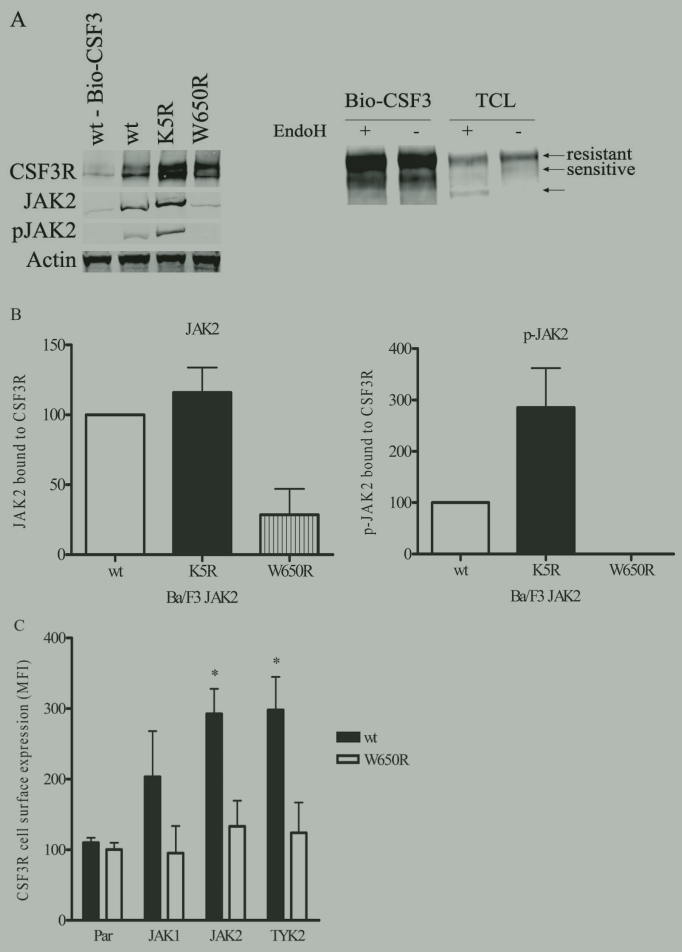

Figure 2: JAK-induced surface expression of CSF3R depends on a tryptophan residue (Trp650) that is critical for JAK binding. (**A**) Left-hand panel: JAK2-overexpressing Ba/F3 cells with the indicated CSF3R variants were stimulated with biotinylated CSF3 (Bio-CSF3) for 30 min. Receptor complexes were isolated using streptavidin pulldown and analysed by Western blotting for the presence of CSF3R, JAK2 or phospho-JAK2. For loading control, blots were stained for actin. A representative blot of three independent experiments is shown. Right-hand panel: Lack of EndoH sensitivity of Bio-CSF3-precipitated plasma-membrane bound CSF3R (left two lanes). CSF3R immunoprecipitates of total cell lysates (TCL, right two lanes) serve as positive controls for EndoH sensitivity of immature CSF3R (lower arrows). (**B**) Quantification of total JAK2 (left-hand panel) and phospho-JAK2 (right-hand 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 with parental Ba/F3 cells expressing wt CSF3R.

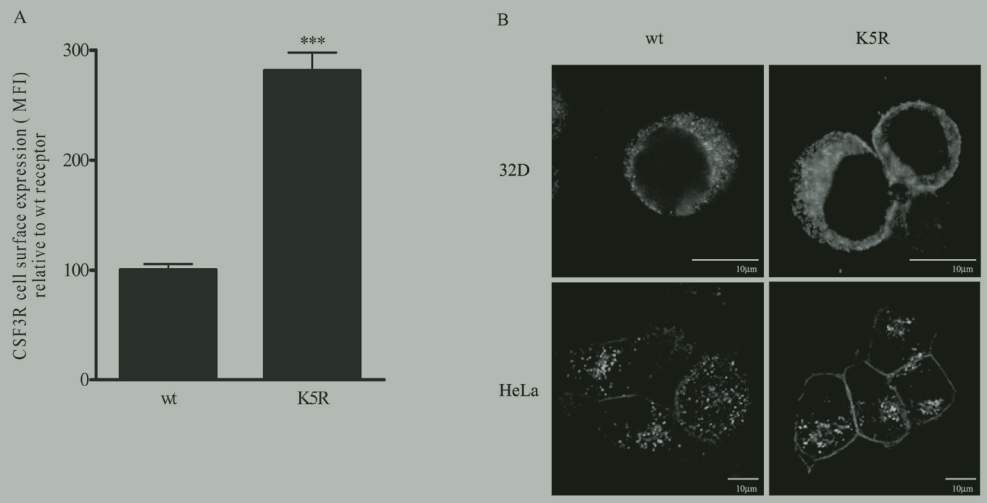

Figure 3: Role of receptor lysine residues in CSF3R cell-surface expression. (**A***)* Sublines of 32D cells were generated with wt or K5R CSF3R. Membrane expression was determined by flow cytometry using CSF3R-PE antibody. Results are shown as MFI ± SEM of 12 clones relative to wt receptor. ***P< 0.001 compared with wt receptor. (**B***)* Confocal microscopy images of 32D clones stably expressing wt or K5R CSF3R (top panels) and HeLa cells transiently transfected with wt or K5R CSF3R constructs (bottom panels).

Because the lysine residues in the cytoplasmic domain of CSF3R are fully conserved between multiple species, we investigated which of these are specifically involved in the negative control of steady-state CSF3R membrane expression. Using a panel of lysine substitution (K→R) and single lysine add-back (mKA-mKE) mutants, we found that Lys^{632} 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 whether increased JAK levels alter the kinetics of CSF3R internalization. As shown in Figure 5(A), none of the JAKs affected the kinetics and magnitude of CSF3R endocytosis. On the other hand, JAK2 and TYK2, and to a lesser extent JAK1, significantly enhanced surface expression of d715 CSF3R and K5R CSF3R (Figure 5B), showing 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, Lys^{632} remains the most prominent lysine residue involved in the downregulation of CSF3R surface expression (Figure 5C, compare to Figure 4C).

JAKs do not inhibit ubiquitination of Lys^{632} in ligand-activated CSF3R

From the studies described above, Lys^{632} emerges as a major negative-determinant in constitutive CSF3R expression. Upon ligand activation of the CSF3R-JAK complex Lys^{632} is ubiquitinated, which is essential for CSF3-induced lysosomal routing of CSF3R [4]. Because JAKs are activated in this setting, we wondered whether increased JAK levels may interfere

with ubiquitination of Lys^{632} to attenuate ligand-induced lysosomal degradation. As shown in Figure 6(A), ubiquitination of Lys^{632}, the only cytoplasmic lysine residue retained in mKA, increased proportionally with elevated CSF3R levels in JAK1-, JAK2- or TYK2-overexpressing cells relative to parental cell controls. Taken together, these data indicate that JAKs do not interfere with the function of Lys^{632} in either forward or retrograde routing of CSF3R.

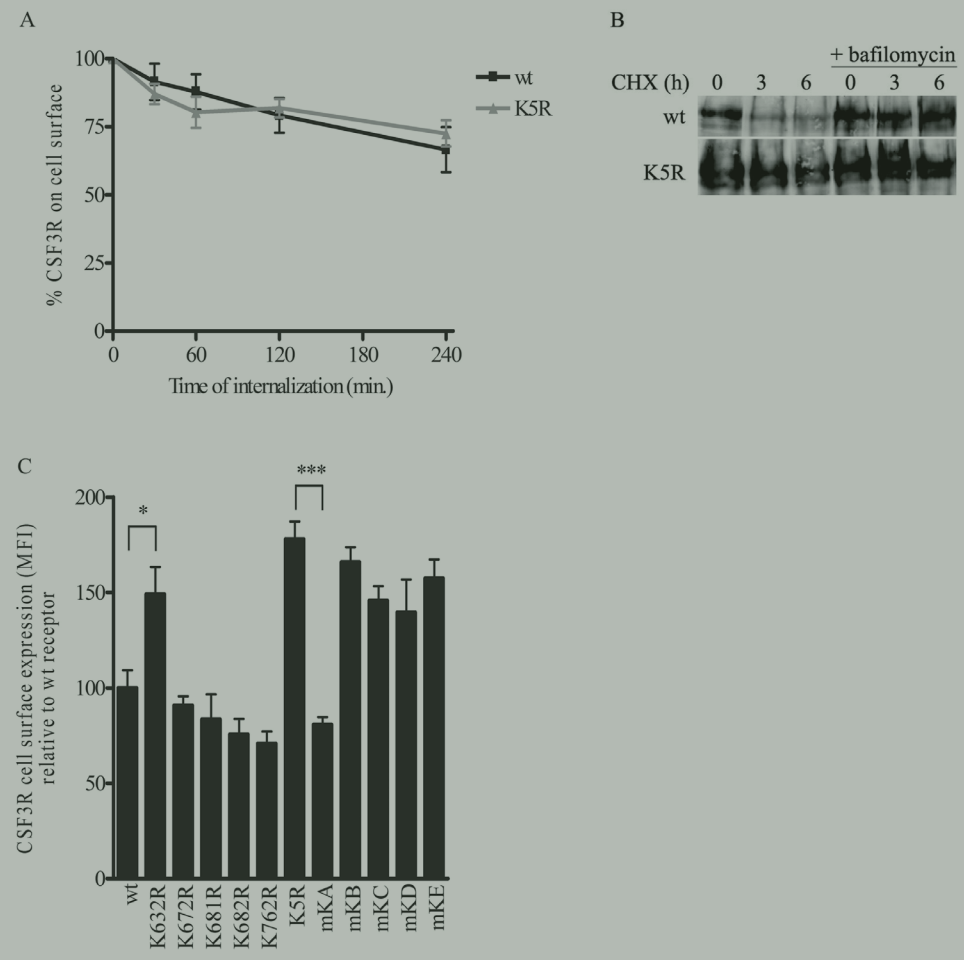

Figure 4: Lysine residues are important for steady-state CSF3R cell-surface expression but are not involved in constitutive internalization. (**A**) 32D cells stably expressing wt or K5R CSF3R were labelled with CSF3R antibody and were allowed to internalize for 4 h in the absence of ligand. CSF3Rs remaining at the cell surface were labelled with goat anti-mouse-PE and analyzed by flow cytometry. Values 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 h. 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 wt 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). Results are shown as MFI ± SEM of four to seven clones relative to wt receptor. *P< 0.05 compared with wt receptor.***P<0.001 mKA compared with K5R CSF3R.

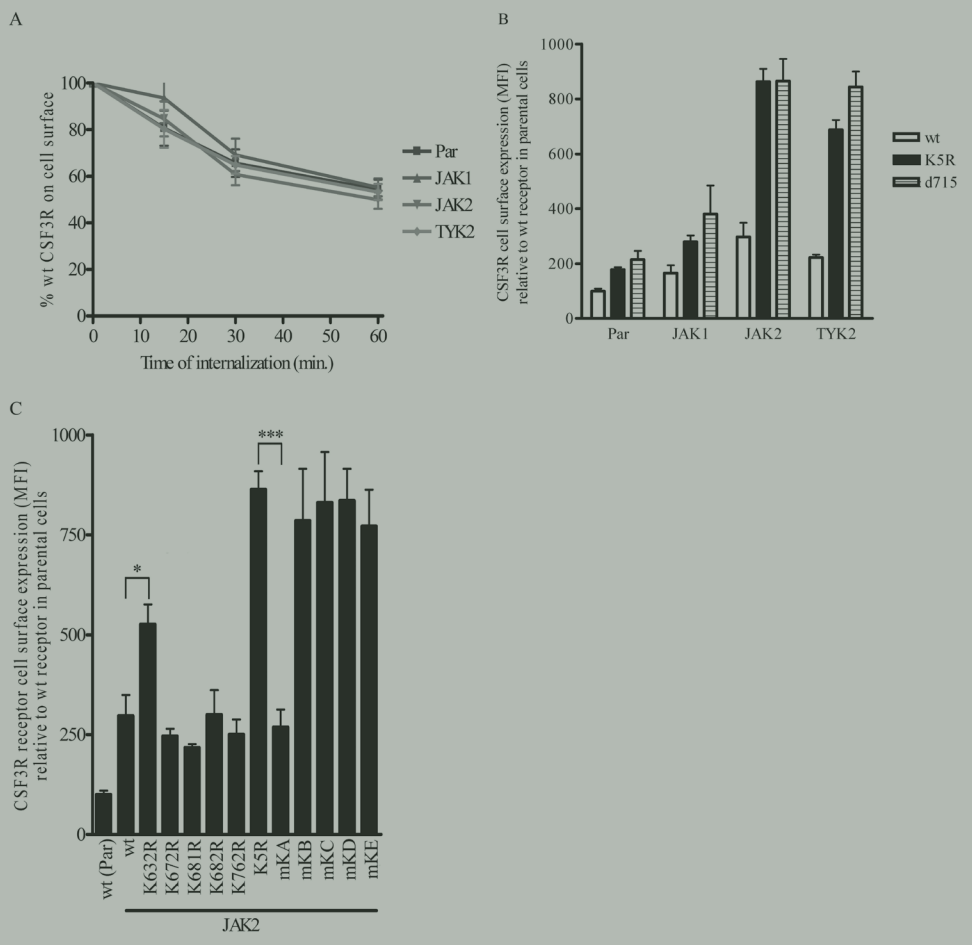

Figure 5: **Enhanced CSF3R cell-surface expression by JAKs is not due to masking of internalization or lysosomal sorting motifs.** (**A**) Ba/F3 parental and JAK overexpressing cells stably expressing wt CSF3R were incubated with CSF3 (100 ng/ml) and at indicated time points stained with anti-CSF3R-PE followed by flow cytometric analysis. Data from three independent experiments are expressed as MFI ± SEM 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 ± SEM of 4-7 clones 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<0.05 K632R compared with wt CSF3R. ***P<0.001 mKA compared with K5R CSF3R.

JAKs enhance protein stability of CSF3R

To determine if JAKs influence CSF3R protein stability, as e.g., shown for the TpoR [8], Phoenix E cells transiently expressing JAK and wt CSF3R were cultured in the presence or absence of CHX for 5 h to block protein synthesis. Lysates were then subjected to immunoprecipitation with anti-CSF3R antibodies. Immunoblots revealed two CSF3R species, the

higher band representing the mature form. The lower band (not to be confused with the glycosylation variant shown in Figure 2A), was sensitive to Endo H treatment thus representing immature CSF3R protein. Overexpression of JAK1, JAK2 and TYK2 predominantly enhanced the stability of the mature form of the wt CSF3R (Figure 6B and results not shown). In contrast, both the mature and immature forms of K5R were stabilized (Figure 6B). These results indicate that JAKs protect both the mature and immature form of CSF3R, provided that receptor lysine-mediated degradation is prevented.

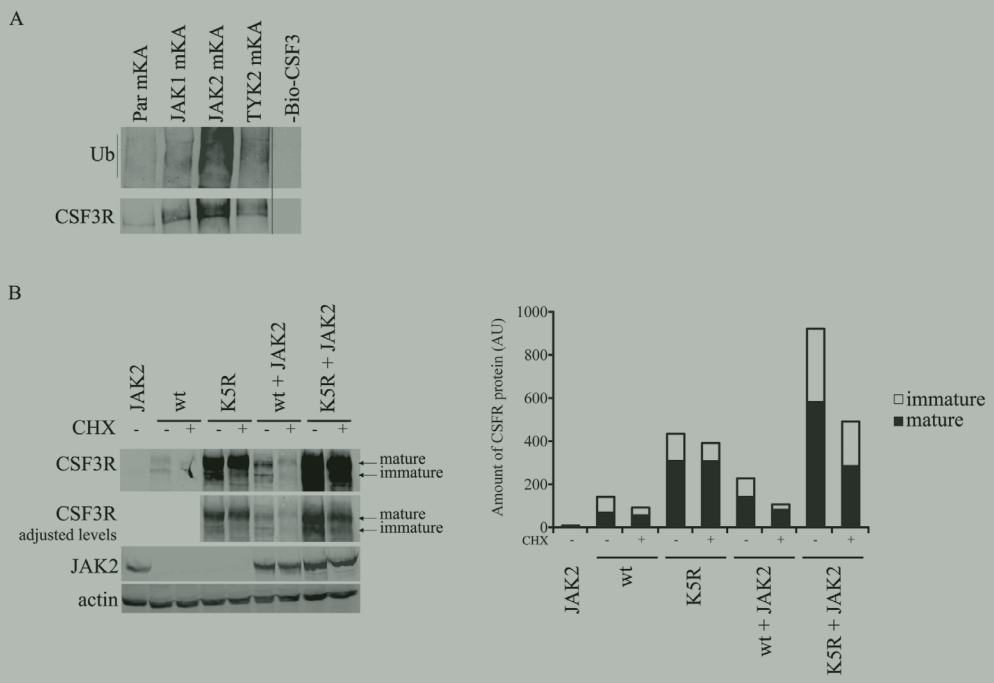

Figure 6: JAKs control CSF3R expression in the biosynthetic pathway irrespective of receptor ubiquitination. (**A**) Ubiquitination status of the lysine residue at position 632 of membrane CSF3R determined in Ba/F3 parental and JAK-overexpressing cells. Cells were stimulated with Bio-CSF3 for 30 min. in the presence of bafilomycin, whereafter CSF3R was pulled down with streptavidin-coated magnetic beads, eluted and subjected to Western blot analysis using anti-ubiquitin antibody P4D1. Vertical line (left-hand side) indicates size variation of receptor-ubiquitin complexes. Blots were restained with anti-CSF3R to determine CSF3R loading. The black line between lanes 4 and 5 indicates that two parts of the blot have been juxtaposed. (**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. The top panels show the relative intensities between CSF3R mutants with and without overexpression of JAK2. In the second panel the intensities are adjusted to show the difference between the mature and immature CSF3R band. Quantification of mature and immature forms of CSF3R is shown in the right-hand panel; 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. The results shown are representative of three independent experiments.

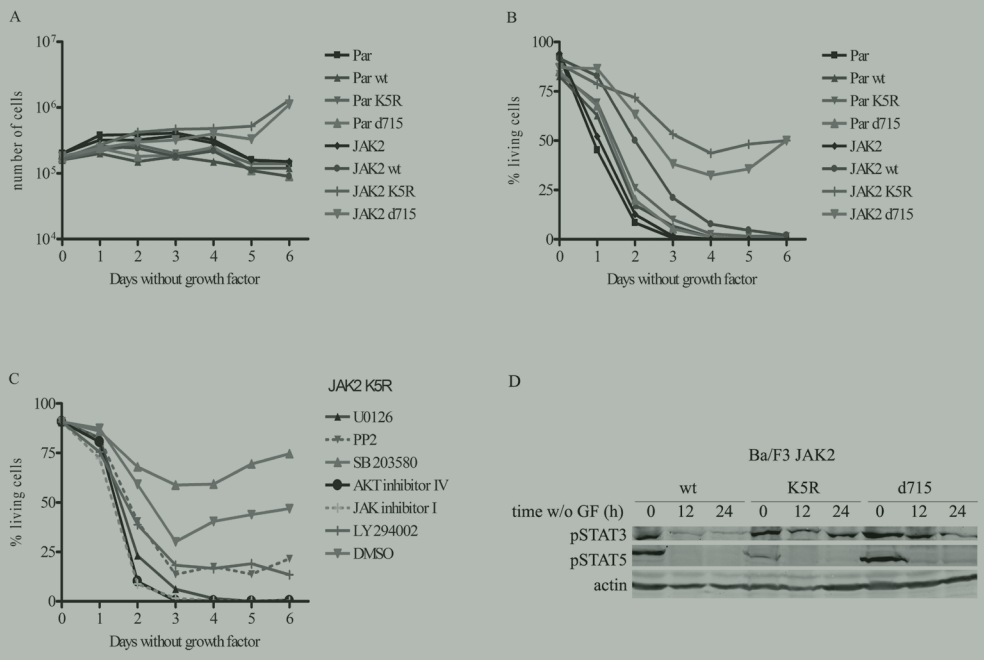

Figure 7: JAK2 overexpression provokes ligand-independent pro-survival signalling by internalization-defective and lysosomal-routing-deficient CSF3R mutants. Parental and JAK2-overexpressing Ba/F3 transfectants were cultured in medium with IL-3 and transferred to medium without growth factors. (**A***)* 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 (time w/o GF) at the indicated time points (h) and subjected to Western blot analysis with antibodies against phosphoSTAT3, phospho-STAT5 and actin as a loading control.

Increased JAK levels confer ligand independent signalling to internalization and lysosomal routing defective CSF3R mutants

Having established that JAKs increase the stability and membrane expression of CSF3R, we wondered whether increased JAK levels can co-operate with mutant CSF3R defective in lysosomal degradation to enhance downstream signalling. As reported previously [24], 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 behaviour. 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 (IC_{50} = 1nM), MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] inhibitor U0126 and Akt (also known as protein kinase B) inhibitor IV completely abolished cell survival. Blocking of PI3K (phosphoinositide 3-kinase), acting upstream of 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 CSF3-induced proliferation and survival [26, 27], we investigated whether these STATs are constitutively activated in JAK2-overexpressing Ba/F3 cells harbouring K5R CSF3R or d715 CSF3R. 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

In the present study, 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 lysine residues, respectively. Major observations were: (i) increased JAKs significantly elevated CSF3R membrane expression and protein levels, (ii) 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 Lys^{632} of CSF3R and (iii) 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 [7, 8, 10, 12, 28], 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 with wt EpoR and EpoR was still expressed on the cell surface of JAK2-deficient embryonic fibroblasts [29]. 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 [10, 11] and similarly JAK1 was found to inhibit internalization of OSMR [7]. In this respect, CSF3R resembles the EpoR, because 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 W650R CSF3R might still suffice to promote cell-surface routing.

Among the five conserved lysine residues in the cytoplasmic tail of CSF3R, the juxtamembrane residue Lys^{632}, in addition to being a major determinant for ligand-induced lysosomal routing [4], also appeared to be uniquely involved in the control of constitutive CSF3R cell-surface expression. Although lysine residues have been reported to regulate ligand-independent internalization of the short isoform of the leptin receptor [30], we could not detect any role of lysine residues in internalization of the CSF3R. Hence, loss of Lys^{632} rather leads to enhanced cell-surface expression by redirection of receptors in the biosynthetic pathway from the ER-lysosomal degradative route towards the plasma membrane. Major players involved in intracellular trafficking of surface membrane proteins are the ESCRT (endosomal sorting complex required for transport) and GGA [Golgi-associated γ-adaptin ear homology domain Arf (ADP-ribosylation factor)-interacting protein] protein complexes, respectively [31]. Because key proteins within these complexes, such as Hrs and GGA, interact with ubiquitinated lysine residues in cargo proteins but simultaneously require interaction with phosphoinositides to increase affinity [32, 33], this might explain why membrane proximal positioning of Lys^{632} is imperative for lysosomal routing of CSF3R. This is supported by recent experiments showing that relocation of Lys^{632} to a more membrane distant position disrupted its role in routing despite the fact that ubiquitination was unaffected (A. Wölfler, M.Irandoust, A.Meenhuis, J.Gits, O,Roovers and I.P. Touw, unpublished work).

How JAKs control basal cell-surface expression of CSF3R remains to be resolved. Because JAKs bind with their N-terminal FERM (4.1/ezrin/radixin/moesin) domain to the juxtamembrane domain of CSF3R that encompasses Lys^{632}, a feasible explanation was that JAK overexpression would mask the function of this lysine residue, 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 Lys^{632} and elevated cell surface levels of mutants K632R and K5R 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 [12].

Abnormal responses to haematopoietic growth factors, including CSF3, have long been suspected to be involved in leukaemic cell growth in AML [34]. The discovery of acquired mutations in *CSF3R* in patients with severe congenital neutropenia that are strongly associated with progression to AML have supported this idea [35]. 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 signalling from lysosomal-routing defective CSF3R via multiple downstream pathways. Such a combinatorial mechanism might, e.g., involve deubiquitinating enzymes that attenuate lysosomal routing by deubiquitination of critical lysine residues 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 [36].

ACKNOWLEDGEMENTS

This work was supported by the Dutch Cancer Society, KWF kankerbestrijding (www.KWFkankerbestrijding.nl). A.W. was supported by the Austrian Science Foundation (FWF) through an Erwin-Schrödinger fellowship (J2536-B05). We thank Stefan Constantinescu for providing Ba/F3 parental and JAK overexpressing cells, human fibrosarcoma cell lines 2C4, γ2A, U4C and 11.1 and the retroviral bicistronic vectors pRex-TYK2-IRES-CD2, pRex Jak1-IRES-CD2. We thank Marieke von Lindern for helpful discussions and critical reading of the manuscript.

REFERENCES

1 Avalos, B. R., Gasson, J. C., Hedvat, C., Quan, S. G., Baldwin, G. C., Weisbart, R. H., Williams, R. E., Golde, D. W. and DiPersio, J. F. (1990). Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. Blood **75**, 851-7

2 Touw, I. P. and Bontenbal, M. (2007). Granulocyte colony-stimulating factor: key (f)actor or innocent bystander in the development of secondary myeloid malignancy? J. Natl. Cancer. Inst. **99**, 183-6

3 Aarts, L. H., Roovers, O., Ward, A. C. and Touw, I. P. (2004). Receptor activation and 2 distinct COOH-terminal motifs control G-CSF receptor distribution and internalization kinetics. Blood **103**, 571-9

4 Irandoust, M. I., Aarts, L. H., Roovers, O., Gits, J., Erkeland, S. J. and Touw, I. P. (2007). Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor. Embo. J.

5 Touw, I. P. and van de Geijn, G. J. (2007). Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. Front. Biosci. **12**, 800-15

6 Ward, A. C., van Aesch, Y. M., Schelen, A. M. and Touw, I. P. (1999). Defective internalization and sustained activation of truncated granulocyte colony-stimulating factor receptor found in severe congenital neutropenia/acute myeloid leukemia. Blood **93**, 447-58

7 Radtke, S., Jorissen, A., de Leur, H. S., Heinrich, P. C. and Behrmann, I. (2006). Three dileucine-like motifs within the interbox1/2 region of the human oncostatin M receptor prevent efficient surface expression in the absence of an associated Janus kinase. J. Biol. Chem. **281**, 4024-34

8 Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J. and Constantinescu, S. N. (2005). Janus kinases affect thrombopoietin receptor cell surface localization and stability. J. Biol. Chem. **280**, 27251-61

9 Tong, W., Sulahian, R., Gross, A. W., Hendon, N., Lodish, H. F. and Huang, L. J. (2006). The membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms. J. Biol. Chem. **281**, 38930-40

10 Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G. and Pellegrini, S. (2003). The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. Embo. J. **22**, 537-47

11 Kumar, K. G., Varghese, B., Banerjee, A., Baker, D. P., Constantinescu, S. N., Pellegrini, S. and Fuchs, S. Y. (2008). Basal ubiquitin-independent internalization of interferon alpha receptor is prevented by Tyk2-mediated masking of a linear endocytic motif. J. Biol. Chem.

12 Huang, L. J., Constantinescu, S. N. and Lodish, H. F. (2001). The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol. Cell **8**, 1327-38

13 Joos, S., Kupper, M., Ohl, S., von Bonin, F., Mechtersheimer, G., Bentz, M., Marynen, P., Moller, P., Pfreundschuh, M., Trumper, L. and Lichter, P. (2000). Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res. **60**, 549-52

14 Rucker, F. G., Bullinger, L., Schwaenen, C., Lipka, D. B., Wessendorf, S., Frohling, S., Bentz, M., Miller, S., Scholl, C., Schlenk, R. F., Radlwimmer, B., Kestler, H. A., Pollack, J. R., Lichter, P., Dohner, K. and Dohner, H. (2006). Disclosure of candidate genes in acute myeloid leukemia with complex karyotypes using microarray-based molecular characterization. J. Clin. Oncol. **24**, 3887-94

15 Barge, R. M., de Koning, J. P., Pouwels, K., Dong, F., Lowenberg, B. and Touw, I. P. (1996). Tryptophan 650 of human granulocyte colony-stimulating factor (G-CSF) receptor, implicated in

the activation of JAK2, is also required for G-CSF-mediated activation of signaling complexes of the p21ras route. Blood **87**, 2148-53

16 Dong, F., van Buitenen, C., Pouwels, K., Hoefsloot, L. H., Lowenberg, B. and Touw, I. P. (1993). Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. Mol. Cell. Biol. **13**, 7774-81

17 van den Akker, E., van Dijk, T., Parren-van Amelsvoort, M., Grossmann, K. S., Schaeper, U., Toney-Earley, K., Waltz, S. E., Lowenberg, B. and von Lindern, M. (2004). Tyrosine kinase receptor RON functions downstream of the erythropoietin receptor to induce expansion of erythroid progenitors. Blood **103**, 4457-65

18 Greenberger, J. S., Eckner, R. J., Sakakeeny, M., Marks, P., Reid, D., Nabel, G., Hapel, A., Ihle, J. N. and Humphries, K. C. (1983). Interleukin 3-dependent hematopoietic progenitor cell lines. Fed. Proc. **42**, 2762-71

19 Palacios, R. and Steinmetz, M. (1985). Il-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell **41**, 727-34

20 Watling, D., Guschin, D., Muller, M., Silvennoinen, O., Witthuhn, B. A., Quelle, F. W., Rogers, N. C., Schindler, C., Stark, G. R., Ihle, J. N. and et al. (1993). Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. Nature **366**, 166-70

21 Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A. G., Barbieri, G., Witthuhn, B. A., Schindler, C. and et al. (1993). The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. Nature **366**, 129-35

22 Velazquez, L., Fellous, M., Stark, G. R. and Pellegrini, S. (1992). A protein tyrosine kinase in the interferon alpha/beta signaling pathway. Cell **70**, 313-22

23 Ward, A. C., Smith, L., de Koning, J. P., van Aesch, Y. and Touw, I. P. (1999). Multiple signals mediate proliferation, differentiation, and survival from the granulocyte colony-stimulating factor receptor in myeloid 32D cells. J. Biol. Chem. **274**, 14956-62

24 Lu, X., Levine, R., Tong, W., Wernig, G., Pikman, Y., Zarnegar, S., Gilliland, D. G. and Lodish, H. (2005). Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. Proc. Natl. Acad. Sci. U S A **102**, 18962-7

25 Weston, C. R., Lambright, D. G. and Davis, R. J. (2002). Signal transduction. MAP kinase signaling specificity. Science **296**, 2345-7

26 McLemore, M. L., Grewal, S., Liu, F., Archambault, A., Poursine-Laurent, J., Haug, J. and Link, D. C. (2001). STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. Immunity **14**, 193-204

27 Dong, F., Liu, X., de Koning, J. P., Touw, I. P., Hennighausen, L., Larner, A. and Grimley, P. M. (1998). Stimulation of Stat5 by granulocyte colony-stimulating factor (G-CSF) is modulated by two distinct cytoplasmic regions of the G-CSF receptor. J. Immunol. **161**, 6503-9

28 Radtke, S., Hermanns, H. M., Haan, C., Schmitz-Van De Leur, H., Gascan, H., Heinrich, P. C. and Behrmann, I. (2002). Novel role of Janus kinase 1 in the regulation of oncostatin M receptor surface expression. J. Biol. Chem. **277**, 11297-305

29 Pelletier, S., Gingras, S., Funakoshi-Tago, M., Howell, S. and Ihle, J. N. (2006). Two domains of the erythropoietin receptor are sufficient for Jak2 binding/activation and function. Mol. Cell. Biol. **26**, 8527-38

30 Belouzard, S. and Rouille, Y. (2006). Ubiquitylation of leptin receptor OB-Ra regulates its clathrin-mediated endocytosis. Embo J. **25**, 932-42

31 Hurley, J. H., Lee, S. and Prag, G. (2006). Ubiquitin-binding domains. Biochem. J. **399**, 361-72

32 Wang, J., Sun, H. Q., Macia, E., Kirchhausen, T., Watson, H., Bonifacino, J. S. and Yin, H. L. (2007). PI4P promotes the recruitment of the GGA adaptor proteins to the trans-Golgi network and regulates their recognition of the ubiquitin sorting signal. Mol. Biol. Cell **18**, 2646-55

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

34 Lowenberg, B. and Touw, I. P. (1993). Hematopoietic growth factors and their receptors in acute leukemia. Blood **81**, 281-92

35 Freedman, M. H., Bonilla, M. A., Fier, C., Bolyard, A. A., Scarlata, D., Boxer, L. A., Brown, S., Cham, B., Kannourakis, G., Kinsey, S. E., Mori, P. G., Cottle, T., Welte, K. and Dale, D. C. (2000). Myelodysplasia syndrome and acute myeloid leukemia in patients with congenital neutropenia receiving G-CSF therapy. Blood **96**, 429-36

36 Migone, T. S., Humbert, M., Rascle, A., Sanden, D., D'Andrea, A. and Johnston, J. A. (2001). The deubiquitinating enzyme DUB-2 prolongs cytokine-induced signal transducers and activators of transcription activation and suppresses apoptosis following cytokine withdrawal. Blood 98, 1935-41


CHAPTER
3

SITE-SPECIFIC UBIQUITINATION DETERMINES LYSOSOMAL SORTING AND SIGNAL ATTENUATION OF THE CSF3 RECEPTOR

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Traffic (2009) 10, 1168–1179

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 (CSF3R), 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 CSF3R 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. CSF3R ubiquitination peaked after endocytosis, was inhibited by methyl-β-cyclodextrin as well as hyperosmotic sucrose and severely reduced in internalization defective CSF3R 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 CSF3R mutants found in severe congenital neutropenia, a hematopoietic disorder with a high leukemia risk.

INTRODUCTION

Granulocyte colony-stimulating factor (CSF3) is the major cytokine involved in the production of neutrophilic granulocytes (1, 2). CSF3 induces the proliferation and survival of myeloid progenitor cells, followed by a cell cycle arrest and neutrophilic differentiation (3). This response depends on the balanced activation and subsequent attenuation of signaling pathways linked to the CSF3 receptor (CSF3R), a member of the class I cytokine receptor superfamily (3). Signal attenuation of CSF3R is severely compromised by mutations causing truncations in the cytoplasmic domain of the CSF3R, which are found in patients with a severe form of neutropenia and a predisposition to acute myeloid leukemia (4). Two major mechanisms implicated in the perturbed signaling functions of these truncated CSF3R mutants are the loss of a dileucine-based internalization motif (5, 6) and the deletion of Y729, the tyrosine residue involved in the recruitment of the suppressor of cytokine signaling protein SOCS3 (7, 8).

Covalent attachment of ubiquitin (Ub) to cytoplasmic lysine residues in activated membrane receptors is one of the major mechanisms involved in downregulation of signaling (9). 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) (10). Upon ligand binding, the ubiquitin ligase (E3) Cbl is recruited to the tyrosine-phosphorylated receptor and induces ubiquitination of lysine residues located within the kinase domain (11). While ubiquitination of the EGFR itself is dispensable for receptor endocytosis, it is crucial for lysosomal routing and degradation of the EGFR (10, 12). 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 (13, 14), 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 CSF3R depends on recruitment of SOCS3 to the activated receptor (15). Besides inhibiting signaling by interfering with JAK kinase activity through its kinase inhibitory region (16), 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) (17). SOCS3-directed lysosomal routing and signal attenuation of the CSF3R was dependent on ubiquitination of lysine residues of the CSF3R as well as on the presence of the SOCS box in SOCS3 (15). A key finding was that SOCS3-induced lysosomal routing of the CSF3R 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 CSF3R (15). This result differed from studies of other receptors showing that endocytosis and lysosomal routing could be triggered by a linear fusion of Ub to the cytoplasmic tail of a membrane receptor (18-21). On the other hand,

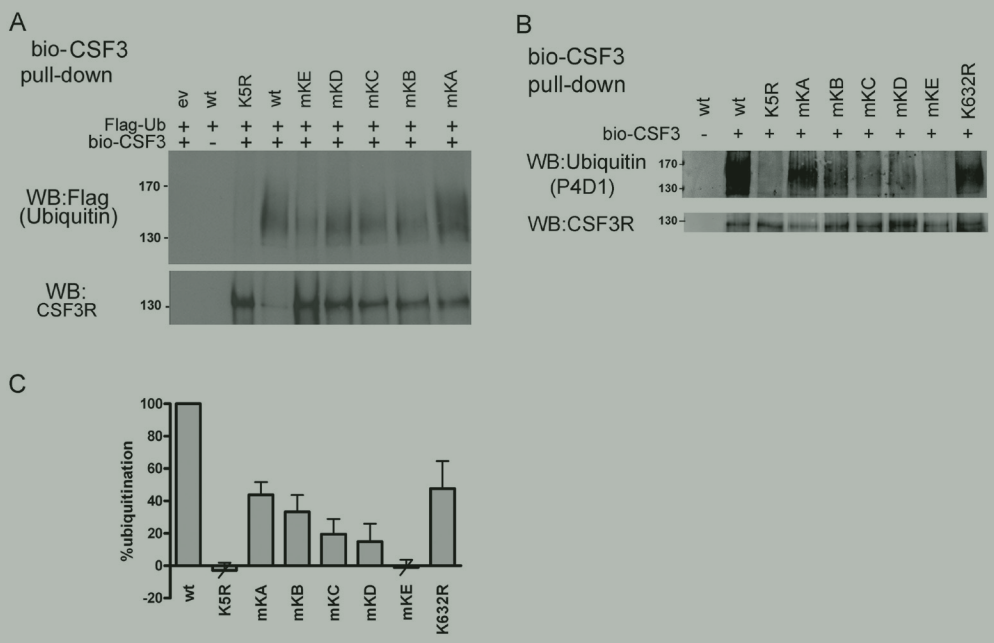


Figure 1: Ubiquitination of cytoplasmic lysine residues after CSF3R activation. (**A**) Phoenix E cells co-transfected with the indicated CSF3R constructs and Flag-Ub were incubated with biotinylated CSF3 (bio-CSF3) for 15 min at 16°C and for 30min at 37°C. Ligand-bound receptors were precipitated using streptavidin-coated beads and analyzed by Western blotting using anti-Flag (upper panel) or anti-CSF3R antibodies (lower panel). (**B**) Ba/F3 cells stably expressing the indicated CSF3R mutants were preincubated with bafilomycin (250nM) for 30 min to inhibit acidification of late endosomes and then stimulated with biotinylated CSF3 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-CSF3R antibodies (lower panel). (**C**) The mean amounts of Ub in the CSF3R 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 CSF3R. Receptor levels were lower for WT and mKA because the lysine on position 632 also regulates steady-state membrane expression of the CSF3R (Meenhuis et al., submitted).

recent reports have suggested that specific positioning of lysines within target proteins can be important for certain ubiquitination-dependent mechanisms, because some E3 ligases preferentially select lysines next to their recruitment site within the target protein (22). For instance, this applies to the F-box protein β-Transducin repeat-containing protein (β-Trcp) (23), which together with the linker Skp1 and Cul1 forms a multi-protein SCF (Skp1-Cul1-F-box) E3 ligase complex. β-Trcp ubiquitinates lysine residues at position 9-13 upstream of its binding motif (24). Targets of β-Trcp include the cytokine receptors interferon α receptor 1 (IFNAR1) (24, 25), prolactin receptor (26) and the erythropoietin receptor (EPOR) (27), but not the CSF3R. 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

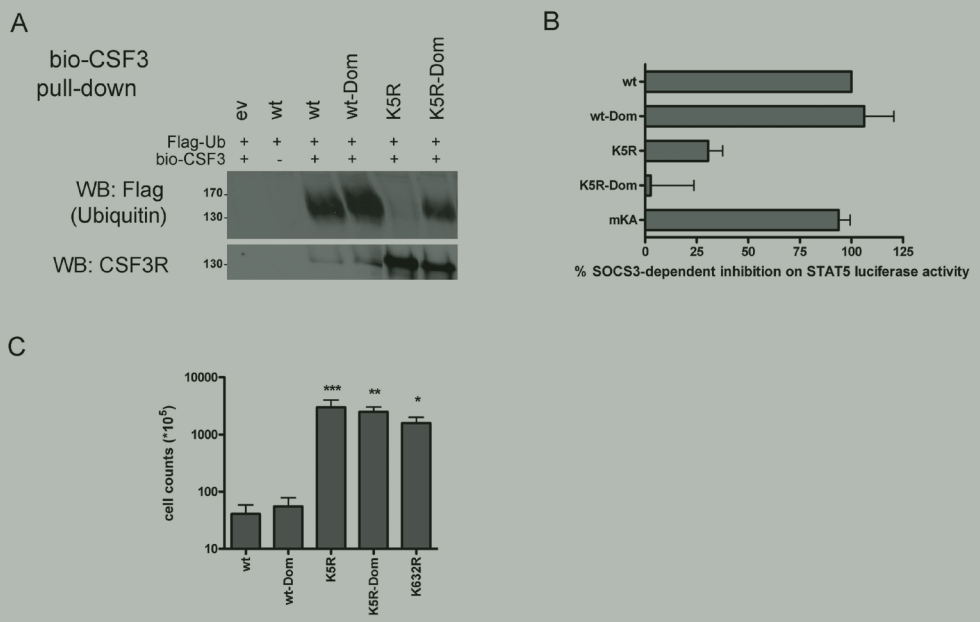


Figure 2: Ubiquitination and function of the K5R-D fusion CSF3R mutant. (**A**) Phoenix E cells were co-transfected with the indicated constructs and incubated with bio-CSF3 as described in Figure 1a. Ligand-bound receptors were precipitated and analyzed by Western blotting using anti-Flag (upper panel) or anti-CSF3R antibodies (lower panel). (**B**) HEK293 cells were transfected with the indicated CSF3R constructs, a STAT5-responsive luciferase construct and with or without SOCS3. SOCS3-dependent inhibition of STAT5 luciferase activity observed by WT-CSF3R was set at 100%. The mean (± SE) of three independent experiments done in triplicate is shown. (**C**) Proliferation of 32D cells expressing the indicated CSF3R constructs in response to CSF3 (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 CSF3R.

critical for binding with a downstream interacting protein, as has recently been implicated to be of major importance to regulate IFNAR1 internalization (14).

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 CSF3R. 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 CSF3R ubiquitination predominantly takes place after receptor internalization. Finally, we demonstrate that a CSF3R deletion mutant (d715) found in patients with neutropenia is severely hampered in ubiquitination, which corroborates the importance of ubiquitination for proper CSF3R function and provides a mechanism for the dysfunction of this receptor mutant associated with leukemic progression of this disease.

RESULTS AND DISCUSSION

Ubiquitination of cytoplasmic lysine residues after CSF3R activation

Although the cytoplasmic domain of the CSF3R contains five highly conserved lysines, SOCS3-induced downregulation of CSF3R signaling via lysosomal routing and degradation criticallly depended on the presence of the juxtamembrane K632 (15). To test whether this relates to differential ubiquitination, we expressed the CSF3R mutant K5R, lacking all cytoplasmic lysine residues, or single lysine add-back mutants (mutants mKA to mKE) together with Flag-tagged Ubiquitin (Ub) in phoenix E cells. After activation with biotinylated CSF3 (bio-CSF3), 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, K681, and K682 after receptor activation (Figure 1a). To rule out that ectopic expression of Ub caused physiologically irrelevant ubiquitination on these lysines (28), we generated Ba/F3 cells stably expressing wt CSF3R and lysine add-back mutants at levels comparable to primary bone marrow cells, without introducing Flag-Ub. Ubiquitination of CSF3R could barely be detected with the Ub antibody P4D1, due to rapid lysosomal degradation (data not shown). However, in the presence of the lysosomal degradation inhibitor bafilomycin (29), we could readily detect a Ub smear with mutants mKA, mKB, mKC and mKD but not with mKE (Figure 1b). This 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 (15) 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).

The detection of Ub smears ranging from 135 to 170 kDa rather than discrete single ubiquitinated forms of the CSF3R 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 (11, 30, 31). The finding that a single lysine (K632) is not only sufficient but also exclusively responsible for direct lysosomal sorting and signal downregulation of the CSF3R differs from the assumption that multiple lysines act as redundant Ub acceptor sites (32). It also challenges the idea that the amount of poly-Ub attached to a receptor, rather than the specific site of ubiquitination, is important for lysosomal sorting (9, 11, 31). Indeed, although ubiquitination levels of mutants mKA and K632R were comparable, their intracellular fate after activation was entirely different, with mKA being sorted to lysosomes with normal signal downregulation, and K632R accumulating in early endosomes accompanied by prolonged signaling (15).

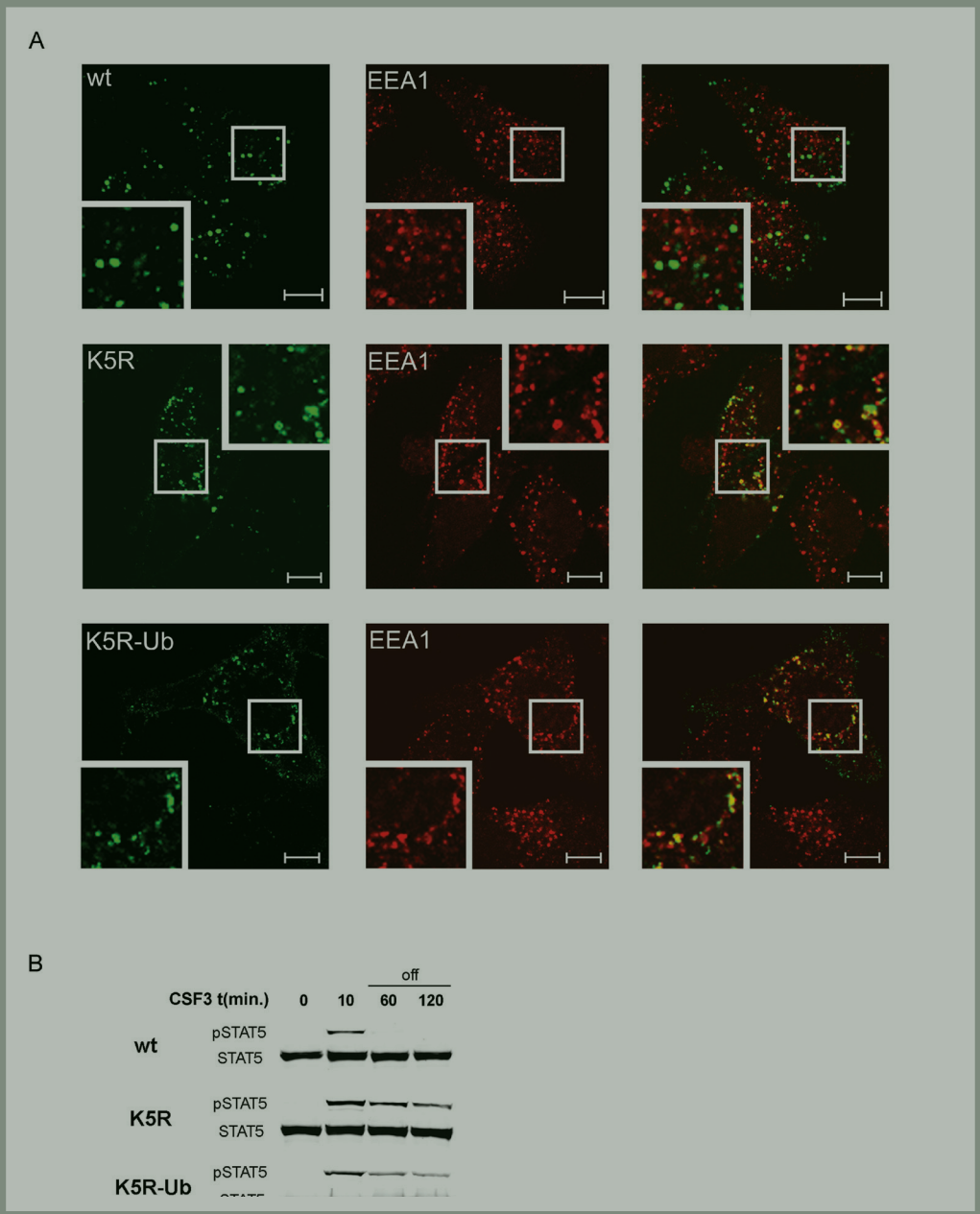


Figure 3: Fusion of ubiquitin to the C-terminus does not restore lysosomal sorting and downregulation STAT5 activation. **(A)** HeLa cells ectopically expressing WT, K5R or K5R-Ub CSF3R were incubated with anti-CSF3R antibodies and CSF3 for 20 min at 16°C, washed and transferred to 37°C for 90 min. Subsequently, cells were fixed and stained for internalized CSF3R (green, left panels) and the early endosomal marker EEA1 (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels indicating colocalization in yellow. Inserts show enlargements of boxed areas. Scale bars indicate 10μm. **(B)** Ba/F3 cells expressing WT or mutant CSF3R were deprived of IL-3 and serum for 4h, incubated with CSF3 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.

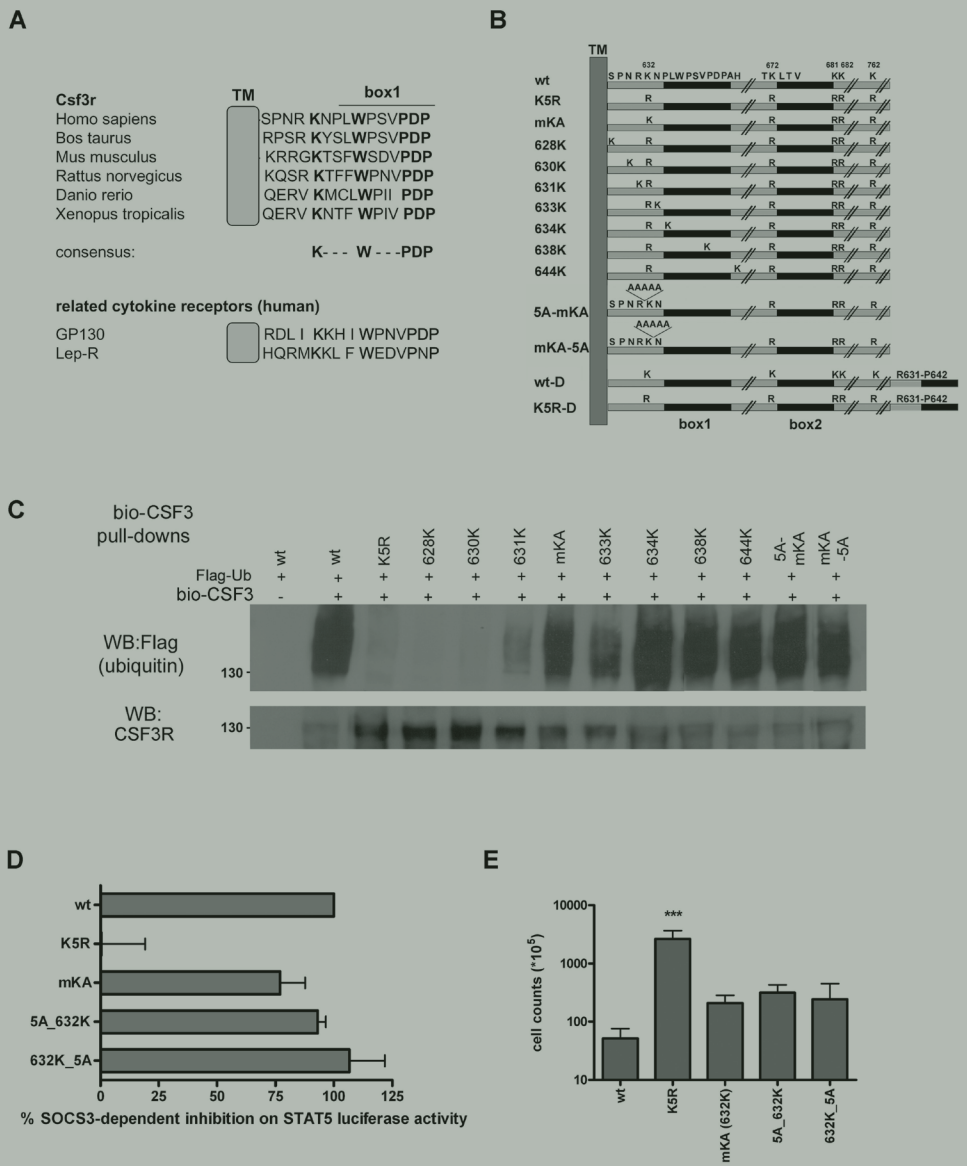


Figure 4: Ubiquitination and signaling function of juxtamembrane alanine-insertion CSF3R mutants. (**A**) Schematic representation showing conservation of juxtamembrane motif in CSF3R and related cytokine receptors gp130 and leptin receptor. TM: transmembrane domain (**B**) Schematic representation of CSF3R mutants used in this study. The SOCS3 binding site is located on position 729. (**C**) Phoenix E cells cotransfected with the indicated CSF3R constructs and Flag-Ub were incubated with bio-CSF3 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-CSF3R antibodies (lower panel). (**D**) 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. (**E**) Proliferation in 32D cells expressing the indicated CSF3R constructs. Numbers (± SE) of cells of three to four independent cell clones per constructs after 10 days of CSF3 treatment are given. *** p<0.001 relative to wt CSF3R.

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

Because the unique involvement of K632 in lysosomal routing of CSF3R 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 encompassing the juxtamembrane domain and box-1 region was fused to the C-terminus of mutant K5R (see also scheme in Figure 4b). We first tested whether the relocated K632 in K5R-D is still ubiquitinated. Indeed, ubiquitination of K5R-D was clearly observed in Flag-Ub expressing Phoenix E cells (Figure 2a). We then studied the functionality of the relocated motif using a STAT5 reporter assay (15). Whereas SOCS3 strongly inhibited STAT5 activation by wt and mKA CSF3R, 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 CSF3 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 CSF3 signaling (Figure 2c).

Fusion of Ub to the CSF3R C-terminus does not restore lysosomal sorting and signal downregulation

Fusion of Ub to either the C-terminus of a degradation-defective EGFR (33) or to an EGFR mutant lacking the whole cytoplasmic domain (18) was shown to be sufficient to restore ligand-dependent internalization and lysosomal sorting of these receptor chimeras. To further dissect a critical role of juxtamembrane positioning of Ub in lysosomal sorting of the CSF3R, we tested whether fusion of Ub directly to the C-terminus could restore lysosomal sorting of K5R. However, contrary to the Ub-tagged EGFR chimeras and wt CSF3R, K5R-Ub accumulated in EEA1-positive (Figure 3a) and Hrs-positive (suppl. Figure 1) early endosomes, as was seen with K5R (15), but did not route to Rab7 positive late endosomes/lysosomes (suppl. Figure 1). Accordingly, prolonged STAT5 phosphorylation in both K5R as well as K5R-Ub expressing Ba/F3 cells was observed after removal of CSF3 (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 CSF3R, further substantiating that site-specific ubiquitination of K632 is critical for lysosomal degradation of the CSF3R.

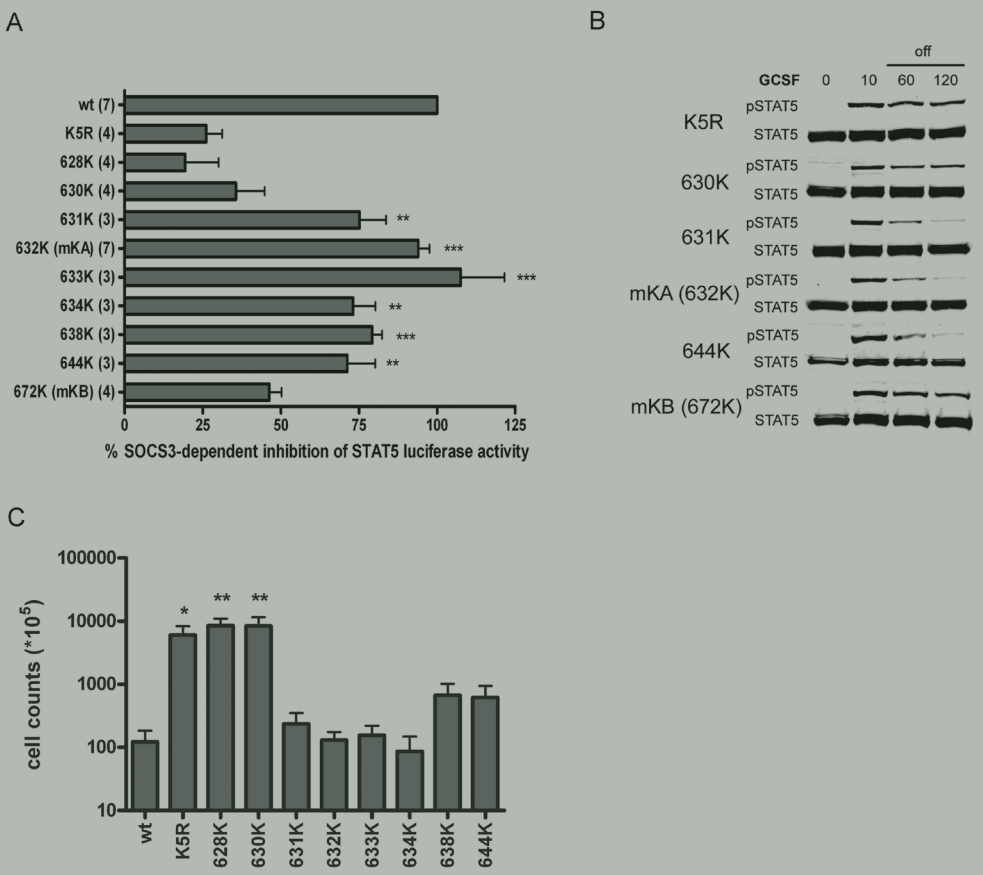


Figure 5: Position of a lysine within the juxtamembrane and box-1 region determines its role in CSF3R signal downregulation. (**A**) STAT5-responsive luciferase assays with or without SOCS3 were done as described in Figure 2c. Mean values ± SE of at least three independent experiments (the total number of experiments for each construct is shown in parenthesis) each done in triplicate are shown. ** p<0.01, *** p<0.001 relative to K5R; (**B**) STAT5 phosphory-lation patterns in Ba/F3 cells expressing indicated mutant CSF3R as described in detail in Figure 3c. (**C**) CSF3-induced proliferation in 32D cells expressing the indicated CSF3R constructs as described in detail in Figure 2d. * p<0.05, ** p<0.01 relative to wt CSF3R.

The reason for the discrepancy to the findings derived from the EGFR model systems is not known, but could relate to experimental differences. Whereas we fused Ub directly to the C-terminus of K5R, Ub was either fused to EGFR lacking the entire cytoplasmic domain (18), leading to its membrane-proximal positioning, or to the C-terminus of the receptor via a flexible linker (33). We therefore also generated a mutant in which the Ub moiety was fused to the C-terminus via a flexible linker. However, introduction of the linker was without any effect: the K5R-GGS(3x)-Ub mutant was retained in early endosomes and, despite very low membrane expression levels, displayed prolonged STAT5 phosphorylation as has been observed for K5R and K5R-Ub (suppl. Figure 2).

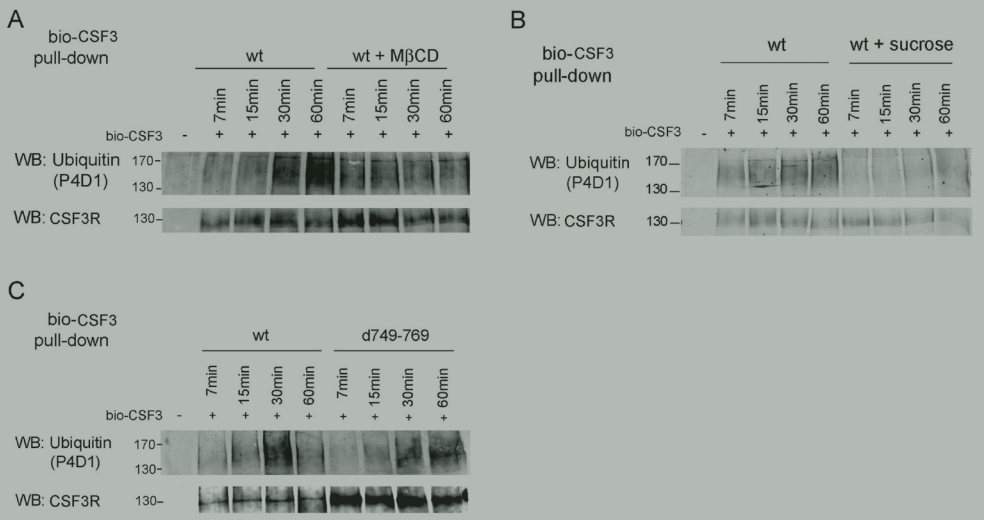


Figure 6: CSF3R ubiquitination is inhibited by methyl-β-cyclodextrin (mßCD) as well as hyperosmotic sucrose and a CSF3R mutant lacking the internalization motif (d749-69) is hampered and delayed in ubiquitination. (**A**) and (**B**) Ba/F3 cells stably expressing wt CSF3R were preincubated with bafilomycin ± mßCD (*A*) or ± sucrose (*B*) and stimulated with bio-CSF3 for the indicated time periods 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-CSF3R antibodies (lower panel). (**C**) Ba/F3 cells stably expressing the indicated CSF3R mutants were preincubated with bafilomycin, stimulated with bio-CSF3 and processed as described for 6a and 6b.

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 CSF3R of different species as well as in related cytokine receptor chains such as gp130 and leptin receptor (Figure 4a). To investigate whether the exact positioning of K632 is imperative 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 SOCS3 dependent inhibition of STAT5 luciferase activity in HEK cells stimulated with CSF3 (Figure 4d). In addition, 32D cells expressing these mutants showed proliferation characteristics comparable to cells expressing mKA (Figure 4e). 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 at position 729 is required for downregulation of CSF3R signaling.

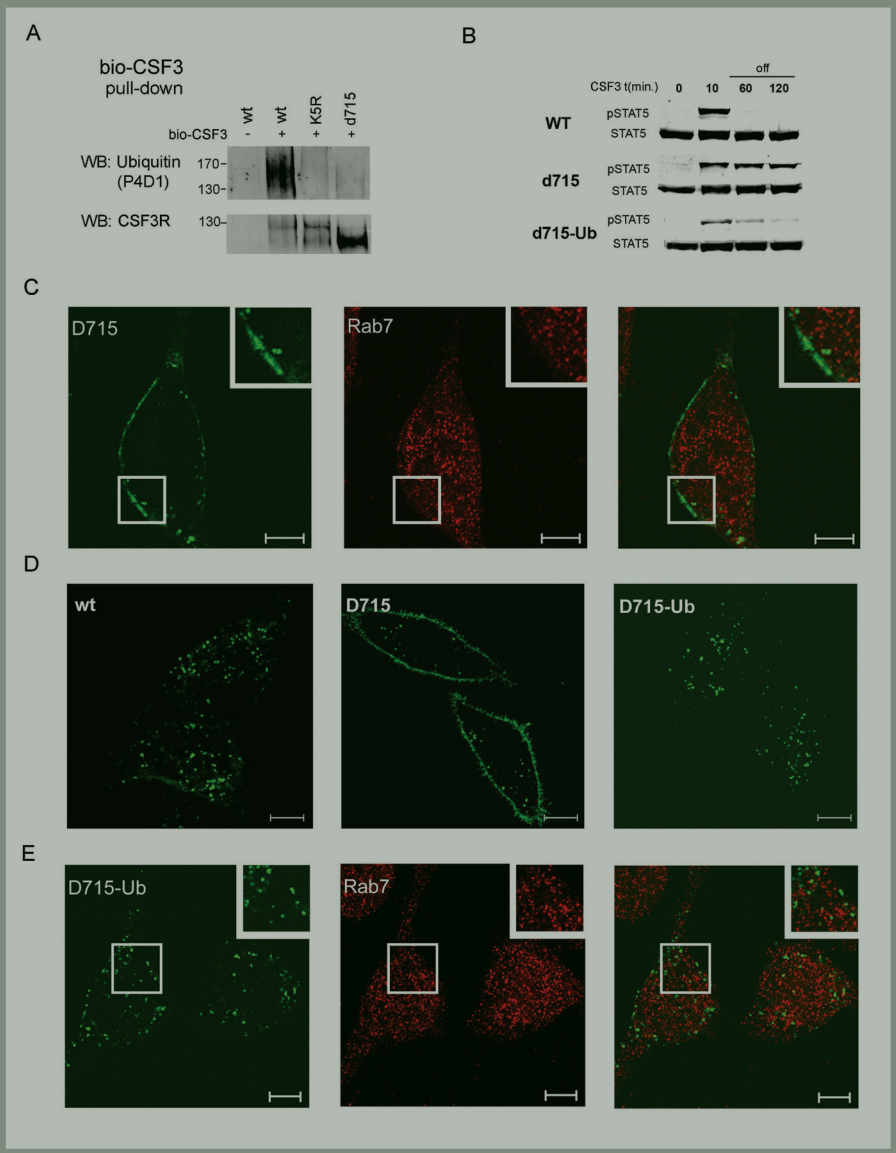


Figure 7: A disease-associated truncated CSF3R (d715) lacks ubiquitination and fusion of ubiquitin to the C-terminus of CSF3R_d715 restores internalization but not lysosomal sorting. (**A**) Ba/F3 cells stably expressing indicated CSF3R mutants were preincubated with bafilomycin and stimulated with bio-CSF3 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-CSF3R antibodies (lower panel). (**B**) STAT5 phosphorylation patterns in Ba/F3 cells expressing indicated CSF3R mutants as described in detail in Figure 3C. (**C**) HeLa cells ectopically expressing CSF3R_d715 mutants were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized CSF3R (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**) HeLa cells ectopically expressing wt, d715 or d715-Ub CSF3R were labeled with anti-CSF3R antibody prior to incubation with CSF3 for 30min. After washing cells were permeabilized, stained with a secondary goat anti-mouse Alexa Fluor 488 and analyzed by CLSM. (**E**) HeLa cells ectopically expressing CSF3R_d715-Ub were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized CSF3R (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.

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 CSF3 stimulation, suggesting that lysines at these positions are not accessible to SOCS3. Consequently, a lysine at position 628 or 630 failed to support SOCS3-mediated inhibition of CSF3-induced STAT5 activation (Figure 5a), affected late endosomal/ lysosomal routing (see suppl. Figure 3), and led to prolonged STAT5 activity upon CSF3 removal (Figure 5b). In addition, 32D cell clones expressing 628K or 630K exhibited hyperproliferation in response to CSF3 comparable to clones expressing K5R (Figure 5c). In contrast, lysines on position 631, 633, 634, 638 and 644 were ubiquitinated (Figure 4c) and these CSF3R mutants showed normal routing (Suppl. Figure 3), SOCS3 dependent downregulation of STAT5 luciferase activity and attenuation of STAT5 phosphorylation (Figure 5a and 5b). The proliferation kinetics of 32D cells expressing CSF3R 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 in response to CSF3 (Figure 5c). 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 across box-1 leads to a rapid decrease of function and a complete loss of function is observed when the lysine is placed within the box-2 region. The reason why there is a loss of function downstream of box-1 despite ubiquitination of the lysine is not clear. One possible explanation is that only a specifc spatial conformation of a membrane-proximal Ub(-chain) in relation to components of lipid membranes, such as phosphoinositides, allows interaction of the ubiquitinated CSF3R with certain effectors of the lysosomal sorting machinery. Several sorting proteins, like Hrs, epsins, and members of the ESCRT complexes contain binding sites for membrane phosphoinositides in addition to their Ub binding domains (34). 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 (28, 35, 36). It is therefore conceivable that interaction of the CSF3R with proteins involved in this sorting step (like EAP45) relies on ubiquitination of a properly positioned juxtamembrane lysine of the CSF3R.

CSF3R ubiquitination increases during endocytosis

Because ubiquitination of receptor lysines is not the key determinant for CSF3R internalization (15), we sought to determine when and in which intracellular compartment(s) ubiquitination of activated CSF3R takes place. In Ba/F3 cells ubiquitination of the wt CSF3R steadily increased over time and was maximal 30 to 60 min after receptor activation (Figure 6a). Because CSF3R endocytosis is completed within minutes (26), these data suggest that CSF3R ubiquitination mainly occurs at early endosomes. This is further substantiated by our finding that ubiquitination of wt CSF3R was significantly inhibited by reagents that impair clathrin-mediated endocytosis of the CSF3R, like methyl-β-cyclodextrin (mβCD) or hyperosmotic sucrose (Figure 6a and 6b). In addition, an internalization defective CSF3R mutant (d749-769), which lacks the critical dileucine-based endocytosis motif (26), showed a markedly delayed and reduced ubiquitination compared to wt CSF3R, despite much higher membrane expression levels (Figure 6c). These results suggest that ubiquitination of CSF3R 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 CSF3R-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) (4, 14, 37). The truncated receptors caused by these mutations, e.g. CSF3R-d715, lack both the internalization motif and the binding site for SOCS3 (Y729). As a consequence, CSF3R-d715 is hardly ubiquitinated (Figure 7a) and is not routed to late endosomes/lysosomes (Figure 7c), thus resulting in prolonged STAT5 phosphorylation after CSF3 washout (Figure 7b).

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 CSF3R (38). These results suggest that internalization of the activated CSF3R requires an intact ubiquitination machinery. This might be interpreted as contradictory to our results, showing that lysineless CSF3R 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 (39). We therefore wondered whether fusion of Ub to the C-terminus of CSF3R-d715, which lacks the STQPLL internalization motif, could restore its internalization. Indeed, attachment of a Ub moiety resulted in rapid endocytosis of CSF3R-d715, with dynamics comparable to wt CSF3R (Figure 7d). These data, in combination with the previous report (38), suggest a combined involvement of an internalization motif and the ubiquitination machinery in CSF3R endocytosis. Comparable results have been reported for leptin receptor (13), MHC I molecules (40) and CD33 (41). These studies commonly establish that Ub can act as an endocytotic signal independent of its positioning. On the other hand, despite its restored capability of endocytosis, mutant d715-Ub still failed

to enter Rab7 positive late endosomes/lysosomes (Figure 7e) and, although to a lesser extent, still displayed prolonged STAT5 signaling (Figure 7b) comparable to what we observed with K5R. 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 CSF3R, 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 CSF3R (position -5 to box-1) is found to be highly conserved in many cytokine receptors, like gp130, leptin receptor, 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 CSF3R, e.g. the EPOR at position -4 to box-1. The finding that the mutant CSF3R-d715 derived from neutropenia patients 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 CSF3R forms and to its predicted role in the leukemic evolution of the disease (42).

MATERIALS AND METHODS

Expression constructs

The constructs of human CSF3R wt, K5R, K632R, mKA, mKB, mKC, mKD, mKE, d715 and d749-769 in pBabe and pLNCX2 vector have been described previously (15, 26). Domain-fusion CSF3R constructs were generated by PCR using GRFR7 as a forward primer (26) and a reverse primer spanning the last 21 nucleotides of the CSF3R 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. CSF3R wt, K5R and K632R were used as templates. PCR fragments were then digested with *Hpa*I and *Bgl*II (26) and ligated in pBabe or pLNCX2 containing wt CSF3R, 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 CSF3R-Ub fusion construct, a first PCR was performed using GRFR7 as a forward primer (26) and a 3' chimeric oligonucleotide (CSF3R/Ub), spanning a 3' segment of the CSF3R and a 5' segment of Ub, as the reverse primer to amplify a C-terminal lysine-less CSF3R fragment using K5R as template. Secondly, to amplify the Ub cDNA, a 5'

chimeric oligonucleotide (Ub/CSF3R), spanning the 5' segment of Ub and the 3' segment of the CSF3R, 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 CSF3R, from which the wt *Hpa*I/*Bgl*II fragment has been removed. The CSF3R-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. The K5R-GGS(3x)-Ub construct was made as K5R-Ub with the exception that instead of primers Ub/CSF3R and CSF3R/Ub, primers Ub/GGS(3x)-CSF3R and CSF3R-GGS(3x)/Ub each containing the linker sequence were used. 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(43).

Antibodies

Flag M2 monoclonal antibody (mAb) was purchased from Sigma (St Louis, MO). mAb anti-human CD114 (CSF3R) and anti-CD114 coupled to phycoerythrin (PE) were from Becton-Dickinson/PharMingen (San Diego, CA). Mouse anti-ubiquitin (P4D1), goat anti-EEA1, rabbit anti-Rab7, mAb anti-HA and rabbit polyclonal anti-STAT5 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).The hrs antibody was a kind gift of H. Stenmark. Secondary goat anti-rabbit Alexa Fluor 546 (for Rab7, rabbit anti-goat Alexa Fluor 546 (for EEA1) 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% CO2. 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 CSF3R expression(44), 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% CO2. 32D.cl8.6 clones and Ba/F3 cells expressing different CSF3R constructs were obtained by retroviral

transduction as described(26). Levels of CSF3R expression were assessed by FACS analysis and clones with comparable CSF3R expression were used in further experiments.

Bio-CSF3 pull downs

CSF3R pull-down was performed with biotinylated CSF3 (bio-CSF3) as described previously(15). Briefly, after bio-CSF3 transfection with CSF3R constructs and Flag-tagged Ub, Phoenix E cells were incubated with (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_3VO4 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(45). 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-CSF3 pull-down experiments with Ba/F3 cells stably expressing indicated CSF3R mutants, cells (1 x 10^7) were washed in HBSS and resuspended in RPMI containing 250 nM bafilomycin (Calbiochem, Invitrogen). In experiments determining the role of endocytosis in CSF3R ubiquitination, methyl-β-cyclodextrin (5 mM) or sucrose (0,45M, both Sigma-Aldrich, Zwijndrecht. The Netherlands) was added concomitantly with bafilomycin. After preincubation for 30 min at 37°C, bio-CSF3 was added for 1 hr. Subsequent procedures were done as described for Phoenix E cells.

Cell proliferation assay and CSF3-induced STAT5 phosphorylation

To investigate the proliferation characteristics of 32D8.6cl clones transduced with various CSF3 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 CSF3. Viable cells were counted and readjusted to 1 x 10^5 cells/ml every second day.

To determine STAT5 phosphorylation off-rate kinetics after CSF3R stimulation, Ba/F3 cells stably expressing the indicated CSF3R mutants were washed twice in HBSS and serum and cytokine starved for 4 hours in RPMI-1640. After stimulation for 10 min with CSF3 (100 ng/ml), cells were washed twice with HBSS to remove CSF3 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(7). Briefly, HEK293 cells were transfected by calcium phosphate precipitation with the indicated CSF3R constructs, with or without a SOCS3 construct (CSF3R 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 CSF3 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 CSF3R after receptor activation was done using confocal laser scanning microscopy (CLSM) as described(15). In brief, Hela cells transiently expressing indicated CSF3R constructs were incubated with CSF3R antibody and CSF3 for 15min at RT, washed twice and incubated at 37° C for 1 hr, if not otherwise stated. Subsequently, cells were permeabilized with saponin, fixed with 3% paraformaldehyde and stained for internalized receptors (DaM-488) and EEA1 (RaG-546) or Rab7 (GaR-546) as a marker for early or late endosomes, respectively (46).

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.

ACKNOWLEDGMENTS

This work was supported by the Dutch Cancer Society (www.KWFkankerbestrijding.nl). A.W. was supported by the Austrian Science Foun-dation (FWF) through an Erwin-Schrödinger fellowship (J2536-B05). We thank Marieke von Lindern for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Akbarzadeh S, Layton JE. Granulocyte colony-stimulating factor receptor: structure and function. Vitam Horm 2001;63:159-194.
2. Avalos BR, Gasson JC, Hedvat C, Quan SG, Baldwin GC, Weisbart RH, Williams RE, Golde DW, DiPersio JF. Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. Blood 1990;75(4):851-857.
3. Touw IP, van de Geijn GJ. Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. Front Biosci 2007;12:800-815.
4. Germeshausen M, Ballmaier M, Welte K. Incidence of CSF3R mutations in severe congenital neutropenia and relevance for leukemogenesis: Results of a long-term survey. Blood 2007;109(1):93-99.
5. Hunter MG, Avalos BR. Deletion of a critical internalization domain in the G-CSFR in acute myelogenous leukemia preceded by severe congenital neutropenia. Blood 1999;93(2):440-446.
6. Ward AC, van Aesch YM, Schelen AM, Touw IP. Defective internalization and sustained activation of truncated granulocyte colony-stimulating factor receptor found in severe congenital neutropenia/acute myeloid leukemia. Blood 1999;93(2):447-458.
7. Hermans MH, van de Geijn GJ, Antonissen C, Gits J, van Leeuwen D, Ward AC, Touw IP. Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells. Blood 2003;101(7):2584-2590.
8. Hortner M, Nielsch U, Mayr LM, Johnston JA, Heinrich PC, Haan S. Suppressor of cytokine signaling-3 is recruited to the activated granulocyte-colony stimulating factor receptor and modulates its signal transduction. J Immunol 2002;169(3):1219-1227.
9. Mukhopadhyay D, Riezman H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 2007;315(5809):201-205.
10. Marmor MD, Yarden Y. Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene 2004;23(11):2057-2070.
11. Huang F, Kirkpatrick D, Jiang X, Gygi S, Sorkin A. Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Mol Cell 2006;21(6):737-748.
12. Huang F, Goh LK, Sorkin A. EGF receptor ubiquitination is not necessary for its internalization. Proc Natl Acad Sci U S A 2007;104(43):16904-16909.
13. Belouzard S, Rouille Y. Ubiquitylation of leptin receptor OB-Ra regulates its clathrin-mediated endocytosis. Embo J 2006;25(5):932-942.
14. Kumar KG, Barriere H, Carbone CJ, Liu J, Swaminathan G, Xu P, Li Y, Baker DP, Peng J, Lukacs GL, Fuchs SY. Site-specific ubiquitination exposes a linear motif to promote interferon-alpha receptor endocytosis. J Cell Biol 2007;179(5):935-950.
15. Irandoust MI, Aarts LH, Roovers O, Gits J, Erkeland SJ, Touw IP. Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor. Embo J 2007;26(7):1782-1793.
16. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 2007;7(6):454-465.

17. Kamura T, Maenaka K, Kotoshiba S, Matsumoto M, Kohda D, Conaway RC, Conaway JW, Nakayama KI. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. Genes Dev 2004;18(24):3055-3065.
18. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol 2003;5(5):461-466.
19. Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu Rev Cell Dev Biol 2003;19:141-172.
20. Raiborg C, Rusten TE, Stenmark H. Protein sorting into multivesicular endosomes. Curr Opin Cell Biol 2003;15(4):446-455.
21. Sigismund S, Polo S, Di Fiore PP. Signaling through monoubiquitination. Curr Top Microbiol Immunol 2004;286:149-185.
22. Hunter T. The age of crosstalk: phosphorylation, ubiquitination, and beyond. Mol Cell 2007;28(5):730-738.
23. Laney JD, Hochstrasser M. Substrate targeting in the ubiquitin system. Cell 1999;97(4):427-430.
24. Fuchs SY, Spiegelman VS, Kumar KG. The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene 2004;23(11):2028-2036.
25. Kumar KG, Tang W, Ravindranath AK, Clark WA, Croze E, Fuchs SY. SCF(HOS) ubiquitin ligase mediates the ligand-induced down-regulation of the interferon-alpha receptor. Embo J 2003;22(20):5480-5490.
26. Aarts LH, Roovers O, Ward AC, Touw IP. Receptor activation and 2 distinct COOH-terminal motifs control G-CSF receptor distribution and internalization kinetics. Blood 2004;103(2):571-579.
27. Meyer L, Deau B, Forejtnikova H, Dumenil D, Margottin-Goguet F, Lacombe C, Mayeux P, Verdier F. beta-Trcp mediates ubiquitination and degradation of the erythropoietin receptor and controls cell proliferation. Blood 2007;109(12):5215-5222.
28. Alam SL, Langelier C, Whitby FG, Koirala S, Robinson H, Hill CP, Sundquist WI. Structural basis for ubiquitin recognition by the human ESCRT-II EAP45 GLUE domain. Nat Struct Mol Biol 2006;13(11):1029-1030.
29. Tanaka Y, Hamano S, Gotoh K, Murata Y, Kunisaki Y, Nishikimi A, Takii R, Kawaguchi M, Inayoshi A, Masuko S, Himeno K, Sasazuki T, Fukui Y. T helper type 2 differentiation and intracellular trafficking of the interleukin 4 receptor-alpha subunit controlled by the Rac activator Dock2. Nat Immunol 2007;8(10):1067-1075.
30. Traub LM, Lukacs GL. Decoding ubiquitin sorting signals for clathrin-dependent endocytosis by CLASPs. J Cell Sci 2007;120(Pt 4):543-553.
31. Umebayashi K, Stenmark H, Yoshimori T. Ubc4/5 and c-Cbl Continue to Ubiquitinate EGF Receptor after Internalization to Facilitate Polyubiquitination and Degradation. Mol Biol Cell 2008.
32. King RW, Deshaies RJ, Peters JM, Kirschner MW. How proteolysis drives the cell cycle. Science 1996;274(5293):1652-1659.
33. Mosesson Y, Shtiegman K, Katz M, Zwang Y, Vereb G, Szollosi J, Yarden Y. Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem 2003;278(24):21323-21326.
34. Slagsvold T, Pattni K, Malerod L, Stenmark H. Endosomal and non-endosomal functions of ESCRT proteins. Trends Cell Biol 2006;16(6):317-326.

35. Slagsvold T, Aasland R, Hirano S, Bache KG, Raiborg C, Trambaiolo D, Wakatsuki S, Stenmark H. Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. J Biol Chem 2005;280(20):19600-19606.

36. Teo H, Gill DJ, Sun J, Perisic O, Veprintsev DB, Vallis Y, Emr SD, Williams RL. ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. Cell 2006;125(1):99-111.

37. Dong F, Brynes RK, Tidow N, Welte K, Lowenberg B, Touw IP. Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. N Engl J Med 1995;333(8):487-493.

38. Kindwall-Keller TL, Druhan LJ, Ai J, Hunter MG, Massullo P, Loveland M, Avalos BR. Role of the proteasome in modulating native G-CSFR expression. Cytokine 2008;43(2):114-123.

39. Govers R, ten Broeke T, van Kerkhof P, Schwartz AL, Strous GJ. Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor. Embo J 1999;18(1):28-36.

40. Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, Lehner PJ. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. Embo J 2006;25(8):1635-1645.

41. Walter RB, Hausermann P, Raden BW, Teckchandani AM, Kamikura DM, Bernstein ID, Cooper JA. Phosphorylated ITIMs enable ubiquitylation of an inhibitory cell surface receptor. Traffic 2008;9(2):267-279.

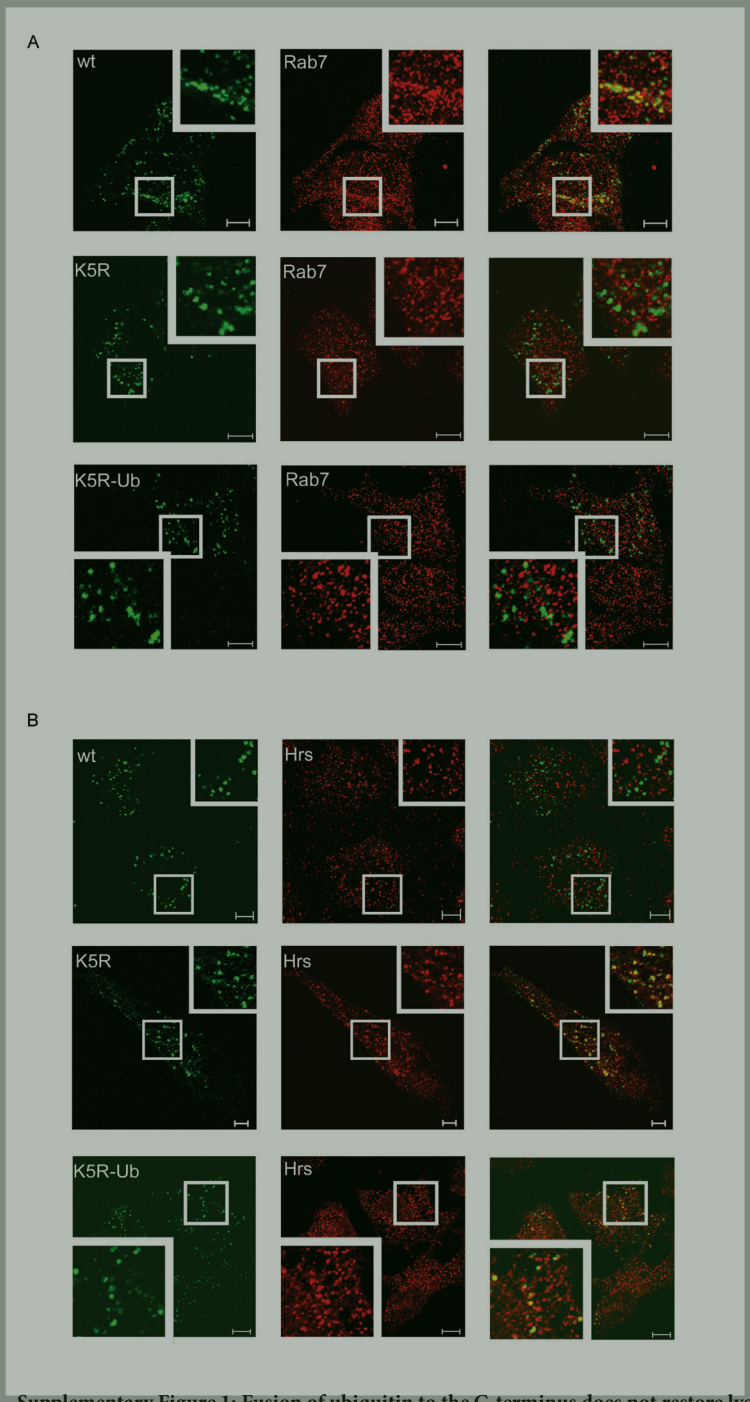
42. Liu F, Kunter G, Krem MM, Eades WC, Cain JA, Tomasson MH, Hennighausen L, Link DC. Csf3r mutations in mice confer a strong clonal HSC advantage via activation of Stat5. J Clin Invest 2008;118(3):946-955.

43. van de Geijn GJ, Gits J, Aarts LH, Heijmans-Antonissen C, Touw IP. G-CSF receptor truncations found in SCN/AML relieve SOCS3-controlled inhibition of STAT5 but leave suppression of STAT3 intact. Blood 2004;104(3):667-674.

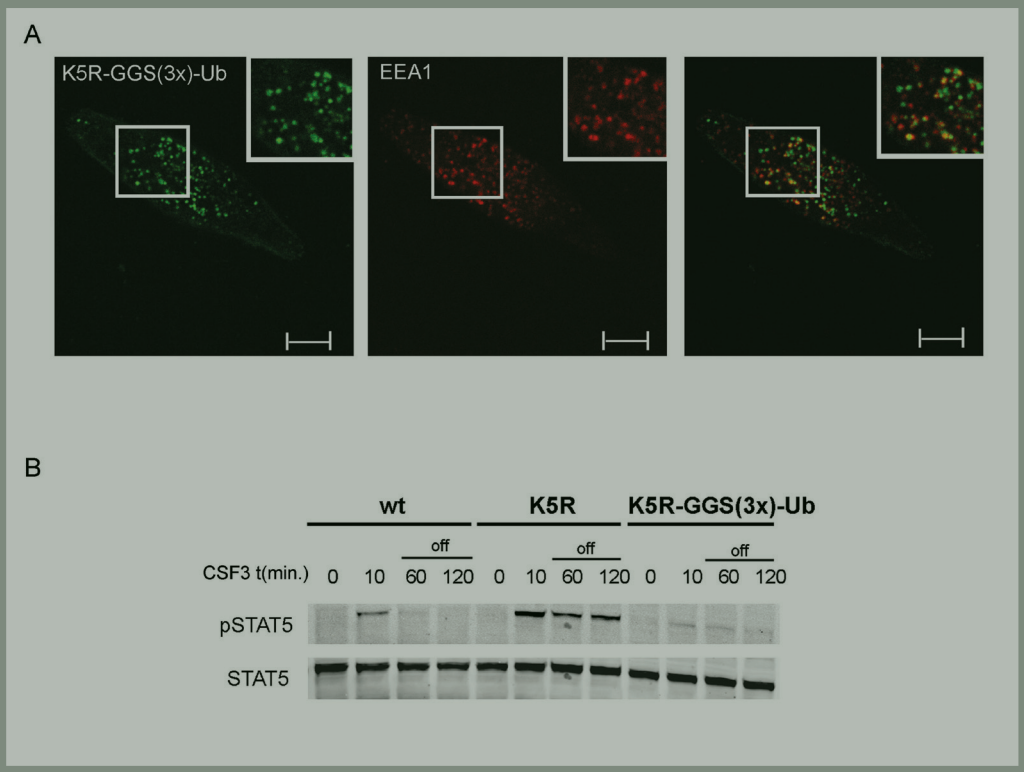
44. de Koning JP, Soede-Bobok AA, Schelen AM, Smith L, van Leeuwen D, Santini V, Burgering BM, Bos JL, Lowenberg B, Touw IP. Proliferation signaling and activation of Shc, p21Ras, and Myc via tyrosine 764 of human granulocyte colony-stimulating factor receptor. Blood 1998;91(6):1924-1933.

45. Hermans MH, Antonissen C, Ward AC, Mayen AE, Ploemacher RE, Touw IP. Sustained receptor activation and hyperproliferation in response to granulocyte colony-stimulating factor (G-CSF) in mice with a severe congenital neutropenia/acute myeloid leukemia-derived mutation in the G-CSF receptor gene. J Exp Med 1999;189(4):683-692.

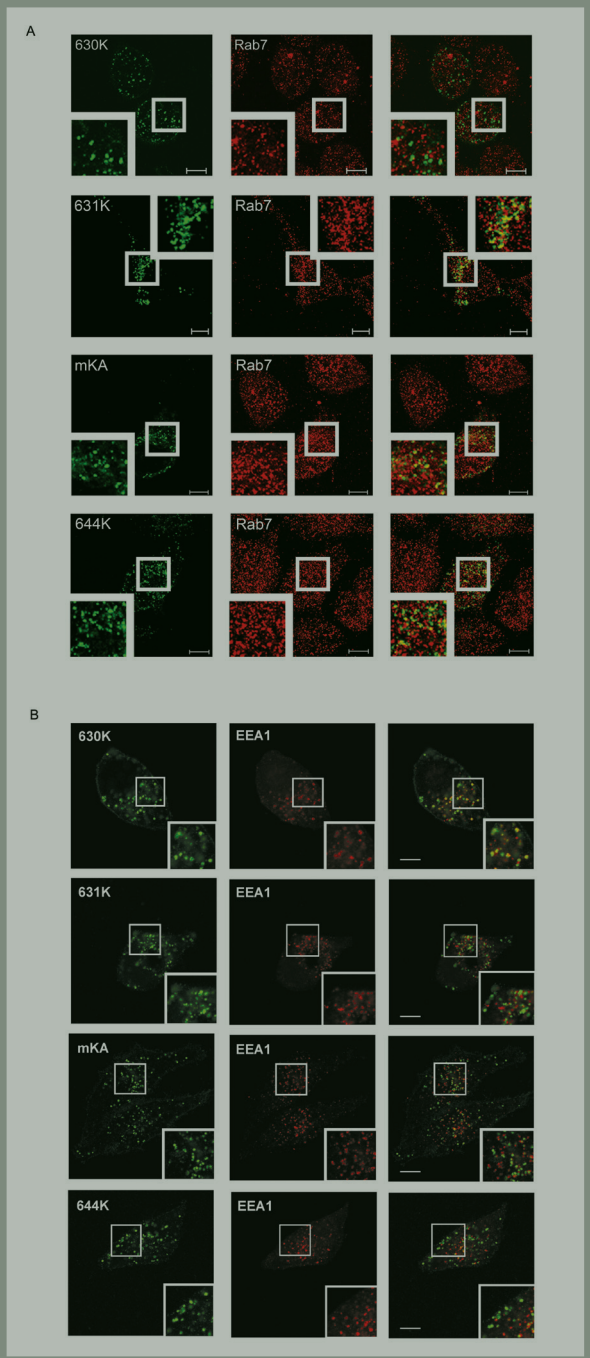
46. Raiborg C, Bache KG, Gillooly DJ, Madshus IH, Stang E, Stenmark H. Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat Cell Biol 2002;4(5):394-398.



Supplementary Figure 1: Fusion of ubiquitin to the C-terminus does not restore lysosomal sorting
HeLa cells ectopically expressing WT, K5R or K5R-Ub CSF3R were incubated with anti-CSF3R antibodies and CSF3 for 20 min at 16°C, washed and transferred to 37°C for 60 min. Subsequently, cells were fixed and stained for internalized CSF3R (green, left panels) and the late endosomal/ lysosomal marker rab7 **(A)** or the early endosomal marker Hrs **(B)** (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels indicating colocalization in yellow. Inserts show enlargements of boxed areas.



Supplementary Figure 2: Introduction of a linker between ubiquitin and the C-terminus of K5R does not restore lysosomal sorting and downregulation STAT5 activation. (A) HeLa cells ectopically expressing K5R-GGS(3x)-Ub CSF3R were incubated with anti-CSF3R antibodies and CSF3 for 20 min at 16°C, washed and transferred to 37°C for 90 min. Subsequently, cells were fixed and stained for internalized CSF3R (green, left panels) and the early endosomal marker EEA1 (red, middle panels) and analyzed by CLSM. Merged pictures are shown in the right panels indicating colocalization in yellow. Inserts show enlargements of boxed areas. **(B)** Ba/F3 cells expressing indicated CSF3R mutants were processed as described (Figure 3b) and STAT5 phosphorylation was monitored. Expression of K5R-GGS(3x)-Ub CSF3R on the cell surface was very low resulting in decreased STAT5 phosphorylation after ten minutes of CSF3 stimulation. However, despite low initial phosphorylation levels, STAT5 phosphorylation was prolonged with K5R-GGS(3x)-Ub CSF3R.



Supplementary Figure 3: Lysosomal routing of selected 'walking' lysine CSF3R mutants. HeLa cells ectopically expressing indicated CSF3R mutants were processed as described (Figure 3a) and analyzed by CLSM after staining for internalized CSF3R (green, left panels) and the late endosomal/lysosomal marker rab7 **(A)** or the early endosome marker EEA1 **(B)** (red, middle panels). Merged pictures are shown in the right panels indicating colocalization in yellow. Inserts show enlargements of boxed areas.

CHAPTER 4

THE DEUBIQUITINATING ENZYME DUB2A ENHANCES CSF3 SIGNALLING BY ATTENUATING LYSOSOMAL ROUTING OF CSF3 RECEPTOR

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Biochem. J. (2011) 434, 343–351

ABSTRACT

Ubiquitination of the colony-stimulating factor 3 receptor (CSF3R) occurs after activated CSF3Rs are internalized and reside in early endosomes. CSF3R ubiquitination is crucial for lysosomal routing and degradation. The E3 ligase suppressor of cytokine signalling 3 (SOCS3) has been shown to play a major role in this process. Deubiquitinating enzymes remove ubiquitin moieties from target proteins by proteolytic cleavage. Two of these enzymes, AMSH and UBPY, interact with the general endosomal sorting machinery. Whether deubiquitinating enzymes control CSF3R trafficking from early towards late endosomes is unknown. In the present study, we asked whether AMSH, UBPY or a murine family of deubiquitinating enzymes could fulfil such a role. This DUB family comprises four members (DUB1, DUB1A, DUB2, and DUB2A), which were originally described as being hematopoietic-specific and cytokine inducible, but their function in cytokine receptor routing and signalling has remained largely unknown. Here, we show that DUB2A expression is induced by CSF3 in myeloid 32D cells and that DUB2 decreases ubiquitination and lysosomal degradation of the CSF3R, leading to prolonged signalling. These data support a model in which CSF3R receptor ubiquitination is dynamically controlled at the early endosome by feedback mechanisms involving CSF3-induced E3 ligase (SOCS3) and deubiquitinase (DUB2A) activities.

INTRODUCTION

To control the duration of signalling, many activated growth-factor receptors are subject to ligand-induced endocytosis and intracellular routing to early endosomes and lysosomes, where the receptor proteins are being degraded [1]. Ubiquitination is the major protein modification involved in this process [1-2]. Colony-stimulating factor 3 (CSF3) stimulates myeloid cell proliferation and differentiation [3]. CSF3 activates the latent transcription factor signal transducer and activator of transcription 3 (STAT3), which induces the expression of *SOCS3* encoding the suppressor of cytokine signalling 3 (SOCS3) protein [4-5]. SOCS3 is recruited to a phosphorylated tyrosine (Y729) motif in the activated CSF3 receptor (CSF3R) [4] and forms an E3 ligase complex via its SOCS box [6]. It was recently shown that SOCS3, by ubiquitination of a juxtamembrane lysine in the CSF3R cytoplasmic domain, promotes CSF3R routing from early to late endosomes, leading to signal termination [7].

Deubiquitinating enzymes remove ubiquitin (Ub) from their target substrates and are therefore potentially important in controlling receptor routing and signalling. Two endosomal deubiquitinating enzymes associated with routing of activated receptors are signal transducing adaptor molecule (STAM) binding protein (STAMBP), also known as associated molecule with the SH3 domain of STAM (AMSH), a member of the JAB1/MPN/mov34-domain-containing (JAMM) family and ubiquitin specific peptidase 8 (USP8) which is also known as ubiquitin isopeptidase Y (UBPY) a member of the ubiquitin-specific proteases (USP) family of deubiquitinating enzymes [8-9]. Both bind to the endosomal sorting complex required for transport (ESCRT)-0 subunit STAM1 via their SH3 domain and to ESCRT-III through their microtubule interacting and transport (MIT) domains [10-13]. AMSH is involved in endosomal trafficking of tyrosine kinase receptors such as the epidermal growth-factor receptor (EGFR) and G-protein coupled receptors like protease activated receptor 2 (PAR_2), δ-opioid peptide receptor (DOR) and CXCR4 [8, 14-17]. UBPY has been shown to be important in stabilizing the ESCRT-0 complex [18-20] and is essential for deubiquitination and degradation of EGFR, PAR_2 and DOR [14-15, 21]. UBPY has been proposed to maintain the pool of free ubiquitin Ub by releasing it from the cargo just prior to lysosomal degradation [22].

The murine DUB family comprises 4 highly homologous USP proteins (DUB1, DUB1A, DUB2 and DUB2A) that were initially identified in hematopoietic cells [23-25]. Their sequence similarity and location on chromosome 7 suggest that they have arisen from a tandem duplication event [24-25]. All four DUBs are rapidly and transiently induced in response to various cytokines [23-26]. For instance, DUB1 expression is induced by interleukin (IL)3, IL5 and CSF2, cytokines that activate receptors with a β common subunit, resulting in a G1 phase cell cycle arrest [25, 27]. For IL3-induced upregulation of DUB1, a critical role for JAK2 and the Ras/Raf/MAPK pathway was shown [28]. However, these pathways appeared necessary but not sufficient, because JAK2 and MAPK activation by the erythropoietin recep-

tor (EPOR) did not result in increased *DUB1* expression [28-29]. DUB1A is IL3 inducible in B-lymphocytes, whereas DUB2 is induced by IL2 in T-cells [26]. DUB2 inhibits apoptosis after IL2 withdrawal and prolongs STAT5 phosphorylation in Ba/F3 cells expressing IL2Rb [24, 30]. The *DUB2A* enhancer element contains one Ets and two AP-1 binding sites that are required for *DUB2A* expression in T-lymphocytes [31]. Finally, DUB1 controls ubiquitination of the dynein heavy chain, which is part of the complex that regulates the movement of cargo along microtubules [32]. Together, these findings suggest that DUBs may modulate a variety of processes at distinct intracellular locations, including endosomal cargo trafficking and processing.

The first human family member found to be homologous to the murine DUB family by data base analysis was DUB3. DUB3 was subsequently shown to be IL4 and IL6 inducible and constitutive expression can block cell proliferation [33]. The official name of DUB3 is now USP17L2. The human USP17 family is reported to be encoded within RS447, a megasatellite repeat, on human chromosome 4 and on chromosome 8 embedded within the copy number variable beta-defensin cluster [34-35]. The multiple family members in different species have evolved as the result of duplication of common ancestral sequence, which is in general replicated after the species diverged [35-36].

Previously, we have shown that CSF3R ubiquitination, predominantly taking place when the receptor resides in early endosomes, triggers its routing towards late endosomes, followed by lysosomal degradation [7]. In view of this negative feedback mechanism involving CSF3-induced *SOCS3* expression followed by lysosomal degradation, we asked whether deubiquitinating enzymes acting on CSF3R at the early endosome may antagonize this inhibitory feedback loop. Here, we show that CSF3 induces *DUB2A* expression in myeloid progenitor cells. Subsequently, DUB2A reduces CSF3R ubiquitination and causes the accumulation of CSF3R in early endosomes, leading to prolonged signalling. These results support a model in which DUB2A acts in a positive feedback loop to counterbalance STAT3-SOCS3-driven lysosomal targeting of the CSF3R.

EXPERIMENTAL

Cell culture

293T, Phoenix E and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Gibco DMEM, Invitrogen, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO2. HeLa cells were plated on glass cover slips and transiently transfected using lipofectamine (Invitrogen, Breda, The Netherlands). Transfection of 293T and Phoenix E cells was done using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA).

Murine myeloid 32D.cl8.6 cells [37] and Ba/F3β/tTA DUB2 [30] were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml murine IL3 at 37°C and 5% CO2. Ba/F3β/tTA DUB2 cells were cultured in presence of 1 μg/ml doxycycline (Dox, Clontech Laboratories, Mountain View, CA, USA). Dox was replenished every 48 hrs. 32D cells and Ba/F3β/tTA DUB2 cells expressing wt CSF3R were obtained by retroviral transduction as previously described [38]. Ba/F3β/tTA DUB2 cells expressing wt CSF3R were selected by sorting for CSF3R positive cells using a FACSAria flow cytometer (Becton Dickinson, San Diego, CA, USA).

Antibodies

Mouse anti-human CSF3R and mouse anti-human CSF3R coupled to phycoerythrin (PE) were purchased from Becton-Dickinson/PharMingen (San Diego, CA, USA); Rabbit anti-FLAG and goat anti-FLAG from Abcam, (Cambridge, UK); Mouse anti-GFP from Roche Applied Sciences (Indianapolis, IN, USA); Goat anti-ACTIN and goat anti-EEA-1 from Santa Cruz Biotechnology inc. (Santa Cruz, CA, USA). Rabbit anti-DUB2 was a gift from James Johnston (University of Belfast) [30]. Secondary goat anti-mouse and goat anti-rabbit either coupled to IRDye680 or IRDye800CW were obtained from LI-COR Biosciences (Lincoln, NE, USA); Donkey anti-goat IRDye800 from Rockland Immunochemicals (Gilbertsville, PA, USA); Donkey anti-goat 488 (DaG-488) and rabbit anti-goat 546 (RaG-546) from Molecular Probes (Invitrogen, Breda, The Netherlands); Donkey anti-mouse Cy5 (DaM-cy5) and donkey anti-rabbit cy3 (DaR-cy3) from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Expression constructs

pBABE expression constructs of wt, mKA and K5R CSF3R, tyrosine substitution mutants and the pCDNA3 construct with Flag tagged ubiquitin (Flag-Ub) have all been described previously [7, 39-40]. pME18S-FLAG-DUB2 was a gift from James Johnston (University of Belfast) [30]. pEGFP-C1-AMSH and pEGFP-C1-UBPY were obtained from Sylvie Urbé (University of Liverpool) and have been previously described [8, 18].

RNA isolation, DNAse treatment and Quantitative Real Time PCR

Cells were washed 3 times with Hanks' balanced salt solution (HBSS) and deprived of serum and factors in RPMI at a concentration of 1.5 million cells/ml for 4 hrs, followed by stimulation with 100 ng/ml CSF3 for the indicated time points. For the inhibitor study, 10 μM LY294002, 10 μM JAK inhibitor I, 10 μM SB203580, 10 μM U0126, 0.5 μM Akt inhibitor IV, 10 μM JNK VIII or 10 μM PP2 (Calbiochem, Gibbstown, NJ, USA ; all dissolved in DMSO), or DMSO as solvent control was added to the medium 30 min prior to the CSF3 stimulation. Cells were harvested, resuspended in TRIzol® (Invitrogen, Breda, The Nether-

lands) and RNA was isolated according to manufacturer's instructions. Removal of genomic DNA, synthesis of cDNA followed by qPCR using Taqman technology (7900HT, PE Applied Biosystems, Foster City, CA, USA) was performed as described before [41]. Primers used for amplification were: mDUB fw222-5'TCTAGCTGACTACATGCTGTC and mDUB rv385-5'GCTTGTGGAAGGCAGAGGTCA, mAMSH-fw1-exon7 5'CCACACGGAGAATGAAGAAG and mAMSH-rv1-exon8 5'TGCGATGGACTCTGGTAAC, mUBPY-fw-2545 5'AAGGTGAAGTGGCAGAAGAA and mUBPY-rv-2713 5'CCATCCATGAGGAACAGAAG.

For *Socs3* and the ribonuclease inhibitor (RI), previously published primers were used [41]. To quantify the relative expression of *Dub, Amsh, Ubpy* and *Socs3*, the Ct values were normalized for endogenous reference (CtSample=CtGene of interest–CtRNase inhibitor) and compared with a calibrator using the ΔΔCt method (ΔΔCtsample=CtSample–CtCalibrator). Expression levels in 32D cells deprived of growth factors for 4 hrs were used as calibrators.

Bio-CSF3 pull downs and CSF3 induced STAT5 phosphorylation

CSF3R pull down with biotinylated CSF3 (Bio-CSF3) and subsequent SDS-polyacrylamide gel electrophoresis and Western blotting was performed as described [42-43]. Detection of proteins was done using fluorescently labeled secondary antibodies and the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Quantification was done using the Odyssey Infrared Imaging System software according to the manufacturer's instructions. For off-rates, Ba/F3*β*/tTA DUB2 cells stably expressing wt CSF3R were grown in the presence or absence of Dox for 60 hrs. Subsequently cells were cytokine and serum deprived followed by stimulation with CSF3 for 10 min CSF3 was then washed away and cells were incubated at 37°C in RPMI for indicated time points.

Confocal microscopy

Lysosomal routing of activated membrane CSF3Rs was visualized by confocal laser scanning microscopy (CLSM) using a Leica SP5 instrument as described [7]. In brief, HeLa cells stably expressing wt CSF3R constructs were transfected with different DUB constructs. After 48 hrs, cells were incubated with CSF3R antibody and CSF3 for 10 min at RT, washed twice and incubated at 37°C for 2 hrs. Subsequently, cells were permeabilized with saponin, fixed with 3% paraformaldehyde and stained for internalized receptors (DaM-cy5), early endosome marker EEA1 (DaG-488 or RaG-546) in combination with or without staining for DUB2 (DaR-cy3).

RESULTS

CSF3 induces *Dub2A* expression

To determine if expression of DUB family members is induced by CSF3, we used myeloid 32D cells expressing human CSF3R. Cells were deprived of serum and IL3 for 4 hrs, followed by CSF3 stimulation for the indicated time points. *Dub* mRNA expression increased, with maximal levels at 60 min of CSF3 stimulation (Figure 1A). Nucleotide sequencing identified *Dub2A* as the single member being induced by CSF3. Because DUB2A is highly homologous to DUB2 (97% identical amino acid sequence), DUB2A was readily detected using a DUB2 antibody, 1 to 3 hrs after initiation of CSF3 stimulation (Figure1C). In contrast, *Ubpy* and *Amsh* expression was not induced by CSF3 (Figure 1A). *Socs3* transcript levels, which were assessed in parallel, were maximal at 30 min of CSF3 stimulation (Figure 1B).

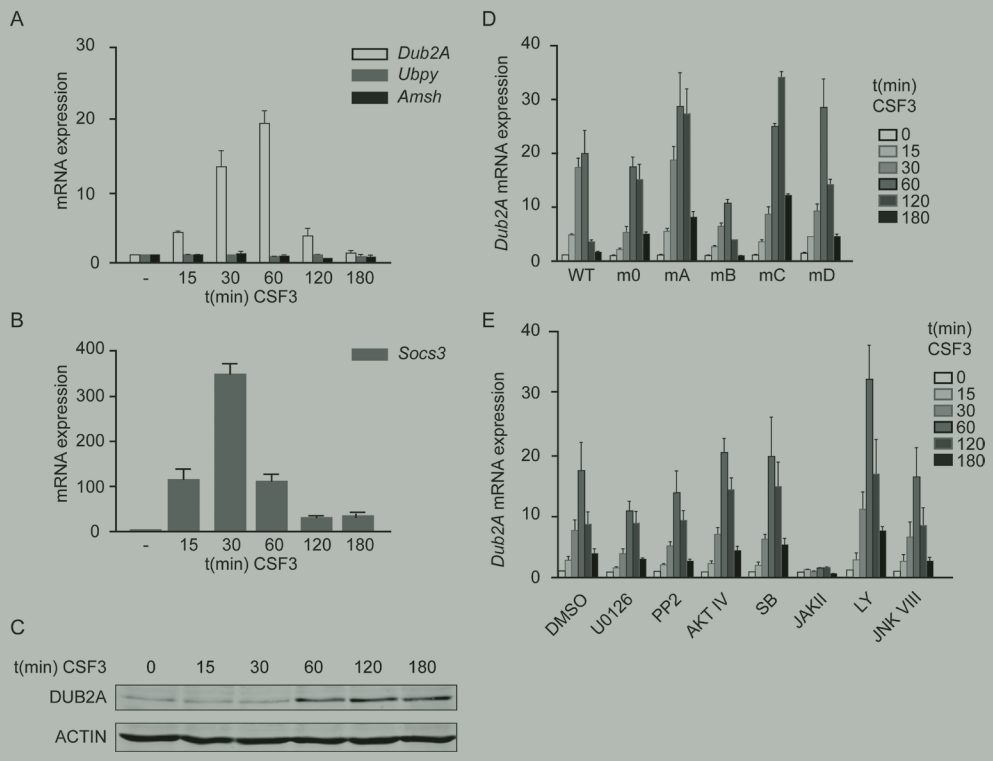

Figure 1: Myeloid 32D cells stably expressing wt CSF3R or receptor tyrosine mutants transiently induce *Dub2A* mRNA after CSF3 stimulation. 32D cells were deprived of growth-factors for 4 hrs (-) and stimulated with CSF3 for the indicated time points. (**A**) *Dub2A, Amsh* and *Ubpy* mRNA expression levels in wt CSF3R expressing cells were determined using RT-PCR and normalized using the ribonuclease inhibitor (RI). *Dub* levels are shown relative to the growth-factor deprived cells. Data represent mean values of three independent experiments ± standard deviation (SD). (**B**) *Socs3* mRNA levels in wt CSF3R were determined as described in A. (**C**) DUB2A protein detected using DUB2 antibody on Western blot of total cell lysates of the 32D cells expressing wt CSF3R stimulated with CSF3 for the indicated time points. (**D**) *Dub2A* mRNA levels in wt, tyrosine null (m0) and single tyrosine add-back CSF3Rs (mA-mD) were determined as in A. *Dub2A* levels are shown relative to growth-factor deprived cells. (**E**) *Dub2A* induction was assessed in the presence of various kinase inhibitors (see text for details) as described in A. Data represent the mean of three independent experiments ± SD.

***Dub2A* induction requires JAK activity**

Previous studies have shown that activation of JAK2 and the Ras/Raf/MAPK pathway is important for IL3 dependent *Dub1* induction [28]. To assess which signalling pathways are vital for CSF3-induced *Dub2A* expression, we first focused on the signalling mechanisms coupled to the CSF3R tyrosines by using a tyrosine (Y) less (m0) and single tyrosine add-back CSF3R mutants (mA, mB, mC and mD) containing respectively Y704, Y729, Y744 and Y764 (Figure 1D). The presence of STAT3 docking sites Y704 or Y744 (mA and mC) resulted in an approximately 1.5 times higher and a prolonged induction of *Dub2A* relative to m0. The presence of SOCS3 recruitment site Y729 (mB) decreased the induction of *Dub2A* expression approximately 2-fold compared to m0. The presence of Y764 (mD), involved in the activation of the Ras/Raf/Erk pathway [37], resulted in only a moderate (1.5-fold) increase of *Dub2A* expression, suggesting that, in contrast to IL3-induced expression of *Dub1*, Erk is not essential for CSF3-induced *Dub2A* expression (Figure 1D). To further investigate which signalling pathways might be involved in this process, we used a panel of chemical inhibitors, including MEK [MAPK (mitogen activated/protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] inhibitor U0126, Src tyrosine kinase inhibitor PP2, Akt inhibitor IV, p38 MAPK inhibitor SB203580, JAK inhibitor I, PI-3K (phosphoinositide-3 kinase) inhibitor LY294002 and JNK inhibitor VIII. JAK inhibitor I completely and MEK inhibitor U0126 partly abolished *Dub2A* upregulation. None of the other compounds showed significant inhibitory effects (Figure 1E).

DUB2 reduces ubiquitination and enhances protein stability of activated CSF3R in the retrograde pathway

Next, we asked if DUB2 affects the CSF3R ubiquitination status and whether this has an impact on CSF3R degradation. Ectopic expression of DUB2 reduced ubiquitination levels of activated wt CSF3Rs (Figure 2A and 2B). CSF3R mutant K5R, which lacks all cytoplasmic lysines and consequently escapes lysosomal degradation [7], was taken along as a relative measure of CSF3R protein stability. Importantly, although the expression of UBPY, AMSH and DUB2 all resulted in reduced CSF3R ubiquitination levels, only DUB2 increased the stability of the wt CSF3R, comparable to the levels of K5R (Figure 2A). In contrast, ectopic expression of AMSH led to increased degradation of the CSF3R, whereas UBPY did not influence CSF3R stability (Figure 2A and 2B).

DUB2 does not affect total levels of nonactivated CSF3R

Subsequently, we wondered if DUBs also influence steady state CSF3R levels. Expression of DUB2 did not elevate the total CSF3R pool, implicating that DUB2-mediated deubiquitination and increased protein stability is specifically associated with the retrograde endocytotic pathway (Figure 2C). Strikingly, expression of AMSH resulted in increased degradation of the total pool of CSF3R, suggesting that AMSH exerts a negative role in CSF3R processing in the biosynthetic pathway. On the other hand, UBPY, like DUB2, did not affect total CSF3R levels.

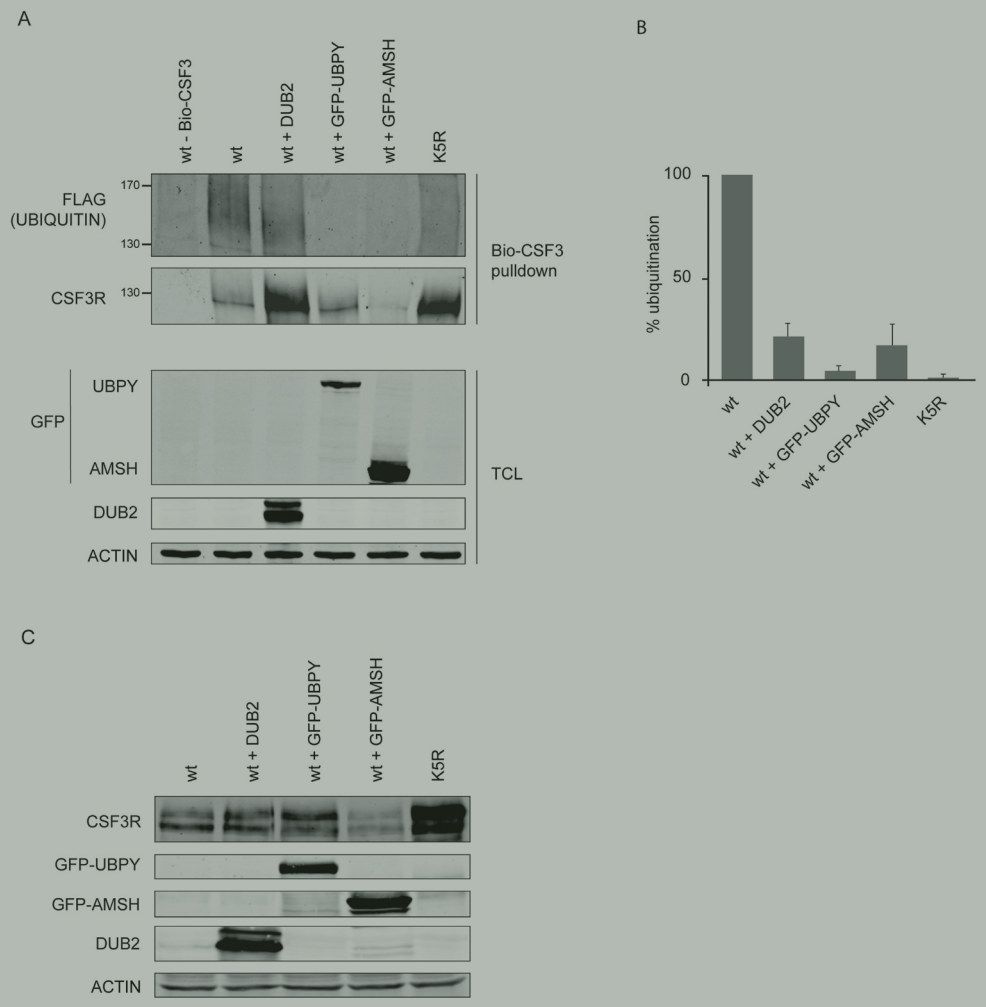

Figure 2: Overexpression of DUB2 or UBPY influences ubiquitination level of wt CSF3R (**A**) 293T cells were transfected with the indicated constructs together with Flag-Ub and after 48 hrs the cells were incubated with biotinylated CSF3 (Bio-CSF3) for 30 min at 37°C. Ligand-bound receptors were precipitated using streptavidin-coated beads and analyzed by Western blotting using Flag and CSF3R antibodies. To confirm the presence of different deubiquitinating enzymes, total cell lysate (TCL) blot was stained using GFP and DUB2 antibodies. TCL blot was also stained for ACTIN as loading control. (**B**) Quantification of three experiments as shown in A. The mean amounts of Ub in the CSF3R immunoprecipitates were normalized to total receptor levels and expressed as a percentage of the ubiquitination status of wt CSF3R ± SD. (**C**) 239T cells were transfected with indicated constructs. After 48h TCL was taken. The blot was stained for CSF3R, GFP, DUB2 and ACTIN as a loading control. Wt: CSF3R wild type; K5R: CSF3R mutant lacking conserved cytosolic lysines.

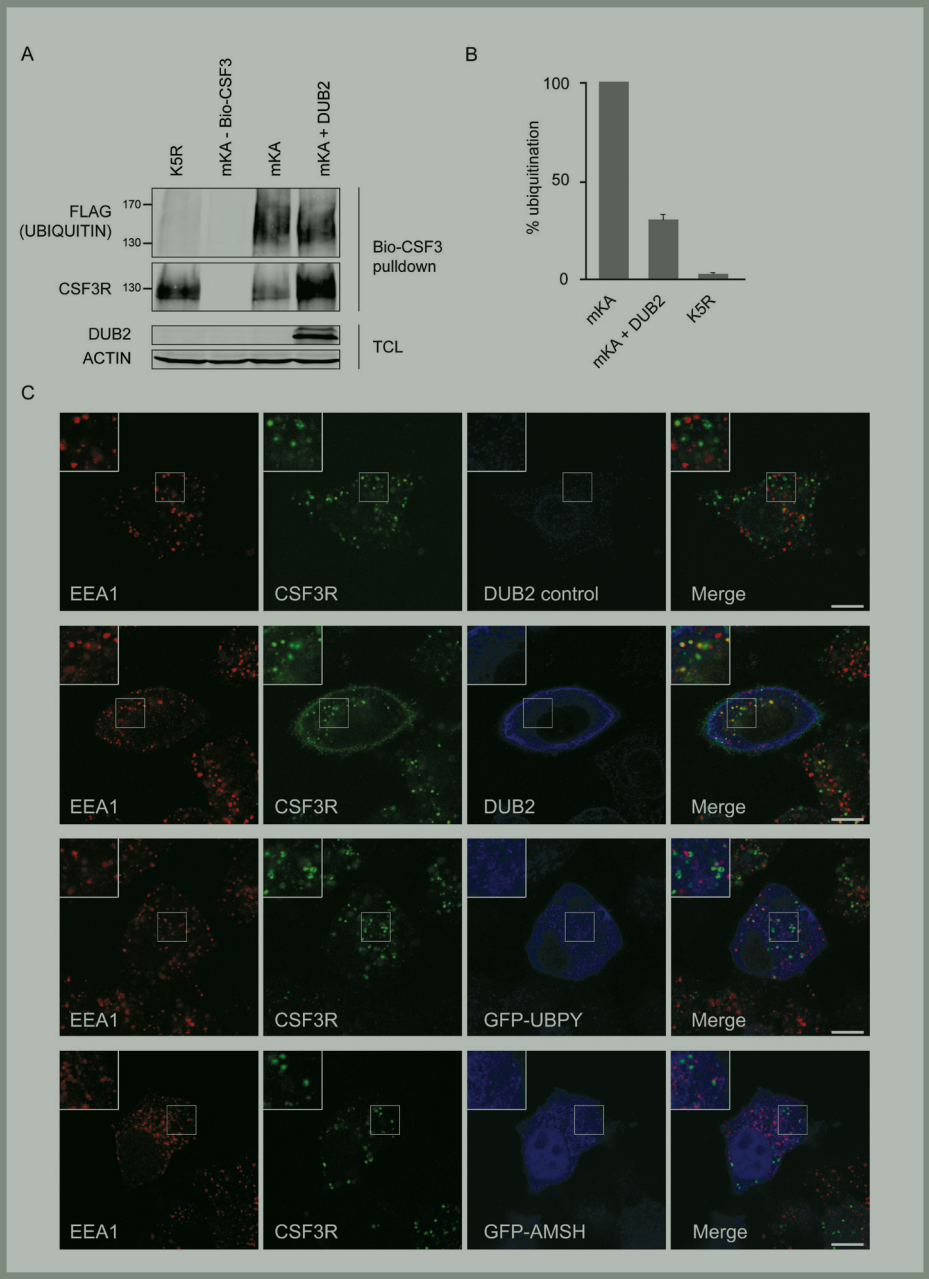

Figure 3: Enhanced levels of DUB2 cause CSF3R accumulation in early endosomes (**A**) and (**B**). Deubiquitination of Lys-632 by DUB2. Experiment performed as described for Figure 2A and 2B. (**C**) HeLa stably expressing wt CSF3R were transfected with ev (first panel), DUB2 (second panel), GFP-UBPY (third panel) or GFP-AMSH (fourth panel). After 48 hrs the cells were incubated with CSF3 and CSF3R antibody for 10 min at RT, washed and put at 37°C for 2 hrs to stimulate lysosomal routing. Subsequently cells were fixed and stained for EEA1 and CSF3R in combination with or without DUB2. Scale Bar, 10μm.

DUB2 promotes retention of activated CSF3R in early endosomes

Previously, we showed that ubiquitination of CSF3R is not required for CSF3R internalization but that ubiquitination of a juxtamembrane lysine residue (Lys-632) is crucial for CSF3R routing from early endosomes towards late endosomes and lysosomes, thereby causing the degradation of the activated CSF3R [7, 43]. Because DUB2 enhances CSF3R protein stability we asked whether DUB2 directly interferes with lysosomal routing by controlling the ubiquitination status of Lys-632. To this end, we employed a mutant, mKA, that retains Lys-632 but lacks the other conserved cytoplasmic lysine residues [7]. DUB2 clearly increased the stability of the mKA CSF3R, providing additional evidence for a role of DUB2 in counteracting lysosomal degradation of CSF3R (Figure 3A and 3B) and further supporting the crucial involvement of Lys-632 and its ubiquitination status in balancing this process.

Next, we studied the effects of DUB2 on the routing dynamics of CSF3R by confocal microscopy. At low endogenous DUB levels, ligand-activated CSF3Rs resided in lysosomes 2 hrs after CSF3 stimulation and were no longer detectable in early endosomes (Figure 3C, upper panel). In contrast, in cells in which DUB2 was ectopically expressed, CSF3R accumulated in early endosomes 2 hrs post CSF3 stimulation (Figure 3C, second panel). Notably, ectopic expression of UBPY or AMSH did not result in increased accumulation of CSF3R in the early endosome compartment, clearly suggesting that these enzymes deubiquitinate CSF3R at a different stage in the retrograde pathway (Figure 3C, third and fourth panel).

DUB2 prolongs CSF3-induced STAT5 phosphorylation

Accumulation of lysine-deficient mutants of CSF3R in early endosomes causes sustained activation of STAT5 upon removal of CSF3 [7]. To investigate whether upregulation of DUB2 has a similar effect on STAT5 activation, Ba/F3β/tTA-DUB2 cells stably expressing wt CSF3R were cultured for 60 hrs with Dox to inhibit and without Dox to induce DUB2 expression. Expression of DUB2 resulted in prolonged STAT5 phosphorylation after CSF3 withdrawal (Figure 4A and 4B). Pull down experiments with bio-CSF3 showed that this was associated with reduced ubiquitination of the CSF3R (Figure 4C and 4D).

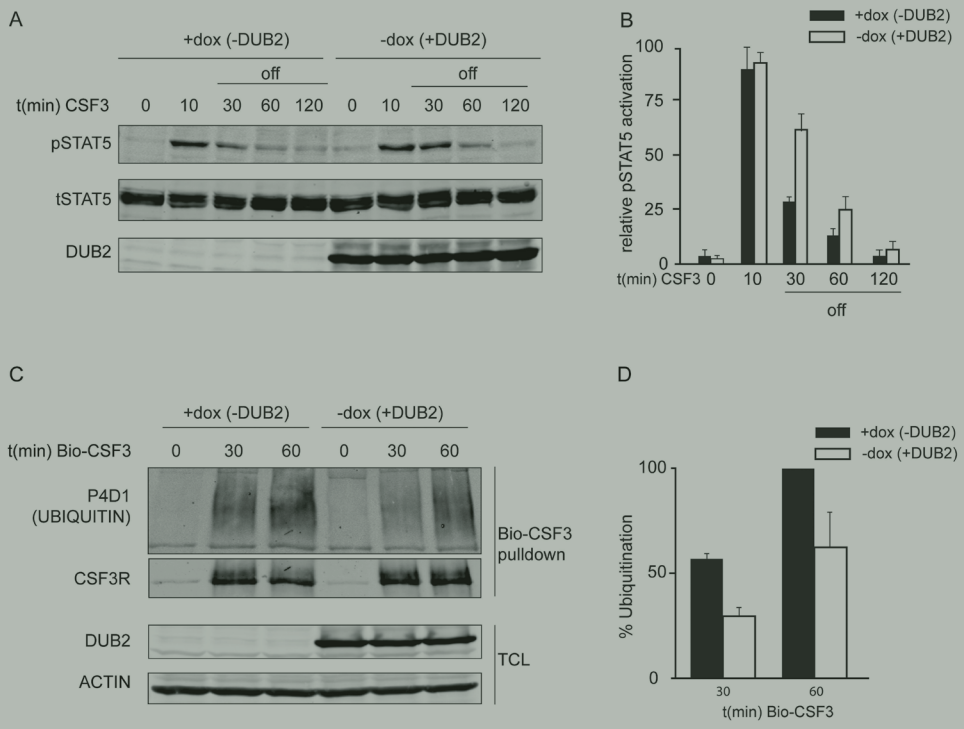

Figure 4: Prolonged STAT5 signalling of wt CSF3R deubiquitinated by DUB2. Ba/F3β/tTA DUB2 cells stably expressing wt CSF3R were grown in the presence or absence of dox for 60 hrs. (**A**) Cells were cytokine and serum deprived for 4 hrs, stimulated for 10 min with CSF3 at 37°C. The CSF3 was washed away and cells were kept in medium without factor at 37°C for the indicated time points. Down regulation of STAT5 phosphorylation was monitored using Western Blot. The blot was stained for total STAT5 as loading control.
(**B**) Quantification of three experiments as shown in A. The mean amounts of pSTAT5 were normalized to total levels and expressed as a percentage of maximum pSTAT5 activation ± SEM. (**C**) Cells were pre-incubated with the lysosomal inhibitor bafilomycin and stimulated with Bio-CSF3 for the indicated time periods. CSF3R were precipitated using streptavidin beads and analyzed by Western blotting for CSF3R and endogenous ubiquitin using P4D1 antibody. TCL blot is shown to confirm upregulation of DUB2 in the absence of dox and stained for actin as a loading control. (**D**) Quantification of 3 independent experiments shown in B. The mean amounts of Ub in the CSF3R immunoprecipitates were normalized to total receptor levels and expressed as a percentage ± SD of ubiquitination status of wt CSF3R after 60 min Bio-CSF3 stimulation in the absence of DUB2 (set at 100%).

DISCUSSION

We have identified the USP family member DUB2A as a major negative regulator of CSF3R ubiquitination, thereby controlling intracellular routing and signalling properties of CSF3R. Among the 4 homologous members of the murine DUB family, only DUB2A was specifically up-regulated in myeloid cells in response to CSF3 stimulation. DUB2A reduced the ubiquitination of the juxtamembrane lysine 632, which was previously shown to be a target for ubiquitination by the E3 ligase SOCS3 [7].

It is currently unknown why DUB2A, but not other family members, is specifically up-regulated in myeloid cells and which signalling pathways and transcriptional regulators are important for its expression. Sequence alignment of the 5' transcription enhancer domains of *Dub1* and *Dub2A* showed that these regions contain comparable transcription factor binding sites, including GATA, ETS, AP1 and CBF sites [23]. Potentially, differential accessibility of promoter/enhancer regions, e.g., due to chromatin structure, could explain why only *Dub2A* and not *Dub1, Dub1A* or *Dub2* is transcribed in myeloid 32D cells in response to CSF3, but this needs further study. Concerning the signalling mechanisms involved, it became clear that activation of JAKs is crucial for CSF3-induced *Dub2A* expression. In contrast, no evidence for a major involvement of other signalling pathways activated by CSF3, e.g., Ras/Raf/Mek1, PI3K/Akt, p38MAPK or Jnk pathways was obtained, either by applying specific inhibitors or from experiments with selective tyrosine mutants of CSF3R known to play a major role in the activation of STAT3 or MAP kinases [37, 39-40, 44].

Although DUB2, UBPY and AMSH all reduced ubiquitination of activated CSF3Rs, only DUB2 enhanced the stability of the CSF3R, through promoting its accumulation in early endosomes. For UBPY, this fits well with its proposed function to retrieve ubiquitin from targeted receptors before they go into MVB [45]. In literature, there are opposing reports about the role of AMSH in EGFR degradation. Whereas one study shows that AMSH is important for EGFR degradation after retrograde transport [16] another demonstrates that AMSH silencing enhances EGFR degradation [8]. We showed that AMSH strongly diminishes the steady-state CSF3R levels independent of receptor activation and internalization. The finding that decreased receptor levels were pulled down with bio-CSF3, might thus be entirely due to reduced membrane expression of CSF3R, rather than elevated degradation after retrograde transport. At this point we cannot discriminate if DUB2 directly or indirectly reduces the ubiquitation of the CSF3R. DUB2 may deubiquitinate members of the endocytotic machinery and thereby influencing the routing and the ubiquitination status of the CSF3 receptor. This is not without precedent because it is known that DUB1 deubiquitinates dynein heavy chain [32]. The dynein heavy chain family, which has two major classes of axonemal and cytoplasmic proteins, mainly associates with microtubule complex and is involved in the regulation of the movement of organelles and vesicles along microtubules [46]. Dynein is

known to be associated with the movement of late endosomes and also for receptor sorting and morphogenesis of early endosomes [47-48].

In conclusion, the data presented here fit into a model in which ubiquitination of activated CSF3R is dynamically controlled by the E3 ligase activity of SOCS3, a direct target of STAT3 [44], and the Ub specific protease activity of DUB2A (Figure 5). Given that receptor ubiquitination has been implicated in the endocytotic routing of numerous membrane receptors involving a variety of E3 ligases [43, 49-52], it is now worth-while to investigate whether and which DUBs are able to delay the lysosomal routing of different growth factor receptor systems. Conceivably, this will give important insights into how these receptor activities are controlled under physiological conditions. Moreover, in view of the increasing association of ubiquitin-mediated pathways with various pathological conditions, the role of DUBs in diseases linked with growth factor receptor dysfunction deserves further study.

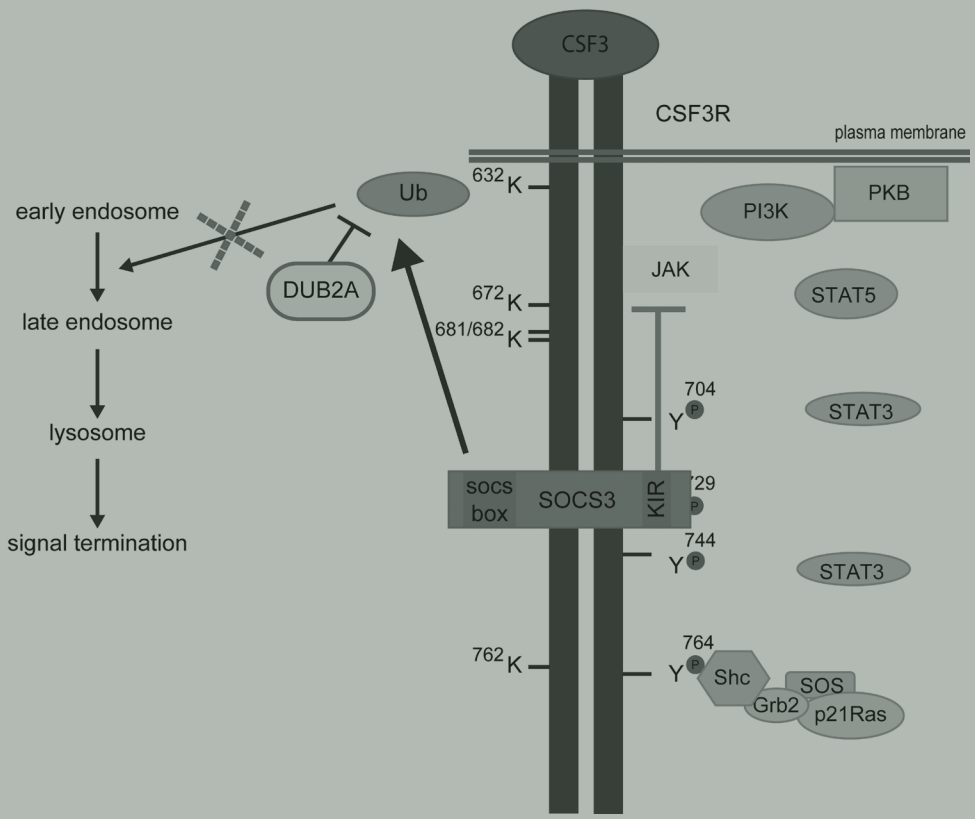

Figure 5: Model of SOCS3 and DUB2A controlled CSF3R routing and signal termination. Following CSF3 stimulation STAT3 is activated which induces SOCS3. SOCS3 is subsequently recruited to Y729 of the CSF3R and forms an E3 ligase complex via its SOCS box that ubiquitinates Lys-632 of the CSF3R (negative feedback loop). This causes CSF3R routing from early endosomes to lysosomes where the receptor is degraded. DUB2A is in addition upregulated by CSF3 via an unknown mechanism which involves activation of JAKs. DUB2A deubiquitinates Lys-632 of the CSF3R, thereby counteracting the SOCS3 ligase complex and inhibiting lysosomal routing (positive feedback loop).

ACKNOWLEDGMENTS

This work was supported by the Dutch Cancer Society, KWF kankerbestrijding(www.KW-Fkankerbestrijding.nl). We are grateful to Dr. James A. Johnston (Belfast, UK) for Ba/F3β/tTA DUB2, pMEF-DUB2 and DUB2 antibodies and to Dr. Sylvie Urbé (Liverpool, UK) for providing pEGFP-C1-AMSH and pEGFP-C1-UBPY constructs.

FUNDING

This work was supported by the Dutch Cancer Society, KWF kankerbestrijding [grant number EMCR 2006-3585].

REFERENCES

1. Zwang, Y. and Yarden, Y. (2009) Systems biology of growth factor-induced receptor endocytosis. Traffic **10**, 349-363
2. Raiborg, C. and Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature **458**, 445-452
3. Lieschke, G.J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., Fowler, K.J., Basu, S., Zhan, Y.F., and Dunn, A.R. (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood **84**, 1737-1746
4. Hortner, M., Nielsch, U., Mayr, L.M., Johnston, J.A., Heinrich, P.C., and Haan, S. (2002) Suppressor of cytokine signaling-3 is recruited to the activated granulocyte-colony stimulating factor receptor and modulates its signal transduction. J. Immunol. **169**, 1219-1227
5. Tian, S.S., Lamb, P., Seidel, H.M., Stein, R.B., and Rosen, J. (1994) Rapid activation of the STAT3 transcription factor by granulocyte colony-stimulating factor. Blood **84**, 1760-1764
6. Babon, J.J., Sabo, J.K., Soetopo, A., Yao, S., Bailey, M.F., Zhang, J.G., Nicola, N.A., and Norton, R.S. (2008) The SOCS box domain of SOCS3: structure and interaction with the elonginBC-cullin5 ubiquitin ligase. J. Mol. Biol. **381**, 928-940
7. Irandoust, M.I., Aarts, L.H., Roovers, O., Gits, J., Erkeland, S.J., and Touw, I.P. (2007) Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor. Embo J. **26**, 1782-1793
8. McCullough, J., Clague, M.J., and Urbe, S. (2004) AMSH is an endosome-associated ubiquitin isopeptidase. J. Cell Biol. **166**, 487-492
9. Kato, M., Miyazawa, K., and Kitamura, N. (2000) A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP. J. Biol. Chem. **275**, 37481-37487
10 Agromayor, M. and Martin-Serrano, J. (2006) Interaction of AMSH with ESCRT-III and deubiquitination of endosomal cargo. J. Biol. Chem. **281**, 23083-23091
11 Kaneko, T., Kumasaka, T., Ganbe, T., Sato, T., Miyazawa, K., Kitamura, N., and Tanaka, N. (2003) Structural insight into modest binding of a non-PXXP ligand to the signal transducing adaptor molecule-2 Src homology 3 domain. J. Biol. Chem. **278**, 48162-48168
12. Row, P.E., Liu, H., Hayes, S., Welchman, R., Charalabous, P., Hofmann, K., Clague, M.J., Sanderson, C.M., and Urbe, S. (2007) The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation. J. Biol. Chem. **282**, 30929-30937
13. Tanaka, N., Kaneko, K., Asao, H., Kasai, H., Endo, Y., Fujita, T., Takeshita, T., and Sugamura, K. (1999) Possible involvement of a novel STAM-associated molecule "AMSH" in intracellular signal transduction mediated by cytokines. J. Biol. Chem. **274**, 19129-19135
14. Hasdemir, B., Murphy, J.E., Cottrell, G.S., and Bunnett, N.W. (2009) Endosomal deubiquitinating enzymes control ubiquitination and down-regulation of protease-activated receptor 2. J. Biol. Chem. **284**, 28453-28466
15. Hislop, J.N., Henry, A.G., Marchese, A., and von Zastrow, M. (2009) Ubiquitination regulates proteolytic processing of G protein-coupled receptors after their sorting to lysosomes. J. Biol. Chem. **284**, 19361-19370

16. Ma, Y.M., Boucrot, E., Villen, J., Affar el, B., Gygi, S.P., Gottlinger, H.G., and Kirchhausen, T. (2007) Targeting of AMSH to endosomes is required for epidermal growth factor receptor degradation. J. Biol. Chem. **282**, 9805-9812

17. Sierra, M.I., Wright, M.H., and Nash, P.D. (2010) AMSH interacts with ESCRT-0 to regulate the stability and trafficking of CXCR4. J. Biol. Chem. **285**, 13990-14004

18. Row, P.E., Prior, I.A., McCullough, J., Clague, M.J., and Urbe, S. (2006) The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. J. Biol. Chem. **281**, 12618-12624

19. Niendorf, S., Oksche, A., Kisser, A., Lohler, J., Prinz, M., Schorle, H., Feller, S., Lewitzky, M., Horak, I., and Knobeloch, K.P. (2007) Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. Mol. Cell Biol. **27**, 5029-5039

20. Mizuno, E., Kobayashi, K., Yamamoto, A., Kitamura, N., and Komada, M. (2006) A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes. Traffic **7**, 1017-1031

21. Alwan, H.A. and van Leeuwen, J.E. (2007) UBPY-mediated epidermal growth factor receptor (EGFR) de-ubiquitination promotes EGFR degradation. J. Biol. Chem. **282**, 1658-1669

22. Naviglio, S., Mattecucci, C., Matoskova, B., Nagase, T., Nomura, N., Di Fiore, P.P., and Draetta, G.F. (1998) UBPY: a growth-regulated human ubiquitin isopeptidase. Embo J. **17**, 3241-3250

23. Baek, K.H., Mondoux, M.A., Jaster, R., Fire-Levin, E., and D'Andrea, A.D. (2001) DUB-2A, a new member of the DUB subfamily of hematopoietic deubiquitinating enzymes. Blood **98**, 636-642

24. Zhu, Y., Lambert, K., Corless, C., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and D'Andrea, A.D. (1997) DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes. J. Biol. Chem. **272**, 51-57

25. Zhu, Y., Pless, M., Inhorn, R., Mathey-Prevot, B., and D'Andrea, A.D. (1996) The murine DUB-1 gene is specifically induced by the betac subunit of interleukin-3 receptor. Mol. Cell Biol. **16**, 4808-4817

26. Baek, K.H., Kim, M.S., Kim, Y.S., Shin, J.M., and Choi, H.K. (2004) DUB-1A, a novel deubiquitinating enzyme subfamily member, is polyubiquitinated and cytokine-inducible in B-lymphocytes. J. Biol. Chem. **279**, 2368-2376

27. Zhu, Y., Carroll, M., Papa, F.R., Hochstrasser, M., and D'Andrea, A.D. (1996) DUB-1, a deubiquitinating enzyme with growth-suppressing activity. Proc. Natl. Acad. Sci. U.S.A. **93**, 3275-3279

28. Jaster, R., Zhu, Y., Pless, M., Bhattacharya, S., Mathey-Prevot, B., and D'Andrea, A.D. (1997) JAK2 is required for induction of the murine DUB-1 gene. Mol. Cell Biol. **17**, 3364-3372

29. Jaster, R., Baek, K.H., and D'Andrea, A.D. (1999) Analysis of cis-acting sequences and trans-acting factors regulating the interleukin-3 response element of the DUB-1 gene. Biochim. Biophys. Acta **1446**, 308-316

30. Migone, T.S., Humbert, M., Rascle, A., Sanden, D., D'Andrea, A., and Johnston, J.A. (2001) The deubiquitinating enzyme DUB-2 prolongs cytokine-induced signal transducers and activators of transcription activation and suppresses apoptosis following cytokine withdrawal. Blood **98**, 1935-1941

31. Baek, K.H., Kim, Y.S., Lee, H.J., and Kang, I. (2004) Essential regions of deubiquitinating enzyme activity and enhancer function for DUB-2A expressed in T-lymphocytes. Arch. Biochem. Biophys. **430**, 191-197

32. Lee, M.Y., Ajjappala, B.S., Kim, M.S., Oh, Y.K., and Baek, K.H. (2008) DUB-1, a fate determinant of dynein heavy chain in B-lymphocytes, is regulated by the ubiquitin-proteasome pathway. J. Cell. Biochem. **105**, 1420-1429

33. Burrows, J.F., McGrattan, M.J., Rascle, A., Humbert, M., Baek, K.H., and Johnston, J.A. (2004) DUB-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. J. Biol. Chem. **279**, 13993-14000

34. Okada, T., Gondo, Y., Goto, J., Kanazawa, I., Hadano, S., and Ikeda, J.E. (2002) Unstable transmission of the RS447 human megasatellite tandem repetitive sequence that contains the USP17 deubiquitinating enzyme gene. Hum. Genet. **110**, 302-313

35. Burrows, J.F., Scott, C.J., and Johnston, J.A. (2010) The DUB/USP17 deubiquitinating enzymes: a gene family within a tandemly repeated sequence, is also embedded within the copy number variable beta-defensin cluster. BMC Genomics **11**, 250

36. Burrows, J.F., McGrattan, M.J., and Johnston, J.A. (2005) The DUB/USP17 deubiquitinating enzymes, a multigene family within a tandemly repeated sequence. Genomics **85**, 524-529

37. de Koning, J.P., Soede-Bobok, A.A., Schelen, A.M., Smith, L., van Leeuwen, D., Santini, V., Burgering, B.M., Bos, J.L., Lowenberg, B., and Touw, I.P. (1998) Proliferation signaling and activation of Shc, p21Ras, and Myc via tyrosine 764 of human granulocyte colony-stimulating factor receptor. Blood **91**, 1924-1933

38. Aarts, L.H., Roovers, O., Ward, A.C., and Touw, I.P. (2004) Receptor activation and 2 distinct COOH-terminal motifs control G-CSF receptor distribution and internalization kinetics. Blood **103**, 571-579

39. Ward, A.C., Hermans, M.H., Smith, L., van Aesch, Y.M., Schelen, A.M., Antonissen, C., and Touw, I.P. (1999) Tyrosine-dependent and -independent mechanisms of STAT3 activation by the human granulocyte colony-stimulating factor (G-CSF) receptor are differentially utilized depending on G-CSF concentration. Blood **93**, 113-124

40. Hermans, M.H., van de Geijn, G.J., Antonissen, C., Gits, J., van Leeuwen, D., Ward, A.C., and Touw, I.P. (2003) Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells. Blood **101**, 2584-2590

41. van de Geijn, G.J., Gits, J., and Touw, I.P. (2004) Distinct activities of suppressor of cytokine signaling (SOCS) proteins and involvement of the SOCS box in controlling G-CSF signaling. J. Leukoc. Biol. **76**, 237-244

42. Hermans, M.H., Antonissen, C., Ward, A.C., Mayen, A.E., Ploemacher, R.E., and Touw, I.P. (1999) Sustained receptor activation and hyperproliferation in response to granulocyte colony-stimulating factor (G-CSF) in mice with a severe congenital neutropenia/acute myeloid leukemia-derived mutation in the G-CSF receptor gene. J. Exp. Med. **189**, 683-692

43. Wolfler, A., Irandoust, M., Meenhuis, A., Gits, J., Roovers, O., and Touw, I.P. (2009) Site-specific ubiquitination determines lysosomal sorting and signal attenuation of the granulocyte colony-stimulating factor receptor. Traffic **10**, 1168-1179

44. Zhuang, D., Qiu, Y., Haque, S.J., and Dong, F. (2005) Tyrosine 729 of the G-CSF receptor controls the duration of receptor signaling: involvement of SOCS3 and SOCS1. J. Leukoc. Biol. **78**, 1008-1015

45. Clague, M.J. and Urbe, S. (2006) Endocytosis: the DUB version. Trends Cell Biol. **16**, 551-559

46. Shima, T., Kon, T., Imamula, K., Ohkura, R., and Sutoh, K. (2006) Two modes of microtubule sliding driven by cytoplasmic dynein. Proc. Natl. Acad. Sci. U.S.A. **103**, 17736-17740

47. Bananis, E., Nath, S., Gordon, K., Satir, P., Stockert, R.J., Murray, J.W., and Wolkoff, A.W. (2004) Microtubule-dependent movement of late endocytic vesicles in vitro: requirements for Dynein and Kinesin. Mol. Biol. Cell **15**, 3688-3697

48. Driskell, O.J., Mironov, A., Allan, V.J., and Woodman, P.G. (2007) Dynein is required for receptor sorting and the morphogenesis of early endosomes. Nat. Cell Biol. **9**, 113-120

49. ernandez-Sanchez, E., Martinez-Villarreal, J., Gimenez, C., and Zafra, F. (2009) Constitutive and regulated endocytosis of the glycine transporter GLYT1b is controlled by ubiquitination. J. Biol. Chem. **284**, 19482-19492

50. Goh, L.K., Huang, F., Kim, W., Gygi, S., and Sorkin, A. (2010) Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J. Cell Biol. **189**, 871-883

51. Marmor, M.D. and Yarden, Y. (2004) Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene **23**, 2057-2070

52. Molfetta, R., Gasparrini, F., Santoni, A., and Paolini, R. (2010) Ubiquitination and endocytosis of the high affinity receptor for IgE. Mol. Immunol. **47**, 2427-2434


CHAPTER
5

PROTEOMIC ANALYSIS OF CSF3R INTERACTION PARTNERS IN EARLY ENDOCYTOTIC TRAFFICKING OF THE ACTIVATED CSF3 RECEPTORS

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ABSTRACT

The CSF3R is the major cytokine receptor involved in neutrophil development. Binding of CSF3 to its receptor induces activation of the JAK/STAT pathway and leads to proliferation and survival of myeloid progenitor cells, followed by cell arrest and terminal neutrophilic differentiation [1]. Downregulation of CSF3 receptors at the plasma membrane by endocytosis is crucial for signal termination. For lysosomal trafficking from early to late endosomes ubiquitination of the CSF3R juxtamembrane lysine K632, mediated by the SOCS3 E3 ligase complex is important [2]. Using mass spectrometry, we identified proteins that bind to the CSF3R complex during trafficking from the plasma membrane to early endosomes. One of these proteins is sequestosome (SQSTM)-1, an ubiquitin binding protein important in autophagy.

INTRODUCTION

Several routes of endocytic uptake of receptors and other cargo proteins into cells exist. Most work has been focused on clathrin mediated endocytosis (CME). The mechanisms by which cargo proteins enter clathrin coated pits (CCPs), that subsequently develop into clathrin-coated vesicles (CCVs) have been studied extensively [3].

Clathrin-independent internalization pathways involve formation of caveolae, macropinocytosis, circular dorsal ruffles, phagocytosis, the clathrin-independent carrier/GPI-AP-enriched early endosomal compartments (CLIC/GEEC), flotillin-dependent invaginations and trans-endocytosis [4].

Receptor trafficking is regulated by ubiquitination of cytoplasmic lysine residues of receptor tyrosine kinases and cytokine receptors after activation. Ubiquitinated receptors are recognized by the HRS-STAM complex also known as the endosomal sorting complex required for transport (ESCRT)-0, and subsequently by ESCRT-I, ESCRT-II and ESCRT-III, which is important for sorting receptors into intraluminal vesicles of multivesicular bodies (MVBs) and degradation in lysosomes [5].

The granulocyte colony-stimulating factor receptor (CSF3R) is rapidly internalized and processed for lysosomal routing after activation by its ligand CSF3. The suppressor of cytokine signalling (SOCS)-3, that binds to the phosphorylated tyrosine (Y)-729 of the CSF3R, involved in ubiquitination and lysosomal routing of the CSF3R [2]. Via its SOCS box, SOCS3 recruits Elongin B and C as well as Cullin 2/5 to form an ECS E3 ligase [6]. Although four out of the five cytoplasmic lysine residues are ubiquitinated after CSF3R activation, a single ubiquitinated lysine (K) located within the juxtamembrane domain, K632, is necessary and sufficient to direct receptors to the lysosomal compartment, where the signal is terminated [2, 7]. CSF3R mutants lacking this important membrane-proximal lysine accumulate in early endosomes [2, 7], suggesting that ubiquitination of this K632 is indispensable for sorting to late endosomes/MVBs. Possibly the interaction of the CSF3R with proteins involved in this sorting step, like HRS and EAP45, relies on ubiquitination of a properly positioned juxtamembrane lysine of the CSF3R. The major players involved in endocytotic routing of ubiquitinated CSF3R are still unknown. To identify candidate proteins involved in this process, specifically those interacting with the ubiquitinated K632 lysine, we performed a proteomics analysis with wt CSF3R, K5R which lacks all five cytoplasmic lysine residues and the single add-back mutant mKA which only contains K632. We also aimed to detect proteins interacting with the CSF3R independent of the lysines, that may also be involved in the endocytotic trafficking or signaling of the CSF3R.

RESULTS AND DISCUSSION

To identify the proteins that interact with the CSF3R during early trafficking, we stimulated Phoenix E cells expressing wt, K5R or mKA CSF3Rs with biotinylated CSF3 (bio-CSF3) for 30 min. Previously it has been shown that the CSF3R reaches the early endosome (EE) in this timeframe [2]. CSF3R complexes were subsequently pulled down using streptavidin beads. To exclude nonspecific protein binding to the beads, the same procedure was performed without adding bio-CSF3. Three independent experiments were performed, which resulted in 12 lists (3 control lists and 9 CSF3R lists).

K632 interacting proteins

We first aimed to identify proteins that bind specifically to K632. To this end we set up the criterion that candidate proteins should at least bind twice to the WT and twice to mKA CSF3R and not to the K5R CSF3R. However we did not detect any ubiquitin binding protein using this approach, even though CSF3R ubiquitination peaks at 30 min [7]. This failure to detect interactions with ubiquitinated K632 likely relates to the low affinity of ubiquitin-binding domain (UBD) interactions which range from 100–500 μM [8-9].

Proteins that interact with the CSF3R during early receptor routing

Next, we focussed on protein binding to the CSF3R, irrespectively of lysines, which resulted in a list of 79 proteins that interacted with the CSF3R at least 5 out of the 9 times. These proteins were classified based on their GO terms and the protein class in protein analysis through evolutionary relationships (PANTHER) classification system http://www.pantherdb.org (Figure 1). Proteins that bind to CSF3R and are involved in signaling or endocytotic trafficking and proteins that could not be classified by GO terms or PANTHER (scored as unknown) are listed in Table1.

Table1: Proteomics analysis of CSF3R complexes

#	MS	HGNC	DESCRIPTION	ABBR
			Endocytosis/Cytoskeleton	
9	1867	HGNC:2092	clathrin heavy chain 1	CLTC
9	1033	HGNC:20771	tubulin, beta, 2	TUBB2C
9	995	HGNC:561	alpha-adaptin A related protein	AP2A1
9	746	HGNC:563	adaptor-related protein complex 2, beta 1 subunit isoform b	AP2B1
9	679	HGNC:562	adaptor-related protein complex 2, alpha 2 subunit	AP2A2
9	589	HGNC:20778	Tubulin, beta	TUBB
9	436	HGNC:564	assembly protein 50	AP2M1
9	410	HGNC:23186	epsin 4	CLINT1
9	336	HGNC:16991	Similar to cytoskeleton-associated protein 4	CKAP4
8	113	HGNC:129	alpha 1 actin precursor	ACTA1
7	2224	HGNC:7579	myosin, heavy polypeptide 9, non-muscle	MYH9
7	1451	HGNC:7568	Myosin-10	MYH10
7	475	HGNC:12012	tropomyosin 3 isoform 2	TPM3
7	441	HGNC:12692	vimentin	VIM
7	307	HGNC:7734	neurofilament 3	NEFM
7	297	HGNC:132	beta actin	ACTB
7	296	HGNC:1488	F-actin capping protein alpha-1 subunit	CAPZA1
7	244	HGNC:7587	myosin light chain 3	MYL6
7	185	HGNC:7596	myosin IB	MYO1B
7	153	HGNC:1491	F-actin capping protein beta subunit	CAPZB
7	94	HGNC:565	clathrin-associated protein	AP2S1
6	609	HGNC:7605	myosin VI	MYO6
6	293	HGNC:169	actin-related protein 2 isoform b	ACTR2
6	194	HGNC:2695	Drebrin	DBN1
6	185	HGNC:144	actin, gamma 1 propeptide	ACTG1
6	178	HGNC:555	AP-1 complex subunit gamma-1	AP1G1
6	174	HGNC:1874	cofilin 1	CFL1
6	143	HGNC:13667	adaptor-related protein complex 1, mu 1 subunit	AP1M1
6	126	HGNC:6207	Junction plakoglobin	JUP
5	448	HGNC:20768	tubulin alpha 6	TUBA1C
5	208	HGNC:3757	flotillin 1	FLOT1
5	103	HGNC:10662	scaffold protein Pbp1	SDCBP
5	70	HGNC:11873	tropomodulin 3 (ubiquitous)	TMOD3
			Signaling	
9	688	HGNC:2439	colony stimulating factor 3 receptor isoform a precursor	CSF3R
9	434	HGNC:6128	insulin receptor substrate 4	IRS4
8	221	HGNC:4566	growth factor receptor-bound protein 2 isoform 1	GRB2
7	298	HGNC:12855	14-3-3 zeta	YWHAZ
6	332	HGNC:12854	14-3-3 theta	YWHAQ
5	197	HGNC:11364	DNA-binding protein	STAT3
5	187	HGNC:12849	14-3-3 beta, isoform C	YWHAB
5	176	HGNC:12852	14-3-3 gamma	YWHAG

Kinase/Phosphatase/Peroxidase				
9	150	HGNC:6190	janus kinase 1	JAK1
8	879	HGNC:29092	OBSL1 protein	OBSL1
6	329	HGNC:9281	protein phosphatase 1, catalytic subunit, alpha isoform 1	PPP1CA
6	176	HGNC:9352	peroxiredoxin 1	PRDX1
6	104	HGNC:9282	protein phosphatase 1, catalytic subunit, beta isoform 1	PPP1CB
5	138	HGNC:17169	thioredoxin peroxidase	PRDX4
Unknown				
9	188	HGNC:25541	transmembrane protein 33	TMEM33
8	221	HGNC:24276	sorting and assembly machinery component 50 homolog (S. cerevisiae)	SAMM50
7	85	HGNC:26058	L antigen family, member 3	LAGE3
6	127	HGNC:1356	SPFH domain family, member 2 isoform 1	ERLIN2
6	110	HGNC:11280	p60	SQSTM1
5	158	HGNC:28184	coiled-coil-helix-coiled-coil-helix domain containing 6	CHCHD6

Number of times found with mass spectrometry (max 9 times)
MS Average Mascot score (Mascot score, S= -10*Log(P), where P is the probability that the observed match is a random event)
HGNC HUGO Gene Nomenclature Committee
ABBR Abbreviation

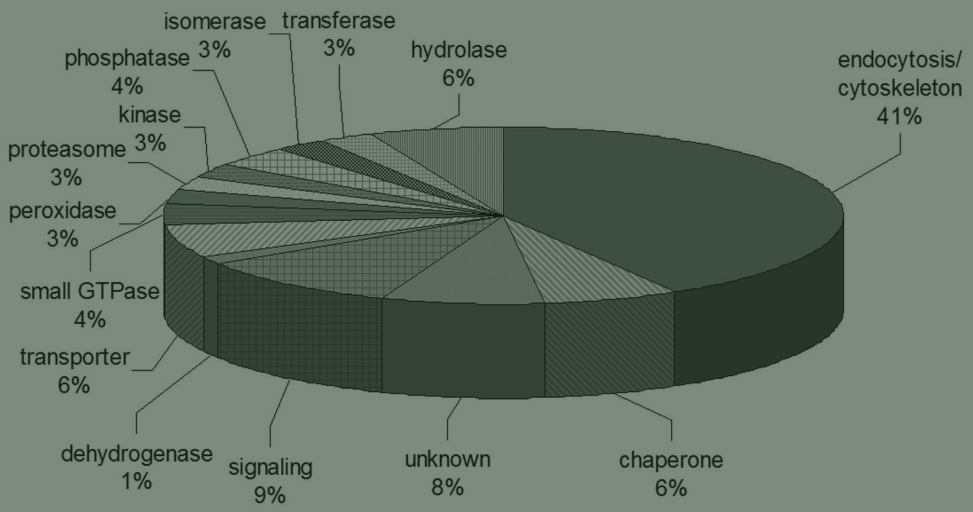

Figure1: Classification of CSF3R interacting proteins found with mass spectrometry analysis based on function.

Endocytosis/cytoskeleton proteins

The protein that was detected most prominently in the bio-CSF3 pulled down complex was clathrin heavy chain 1, indicating that the CSF3R at least partly internalizes via CCPs. The adaptor protein complex (AP)2 is the principle non-clathrin constituent of endocytic CCV which links the cargo to nucleating clathrin. AP2 is a stable complex consisting of four different subunits α- subunit, μ2- subunit, β2- subunit and a σ2- subunit [10-11], which were all present in the CSF3R complex. Dileucine- and tyrosine-based motifs on the cytoplasmic domain of cargo molecules are directly sensed by separate sites of the AP2 complex. The μ2-subunit recognizes the YXXΦ motif in the cargo. The CSF3R contains a YENL AP2 binding site at position 764-767 [12]. The α-subunit and a σ2- subunit recognize the [DE]XXXL[LI] which is present at position 749-755 as a STQPLL motif. The phosphorylated serine can be substituted by an aspartic acid (D) [12].

Flotillin proteins are not involved in CME but oligomerize in distinct membrane microdomains (64). Flotillins have a topology similar to caveolin 1 and generate structures that resemble caveolae [13-14]. Flotillin1 was found in the CSF3R complex, and is also known to be a CSF3 inducible target during neutrophilic differentiation [15].

Signaling proteins

The family of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHA), also known as 14-3-3 proteins, are highly conserved acidic proteins recognized as discrete phosphoserine/threonine binding modules in different cytokine receptors such as the CSF2-, interleukin (IL)3- and IL5 receptors [16]. This binding was important for IL3 induced phosphatidylinositol 3 kinase (PI3K), AKT signaling and cell survival [16]. The YWHA proteins are known to bind to RXXX(S/T)XP motif [17] that is also found in the CSF3R as RCDSTQPLL. Five of the family members bind to the CSF3R.

Sequestosome-1

There were 6 proteins with unknown function according to PANTHER classification and GO terms and one member is sequestosome (SQSTM)-1. SQSTM1 is a 62kDa protein which contains an ubiquitin interacting motif called ubiquitin associated (UBA) domain at its C-terminus [18]. SQSTM1 knock-out mice develop mature-onset obesity and leptin resistance hypothesized by lack of ERK down modulation [19]. Recent data indicate both oncogenic and tumour suppressor functions for SQSTM1 in various types of cancer [20-21]. SQSTM1 is implicated in a number of cellular functions including regulation of many signaling pathways such as mitogen-activated protein (MAP) kinases and NF-κB [22-24] but also in nerve growth factor receptor (TrkA) interaction with proteasomes, and transportation of poly-ubiquitinated proteins destined for degradation by the proteasome and autophagy systems [25-26]. Earlier, SQSTM1 was found to colocalize robustly with the epidermal growth factor receptor (EGFR) in late endosomes and lysosomes [25]. Based on the multiple functions

of SQSTM1, we wondered whether this protein might be involved in CSF3R signaling and routing. These studies will be discussed in the next chapter of this thesis.

MATERIAL EN METHODS

Cell culture

Phoenix E cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco DMEM, Invitrogen, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO2.

Sample preparation for mass spectrometry

Phoenix E cells were transiently transfected with wt, mKA and K5R CSF3R constructs using lipofectamine (Invitrogen) [2]. After 48 hours the cells were stimulated for 30 min with biotinylated CSF3 (Bio-CSF3) at 37°C. Cells were subsequently lysed in Carin buffer (20 mM Tris-HCl pH8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% v/v NP-40, 10% v/v Glycerol) with 50 µg/ml aprotinin, 1 mM pefablock, 2 mM Na3VO4, 20 µg/ml DNase and 30 µg/ml RNase. Activated CSF3R complexes were isolated using streptavidin coated beads (Invitrogen) for 3h at 4°C. As control unstimulated cells were taken along. Beads were incubated in Laeµmli buffer for 5 min. at 95°C and then subjected to SDS-polyacrylamide gel electrophoresis on a 4-12% NuPage Bis-Tris gel (Invitrogen).

Mass spectrometric analysis

One-dimensional SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described [27]. Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the NCBInr database (release

NCBInr_20080718; taxonomy: mammalian). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table. Proteins that based on their localisation without cell lysis could not interact with the CSF3R, such as nuclear and mitochondrial proteins, were removed from the lists.

ACKNOWLEDGEMENTS

The authors would like to thank dr. T. Jevdjovic for fruitful discussions about the mass spectrometry list and J. van Bolhuis and R. Beekman for help with analyzing the data.

REFERENCES

1. Touw, I.P. and G.J. van de Geijn, *Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders.* Front Biosci, 2007. **12**: p. 800-15.
2. Irandoust, M.I., et al., *Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor.* EMBO J, 2007. **26**(7): p. 1782-93.
3. Sorkin, A., *Cargo recognition during clathrin-mediated endocytosis: a team effort.* Curr Opin Cell Biol, 2004. **16**(4): p. 392-9.
4. Doherty, G.J. and H.T. McMahon, *Mechanisms of endocytosis.* Annu Rev Biochem, 2009. **78**: p. 857-902.
5. Raiborg, C., et al., *Differential functions of Hrs and ESCRT proteins in endocytic membrane trafficking.* Exp Cell Res, 2008. **314**(4): p. 801-13.
6. Kamura, T., et al., *VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases.* Genes Dev, 2004. **18**(24): p. 3055-65.
7. Wolfler, A., et al., *Site-specific ubiquitination determines lysosomal sorting and signal attenuation of the granulocyte colony-stimulating factor receptor.* Traffic, 2009. **10**(8): p. 1168-79.
8. Sundquist, W.I., et al., *Ubiquitin recognition by the human TSG101 protein.* Mol Cell, 2004. **13**(6): p. 783-9.
9. Slagsvold, T., et al., *Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain.* J Biol Chem, 2005. **280**(20): p. 19600-6.
10. Bonifacino, J.S. and L.M. Traub, *Signals for sorting of transmembrane proteins to endosomes and lysosomes.* Annu Rev Biochem, 2003. **72**: p. 395-447.
11. Owen, D.J., B.M. Collins, and P.R. Evans, *Adaptors for clathrin coats: structure and function.* Annu Rev Cell Dev Biol, 2004. **20**: p. 153-91.
12. Aarts, L.H., et al., *Receptor activation and 2 distinct COOH-terminal motifs control G-CSF receptor distribution and internalization kinetics.* Blood, 2004. **103**(2): p. 571-9.
13. Frick, M., et al., *Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding.* Curr Biol, 2007. **17**(13): p. 1151-6.
14. Glebov, O.O., N.A. Bright, and B.J. Nichols, *Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells.* Nat Cell Biol, 2006. **8**(1): p. 46-54.
15. Iida, S., et al., *Identification of CCR2, flotillin, and gp49B genes as new G-CSF targets during neutrophilic differentiation.* J Leukoc Biol, 2005. **78**(2): p. 481-90.
16. Guthridge, M.A., et al., *Site-specific serine phosphorylation of the IL-3 receptor is required for hemopoietic cell survival.* Mol Cell, 2000. **6**(1): p. 99-108.
17. Yaffe, M.B., *How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis.* FEBS Lett, 2002. **513**(1): p. 53-7.
18. Ciani, B., et al., *Structure of the ubiquitin-associated domain of p62 (SQSTM1) and implications for mutations that cause Paget's disease of bone.* J Biol Chem, 2003. **278**(39): p. 37409-12.
19. Rodriguez, A., et al., *Mature-onset obesity and insulin resistance in mice deficient in the signaling adapter p62.* Cell Metab, 2006. **3**(3): p. 211-22.
20. Mathew, R., et al., *Autophagy suppresses tumorigenesis through elimination of p62.* Cell, 2009. **137**(6): p. 1062-75.
21. Moscat, J. and M.T. Diaz-Meco, *p62 at the crossroads of autophagy, apoptosis, and cancer.* Cell, 2009. **137**(6): p. 1001-4.

22. Lee, S.J., et al., *A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis.* EMBO Rep, 2010. **11**(3): p. 226-32.
23. Duran, A., et al., *The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis.* Cancer Cell, 2008. **13**(4): p. 343-54.
24. Martin, P., M.T. Diaz-Meco, and J. Moscat, *The signaling adapter p62 is an important mediator of T helper 2 cell function and allergic airway inflammation.* EMBO J, 2006. **25**(15): p. 3524-33.
25. Sanchez, P., et al., *Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62.* Mol Cell Biol, 1998. **18**(5): p. 3069-80.
26. Geetha, T., et al., *p62 serves as a shuttling factor for TrkA interaction with the proteasome.* Biochem Biophys Res Commun, 2008. **374**(1): p. 33-7.
27. Wilm, M., et al., *Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry.* Nature, 1996. **379**(6564): p. 466-9.


CHAPTER
6

MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice

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Blood, in press

ABSTRACT

MicroRNAs (miRNAs) are pivotal for regulation of hematopoiesis but their critical targets remain largely unknown. Here, we show that ectopic expression of *miR-17,-20,-93* and *-106*, all AAAGUGC seed-containing miRNAs, increases proliferation, colony outgrowth and replating capacity of myeloid progenitors and results in enhanced P-ERK levels. We found that these miRNAs are endogenously and abundantly expressed in myeloid progenitors and downregulated in mature neutrophils. Quantitative proteomics identified sequestosome 1 (SQSTM1), an ubiquitin-binding protein and regulator of autophagy-mediated protein degradation, as a major target for these miRNAs in myeloid progenitors. In addition, we found increased expression of *sqstm1* transcripts during CSF3-induced neutrophil differentiation of 32D-CSF3R cells and an inverse correlation of SQSTM1 protein levels and *miR-106* expression in AML samples. ShRNA-mediated silencing of *Sqstm1* phenocopied the effects of ectopic *miR-17/20/93/106* expression in hematopoietic progenitors *in vitro* and in mice. Further, SQSTM1 binds to the ligand-activated colony-stimulating factor 3 receptor (CSF3R) mainly in the late endosomal compartment, but not in LC3 positive autophagosomes. SQSTM1 regulates CSF3R stability and ligand-induced mitogen-activated protein kinase signalling. We demonstrate that AAAGUGC seed-containing miRNAs promote cell expansion, replating capacity and signalling in hematopoietic cells by interference with SQSTM1-regulated pathways.

INTRODUCTION

MiRNAs are transcribed as long primary transcripts that are processed by RNaseIII endonucleases DROSHA and DICER into single stranded RNAs of ~22nt [1]. The nucleotides 2-7 at the 5'-end of miRNAs, referred to as the miRNA "seed" region, are important for miRNA target recognition [2]. MiRNAs regulate gene expression by pairing with the seed complementary sequences in the 3' untranslated region (UTR) of mRNAs. Most mammalian miRNAs both repress translation and enhance decay of their target transcript [3-4]. MiRNAs containing homologous seeds such as e.g., the *Let-7* family of miRNAs, are believed to regulate the same targets [2].

Involvement of miRNAs in hematopoiesis is strongly suggested by the position of miRNA genes near translocation breakpoints and by their presence in loci targeted for deletion in human leukemias [5]. Furthermore, expression profiling data suggests a major role for miRNAs in regulation of hematopoietic cell commitment, proliferation, apoptosis, survival, and differentiation [6-9]. The importance of miRNAs during hematopoiesis has been shown by disruption of miRNA biogenesis in mice. For instance, Dicer deleted hematopoietic stem cells are unable to reconstitute the hematopoietic system [10]. Further, conditional deletion of Dicer in T-and B-cells results in strong reduction of lymphocytes and diminished cell survival and functions [11-13]. Argonaute-2 knock-out in hematopoiesis results in impaired differentiation of B-lymphocytes and erythroid cells [14-15]. MiRNAs can be expressed in a cell type or tissue specific manner. For instance, *miR-223* and *miR-142* are almost exclusively expressed in hematopoietic cells [16]. *MiR-223* is transcriptionally controlled by CCAAT/enhancer-binding protein alpha (CEBPA) and suppresses the myeloid transcription factor MEF2C, a major regulator of progenitor cell proliferation and granulocyte specific functions [17-18]. In addition, specific miRNAs control cellular processes important for proliferation, survival, cytokine production and cell lineage decisions of developing T and B cells [8, 12]. In hematopoietic stem cells, sustained expression of miR-155 cells causes a myeloproliferative disorder in mice [19]. Furthermore, forced miR-29a in hematopoietic precursors induces aberrant self-renewal and acute myeloid leukemia by still unidentified mechanisms [20]. These examples illustrate the role of miRNAs as regulators of critical pathways determining normal hematopoietic cell fate and differentiation. However, there is a lack of data concerning the mRNA targets of miRNAs that are expressed in hematopoiesis.

Here, we have used a newly developed bar-coded miRNA expression library to screen for miRNAs that control normal granulopoiesis and found that AAAGUGC seed-containing miRNAs potently enhance expansion of myeloid 32D cells and primary hematopoietic progenitors. Using quantitative proteomics, we identified *Sequestosome1* (*Sqstm1*) as a prominent target of the AAAGUGC seed-containing miRNAs in myeloid cells. We show that SQSTM1 controls myeloid cell expansion and replating capacity, mitogen-activated protein (MAP) kinase activity and CSF3R stability.

METHODS

Generation of a Bar-coded retroviral expression vector (MSCV-BC-miRNA)

The MSCV-BC constructs were generated using standard molecular biology techniques. Maps, sequences and cloning information are available on the Hynes lab web site. Ninety-six unique BC sequences were cloned in the MSCV vector, which are anti-sense to the sequences coupled to xMAP beads (Luminex). MiRNA plus ~250 flanking sequences were amplified by PCR and cloned in the MSCV-BC vector.

Cell culture and gene transfer

The IL-3-dependent murine myeloid cell lines 32D and Ba/F3 containing the human wild-type CSF3R were expanded, differentiated and analyzed as described [21]. Stem cells were expanded as described [22]. The angiopoietin-like-2 vector was a kind gift from Cheng Cheng Zhang, Whitehead Institute and the RAB7-GFP was a gift of Peter van der Sluijs, Department of Cell Biology, University Medical Center Utrecht, the Netherlands [23]. MSCV virus particles were generated as described [24]. The 32D cells, Ba/F3 cells and BM derived progenitors and HSCs were infected with pMSCV-BC-miRNA virus using retroNectin™ (Takara Bio Inc.) according to manufacturer's instruction and selected for GFP expression with FACSaria cell sorter (Becton Dickinson). Hek293 and HeLa cells were grown in DMEM medium supplemented with FCS (10%) and under standard conditions. For SQSTM1 knock down SQSTM1 ON-targetplus smartpool siRNAs (Cat #L-010230-00-0005), SiGLO-cyclophilinB (D-00161001) and control siRNA pool (D-001810-10-05, Dharmacon), at a final concentration of 10 nM, were transfected into HeLa cells with Dharmafect-I (Dharmacon) according to manufacturer's protocols

Luminex experiments

Sorted MSCV-BC-miRNA 32D cell populations were mixed with empty MSCV-BC control cells in a 1:1 ratio and switched to CSF3-containing medium. Genomic DNA was isolated at different time points and BC sequences were amplified by PCR with primers: reverse primer 5'-Bio-CAGAGAACTATCATTGCATATACAC-3' and forward primer 5'-CTAATACGACTCACTATAGGGA-GAACGC-3', labelled with streptaviding-Phycoerythrin (2 μg/mL) and analyzed on a Luminex machine according to manufacturer's instruction (Luminex).

Colony assays and competitive reconstitution in mice

Lineage negative hematopoietic cells were harvested from the femurs and tibiae of 8-12 week-old C57BL/6 mice (The Jackson Laboratory) as described [16]. For the colony assays, 10^4 MSCV transduced BM cells were plated in triplicate in methocult (M3231, Stem Technologies), either supplemented with human CSF3 (100 ng/mL) and puromycin (1.5 μg/mL) for CFU-G assay or IL-6 (10 ng/mL), IL-3 (supernatant 1/1000), SCF (10 ng/mL), GM-CSF

(10 ng/mL and puromycin (1.5 μg/mL) for replating assays. For competitive reconstitution experiments, 8-10 weeks old recipient mice were lethally irradiated with 8.5 Gy and tail vein injected with 1-5x10^5 transduced Lin- cells. Peripheral blood was obtained by tail and total BM was analyzed as described [25].

Luciferase experiments

The wild type and mutant full length human *Sqstm1* 3'UTR were cloned behind the Firefly Luciferase gene of the pGL3-Promoter vector (Promega). HEK293 cells were transfected with pGL3-SQSTM1 3'UTR and Renilla control vectors with Fugene6 transfection reagent (Roche). For dual Luciferase reporter assay, cells were lysed and analyzed according to manufacturer's instruction (Promega) with a VICTOR™ multi label counter.

Real-time quantitative PCR

Human AML cells were obtained following informed consent and were purified as previously described [26]. BM samples were collected from healthy individuals following the declaration of Helsinki principles. Different stages of neutrophil differentiation, that is, myeloblasts/promyelocytes, metamyelocytes and neutrophils, were FACS-sorted using cell type specific markers [27], i.e. CD10-APC, CD11b-APC-Cy7, CD34-Pe-Cy7, CD45-PerCP, CD117-PE (Becton Dickinson) and CD36-FITC (Beckman Coulter). The purity of sorted samples was determined by immunophenotyping and morphology of the cytospins, stained with May Grünwald Giemsa. Total RNA was extracted and miRNAs were detected as described [28].

See also Suppl. methods:

Quantitative mass spectrometry (SILAC samples), Mass spectrometric analysis (Bio-CSF3 pull-down samples), Western blotting, Confocal and Spinning-disk live cell imaging.

RESULTS

Ectopic expression of *miR-292* or *miR-93* interferes with CSF3-induced myelopoiesis

We developed a bar-coded (BC) retroviral (Murine Stem Cell Virus, MSCV) miRNA expression library, MSCV-BC-miRNA, that allows for functional analysis of miRNAs on a large scale (Fig.1A, Suppl.Table1). We have previously developed 32D clones expressing human colony-stimulating factor 3 receptor (CSF3R) as a model to study neutrophilic differentiation [21]. 32D-CSF3R cells remain immature when cultured in interleukin (IL)-3-containing medium. However, upon transfer to CSF3-containing medium these cells initially proliferate and then undergo terminal neutrophilic differentiation [21]. We used the 32D-CSF3R cell line as a model to identify miRNAs that control granulopoiesis and to examine whether particular miRNAs when overexpressed would interfere with the balance of cell expansion and differentiation. We infected 32D-CSF3R cells with retroviruses expressing miRNAs as well as an empty vector (EV) MSCV-BC-A1 (BCA1) as control. Up to ten cell populations, each expressing a different miRNA from a BC retroviral vector and a BCA1 vector control, were mixed in a 1:1 ratio and transferred to CSF3-containing medium. While expression of most miRNAs tested (see library Table 1) did not change the miRNA BC/EV BC ratios after nine days of CSF3 treatment, BC signals A8 (*miR-292*) and F5 (*miR-93*) increased relative to the BCA1 (Fig.1B, 1C), indicating an enhanced cell expansion when *miR-292* or *miR-93* are expressed. To substantiate the effect of these miRNAs on myelopoiesis, we repeated the experiment with individual *miR-292* and *miR-93* expressing cell populations and compared growth and differentiation capacities of these cells with EV infected controls. When 32D-*miR-292* and 32D-*miR-93* cells were switched to CSF3-containing medium, cells with a blast-like morphology persisted in culture and expanded continuously under CSF3 conditions, whereas the wild type 32D-CSF3R cells and empty vector infected control cells stopped dividing and differentiated after 7 days of culture (Fig.1D). Thus, ectopic expression of *miR-292* and *miR-93* enhances the expansion of blast-like 32D-CSF3R cells at the expense of myeloid differentiation.

MiR-93-related miRNAs are endogenously expressed in myeloid cells

MiR-292 (A**AAGUGC**CGCCAGGUUUUGAGUGU) and *miR-93* (C**AAAGUGC**UGUUCGUGCAGGUAG) contain remarkably similar seeds. The previous experiments triggered the question whether *miR-292* and *miR-93* or other sequence related miRNAs are expressed in myelopoiesis. Sixteen murine miRNAs containing (A)AAGUGC seed sequences were found in the miRbase database (http://www.mirbase.org/) and are listed in Suppl.Table-2. Notably, expression of *miR-292*-related miRNAs (*miR-302a-d*, *-290-3p, -291a-3p, -291b-3p, -294, -295* and human homologues miRNAs *miR-302a-d, -372, -373, -512, -515-3p, -519a-e, -520a-e, -526b,* could not be detected by qPCR in normal hematopoietic cells (data not shown). Only the *miR-17, -20, -93* and *-106,* which are highly conserved between species, are expressed in hematopoietic cells at different stages of myeloid development from both mouse and human origin (Fig.2). *MiR-17* and *miR-20a* were the most abundant miRNAs followed by *miR-106a/b* and *miR-93* with a ~2-4 fold lower expression. Strikingly, miR-20b was clearly the

lowest expressed in 32D progenitors. Expression levels of *miR-17, -20a -20b, -106a* and *-106b* were reduced during differentiation of 32D-CSF3R cells (Fig.2A) and *miR-17, -20a, -20b* significantly declined in human mature neutrophils (Fig.2B). Collectively, *miR-17, -20, -93* and *-106* contain the same "seed" sequence, are conserved across species and are expressed in hematopoietic cells. Therefore, these miRNAs were selected for further analysis.

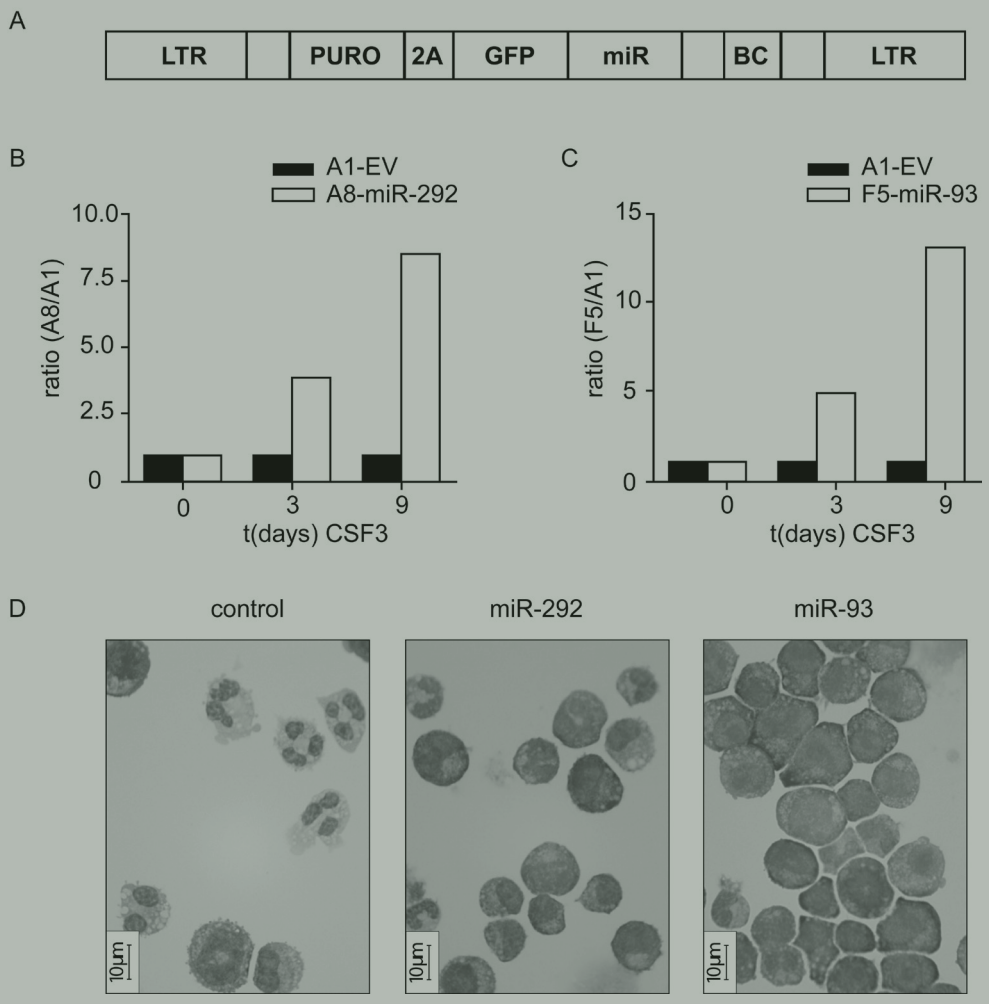


Figure 1: Functional investigation of miRNAs in 32D cells. (**A**) Overview of the bar-coded retroviral miRNA expression vector MSCV-BC-miRNA. MiRNA expression is driven by the viral LTR promoter. This vector contains the miRNA and ~250 bp endogenous flanking sequences. To allow selection of infected cells, we incorporated a dual selection cassette that consist of the puromycin-N-acetyltransferase gene fused to a segment of the FMDV 2A peptide followed by the gene coding for GFP and a 24 BP barcode (BC) sequence.
(**B**, **C**) Murine myeloid 32D cells were infected with MSCV-BC vectors containing different miRNAs or no miRNA as control (A1, EV) and sorted for GFP expression by flow cytometry. Equal number of cells were mixed and switched from IL-3- to CSF3-containing medium. Cell samples were taken at indicated time points and genomic DNA was isolated. The abundance of the different BC sequences was measured with the Luminex technology. The ratios of the bar-code A8 (*miR-292*) and bar-code F5 (*miR-93*) signals to the A1 bar-code (EV) signal of a representative result (out of 2 experiments for *miR-93* and 3 experiments for *miR-292*) are shown.
(**D**) Micrographs showing morphology of control 32D cells (A1-EV), 32D-A8-miR-292 and 32D-F5-miR-93 cells on day 7 of CSF3 treatment. Bar indicates 10 µM.

Suppl. Table 1: List of miRNAs cloned in MSCV-BC vector as shown in Figure 1A

BCA10-miR-135	BCC5-let-7a-2	BCB12-miR-425	BCF10-miR-128-1
BCA10-miR-294	BCC5-miR-186	BCB12-miR-7b	BCF10-miR-142
BCA11-miR-124a-2	BCC6-let-7c-1	BCB1-miR-328	BCF11-miR-143
BCA11-miR-141	BCC6-miR-151	BCB1-miR-365	BCF12-miR-214
BCA11-miR-215	BCC7-let-7d	BCB1-miR-431	BCF1-let-7a-1
BCA12-miR-1-2	BCC7-miR-216	BCB2-miR-331	BCF1-miR-129-1
BCA12-miR-184	BCC7-miR-296	BCB2-miR-365-2	BCF1-miR-467
BCA12-miR-298	BCC7-miR-297-1	BCB2-miR-448	BCF2-let-7c-2
BCA1-miR-196b	BCC8-miR-224	BCB3-miR-471	BCF2-miR-10b
BCA1-miR-21	BCC9-let7f-1	BCB3-miR-483	BCF2-miR-130b
BCA1-miR-29a	BCD10-miR-10b	BCB4-miR-19a	BCF2-miR-219-2
BCA2-miR-199a-2	BCD10-miR-199a	BCB4-miR-33	BCF3-let-7g
BCA2-miR-222	BCD11-miR-101a	BCB4-miR-379	BCF3-miR-127
BCA2-miR-29b-1	BCD11-miR-125a	BCB5-miR-340	BCF3-miR-132
BCA3-miR-223	BCD12-miR-143	BCB5-miR-380	BCF4-miR-133a-1
BCA3-miR-300	BCD1-miR-124a	BCB5-miR-452	BCF4-miR-199b
BCA4-miR-199a-1	BCD1-miR-133a	BCB6-miR-200b	BCF5-miR-103-2
BCA4-miR-22	BCD2-miR-139	BCB6-miR-341	BCF5-miR-133a-2
BCA4-miR-301	BCD2-miR-150	BCB6-miR-464	BCF5-miR-7a-1
BCA5-miR-18	BCD2-miR-215	BCB7-miR-202	BCF5-miR-93
BCA5-miR-19b-2	BCD3-miR-15b	BCB7-miR-342	BCF6-miR-101a
BCA5-miR-24-2	BCD3-miR-29b	BCB7-miR-466	BCF6-miR-134
BCA6-miR-290	BCD4-miR-133b	BCB8-miR-205	BCF6-miR-206
BCA6-miR-382	BCD4-miR-127	BCB8-miR-345	BCF7-miR-136
BCA6-miR-7a-1	BCD4-miR-133a	BCB8-miR-409	BCF7-miR-30c-2
BCA7-miR-169-2	BCD5-let-7c	BCB9-miR-346	BCF8-miR-107
BCA7-miR-201	BCD5-miR-125b	BCB9-miR-410	BCF8-miR-139
BCA7-miR-291	BCD5-miR-199b	BCB9-miR-468	BCF8-miR-26b
BCA8-miR-292	BCD6-miR-16-1	BCC10-miR-378	BCF9-miR-124a-1
BCA8-miR-384	BCD6-miR-26b	BCC10-miR-484	BCF9-miR-140
BCA9-miR-293	BCD7-miR-128a	BCC11-miR-1-2	BCF9-miR-149
BCA9-miR-429	BCD7-miR-154	BCC12-miR-339	BCG10-miR-181b-2
BCA9-miR-92_2	BCD8-let-7-1a	BCC1-miR-377	BCG11-miR-187
BCB10-miR-208	BCD8-miR-129-1	BCC1-miR-9	CG12-miR-192
BCB10-miR-211	BCD9-miR-15a	BCC2-miR-27b	BCG1-miR-144
BCB10-miR-34b	BCD9-miR-206	BCC2-miR-485	BCG1-miR-194-1
BCB10-miR-411	CE2-miR-155	BCC2-miR-92-2	BCG1-miR-195
BCB10-miR-470	BCE3-miR-143	BCC3-miR-146	BCG2-miR-145
BCB11-miR-217	BCE6-miR-130b	BCC3-miR-98	BCG2-miR-215
BCB11-miR-34c	BCE7-miR-31	BCC4-miR-99	BCG3-miR-150
BCB11-miR-412	BCE8-miR-451	BCG8-miR-15b	BCG3-miR-29b-2
BCB11-miR-434	BCF10-miR-124-2	BCG7-miR-15a	BCG4-miR-152
BCB12-miR-26a-2	BCB12-miR-361	BCG6-miR-154	BCG5-miR-153

Suppl. Table 2: AAAGUGC seed miRNAs of which the expression of some are shown in Figure 2

mouse microRNAs	mature miR sequence	Genome context	host gene
mmu-miR-17*	CAAAGUGCUUACAGUGCAGGUAG	14: 115442893-115442976 [+]	intergenic
mmu-miR-20a*	UAAAGUGCUUAUAGUGCAGGUAG	14: 115443379-115443485 [+]	intergenic
mmu-miR-106b*	UAAAGUGCUGACAGUGCAGAU	5: 138606965-138607046 [-]	MCM-7
mmu-miR-93*	CAAAGUGCUGUUCGUGCAGGUAG	5: 138606751-138606838 [-]	MCM-7
mmu-miR-106a*	CAAAGUGCUAACAGUGCAGGUAG	X: 50095680-50095744 [-]	intergenic
mmu-miR-20b*	CAAAGUGCUCAUAGUGCAGGUAG	X: 50095290-50095369 [-]	intergenic
mmu-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG	3: 127248146-127248219 [+]	Larp-7
mmu-miR-302c	AAGUGCUUCCAUGUUUCAGUGG	3: 127248281-127248348 [+]	Larp-7
mmu-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	3: 127248414-127248482 [+]	Larp-7
mmu-miR-302d	UAAGUGCUUCCAUGUUUGAGUGU	3: 127248542-127248607 [+]	Larp-7
mmu-miR-290-3p	AAAGUGCCGCCUAGUUUUAAGCCC	7: 3218627-3218709 [+]	intergenic
mmu-miR-291a-3p	AAAGUGCUUCCACUUUGUGUGC	7: 3218920-3218709 [+]	intergenic
mmu-miR-292-3p	AAAGUGCCGCCAGGUUUUGAGUGU	7: 3219190-3219271 [+]	intergenic
mmu-miR-291b-3p	AAAGUGCAUCCAUUUUGUUUGU	7: 3219483-3219561 [+]	intergenic
mmu-miR-294	AAAGUGCUUCCCUUUUGUGUGU	7: 3220642-3220725 [+]	intergenic
mmu-miR-295	AAAGUGCUACUACUUUUGAGUCU	7: 3220774-3220842 [+]	intergenic
Human microRNAs	mature miR sequence	Genome context	host gene
hsa-miR-17*	CAAAGUGCUUACAGUGCAGGUAG	13: 92002859-92002942 [+]	intergenic
hsa-miR-20a*	UAAAGUGCUUAUAGUGCAGGUAG	13: 92003319-92003389 [+]	intergenic
hsa-miR-106b*	UAAAGUGCUGACAGUGCAGAU	7: 99691616-99691697 [-]	MCM-7
hsa-miR-93*	CAAAGUGCUGUUCGUGCAGGUAG	7: 99691391-99691470 [-]	MCM-7
hsa-miR-106a*	AAAAGUGCUUACAGUGCAGGUAG	X: 133304228-133304308 [-]	intergenic
hsa-miR-20b*	CAAAGUGCUCAUAGUGCAGGUAG	X: 133303839-133303907 [-]	intergenic
hsa-miR-512-1-3p	AAGUGCUGUCAUAGCUGAGGUC	19: 54169933-54170016 [+]	intergenic
hsa-miR-512-2-3p	AAGUGCUGUCAUAGCUGAGGUC	19: 54172411-54172508 [+]	intergenic
hsa-miR-520e	AAAGUGCUUCCUUUUUGAGGG	19: 54178965-54179051 [+]	intergenic
hsa-miR-519e	AAGUGCCUCCUUUUAGAGUGUU	19: 54183194-54183277 [+]	intergenic
hsa-miR-520f	AAGUGCUUCCUUUUAGAGGGUU	19: 54185413-54185499 [+]	intergenic
hsa-miR-515-3p	GAGUGCCUUCUUUUGGAGCGUU	19: 54188263-54188345 [+]	intergenic
hsa-miR-519c-3p:	AAAGUGCAUCUUUUUAGAGGAU	19: 54189723-54189809 [+]	intergenic
hsa-miR-520a-3p	AAAGUGCUUCCCUUUGGACUGU	19: 54194135-54194219 [+]	intergenic
hsa-miR-526b	GAAAGUGCUUCCUUUUAGAGGC	19: 54197647-54197729 [+]	intergenic
hsa-miR-519b-3p	AAAGUGCAUCCUUUUAGAGGUU	19: 54198467-54198547 [+]	intergenic
hsa-miR-520b	AAAGUGCUUCCUUUUAGAGGG	19: 54204481-54204541 [+] i	intergenic
hsa-miR-520c-3p	AAAGUGCUUCCUUUUAGAGGGU	19: 54210707-54210793 [+]	intergenic
hsa-miR-519d:	CAAAGUGCCUCCCUUUAGAGUG	19: 54216601-54216688 [+]	intergenic
hsa-miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU	19: 54223350-54223436 [+]	intergenic
hsa-miR-519a-1	AAAGUGCAUCCUUUUAGAGUGU	19: 54255651-54255735 [+]	intergenic
hsa-miR-519a-2	AAAGUGCAUCCUUUUAGAGUGU	19: 54265598-54265684 [+]	intergenic
hsa-miR-372	AAAGUGCUGCGACAUUUGAGCGU	19: 54291144-54291210 [+]	intergenic
hsa-miR-373	GAAGUGCUUCGAUUUUGGGGUGU	19: 54291959-54292027 [+]	intergenic
hsa-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG	4: 113569641-113569713 [-]	Larp-7
hsa-miR-302c	UAAGUGCUUCCAUGUUUCAGUGG	4: 113569519-113569586 [-]	Larp-7
hsa-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	4: 113569339-113569407 [-]	Larp-7
hsa-miR-302d	UAAGUGCUUCCAUGUUUGAGUGU	4: 113569030-113569097 [-]	Larp-7

AAAGUGC seed-containing miRNAs are listed. * Are expressed in hematopoietic cells.

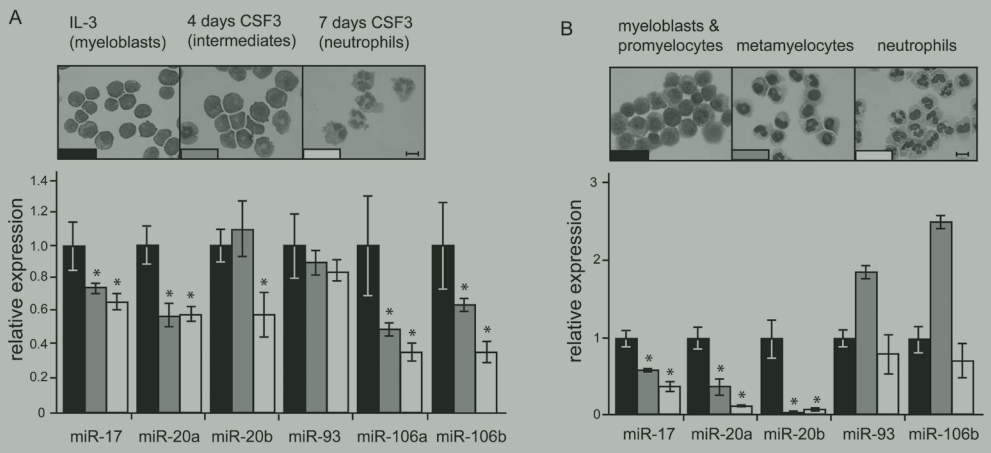


Figure 2: *MiR-17/20/93/106* expression levels during mouse and human neutrophil differentiation. (**A**) 32D-CSF3R cells were switched from IL-3 to CSF3-containing medium on day t=0. Micrographs show the morphology of 32D-CSF3R cells on indicated time points of CSF3 treatment. Bars indicate 10 μm. Total RNA was isolated at indicated time points. Expression of indicated mature miRNAs was measured in triplicate with quantitative reverse-transcription-polymerase chain reaction (qRT-PCR). The miRNA expressions relative to SnoRNA-234 were measured from immature (steady state, IL-3) samples and set to 1. The average fold expression (n=3) at indicated time points relative to the IL-3 condition are shown. Error bars represent standard deviation. Significance was calculated by comparing the samples of steady state and the different time points of CSF3 treatment with the Mann-Whitney test (Asymp. Sig. (2-tailed)). * $p < 0.05$. (**B**) Indicated myeloid cell fractions from human cord blood were stained with cell type specific antibodies and isolated using FACS sort. Micrographs show morphology of sorted cell fractions. Bars indicate 10 μm. MiRNA levels and significance were measured and calculated as described in A.

Ectopic expression of *miR-17*, *-20*, *-93* and *-106* promotes expansion and replating capacity of myeloid progenitors and enhances MAP kinase signalling

Next, we tested whether ectopic expression of these miRNAs would also affect the outgrowth of myelo-neutrophilic progenitor cells (granulocyte-colony-forming unit [CFU-G]). Mouse lineage marker negative (Lin-) hematopoietic progenitor cells isolated from the bone marrow (BM) were transduced with MSCV-miR-17, -20, -93, -106 or MSCV-EV control viruses. We observed an up to 2.5 -fold increase in the number of CSF3-induced colonies which are formed by Lin- cells transduced with MSCV-miR-17, -20, -93 and -106 containing vectors as compared to control infected cells (Fig.3A). Notably, not only the number of colonies, also colony size increased markedly when these miRNAs are overexpressed (Fig.3A). These differentiated cells appeared morphologically normal (Suppl. Fig 1A). Next, we asked whether enhanced expression of *miR-17*, *-20*, *-93* and *-106* has an effect on replating capacity of hematopoietic cells. To study this, we transduced Lin- cells isolated from the BM and performed a progenitor replating assay. We observed markedly more and bigger colonies of MSCV-miR-17, -20, -93 and -106 transduced cells compared to control cells after the second plating (Fig.3B), indicating an enhanced self-renewal capacity of cells that ectopically express the latter AAAGUGC-seed miRNAs. No colony formation was observed after the third plating of 10^4 cells, demonstrating that these cells do not self-renew *ad infinitum* under these conditions. To assess the effects of AAAGUGC seed miRNAs expression also *in vivo*, MSCV-miR-17, -20, -93, -106 and MSCV-EV virus infected Lin- GFP^+ BM cells (~20%) mixed with wt Lin- cells (~80%) were transplanted in lethally irradiated recipient mice. There was a considerable proliferation advantage for the multi-potent progenitors (Lin^-, $Sca\text{-}I^+$, $c\text{-}Kit^{+/-}$) expressing *miR-17*, *-20*, *-93* and *-106* over wt cells compared to EV-transduced cells at 6 weeks post-transplantation as is evident from the Fold Induction of miRNA containing GFP^+ cells (Fig.3C). Immunophenotypic analyses of *miR-17*, *-20*, *-93* and *-106* expressing BM and blood cells showed no aberrant myeloid differentiation (Suppl.Fig.1B, 1C). To investigate whether AAAGUGC seed-containing miRNAs control expansion of granulocytic progenitors through the regulation of cytokine-induced signalling, we analyzed CSF3-induced phosphorylation of several signalling intermediates in presence and absence of AAAGUGC miRNAs. Hematopoietic Ba/F3 cells co-expressing the CSF3R and *miR-17*, *-20, -93* and *-106* were factor deprived and restimulated with CSF3. Phosphorylation of extracellular signal-regulated kinase (ERK) was enhanced in cells expressing the different AAAGUGC seed-containing miRNAs compared to control cells (Fig.3D). Strikingly, enhanced phosphorylation levels were not observed for other signalling molecules such as STAT5, STAT3 and AKT (data not shown), suggesting the specific regulation of mitogen-activated protein (MAP) kinase activity. Collectively, increased expression of AAAGUGC-seed containing miRNAs in Lin- BM cells promotes replating capacity and expansion of myeloid progenitors and CSF3-induced MAP kinase signallin

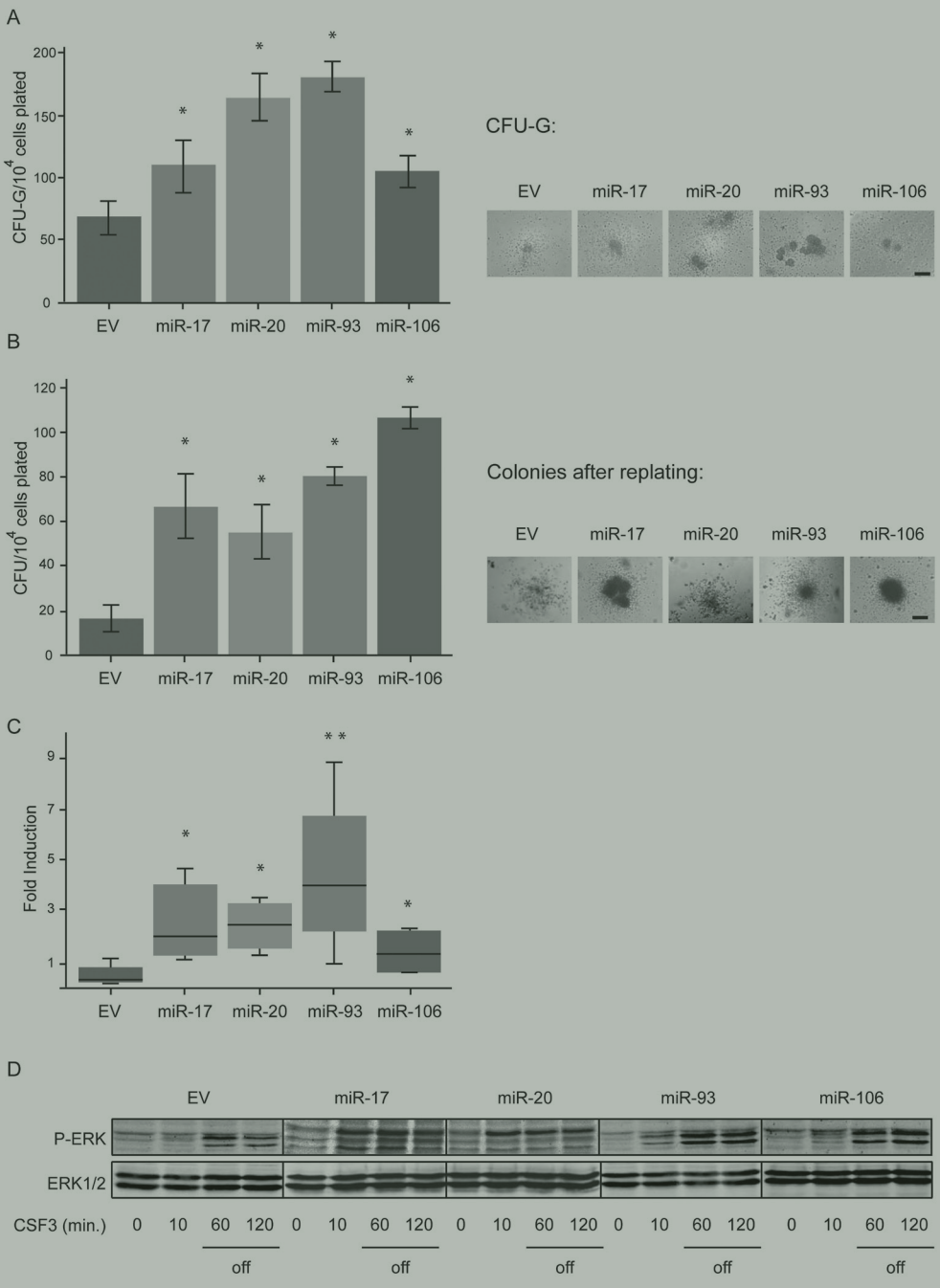

A
CFU-G/10^4 cells plated
200
150
100
50
0
EV
miR-17
miR-20
miR-93
miR-106
CFU-G:
EV
miR-17
miR-20
miR-93
miR-106
B
CFU/10^4 cells plated
120
100
80
60
40
20
0
EV
miR-17
miR-20
miR-93
miR-106
Colonies after replating:
EV
miR-17
miR-20
miR-93
miR-106
C
Fold Induction
9
7
5
3
1
EV
miR-17
miR-20
miR-93
miR-106
D
EV
miR-17
miR-20
miR-93
miR-106
P-ERK
ERK1/2
CSF3 (min.)
0 10 60 120 0 10 60 120 0 10 60 120 0 10 60 120 0 10 60 120
off
off
off
off
off

Figure 3: Functional analysis of *miR-17/20/93/106* in primary mouse Lin⁻ BM cells. (**A**) CFU-G assay of mouse lin- BM progenitors infected with MSCV-miR-17, -miR-20, -miR-93, miR-106 or empty vector control virus. Cells were plated in triplicate at densities of $1x10^4$ cells per dish in 1 mL methylcellulose medium containing CSF3 (100 ng/mL). Colonies consisting of more than 50 cells were counted after 7 days of growth. Micrographs show size of CFU-CSF3 on day 7 after plating. Bar indicates 100 μm. Significance was calculated by comparing the samples with EV control and the different miRNAs with the Mann-Whitney test (Asymp. Sig. (2-tailed)). * $p< 0.05$. (**B**) Replating assay of mouse lin- BM progenitors infected with MSCV-miR-17, -miR-20, -miR-93, miR-106 or empty vector control virus. Cells were plated in triplicate at densities of $1x10^4$ cells per dish in 1 mL methylcellulose medium containing IL-6 (10 ng/mL), IL-3 (supernatant 1/1000), SCF (10 ng/mL), CSF2 (10 ng/mL). Cells were isolated from dishes,counted and replated under the same conditions. Colonies from the second plating were counted after 7 days of growth. Micrographs show size of CFU's on day 7 after plating. Bar indicates 100 μm. Significance was calculated as described in A. (**C**) Mouse lineage negative progenitor cells isolated from the BM of C57BL/6 mice were infected with MSCV-miR-17, -miR-20, -miR-93, miR-106 or empty vector control virus. Recipient C57BL/6 mice were irradiated (8.5 Gy) and reconstituted with ~20% GFP positive cells and ~80% WT cells. Six weeks post-transplantation, mice were sacrificed and BM cells were isolated. Lin- cells were stained for flow cytometry analysis. The fold induction of the percentage GFP^+ control (n=4), miR-17 (n=6), miR-20 (n=4), miR-93 (n=4), miR-106 (n=6) Lin-; $Sca\text{-}I^+$; $c\text{-}Kit^{+/-}$ cells in the BM compared to the input are shown. Significance was calculated by comparing the samples of mice transplanted with EV control and the different miRNAs with the Mann-Whitney test (Asymp. Sig. (2-tailed)). * $p< 0.05$ and ** $p=0.057$. (**D**) Ba/F3-CSF3R cells were infected with MSCV-miR-17, miR-20, miR-93, miR-106 or EV control viruses. Cells were factor deprived for 4 hours (t=0) followed by CSF3 (100 ng/mL) stimulation for 10 minutes. Cells were washed 2 times with PBS and incubated in RPMI medium for 60 or 120 minutes. Samples for cell lysates were taken at indicated time points and analyzed by Western blotting using total and phosphor- specific antibodies against ERK.

MiR-17, *-20*, *-93*, and *-106* regulate SQSTM1 levels in hematopoietic cells

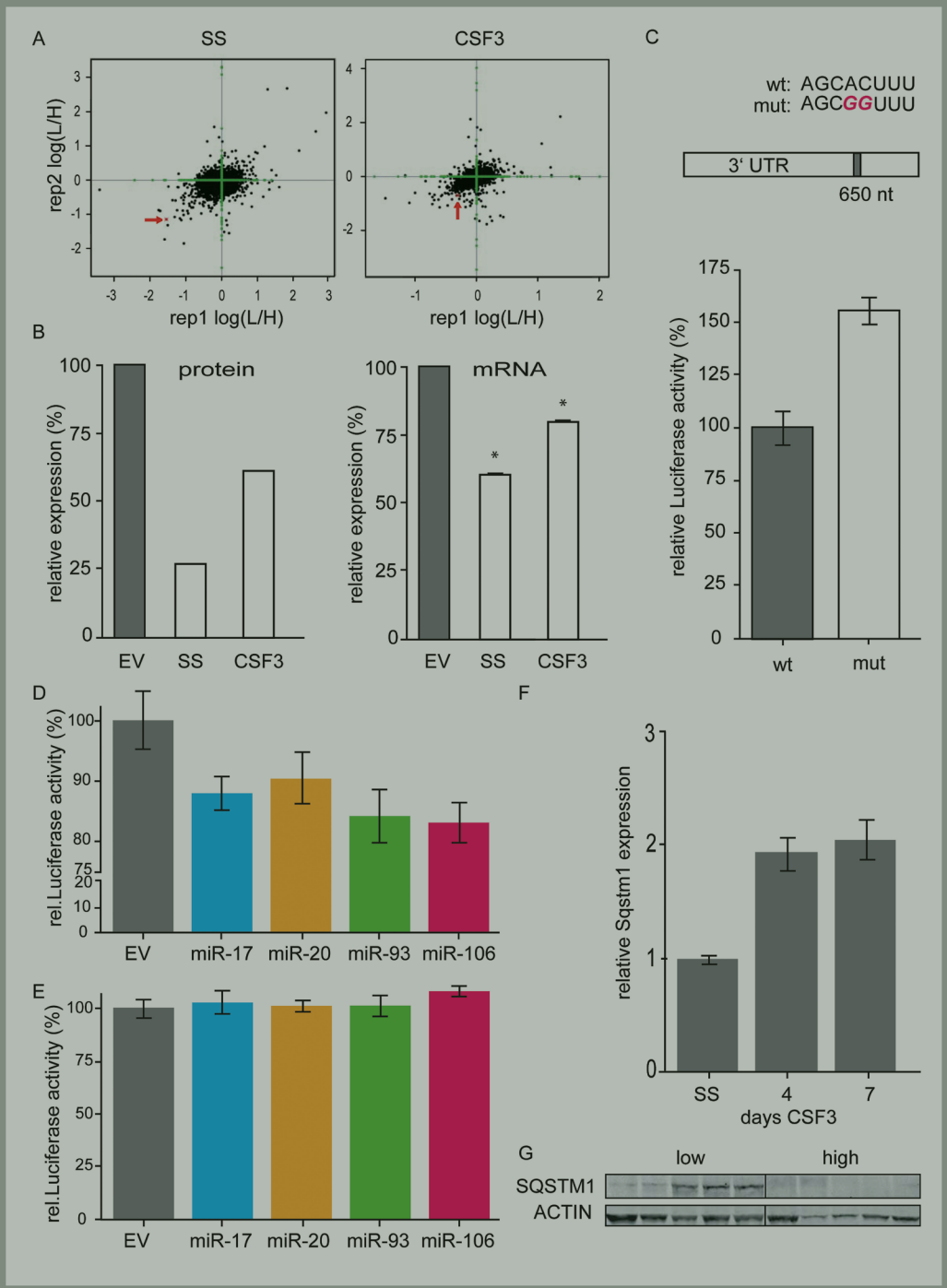
We investigated how *miR-93* promotes cell expansion by identification of the target transcripts in 32D-CSF3R cells. To this end, we analyzed the proteome of 32D-CSF3R cells expressing *miR-93* compared to EV control cells using the SILAC quantitative proteomics approach. 32D-CSF3R-EV control cells were grown in medium containing $^{13}C_6$-labeled (heavy) Lysine (Lys6) and Arginine (Arg6) and 32D-CSF3R-miR-93 cells in medium containing $^{12}C_6$ (regular) Lys and Arg. In addition, these cells were switched to CSF3-containing medium for 4 days. For all the samples measured we identified at least 3400 unique proteins with 2 or more peptides per protein (for top-100 downregulated proteins see Suppl.Table 3 and 4). Most predicted targets for *miR-93* by Target scan (http://www.targetscan.org) are not or slightly downregulated (Suppl.Table 3 and 4). Sequestosome 1 (SQSTM1) was identified as the only protein from the top-20 downregulated hits for *miR-93* with a predicted recognition site for *miR-17/20/93/106/519d* in the 3'UTR (Suppl. Table 3, 4 and Fig.4A). SQSTM1 was identified based on 9 unique peptides. In addition to reduced protein levels, diminished *Sqstm1* mRNA was detected by QPCR in cells expressing *miR-93* (Fig.4B).

Sqstm1 is a predicted target of *miR-17/20/93/106* by target scan as its transcript contains a well conserved 8-mer site at position 650 (AGCACUUU) of the *Sqstm1* 3'UTR (Fig.4C). To test whether this putative miRNA binding site controls SQSTM1 expression, we cloned the full length *Sqstm1* 3'UTR behind a Luciferase reporter and compared Luciferase activity of the reporters containing a wt *Sqstm1* 3'UTR with a mutated 3' UTR lacking the 8-mer miRNA recognition site. Indeed, a two nucleotide mutation of the miRNA binding site resulted in 60% increased Luciferase activity compared to the wt control 3'UTR (Fig.4C), indicating regulatory functions of this sequence on protein expression. Enhanced expression of *miR-17*, *-20*, *-93* and *-106* resulted in an additional 15% downregulation of Luciferase activity using the wt *Sqstm1* 3'UTR (Fig.4D), which was not observed with the mutant 3'UTR (Fig.4E).

Figure 4: Identification of SQSTM1 as a target for AAAGUGC seed-containing miRNAs. The myeloid cell line 32D was infected with miR-93 or empty vector (EV) control viruses. The EV control cells were grown in RPMI media containing ^{13}C-labeled Lys and Arg (heavy) and 32D-miR-93 cells in medium containing regular Lys and Arg (light). Cells were counted and *miR-93* containing cells were either mixed with EV control cells in a 1:1 ratio for proteomics analysis or subjected for mRNA isolation. (**A**) Samples were prepared and analyzed by quantitative proteomics. The ratios of protein expression in steady state (ss) conditions and at four days of CSF3 treatment are plotted. The correlation of the average log (L/H) values (n=2 independent measurements) of a biological duplicate (rep1=X-axis and rep2=Y-axis) of all the identified proteins are indicated by the black dots. Single identifications from one experiment are indicated by the green dots. SQSTM1 is indicated by the red arrow and dot. (**B**) The average SQSTM1 protein levels (n=2 independent measurements) of a biological duplicate and the avarage mRNA abundances relative to GAPDH and EV control cells in steady state (ss) conditions and at four days of CSF3 treatment are shown (n=3). Error bars represent standard deviation (mRNA). Significance was calculated by comparing the EV control with the steady state and CSF3 condition with the Mann-Whitney test (Asymp. Sig. (2-tailed)). * $p< 0.05$. (**C**) Luciferase reporter plasmids containing the full length 3'UTR sequence of *Sqstm1* with a wt or mutated miRNA binding site were generated and transfected with the Firefly control vector into HEK293 cells. Two days after transfection, cells were lysed and assayed for Luciferase activity. Luciferase values were normalized against Firefly activity. Normalized values for wt 3'UTR were put to 100%. The Luciferase activity values of the mutant 3'UTR relative to the wt is shown. Error bars represent standard deviation of three experiments. (**D**) Same as C. Now the Luciferase reporter plasmids containing the full length 3'UTR sequence of *Sqstm1* were cotransfected with indicated MSCV-miRs. The Luciferase activity values of the miRNA expressing cells relative to the Empty vector control are shown. Error bars represent standard deviation of three experiments. (**E**) Same as D. Now the Luciferase reporter plasmids containing the mutant 3'UTR sequence of *Sqstm1* were cotransfected with indicated MSCV-miRs. (**F**) 32D-CSF3R cells were switched from IL-3 to CSF3-containing medium on t=0 days (steady state, SS). Total RNA was isolated at indicated time points. *Sqstm1* levels were measured by qPCR in triplicate. *Sqstm1* expression relative to *Gapdh* and to SS condition is shown. (**G**) Western blot analysis of normal karyotype AML samples exhibiting low and high *miR-106* with monoclonal anti-SQSTM1 antibodies. ACTIN was stained for loading control.

These results indicate the predicted target-site as the important determinant for miR-17/20/93/106-mediated regulation of SQSTM1 expression. However, the 3'parts of the miRNAs tested might still modulate the action of the miRNAs. In addition, we found increased expression of *sqstm1* transcripts during CSF3-induced neutrophil differentiation of 32D-CSF3R cells (Fig.4F) and an inverse correlation of SQSTM1 protein levels and *miR-106* expression in AML samples (Fig.4G).

We reasoned that, if AAAGUGC miRNAs promote cell expansion by repressing the expression of SQSTM1, this effect should be at least partially pheno-copied by shRNA-mediated knockdown of SQSTM1. Indeed, we observed bigger colonies and an enhanced replating capacity, using two different specific shRNAs against *Sqstm1* compared to control cells (Fig.5A). In addition, we noted an enhanced cell expansion of *Sqstm1*-shRNA expressing progenitor cells compared to controls in mice (Fig.5B). These results reveal SQSTM1 as a key effector of AAAGUGC-seed containing miRNAs in myeloid cells at the advantage of cell expansion.


A
SS
CSF3
rep2 log(L/H)
rep1 log(L/H)
rep1 log(L/H)
B
protein
mRNA
relative expression (%)
EV
SS
CSF3
C
wt: AGCACUUU
mut: AGCGGUUU
3‘ UTR
650 nt
relative Luciferase activity (%)
wt
mut
D
rel.Luciferase activity (%)
EV
miR-17
miR-20
miR-93
miR-106
E
rel.Luciferase activity (%)
F
relative Sqstm1 expression
SS
4
7
days CSF3
G
low
high
SQSTM1
ACTIN

Suppl. Table3: TOP100 downregulated proteins. SQSTM1 is further described in Figure 4

sample:	miR-93 SS					
name	ratio miR-93/EV	miR-17-5p_20_93 mr_106_519.d	miR-290-3p_ 292-3p_467a	miR-291b-3p_519a_ 519b-3p_519c-3p	miR-302ac_ 520f	miR-106_302
Epx	0.189640378	FALSE	FALSE	FALSE	FALSE	FALSE
Cyp11a1;Cyp11a	0.244300303	FALSE	FALSE	FALSE	FALSE	FALSE
Ctsg	0.252177483	FALSE	FALSE	FALSE	FALSE	FALSE
Sqstm1;A170;STAP	0.264919766	TRUE	FALSE	FALSE	FALSE	FALSE
Cfp;Pfc	0.292476385	FALSE	FALSE	FALSE	FALSE	FALSE
Ero1l	0.293427764	FALSE	FALSE	FALSE	FALSE	FALSE
Fcgr2;Fcgr2b;Ly-17	0.303957203	FALSE	FALSE	FALSE	FALSE	FALSE
Avil;Advil	0.316909605	FALSE	FALSE	FALSE	FALSE	FALSE
P4ha1	0.318471884	FALSE	FALSE	FALSE	FALSE	FALSE
S100a10;Cal1l	0.350321012	FALSE	FALSE	FALSE	FALSE	FALSE
Niban;Fam129a	0.353289073	FALSE	FALSE	TRUE	FALSE	FALSE
B4galnt1;Galgt;Galgt1;Ggm2	0.371788003	FALSE	FALSE	FALSE	FALSE	FALSE
Anxa3;Anx3	0.373166013	FALSE	FALSE	FALSE	FALSE	FALSE
Ccnh	0.393986566	FALSE	FALSE	FALSE	FALSE	FALSE
Serpinb1a;Serpinb1	0.394392212	FALSE	FALSE	FALSE	FALSE	FALSE
Alas1	0.403828753	FALSE	FALSE	FALSE	FALSE	FALSE
Fxyd5;Oit2	0.411707164	FALSE	FALSE	FALSE	FALSE	FALSE
Gng2	0.424553102	FALSE	FALSE	FALSE	FALSE	FALSE
Tf;Trf	0.438379626	FALSE	FALSE	FALSE	FALSE	FALSE
Ghitm	0.439506546	FALSE	FALSE	FALSE	FALSE	FALSE
Il2ra;Il2r	0.448603787	FALSE	FALSE	FALSE	FALSE	FALSE
Gm2a	0.44962593	FALSE	FALSE	FALSE	FALSE	FALSE
Lonp1;Prss15	0.452361812	FALSE	FALSE	FALSE	FALSE	FALSE
Degs1;Degs;Des1;Mdes	0.459953188	FALSE	FALSE	FALSE	FALSE	FALSE
Cd82;Kai1	0.470799852	FALSE	FALSE	FALSE	FALSE	FALSE
Mthfd2;Nmdmc	0.479797808	FALSE	FALSE	FALSE	FALSE	FALSE
Glipr2;Gapr1	0.480884429	FALSE	FALSE	FALSE	FALSE	FALSE
Kif23;KIF23	0.48660962	TRUE	FALSE	TRUE	FALSE	FALSE
Apob48r	0.490503564	FALSE	FALSE	FALSE	FALSE	FALSE
Plac8	0.510975583	FALSE	FALSE	FALSE	FALSE	FALSE
S100a6;Cacy	0.516757688	FALSE	FALSE	FALSE	FALSE	FALSE
Asph	0.52066461	FALSE	FALSE	FALSE	FALSE	FALSE
Hk3	0.523998228	FALSE	FALSE	FALSE	FALSE	FALSE
Gla;Ags	0.526959298	FALSE	FALSE	FALSE	FALSE	FALSE
Galnt6	0.532014842	FALSE	FALSE	FALSE	FALSE	FALSE
Dnajc6;Kiaa0473	0.5335619	FALSE	FALSE	FALSE	FALSE	FALSE
Incenp	0.539419464	FALSE	FALSE	FALSE	FALSE	FALSE
Pck2	0.542899179	FALSE	FALSE	FALSE	FALSE	FALSE
Sc4mol	0.547020613	FALSE	FALSE	FALSE	FALSE	FALSE
Tmx4;Txndc13	0.552762023	FALSE	FALSE	FALSE	FALSE	FALSE
Zfp62	0.553934155	FALSE	FALSE	FALSE	FALSE	FALSE
Asns	0.556837579	FALSE	FALSE	FALSE	FALSE	FALSE
Zbtb11	0.563517348	FALSE	FALSE	FALSE	TRUE	TRUE
Arhgap15	0.564434529	FALSE	FALSE	FALSE	FALSE	FALSE
Pdk1	0.566496115	FALSE	FALSE	FALSE	FALSE	FALSE
Tbc1d9b;Kiaa0676	0.572279835	FALSE	FALSE	FALSE	FALSE	FALSE
Anxa2;Anx2;Cal1h	0.574160214	FALSE	FALSE	FALSE	FALSE	FALSE
Gpt2;Aat2	0.5808154	FALSE	FALSE	FALSE	FALSE	FALSE

Csf2rb2;Ai2ca;Il3r;Il3rb2	0.583136327	FALSE	FALSE	FALSE	FALSE	FALSE
Ttc3;mKIAA4119	0.58406904	FALSE	FALSE	FALSE	FALSE	FALSE
Dhrs1;D14ertd484e	0.58602669	FALSE	FALSE	FALSE	FALSE	FALSE
Tm9sf1	0.594048025	FALSE	FALSE	FALSE	FALSE	FALSE
Vps13c	0.596341758	FALSE	FALSE	FALSE	FALSE	FALSE
Ak3l1;Ak-4;Ak3b;Ak4	0.600114777	FALSE	FALSE	FALSE	FALSE	FALSE
Golm1;Golph2	0.600359885	FALSE	FALSE	FALSE	FALSE	FALSE
Arhgap5;Rhogap5	0.601314624	FALSE	FALSE	FALSE	FALSE	FALSE
Aurkb;Ark2;Stk1;Stk12;Stk5	0.602231739	FALSE	FALSE	FALSE	FALSE	FALSE
Rab27a	0.603483144	FALSE	FALSE	FALSE	FALSE	FALSE
Man2a1;Mana2	0.609888608	FALSE	FALSE	FALSE	FALSE	FALSE
Arhgap6	0.611720454	FALSE	FALSE	FALSE	FALSE	FALSE
Hspa9;Grp75;Hsp74;Hspa9a	0.613409313	FALSE	FALSE	FALSE	FALSE	FALSE
Slc3a2;Mdu1	0.615283413	FALSE	FALSE	FALSE	FALSE	FALSE
Hmmr;Ihabp;Rhamm	0.61612359	FALSE	FALSE	FALSE	FALSE	FALSE
Unc84b;Sun2;mKIAA0668	0.616208459	FALSE	FALSE	FALSE	FALSE	FALSE
Acsl4;Acs4;Facl4	0.616924572	TRUE	FALSE	TRUE	FALSE	FALSE
Kif22	0.62194948	FALSE	FALSE	FALSE	FALSE	FALSE
Myo1f;mFLJ00395	0.622471604	FALSE	FALSE	FALSE	FALSE	FALSE
Cat;Cas-1;Cas1	0.623515192	FALSE	FALSE	FALSE	FALSE	FALSE
Sgpl1	0.624429986	FALSE	FALSE	FALSE	FALSE	FALSE
Fdxr	0.62487883	FALSE	FALSE	FALSE	FALSE	FALSE
Hip1r	0.626153395	FALSE	FALSE	FALSE	FALSE	FALSE
Prc1	0.627284107	FALSE	FALSE	FALSE	TRUE	FALSE
Ormdl1;Ormdl2	0.627718107	FALSE	FALSE	FALSE	FALSE	FALSE
Man2a2	0.62887054	FALSE	FALSE	FALSE	FALSE	FALSE
Ncf4	0.630539148	FALSE	FALSE	FALSE	FALSE	FALSE
Man1a1;Man1a	0.631512079	FALSE	FALSE	FALSE	FALSE	FALSE
Slc9a3r1;Nherf;Nherf1	0.631764379	FALSE	FALSE	FALSE	FALSE	FALSE
H2-D1;H2-K1;H2-K;H2-Q2	0.632868208	FALSE	FALSE	FALSE	FALSE	FALSE
Idua	0.633757121	FALSE	FALSE	FALSE	FALSE	FALSE
Atg7;Apg7l	0.634682752	FALSE	FALSE	FALSE	FALSE	FALSE
Flnb	0.636318942	FALSE	FALSE	FALSE	FALSE	FALSE
Rdh11;Arsdr1;Mdt1;Psdr1	0.637106786	FALSE	FALSE	FALSE	FALSE	FALSE
Tpm4	0.637515585	FALSE	FALSE	FALSE	FALSE	FALSE
Osbpl8;mKIAA1451	0.641548106	FALSE	FALSE	FALSE	TRUE	FALSE
Pgrmc1;Pgrmc	0.641603095	FALSE	FALSE	FALSE	FALSE	FALSE
Ptk2b;Fak2;Pyk2;Raftk	0.644220202	FALSE	FALSE	FALSE	FALSE	FALSE
Cep55	0.645023164	FALSE	FALSE	FALSE	FALSE	FALSE
Tgfb1	0.645130095	FALSE	FALSE	FALSE	FALSE	FALSE
Fcer1g;Fce1g	0.648404683	FALSE	FALSE	FALSE	FALSE	FALSE
Ipo4;Imp4a;Ranbp4	0.651553222	FALSE	FALSE	FALSE	FALSE	FALSE
Acot2;Mte1;Acot1;Cte1	0.651729173	FALSE	FALSE	FALSE	FALSE	FALSE
H2-T23	0.652539929	FALSE	FALSE	FALSE	FALSE	FALSE
Tax1bp1	0.653239227	FALSE	FALSE	FALSE	FALSE	FALSE
Dnaja3;Tid1	0.654756406	FALSE	FALSE	FALSE	FALSE	FALSE
Ikzf2;Helios;Zfpn1a2;Znfn1a2	0.65509353	FALSE	TRUE	TRUE	TRUE	TRUE
Lamp2	0.655347117	FALSE	FALSE	FALSE	FALSE	FALSE
Mgat2	0.655920949	FALSE	FALSE	FALSE	FALSE	FALSE
Csnk2b;Ck2n	0.658935902	FALSE	FALSE	FALSE	FALSE	FALSE
Tmx3;Kiaa1830;Txndc10	0.659932703	FALSE	FALSE	FALSE	FALSE	FALSE
Kif11	0.661081818	FALSE	FALSE	FALSE	FALSE	FALSE

Suppl. Table 4 : TOP100 downregulated proteins

sample: miR-93 CSF3

name	measurement 1	measurement 2	miR-17-5p_20_93 mr_106_519.d	miR-290-3p_ 292-3p_467a	miR-291b-3p_519a_ 519b-3p_519c-3p	miR-302ac_ 520f	miR-106_302	Predicted Target	Diff_max
Bcr;Kiaa3017	0.005480353	0.002691935	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
S100a6;Cacy	0.226639168	0.448994253	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Gm2a	0.335683115	0.379434642	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Lilrb4;Gp49b	0.487329435	0.362004054	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Cst7	0.548125411	0.32011268	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Ctss;Cats	0.367822857	0.548486178	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Hsd17b10;Erab	0.50193244	0.420716059	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Avil;Advil	0.48809059	0.469461528	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Tgm2	0.53783682	0.426566566	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Arsb;As1;As1-s	0.624336642	0.342641768	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Tpm4	0.466352656	0.531943188	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Trpv2;Grc	0.503676841	0.511221308	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Ak3l1;Ak-4;Ak3b;Ak4	0.561639989	0.487305687	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Pgm2l1	0.570125428	0.508130081	TRUE	FALSE	TRUE	FALSE	FALSE	TRUE	TRUE
Nfkb1	0.670286212	0.431704369	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
P2rx4;P2x4	0.600781015	0.541946672	TRUE	FALSE	FALSE	TRUE	TRUE	TRUE	TRUE
Napsa;Kdap;Nap	0.598909984	0.546657191	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Ltb4r;Bltr;Ltb4r1	0.636658815	0.521593991	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
C3	0.835770999	0.323540831	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Scin	0.62535176	0.539490721	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Casp6	0.755287009	0.43761761	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ero1l	0.667645881	0.538358008	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Stom;Epb7.2;Epb72	0.817460966	0.398676394	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Crot;Cot	0.723431961	0.497265042	TRUE	FALSE	FALSE	TRUE	TRUE	TRUE	TRUE
Ccdc115	0.54564304	0.68231441	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Sqstm1;A170;STAP	0.735889322	0.500876534	TRUE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE

Arhgap6	0.645953104	0.59970015	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Fcgr2;Fcgr2b;Ly-17	0.76540375	0.486499635	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Acaa2;mCG_22649	0.709169562	0.557631183	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Dhrs1;D14ertd484e	0.664451827	0.605436823	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Cd84;Slamf5	0.62119518	0.65269891	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Fam162a;E2ig5	0.679301678	0.596267366	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Cd68	0.853679358	0.423728814	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pecam1;Pecam;Pecam-1	0.709975151	0.568084986	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Creg1;Creg	0.706464147	0.580787548	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Sema4a;Semab;SemB	0.614703713	0.67585834	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Gsn;Gsb	0.756773119	0.541917303	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Lta4h	0.813206473	0.490725292	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Hexb	0.718029726	0.588962836	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Niban;Fam129a	0.738170813	0.571004397	FALSE	FALSE	TRUE	FALSE	FALSE	TRUE	TRUE
Prkca	0.641848524	0.669612964	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pon3	0.726902668	0.584624379	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Endod1;Kiaa0830	0.672178531	0.651126449	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Anxa6;Anx6	0.621929224	0.708516367	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Cyp11a1;Cyp11a	0.70318543	0.62984191	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Cstb;Cst6;Stfb	0.738170813	0.600672753	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Arhgap5;Rhogap5	0.81366965	0.526371197	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Eea1	0.701852892	0.639386189	TRUE	FALSE	TRUE	FALSE	FALSE	TRUE	TRUE
Ppt1;Ppt	0.731314904	0.612519907	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE
Myo1f;mFLJ00395	0.822503701	0.525955925	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Fuca1;Fuca	0.694107031	0.663614042	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Hsd17b11;Dhrs8;Pan1b	0.703680248	0.656685054	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Gba	0.782778865	0.577934462	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Q9ERY9	0.732439757	0.641519117	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE

Suppl. Table 4 : TOP100 downregulated proteins continued

sample miR-93 CSF3

name	measurement 1	measurement 2	miR-17-5p_20_93mr_106_519.d	miR-290-3p_292-3p_467a	miR-291b-3p_519a_519b-3p_519c-3p	miR-302ac_520f	miR-106_302	Predicted Target	Diff_max
Pfkl;Pfk-l;Pfkb	0.716229767	0.658761528	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ggh	0.790763878	0.586957798	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Prdx5;Prdx6	0.769408325	0.615422488	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Gstt3	0.78057919	0.606023877	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Tf;Trf	0.552791598	0.837520938	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Fxyd5;Oit2	0.674581759	0.716897269	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Glb1;Bgl;Glb-1	0.701459035	0.690894017	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Hk3	0.730833881	0.662954124	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Tapbp;Tapa	0.729235032	0.666888963	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
B2m	0.696767001	0.701951425	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ctsa;Ppgb	0.759647524	0.646872372	TRUE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE
Fdxr	0.729713952	0.683386865	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ctsg	0.885034074	0.529464711	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ftl1;Ftl;Ftl-1;Ftl2;Ftl-2	0.80762397	0.609161793	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Tpi1;Tpi	0.714336738	0.704027035	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Xpc	0.796368559	0.623480267	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pklr	0.599592277	0.822503701	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Diaph2;Diap2	0.774653343	0.658718135	FALSE	TRUE	FALSE	FALSE	FALSE	TRUE	FALSE
Anxa2;Anx2;Cal1h	0.659891778	0.775374118	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Unc84b;Sun2;mKIAA0668	0.897182846	0.539025442	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Osbpl7	0.763067531	0.677460877	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Itgal;Lfa-1;Ly-15	0.702691308	0.74261102	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pdxk;Pkh	0.722178089	0.725531452	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Arrb1	0.704423781	0.744657085	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Bin3	0.661463157	0.795102171	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE

Lxn	0.817460966	0.645411127	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Idua	0.757174226	0.705766109	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Myo1g	0.785175879	0.679255536	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Rock2	0.798020908	0.668806849	FALSE	FALSE	TRUE	FALSE	FALSE	TRUE	FALSE
Tes;mCG_11251	0.772260406	0.694927033	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Rps6ka5;Msk1	0.719217491	0.748055057	TRUE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE
Fth1;Fth	0.745545366	0.723955694	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pdia4;Cai;Erp72	0.82176021	0.6477523	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Mboat7;Bb1;Leng4;Oact7	0.80925791	0.662998077	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ppib	0.767224183	0.709119274	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Fmnl1;Frl	0.771366862	0.706364343	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
H2-D1;H2-K1;H2-K;H2-Q2	0.837871806	0.64008193	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Mif	0.752219046	0.725742071	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Aga	0.830702775	0.648929267	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Slc25a10;Dic	0.82781457	0.652613718	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
S100a8;Caga;Mrp8	1.154121367	0.327911857	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Ankrd44	0.785360873	0.697885407	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Hexa	0.753295669	0.733568075	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Lrpap1	0.796685787	0.694685655	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pld4	0.780213779	0.712199986	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
Pcyox1l	0.844808651	0.650068257	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE

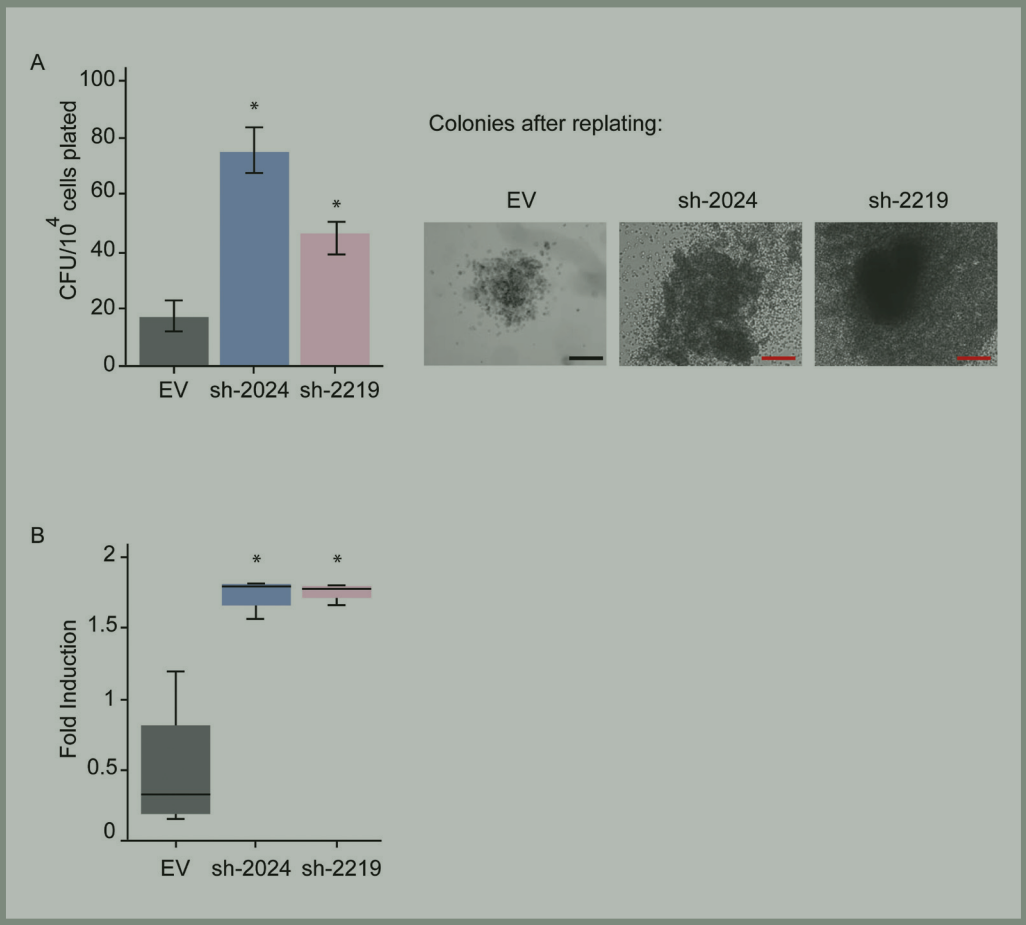


Figure 5: ShRNA-mediated silencing of SQSTM1 phenocopies miRNA overexpression. (**A**) Replating assay of mouse lin- BM progenitors infected with two different hairpin vectors against SQSTM1, pSM2C-SH2024 and pSM2C-SH-2219, or empty vector control virus. Cells were plated in triplicate at densities of $1x10^4$ cells per dish in 1 mL methylcellulose medium containing IL-6 (10 ng/mL), IL-3 (supernatant 1/1000), SCF (10 ng/mL), CSF2 (10 ng/mL). Cells were grown for a week, isolated and replated in a secondary CFU-assay (10^4 cells per plate). Colonies from the second plating were counted after 7 days of growth. Micrographs show size of CFUs on day 7 after plating. Black bar indicates 100 μm, red bars indicate 200 μm. Significance was calculated by comparing the samples with EV control and the different shRNAs with the Mann-Whitney test (Asymp. Sig. (2-tailed)). * $p< 0.05$. (**B**) Mouse lineage negative progenitor cells isolated from the BM of C57BL/6 mice were infected with pSM2C-sh-2024 and pSM2C-sh-2219, empty vector control virus or with GFP containing control virus. Cells were selected in PURO 1.5 (μg/mL) containing expansion medium for 2 days. Recipient C57BL/6 mice were lethally irradiated (8.5 Gy) and reconstituted with 45% GFP positive cells mixed with shRNA or EV control infected cells. Six weeks post-transplantation, mice were sacrificed and BM cells were isolated. Lin- cells were stained for flow cytometry analysis. The fold induction of the percentage of GFP^- Lin-; Sca-I^+; c-Kit^+ cells in the BM of pSM2C-EV (n=4), sh-2024 (n=5) and sh-2219 (n=4) relative to the input are shown. Significance was calculated by comparing the samples of mice transplanted with EV control and the different shRNAs with the Mann-Whitney test (Asymp. Sig. (2-tailed)). * $p< 0.05$.

Role of SQSTM1 in regulation of ligand-induced CSF3R routing, stability and signalling

Signalling kinetics of the CSF3R govern the balance between cellular expansion and differentiation of progenitors [29]. Ubiquitination of activated CSF3R controls intracellular receptor routing to lysosomal degradation and signal duration [30]. To identify proteins that bind to the ligand-activated CSF3R complex, we performed a proteomics analysis of CSF3R complexes pulled down on streptavidin-coated magnetic beads at 30 minutes post-stimulation with biotinylated CSF3 (Bio-CSF3) (Meenhuis A and Touw IP, unpublished data). SQSTM1 was one of the proteins that were consistently present in the activated CSF3R complex. CSF3R-SQSTM1 interaction was confirmed by Western blot analysis in HeLa cells that stably express hu-CSF3Rs (HeLa-CSF3R cells) (Fig.6A). SQSTM1 binding to the CSF3R was not detectable above background by Western blotting at early time-points but was obvious at 2 hours post-stimulation. Notably, the enhanced binding of SQSTM1 to the receptor correlated with CSF3R degradation (Fig.6A). SQSTM1 binding to the CSF3R was dependent on receptor lysines, as the CSF3R lysine null mutant (K5R) [31] showed a strongly reduced SQSTM1 binding capacity compared to the wt CSF3R (Fig.6A). In full agreement with these observations, confocal microscopy analyses showed increasing CSF3R colocalization with endogenously expressed SQSTM1 at 2 hours post-stimulation compared to earlier time points (Fig.6B). Although SQSTM1 has been associated with autophagy, no colocalization has been observed between the CSF3R and the LC3 marker for autophagosomes at different time-points post-stimulation (Fig.6B). To study the effects of SQSTM1 binding for CSF3R routing we silenced SQSTM1 expression by RNAi and analyzed receptor localization in living cells up to 2 hours post-stimulation. We detected a 100% colocalization of CSF3Rs with RAB7, an intracellular marker for late endosomes after 2 hours (Suppl.Fig.2), indicating that siRNA-mediated knock-down of SQSTM1 has no effect on normal routing of the CSF3R to the endosomal compartment after ligand stimulation in our model. Since SQSTM1 targets ubiquitinated proteins for degradation, we asked whether SQSTM1 controls CSF3R stability. Similar ligand-activated CSF3R protein levels were detected at 15 minutes post-treatment with Bio-CSF3 (Fig.7B). In contrast, a ~75% siRNA-mediated silencing of SQSTM1 in HeLa-CSF3R cells resulted in small, but consistently enhanced CSF3R levels (~30%) at 2 hours post-stimulation, compared to control siRNA treated cells (Fig.7A, B). The enhanced CSF3R level was concomitant with increased ligand-induced P-ERK levels (Fig.7C). Thus, the interaction of SQSTM1 to the stimulated CSF3R adjusts receptor degradation via the late endosomal compartment and regulates cellular P-ERK levels.

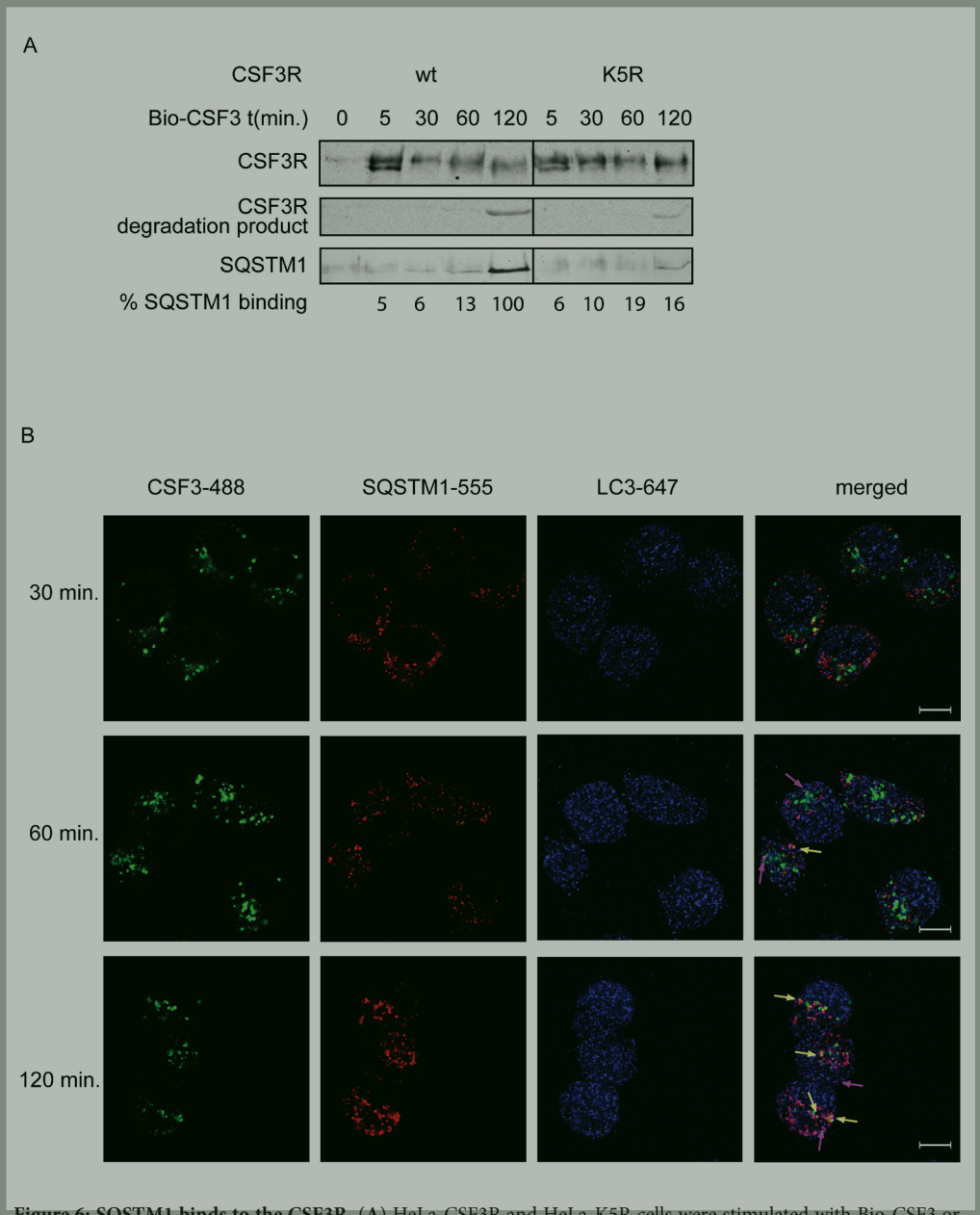
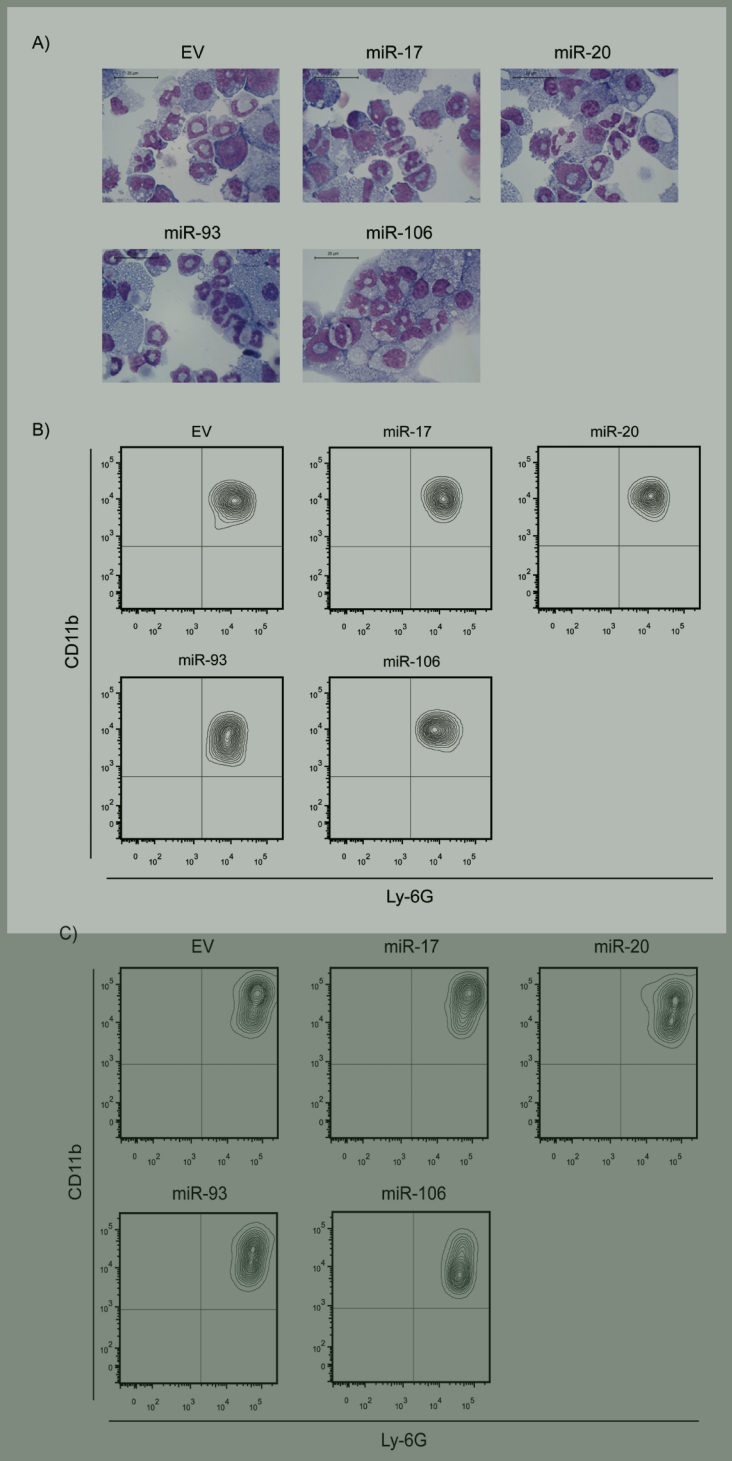
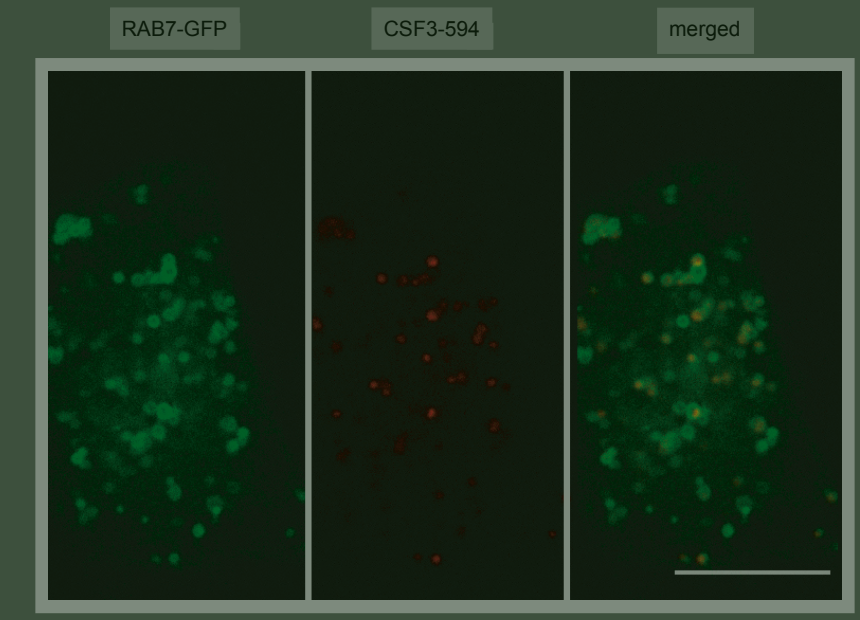


Figure 6: SQSTM1 binds to the CSF3R. (**A**) HeLa-CSF3R and HeLa-K5R cells were stimulated with Bio-CSF3 or left unstimulated (t=0) for 10 minutes at RT, washed with RPMI and transferred to 37˚C for indicated time points. CSF3R binding proteins were pulled down with streptavidin-coated beads from cell lysates and analyzed by Western blotting using monoclonal antibodies against CSF3R and SQSTM1. The percentage SQSTM1 binding relative to the amount of CSF3Rs is given. (**B**) HeLa-CSF3R cells were stimulated with CSF3-488 for 10 minutes at RT, washed and transferred to 37˚C for indicated time-points. Subsequently, cells were fixed and stained for SQSTM1 (red) and LC3 (blue). Colocalization of CSF3R and SQSTM1 is indicated by the orange-yellow dots and yellow arrows. Colocalization of SQSTM1 with LC3 is indicated by the pink dots and arrows.



Suppl. Figure 1: Differentiation capacity of miR-17/20/106/93 overexpressing cells compared to EV controls. (**A**) Micrographs showing morphology of cells expressing indicated miRNAs or control cells (EV) isolated from CFU-G assay. Bar indicates 25 µm. (**B,C**) BM and blood were isolated from transplanted mice that received stem cells/progenitors transduced with miR-17/20/93/106 or control viruses. Plots show GFP+ granulocytes that are stained with antibodies against CD11b and Ly6G.



Suppl. Figure 2: Ligand-stimulated CSF3Rs colocalize with RAB7-EGFP (control experiment for Figure 4). Cells were plated on 25mm #1 glass coverslips and grown in DMEM supplemented with 10% FCS. CSF3-594 was added to the medium for 15 minutes. Cells were washed 2 times and fresh medium was added. CSF3R (Red) and RAB7-GFP (green) are shown at 2 hours post-stimulation. Colocalization of CSF3R and RAB7-GFP is indicated by the orange-yellow dots. Bar indicates 10 um.

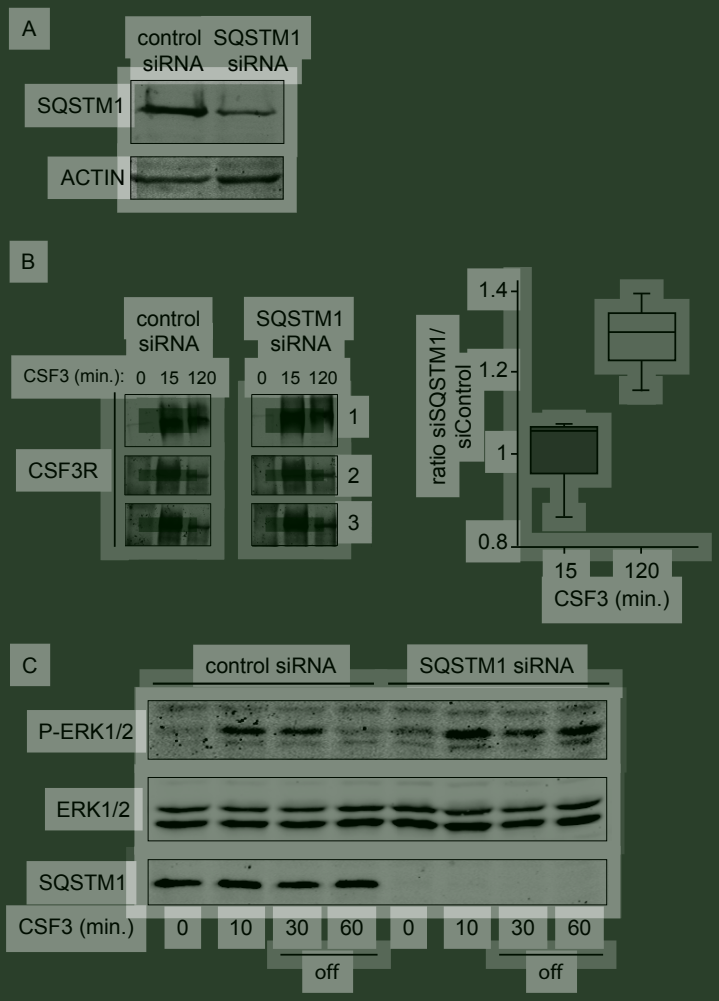


Figure 7: Role of SQSTM1 in regulation of ligand-induced CSF3R routing, stability and signalling. (**A**) HeLa-CSF3R cells were transfected with siRNAs against *Sqstm1* or control siRNAs. Western blotting showed a ~75% knock-down (KD) in cells transfected with *Sqstm1* siRNAs compared to cells transfected with control siRNAs. (**B**) Transfected cells were stimulated with Bio-CSF3 or left unstimulated for 10 minutes at RT (t=0), washed with RPMI and transferred to 37˚C for indicated time-points. CSF3R proteins were pulled down and analyzed as described in A. The ratio of detected CSFR protein levels siSQSTM1 /siControl samples (n=3) was calculated for 15 and 120 minutes CSF3 treatment and is depicted. The significance of the difference between the calculated ratios of time point 120 minutes and 15 minutes was calculated with the Mann-Whitney test (Asymp. Sig. (2-tailed). ($p<0.10$). (**C**) HeLa-CSF3R cells were transfected with siRNAs against *Sqstm1* (+) or control siRNAs (-). Western blotting showed a > 95% knock-down (KD) in cells transfected with siRNAs targeting *Sqstm1* transcripts compared to cells transfected with control siRNAs. Cells were serum starved for 24 hours followed by CSF3 (100 ng/mL) treatment as described in A. Samples for cell lysates were taken at indicated time points and analyzed by Western blotting with total and/or phosphor-specific antibodies against ERK.

DISCUSSION

Role of AAAGUGC seed-containing miRNAs in hematopoiesis

miRNAs play pivotal regulatory roles in homeostatic physiological processes including hematopoiesis. Hematopoiesis is characterized by the tight regulation of cellular renewal, proliferation and differentiation with the objective of adjusting blood cell production according daily needs. Here, we present a series of experiments that identify a set of miRNAs that share identical AAAGUGC seed sequences and also share functions in hematopoietic progenitor cells. Our data from 32D cells, mouse progenitor assays and *in vivo* transplantation studies consistently indicate a role for *miR-17/20/93/106* in hematopoietic cell expansion and replating capacity. We show that *miR-17, -20, -93* and *-106* are abundantly expressed in hematopoietic progenitors of human and mouse origin and their expression declines considerably during myeloid differentiation. Consistent with this, expression of *miR-17* and *miR-106* were previously detected in human $CD34^+$ progenitor cells and were shown to be significantly downregulated during *in vitro* differentiation towards mature megakaryocytes and monocytes [7]. The *miR-17-5p*, *-20* and *-106* were also found to be downregulated in monocytopoiesis [32]. Collectively, these results suggest a more general role for these miRNAs at an early stage of myeloid development. Perturbed maturation of primary hematopoietic progenitors was not observed in CFU-assays and in transplantation experiments in mice, indicating that the effect of miRNAs is a genuinely enhanced expansion of hematopoietic precursors, and not proliferation due to impaired differentiation.

The AAAGUGC seed-containing miRNAs target SQSTM1 and control CSF3R stability, MAP kinase activity and cell expansion

Whereas most proteins detected by quantitative proteomics were not or moderately changed in their expression by *miR-93* in 32D cells, SQSTM1 expression was strongly decreased. SQSTM1 is a multifunctional signal adaptor protein controlling a variety of cellular events such as e.g., osteoclastogenesis, T-cell and adipocyte differentiation, regulation of the NF-κB pathway, nerve growth factor receptor (NGFR) internalization, and transportation of poly-ubiquitinated proteins destined for degradation by the proteasome and autophagy systems [33-37]. We show for the first time that SQSTM1 expression is post-transcriptionally regulated by miRNAs.

SQSTM1 was formerly found to colocalize robustly with the EGFR and the late endosomal markers RAB7 and LAMP1, implicating functions of SQSTM1 predominantly in late endosomes and lysosomes [38]. Ubiquitination of the lysine in the juxtamembrane part of the CSF3R is pivotal for trafficking of the receptor from early to late endosomes, and impaired lysosomal targeting results in enhanced proliferation of progenitors [30]. We observed SQSTM1 binding to the activated CSF3R complex 30 minutes post-stimulation and this interaction was the most abundant after 2 hours. This interaction was concomitant with

the amount of CSF3R degradation products on the gel. The K5R mutant of the CSF3R binds reduced levels of SQSTM1 compared to wt CSF3R. This receptor mutant accumulates in the early endosomes [31]. Therefore, it's still unclear, whether ubiquitination of the CSF3R itself is crucial for SQSTM1-mediated functions. Does SQSTM1 binding affect CSF3R localization? SiRNA-mediated silencing of SQSTM1 expression showed no role of SQSTM1 in CSF3R routing to the late endosomal compartment. However, knock-down of SQSTM1 resulted in enhanced CSF3R stability, which can be explained by inefficient receptor routing to the lysosomes. Studies with SQSTM1 mutants are needed to elucidate the binding specificities of SQSTM1 to the CSF3R complex.

We showed enhanced P-ERK levels when SQSTM1 expression is silenced. This could be explained by two possible mechanisms which are not mutually exclusive. First, several studies demonstrate the importance of SQSTM1 in regulation of the activity of MAPK family members by inhibition and direct sequestration of ERKs [39-41]. Second, recent data show that the MEK-ERK pathway is anchored to the late endosomes [42]. Defective routing of the CSF3R to lysosomal degradation presumably results in prolonged localization of active receptors in late endosomal compartment, and is a plausible explanation of the enhanced ERK activation.

The question emerging from these data is, whether the proliferative effects of SQSTM1 silencing could be explained by enhanced P-ERK levels? In fact, CSF3-induced activation of the ERK kinases is known to be involved in stimulation of proliferation [29]. In addition, the tyrosine at amino acid position 764 of the CSF3R is a docking site for signalling proteins implicated in the activation of P21RAS/MAP kinase pathway and controls cell expansion [43]. Thus, the proliferative phenotype of hematopoietic cells with enhanced levels of AAAGUGC seed-containing miRNAs could be at least partially explained by the enhanced activation of MAP kinases caused by reduced SQSTM1 action. In conclusion, AAAGUGC-seed containing miRNAs in hematopoietic cells control cell expansion, self-renewal and MAP-kinase signalling by interference with SQSTM1-regulated pathways.

Role of AAAGUGC seed-containing miRNAs in myeloid leukemia

There is strong evidence for a role of AAAGUGC seed-containing miRNAs in cancer. For instance, the *miR-372* and *miR-373* have been implicated as oncogenes in testicular germ cell tumours through reduction of LATS2 tumour suppressor levels [44]. Elevated expression of *miR-17* was found in tumours of different origins e.g., hematopoietic, colorectal, and lung tumours and transgenic mice that overexpress the *miR-17~92* polycistron are more susceptible to cancer [45-47]. Further, increased *miR-106* level is significantly correlated with tumour stage, metastasis and invasion in gastric carcinoma and cancer cell lines [48]. *MiR-93*, *miR-17*, *miR-20* and *miR-106* are abundantly expressed in a large subset of AML samples [28]. In a genome wide miRNA expression analysis of 52 AML samples with common translocations, m*iR-17* and *miR-20* in combination with 5 other miRNAs could

discriminate MLL-rearrangement AMLs from CBFs and t(15;17) rearrangement positive leukemias [49]. Similar to our observations in normal progenitors, forced expression of the *miR-17~92* cluster, which contain *miR-17* and *miR-20*, in MLL-transformed cells results in enhanced colony forming capacity of BM progenitors, particularly in cooperation with MLL fusions [50]. Accordingly, leukemic stem cell potential and self-renewal is enhanced in MLL transformed cells when *miR-17~92* is abundantly expressed, in part by the modulation of P21 expression [51]. We identified P21 by mass spectrometry, but unfortunately these results did not match the selection criteria as described in Suppl. Methods. Therefore, this protein had been discarded for further analysis. Strikingly, SQSTM1 has not been identified as a target in the study of Mi et al.. There are two explanations feasible for this discrepancy: 1. SQSTM1 is due to still unidentified mechanisms not regulated in MLL transformed tumor stem cells. 2. SQSTM1 protein expression is down-regulated by the miRNAs, but the difference in mRNA expression in the MLL transformed cells compared to controls might be minor and too small to be picked up by the microarrays used in the study. The second hypothesis is in full agreement to our data, because we found that overexpression of AAAGUGC-seed miRNAs strongly affects SQSTM1 protein expression and not so much the mRNA stability (<40%). To our opinion, it is still feasible that both experimentally identified targets collaborate in the control of leukemic and normal hematopoietic precursor expansion.Interestingly, a multivariable cox regression analysis on data from 85 cytogenetically normal cases of AML, accounting for known confounders (age of the patient, white blood cell count, mutations in genes coding for NPM1, CEBPA and FLT3) indicated that high *miR-106* expression significantly correlates with adverse survival (p=0.018, Hazard Ratio = 1.69, Table 1). We found that SQSTM1 protein levels were markedly downregulated in normal karyotype AML samples exhibiting high expression of *miR-106.* Recent data indicate both oncogenic and tumour suppressor functions for SQSTM1 in various types of cancer [52-54]. Our patient data suggest that AAAGUGC seed-containing miRNAs play a role in leukemogenesis by deregulation of SQSTM1-controlled mechanisms. Additional studies are needed to further test this hypothesis and to determine if *miR-106* could be a potential biomarker for diagnosis of this particular group of AML patients. In conclusion, AAAGUGC-seed containing miRNAs in hematopoietic cells control cell expansion, replating capacity and MAP-kinase signalling, in part by the interference with SQSTM1-regulated pathways.

Table 1: Multivariate analysis for overall survival (OS) in cytogenetically normal AML

Variables	HR	95% CI	*P* *value
Overall survival			
miRNA-106$^{\alpha}$	1.69	1.09-2.62	0.018*
FLT3 $^{ITD\beta}$	2.34	1.16-4.69	0.017*
NPM1$^{\beta}$	0.21	0.09-0.48	<0.0001
CEBPA$^{\beta}$	0.92	0.44-1.92	0.82
WBC count$^{\delta}$ (x109/L)	1.52	1.14-2.02	0.004*
Age$^{\alpha}$	1.03	1.00-1.06	0.036*

Cox's proportional hazard model for multivariable analyses of *miRNA-106* as prognostic marker for overall survival. Analyses included 85 cytogenetically normal acute myeloid leukemia patients with age ≤ 60. Abbreviations: HR, hazard ratio; 95% CI, confidence interval; *FLT3* ^{ITD}, *FLT3* Internal Tandem Duplications; *NPM1* Nucleophosmin; *CEBPA*, CCAAT-enhancer binding protein alpha.

P * value ≤ 0.05
α *miRNA-106* expression is used as continues variable
β *FLT3* ^{ITD} versus no *FLT3*^{ITD}
β *NPM1* mutation versus no *NPM1* mutation
β *CEBPA* mutation versus no *CEBPA* mutation
δ WBC count, white blood cell count higher than 20x10^{9}/L versus lower than 20 x 10^{9}/L
α age is used continues variable

ACKNOWLEDGEMENTS

The authors thank P. van Geel and Dr. E. Rombouts for sorting cell populations and E. Simons for assistance with the preparation of figures. We thank Dr. T. Jevdovic, O. Roovers, C. Verwijmeren, S. Hoefnagels, L. Schulte, M.A. Sanders and V. Rockova for technical and bioinformatics assistance. We thank Drs. M. von Lindern, E. Bindels, J. van Bergen, F. Ossendorp for critical reading of the manuscript and discussions. This work was supported by grants from the Netherlands Organisation for Scientific Research (NWO-VENI) and the Dutch Cancer Society (KWF). This work was also supported by United States Public Health Service grants RO1-GM34277 from the National Institutes of Health, PO1-CA42063 from the National Cancer Institute to PAS and partially by Cancer Center Support (core) grant P30-CA14051 from the National Cancer Institute.

REFERENCES

1. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function*. Cell, 2004. 116(2): p. 281-97.
2. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions*. Cell, 2009. 136(2): p. 215-33.
3. Petersen, C.P., et al., *Short RNAs repress translation after initiation in mammalian cells*. Mol Cell, 2006. 21(4): p. 533-42.
4. Pillai, R.S., S.N. Bhattacharyya, and W. Filipowicz, *Repression of protein synthesis by miRNAs: how many mechanisms?* Trends Cell Biol, 2007. 17(3): p. 118-26.
5. Calin, G.A., et al., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers*. Proc Natl Acad Sci U S A, 2004. 101(9): p. 2999-3004.
6. Baltimore, D., et al., *MicroRNAs: new regulators of immune cell development and function*. Nat Immunol, 2008. 9(8): p. 839-45.
7. Garzon, R., et al., *MicroRNA fingerprints during human megakaryocytopoiesis*. Proc Natl Acad Sci U S A, 2006. 103(13): p. 5078-83.8. Neilson, J.R., et al., *Dynamic regulation of miRNA expression in ordered stages of cellular development*. Genes Dev, 2007. 21(5): p. 578-89.
9. Cobb, B.S., et al., *A role for Dicer in immune regulation*. J Exp Med, 2006. 203(11): p. 2519-27.
10. Guo, S., et al., *MicroRNA miR-125a controls hematopoietic stem cell number*. Proc Natl Acad Sci U S A. 107(32): p. 14229-34.
11. Koralov, S.B., et al., *Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage*. Cell, 2008. 132(5): p. 860-74.
12. Muljo, S.A., et al., *Aberrant T cell differentiation in the absence of Dicer*. J Exp Med, 2005. 202(2): p. 261-9.
13. Cobb, B.S., et al., *T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer*. J Exp Med, 2005. 201(9): p. 1367-73.
14. O'Carroll, D., et al., *A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway*. Genes Dev, 2007. 21(16): p. 1999-2004.
15. Rasmussen, K.D., et al., *The miR-144/451 locus is required for erythroid homeostasis*. J Exp Med, 2010. 207(7): p. 1351-8.
16. Chen, C.Z., et al., *MicroRNAs modulate hematopoietic lineage differentiation*. Science, 2004. 303(5654): p. 83-6.
17. Johnnidis, J.B., et al., *Regulation of progenitor cell proliferation and granulocyte function by microRNA-223*. Nature, 2008. 451(7182): p. 1125-9.
18. Fazi, F., et al., *A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis*. Cell, 2005. 123(5): p. 819-31.
19. O'Connell, R.M., et al., *Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder*. J Exp Med, 2008. 205(3): p. 585-94.
20. Han, Y.C., et al., *microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia*. J Exp Med, 2010. 207(3): p. 475-89.
21. de Koning, J.P., et al., *STAT3-mediated differentiation and survival and of myeloid cells in response to granulocyte colony-stimulating factor: role for the cyclin-dependent kinase inhibitor p27(Kip1)*. Oncogene, 2000. 19(29): p. 3290-8.

22. Zhang, C.C., et al., *Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells*. Nat Med, 2006. 12(2): p. 240-5.
23. Bottger, G., B. Nagelkerken, and P. van der Sluijs, *Rab4 and Rab7 define distinct nonoverlapping endosomal compartments*. J Biol Chem, 1996. 271(46): p. 29191-7.
24. Cherry, S.R., et al., *Retroviral expression in embryonic stem cells and hematopoietic stem cells*. Mol Cell Biol, 2000. 20(20): p. 7419-26.
25. Wolfler, A., et al., *Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors*. Blood, 2010. 116(20): p. 4116-25.
26. Lowenberg, B., et al., *Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia*. N Engl J Med, 1993. 328(9): p. 614-9.
27. van Lochem, E.G., et al., *Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts*. Cytometry B Clin Cytom, 2004. 60(1): p. 1-13.
28. Jongen-Lavrencic, M., et al., *MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia*. Blood, 2008. 111(10): p. 5078-85.
29. Touw, I.P. and G.J. van de Geijn, *Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders*. Front Biosci, 2007. 12: p. 800-15.
30. Wolfler, A., et al., *Site-specific ubiquitination determines lysosomal sorting and signal attenuation of the granulocyte colony-stimulating factor receptor*. Traffic, 2009. 10(8): p. 1168-79.
31. Irandoust, M.I., et al., *Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor*. Embo J, 2007.
32. Fontana, L., et al., *MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation*. Nat Cell Biol, 2007. 9(7): p. 775-87.
33. Duran, A., et al., *The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis*. Dev Cell, 2004. 6(2): p. 303-9.
34. Martin, P., M.T. Diaz-Meco, and J. Moscat, *The signaling adapter p62 is an important mediator of T helper 2 cell function and allergic airway inflammation*. Embo J, 2006. 25(15): p. 3524-33.
35. Rodriguez, A., et al., *Mature-onset obesity and insulin resistance in mice deficient in the signaling adapter p62*. Cell Metab, 2006. 3(3): p. 211-22.
36. Geetha, T., J. Jiang, and M.W. Wooten, *Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling*. Mol Cell, 2005. 20(2): p. 301-12.
37. Seibenhener, M.L., et al., *Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation*. Mol Cell Biol, 2004. 24(18): p. 8055-68.
38. Sanchez, P., et al., *Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62*. Mol Cell Biol, 1998. 18(5): p. 3069-80.
39. Lee, S.J., et al., *A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis*. EMBO Rep, 2010. 11(3): p. 226-32.
40. Pursiheimo, J.P., et al., *Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62*. Oncogene, 2009. 28(3): p. 334-44.
41. Moscat, J., M.T. Diaz-Meco, and M.W. Wooten, *Signal integration and diversification through the p62 scaffold protein*. Trends Biochem Sci, 2007. 32(2): p. 95-100.
42. Nada, S., et al., *The novel lipid raft adaptor p18 controls endosome dynamics by anchoring the MEK-ERK pathway to late endosomes*. Embo J, 2009. 28(5): p. 477-89.

43. Hermans, M.H., et al., *Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells*. Blood, 2003. 101(7): p. 2584-90.
44. Voorhoeve, P.M., et al., *A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors*. Cell, 2006. 124(6): p. 1169-81.
45. Ota, A., et al., *Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma*. Cancer Res, 2004. 64(9): p. 3087-95.
46. James, C., et al., *A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera*. Nature, 2005. 434(7037): p. 1144-8.
47. Hayashita, Y., et al., *A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation*. Cancer Res, 2005. 65(21): p. 9628-32.
48. Xiao, B., et al., *Detection of miR-106a in gastric carcinoma and its clinical significance*. Clin Chim Acta, 2009. 400(1-2): p. 97-102.
49. Li, Z., et al., *Distinct microRNA expression profiles in acute myeloid leukemia with common translocations*. Proc Natl Acad Sci U S A, 2008. 105(40): p. 15535-40.
50. Mi, S., et al., *Aberrant overexpression and function of the miR-17-92 cluster in MLL-rearranged acute leukemia*. Proc Natl Acad Sci U S A, 2010. 107(8): p. 3710-5.
51. Wong, P., et al., *The miR-17-92 microRNA polycistron regulates MLL leukemia stem cell potential by modulating p21 expression*. Cancer Res, 2010. 70(9): p. 3833-42.
52. Mathew, R., et al., *Autophagy suppresses tumorigenesis through elimination of p62*. Cell, 2009. 137(6): p. 1062-75.
53. Moscat, J. and M.T. Diaz-Meco, *p62 at the crossroads of autophagy, apoptosis, and cancer*. Cell, 2009. 137(6): p. 1001-4.
54. Jaakkola, P.M. and J.P. Pursiheimo, *p62 degradation by autophagy: another way for cancer cells to survive under hypoxia*. Autophagy, 2009. 5(3): p. 410-2.

SUPPL. METHODS

SILAC Quantitative mass spectrometry

Sample preparation

32D-EV control cells were grown in RPMI media containing 13C6-labeled Lys and 13C6-labeled Arg (heavy) and 32D-miR-93 cells in medium containing regular Lys and Arg (light) (SILACTM Protein ID & Quantification Media Kit, Invitrogen). Equal numbers of cells from each fraction were combined and cells were washed with PBS to remove the growth medium. This was done at steady state (SS, IL-3 condition) and after 4 days CSF3 treatment. Biological experiments were performed in duplicate. Subsequently, the cell pellet was dissolved in a ten fold volume of SDT-lysis buffer as described in the FASP protocol 1, with the use of 30 kDa Microcon filters. Following the FASP work-up, salts were removed by solid phase extraction using Oasis C18 columns (Waters). Samples were separated by iso-electric focusing (IEF). Samples were loaded onto 13 cm/12 fraction Immobilin DryStrips pH 3-10 (GE Healthcare) in 1 M urea and focused up to 20 kVh with a 3100 OFFGEL fractionator (Agilent Technologies). The resulting 12 IEF fractions were desalted by SPE as described above and freeze-dried.

On-line nanoHPLC-tandem mass spectrometry

The IEF fractions were analyzed by nanoflow liquid chromatography using an Agilent 1100 HPLC binary system (Agilent Technologies), using the set-up of Meiring 2 and coupled on-line to a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific). The end of the nanocolumn was drawn to a tip (internal diameter ~5 μm), from which the eluent was sprayed into the mass spectrometer. Peptides were trapped at 5 μl/min on a 1 cm column (100-μm ID; ReproSil-Pur C18-AQ, 3 μm) and eluted to a 15 cm column (50-μm ID; ReproSil-Pur C18-AQ, 3 μm) at 150 nl/min in a 120 minutes gradient from 0 to 50% acetonitrile in 0.1% formic acid. All chromatographic columns were prepared in-house. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. The five most intense ions were then isolated for collision induced dissociation in the linear ion trap at a target value of 10,000, and placed on the dynamic exclusion list for 45 seconds. NanoHPLC MS/MS experiments of the biological replicates were run in duplicate.

Raw files were submitted to the MaxQuant package (version 1.0.13.13) for relative quantitation, using the default settings of the package, using IPImouse version 3.66 as the database 3, 4. MSM-files produced by the Quant program of the MaxQuant package were submitted for database searching using mascot version 2.1.04. Application of the Identify program of the MaxQuant package to the combined LC-MS/MS runs of day 0 (SS) and 4 (CSF3) yielded approximately 4300 proteins with at least 2 peptides per protein and a false discovery rate of 1% for both samples.

Statistical analysis

The Benjamini-Hochberg approach (FDR<5%) was used on significance B of the MaxQuant output 5. Proteins that were significantly up- or downregulated in both biological replicates were considered for further analysis. Microsoft Excel 2007 and SPSS Statistics 17.0 were used for further processing. To correlate the protein names-output from the proteomics data with the expression data-based gene names on the MMU array we developed a software package that scans the NCBI, Ensembl and Swiss- Prot databases. In this way 97% of the MaxQuant-identified proteins could be linked to the expression data. Normalization and mapping were performed by the use of BrainArray annotation. Associations to predicted targets of related miRNA's were inferred using TargetScan.

Mass spectrometric analysis of Bio-CSF3 pull-down samples

HEK293 cells were transiently transfected with pBABE hu-CSF3R expression constructs 6 using FuGENE 6 (Roche) according to manufactures protocol, and stimulated for 30 minutes with Bio-CSF3 at 37°C. Cells were subsequently lysed and activated CSF3R complexes were isolated using streptavidin coated beads as described 6 and loaded on a SDS-PAGE gel. 1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to ingel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described previously 7. Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr. Maisch GmbH; column dimensions 1.5 cm × 100 μm, packed in-house) at a flow rate of 8 μl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 μm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were a tomatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the NCBInr database (release NCBInr_20080718; taxonomy: mammalian). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications

using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table. Database analysis was performed on an in-house Mascot server.

Spinning-disk live cell imaging

Cells were plated on 25mm #1 glass coverslips (D-M, Germany) and grown in DMEM supplemented with 10% FCS. CSF3 was conjugated to Alexa594 (according to the manufacturer's instructions, Molecular Probes, Invitrogen). CSF3-594 was added to the medium for 15 minutes. Cells were washed 2 times and fresh medium was added. CSF3R internalization and localization were monitored for 2 hours. During imaging cells were maintained at 37°C in the stage top incubator (model INUG2EZILCS, Tokai Hit). Live cell imaging was performed on an inverted microscope Nikon Eclipse Ti-E (Nikon) with perfect focus system (PFS) (Nikon), equipped with Nikon CFI Apo TIRF 100x 1.49 N.A. oil objective (Nikon), CSU-X1-A1 Spinning Disc (Yokogawa), QuantEM 512SC EMCCD camera (Roper Scientific) and controlled with MetaMorph 7.5 software (Molecular Devices). For excitation of Rab7-GFP and CSF3-594 we used 491nm 50mW Calypso (Cobolt) and 561nm 50mW Jive (Cobolt) lasers. For simultaneous imaging of Rab7-GFP and CSF3-594 we used ETmCherry/GFP filter set (59022, Chroma) together with DualView (DV2, Roper) equipped with dichroic filter 565dcxr (Chroma) and HQ530/30m and HQ630/50m emission filters (Chroma). The Spinning Disc was equipped with 405-491-561 tipple band mirror and ET-GFP, ET-mCherry and ETGFP/mCherry emission filters (Chroma). The 16-bit images were projected onto the CCD chip with intermediate lens 2.0X (Edmund Optics) at a magnification of 0.068 mkm/pixel. Images were prepared for publication using MetaMorph.

REFERENCES

1. Wisniewski, J.R., Zougman, A., Nagaraj, N. & Mann, M. Universal sample preparation method for proteome analysis. Nat Methods 6, 359-362 (2009).
2. Meiring, H.D., van der Heeft, E., ten Hove, G.J. & de Jong, A.P.J.M. Nanoscale LC-MS(n): technical design and applications to peptide and protein analysis. Journal of Separation Science 25, 12 (2002).
3. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26, 1367-1372 (2008).
4. Cox, J. et al. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. Nat Protoc 4, 698-705 (2009).
5. Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N. & Golani, I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 125, 279-284 (2001).
6. Irandoust, M.I. et al. Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor. Embo J (2007).
7. Wilm, M. et al. Femtomole sequencing of proteins from polyacrylamide gels by nanoelectrospray mass spectrometry. Nature 379, 466-469 (1996).

CHAPTER 7

General discussion and Summary

DISCUSSION

1. Cytokine receptors and the JAK-STAT pathway

The four-α-helix bundle hematopoietic growth factor superfamily includes amongst others erythropoietin, growth hormone, prolactin, trombopoietin, oncostatin M, leukemia inhibitory factor, granulocyte-colony stimulating factor and leptin (Structural Classification of Proteins (SCOP: 47266)). These cytokines exert their function by binding to their cognate receptors, which lack intrinsic kinase activity and rely on one or more JAK and STAT family members for signal transduction [1]. Although there is only a limited number of JAK and STAT proteins that are activated by these receptors, activation of the different cytokine receptors leads to unique biological responses. After receptor activation, STAT molecules bind to the phosphorylated tyrosine residues with their SH2 domains or they interact directly with JAKs. Post-translational modifications of STAT proteins and formation of STAT hetero- and homodimers or tetramers expands the range of different DNA binding site selections and hence transcription [2, 3]. Furthermore, transcription is refined by genomic accessibility of the genes and by cofactors involved which can be cell type specific [4]. However, these mechanisms do not entirely explain how specificity in gene expression is controlled by cytokine receptors that in a single cell type may activate the same JAKs and STATs. Although it has been firmly established that CSF3 activates JAK1, JAK2 and TYK2, the specific roles of these kinases in CSF3 signaling are yet not clear.

2. Increased JAK levels confer ligand-independent signaling to internalization and lysosomal routing defective CSF3R mutants

JAK overexpression has been reported to enhance cell-surface expression of a number of the cytokine receptors such as the EPOR, GHR, TPOR and OSMR [5-8]. The mechanisms involved are heterogeneous and how endogenous JAKs control receptor trafficking is still controversial. The data presented in Chapter 2 indicate that increased JAK1, JAK2 or TYK2 levels enhance CSF3R membrane expression but do not affect CSF3R internalization and endocytosis, which implicate a role of the JAKs in CSF3R forward routing. The exact mechanism by which JAKs control forward routing of CSF3R still remains to be elucidated. Cell surface expression of the W650R-CSF3R mutant, which fails to activate JAKs and is severely hindered in JAK binding [9], is comparable to wt CSF3R, implying that JAKs are not per se required for membrane expression. Intriguingly, increased levels of JAK2 in combination with a lysosomal-routing-defective K5R-CSF3R- or an internalization-defective d715-CSF3R mutant resulted in a pronounced increase in growth factor independent survival of Ba/F3 cells, that normally fully dependent on interleukin (IL)-3 for proliferation and survival and die within 4 hrs after (IL)-3 withdrawal. Prominent ligand-independent activation of STAT3 was observed in these cells. Perturbed signaling by activated d715-CSF3R truncation mutants, found in patients with AML preceded by severe chronic neutropenia, is to a large extent due

to the inability of the receptors to undergo endocytosis and thereby signal termination [10]. Because CSF3R mutations are rare in de novo AML, an alternative mechanism of deregulated CSF3R signaling might involve increased levels of JAK, which could confer spontaneous pro-survival signaling from CSF3Rs perturbed in endosomal trafficking.

Previously, it was reported that JAK overexpression is a key genetic event in the transformation of cytokine dependent cells into spontaneously proliferating cells in the presence of a proliferation defective mutant IL-9 receptor [11]. Autonomous cell growth of these cells correlated with phosphorylation of STAT5, ERK1 and 2 and AKT. Based on these observations, JAK overexpression can be considered as an oncogenic event leading to constitutive activation of the JAK-STAT pathway. In the last decade, several acquired JAK mutations have been identified in different malignancies. These mutations usually result in a gain of kinase function. The best studied example is the JAK2V617F mutation, which is associated with multiple myeloproliferative disorders [12-14]. JAK2V617F is a mutation in the pseudokinase domain that retains the ability of the JAK2 to interact with cytokine receptors via its intact FERM domain [15]. This is important because at physiological levels of expression, JAK2V617F needs to be associated to a JAK2 binding dimerized homodimeric type I cytokine receptors such as the EPOR, TPOR or the CSF3R to allow constitutive signaling [16, 17]. The ability of JAK2V617F to bind and activate these cytokine receptors provides a molecular basis for the prevalence of JAK2V617F in diseases of myeloid-lineage cells such as polycythemia vera (PV), essential thrombocythemia (ET), and idiopathic myelofibrosis (IMF), however it is rarely found in AML [18, 19]. Expression of the JAK2V617F mutant in mice resulted in erythrocytosis, neutrophilia, trilineage hyperplasia in bone marrow and splenomegaly [20]. In conclusion, constitutive signaling or prolonged signaling can be provoked by mutations increasing the JAK kinase activity or by increased levels of JAKs. In the latter case, the effects on signaling are significantly enhanced when receptor internalization and lysosomal routing is blocked, as is the case with the SCN-derived internalization-defective CSF3R mutants, or with the lysine-less experimental variant K5R-CSF3R (Chapter 2).

3. Signal termination by endocytosis

The immediate consequence of endocytosis is that signaling receptors disappear from the plasma membrane, thereby limiting the access of receptors to its growth factor ligand. However the differential distribution of signaling receptors and their effectors between the plasma membrane and the different endosomal compartments also functions to regulate signaling output. Endosomes can sustain the signals that originate from the plasma membrane but also generate unique signals that are prohibited at the plasma membrane, which eventually adds up to signal diversification and specificity [21]. Ultimately, the receptors route to lysosomes where they are degraded by proteases [22]. Several receptor internalization mechanisms using the clathrin-coated pit (CCP) pathway or clathrin-independent pathways have been described [23, 24]. The proteomics studies presented in Chapter 5 suggest that

CSF3R internalizes at least partly through CCP. Much of the current knowledge about the role of endocytosis and endosomal sorting of membrane receptors comes from studies on the EGFR [25]. Upon ligand activation, EGFR undergoes both multiple mono- as well as K63-linked poly-ubiquitination which results in its localization to clathrin coated pits, where it can interact with ubiquitin-binding adaptor proteins such as Eps15 and Epsin-1 [26, 27]. The E3 ligase involved in EGFR ubiquitination is Cbl, which is recruited to a phosphorylated tyrosine of EGFR as well as to GRB2, which itself binds to the same phosphorylated tyrosine of EGFR. Cbl induces ubiquitination of lysine residues located within the EGFR kinase domain in cooperation with the E2 conjugating enzyme ubc4/5 [28, 29]. Ubiquitination of the EGFR is important but not crucial for EGFR internalization. The EGFR receptor mutant that lacks the Cbl-binding site is not hampered in internalization, indicating that ubiquitination is not essential for receptor internalization [30, 31]. Cbl has been reported to play a role in CSF3R PI3K signaling [32, 33] However, we were unable to show an interaction of the CSF3R with Cbl using different techniques such as co-immunoprecipitation and mammalian protein-protein interaction trap assay. Thus, it remains controversial whether Cbl binds to CSF3R and whether CSF3R may be a substrate for the E3 ligase activity of Cbl.

4. SOCS proteins in downmodulation of cytokine receptor signaling

SOCS protein expression is rapidly induced in response to several cytokines [1, 34]. Many SOCS proteins function in a classic negative feedback loop, implying that they downmodulate the signaling pathway of the same receptor that stimulates their production. Eight SOCS proteins have been shown to be involved in signal termination of different cytokine receptors [1, 35-37].

SOCS proteins are known to control the duration and intensity of cytokine responses by blocking different aspects of the cytokine signaling pathways. SOCS proteins contain a central SH2 domain which binds to a phosphorylated tyrosine-containing motif of the target protein thereby competing with other signaling molecules in binding [38]. SOCS1 and SOCS3 have an additional domain called the kinase inhibitory region (KIR) by which they directly downregulate JAK activity [39, 40]. Furthermore all SOCS proteins contain a SOCS-box which can function as a E3 ligase complex together with Rbx1/2, Cul2/Cul5, Elongin B/C, [41, 42]. Following ubiquitination, receptor proteins can be targeted either for proteasomal or lysosomal degradation. In case of the EPOR and the GHR, the SOCS protein CIS was found to mainly induce proteasome-dependent degradation [43]. Additionally, CIS plays a role in internalization of the GHR; however the lysine residues within GHR are not involved in this process [44, 45]. Besides proteasomal degradation, SOCS proteins can also direct lysosomal routing, as was e.g., shown for CSF3R [37].

4.1. SOCS3 dependent site-specific ubiquitination of the CSF3R K632 involved in endosomal routing

A key finding of our previous study was that SOCS3-induced downregulation of STAT5 activity and lysosomal routing of the CSF3R depends on K632, even though there are in total five conserved lysines present in the cytoplasmic domain of the CSF3R that can be ubiquitinated [37]. We studied why especially this highly conserved lysine residue is required for STAT5 signal termination and routing of the CSF3R from early to late endosomes (Chapter3). By changing the position of K632 we showed that the lysine is not part of a conserved and rigid spatial motif and that neither a strict proximity of the lysine to the cell membrane nor a strict positioning relative to the SOCS3 binding site is required for CSF3R signal downregulation. However, moving the lysine beyond the conserved Box-1 region led to a rapid decrease in functionality, emphasizing the importance of membrane-proximity of the lysine for lysosomal routing. Previously it was shown that the CSF3R lysines are not implicated in CSF3 induced receptor internalization. For instance, this was evident from the observation that the K5R mutant internalizes with the same kinetics as wt CSF3R [37]. However, artificial attachment of Ub to the internalization defective mutant d715-CSF3R did result in increased internalization, but failed to guide this mutant to lysosomes. These results show that there is a striking difference between Ub-mediated internalization which is insensitive to the Ub positioning and Ub-mediated receptor routing which is dependent on the juxtamembrane localization of Ub.

Interaction of a juxtamembrane lysine with ubiquitin-binding adaptor proteins present at the plasma membrane is therefore likely to be important. Several proteins involved in receptor routing such as Hrs, STAM, Epsin and EAP45 contain UBD [46, 47]. Using a mass-spectrometry approach (Chapter 5) we were unable to identify UBD-containing protein that interacted with K632 of the CSF3R. Previously, we were also unable to show any interaction of the CSF3R with STAM and Hrs using co-immunoprecipitation. A complicating factor is that ubiquitin-interacting domains have a low affinity for ubiquitin, usually in the micromolar range and that interactions will often be transient, which will make them difficult to detect.

5. Types of ubiquitin chains in CSF3 induced receptor endocytosis

Different types of ubiquitination may occur which have different functional roles: monoubiquitination, multiubiquitination or polyubiquitination (Figure 1). In case of polyubiquitination, chains of multiple Ub moieties linked via one or more of its 7 internal lysines are affixed to the target protein. These chains can either be linear or branched. In addition, linear polyUb chains can be formed between the C-terminal glycine residue of Ub and the N-terminal methionine residue of the next Ub molecule [48]. Polyubiquitination involving K48 linked chains targets proteins for degradation via the 26S proteasome, whereas K63 linked chains have been implicated in DNA repair, transcriptional activation of protein kinases and endocytosis [48, 49].

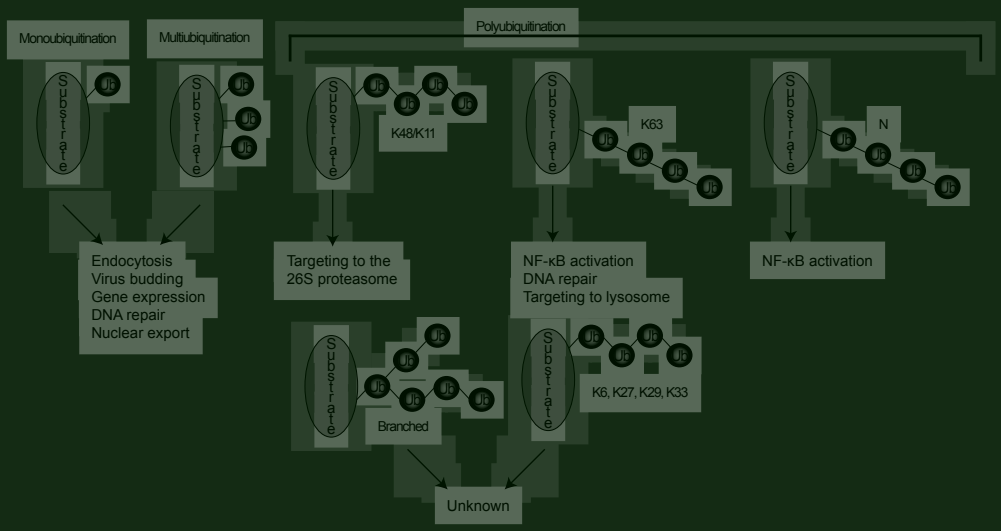


Figure 1: Different types of ubiquitin conjugations and their biological effects.

To study the Ub chain linkage attached to the activated CSF3R we used Western blot analysis with antibodies specific for two different types of ubiquitin linked chains, K48 and K63 Ub-chains. Ubiquitination of wt CSF3R could be detected with an antibody against K63-linked chains (Figure 2A). In contrast, no ubiquitination could be detected with the K48-antibody. Ubiquitination of the mKA mutant in which all the lysines are mutated to arginines except for K632 was readily detected with the K63 recognizing antibody (Figure 2B), indicating that K632, crucial for endocytic routing, is modified with K63-Ub chains.

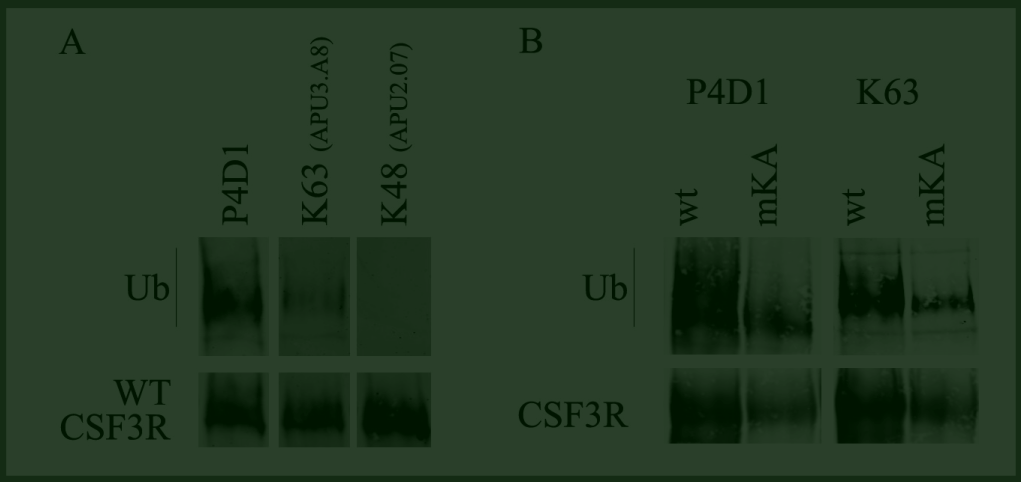


Figure 2: Activated CSF3Rs are modified with K63-linked Ub chains. (**A**) HEKT cells expressing wt CSF3R were stimulated for 30 min. at 37°C with Bio-CSF3. Receptor complexes were pulled down using streptavidin beads and analyzed by Western blotting using different Ub antibodies, P4D1 which recognizes all ubiquitin-conjugated proteins, APU3.A8 for K63-linked- and APU2.07 for K48-linked Ub-chains. (**B**) Same experiments as in A including the mKA CSF3R which only contains K632.

For the neurotrophic tyrosine kinase receptor A (TrkA) receptor it is shown that trafficking of the activated receptor and lysosomal degradation depends on K63-polyubiquitination of its K485. Blocking lysosomal degradation using different inhibitors resulted in the accumulation of TrkA receptors, that were not ubiquitinated. Blocking the proteasome with inhibitor lactacystin blocked TrkA receptor deubiquitination and lysosomal degradation. The model is that proteasomal deubiquitinating enzymes trim K63-ubiquitin chains from the TrkA receptor prior to its delivery to lysosomes for degradation [50]. A similar model has been proposed for the activated EPOR in which the proteasome first removes the C-terminal intracellular domain. In this study it was not determined if and which type of ubiquitination is involved in this proteasomal degradation. Subsequently, the remaining part is routed towards the lysosomes where it is degraded [51]. The TPOR was recently found to be ubiquitinated by Cbl on both of its cytoplamic lysine residues K553 and K573, followed by lysosomal and proteasomal degradation [52]. A CSF3R degradation product was detected 1 to 2 hours post CSF3 stimulation (Chapter 6). It remains to be established whether this is a physiologic proteosomal degradation product and whether its formation depends on ubiquitination of receptor lysines. If so, it would be interesting to determine if this lysine if modified with K63-linked Ub-chains or another type of Ub-chain and if other E3 ligases implicated in lysosomal degradation of cytokine receptors, such as β-Transducin repeat-containing protein (β-Trcp), might be involved in this process.

6. E2 ubiquitin conjugating enzymes involved in SOCS3-mediated CSF3R ubiquitination

The conjugation of Ub to an ε-NH2 group of a lysine residue in the substrate protein is usually achieved by the sequential actions of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [53]. The E2 is responsible both for E3 selection, the type of Ub linkage involved and is therefore responsible for much of the diversity of Ub involved signaling [54]. Active E2s contain a core ubiquitin-conjugating domain which contains a cysteine residue that interacts with the E1. After being charged with ubiquitin E2s interact with E3s to catalyze ubiquitination of the substrate [49]. A single E2 can interact with several E3s and a given E3 may interact with numerous E2s. The first step is initiated by the transfer of ubiquitin to a lysine residue in the target protein, thereafter chain elongation occurs. For some E2 conjugating enzymes it is known that they specifically elongate certain type of chains e.g. UBE2N-UBE2V1 are K63-specific elongating E2s while UBE2S specifically elongates K11-chains and UBE2K K48-chains [55-59]. It is thought that this preference arises by the fact that the E2 orients the acceptor Ub in such a manner that the preferential lysine is exposed to its active site [60, 61]. Determining E2-E3 pairs in human cells is difficult. However, directed yeast two-hybrid (Y2H) approaches have been fairly successful in identifying E2–E3 pairs using RING type E3s [62, 63]. In a large Y2H screen Rbx1 was shown to interact with UBE2M [62]. However, UBE2M is implicated in NEDD8ylation rather than ubiquitination [64]. The E2 ubiquitin-conjugating enzyme(s) involved in SOCS3

dependent ubiquitination of CSF3R have not yet been identified. Screening of the ~38 different members of E2 family in an Y2H assay in which all components of the ECS complex are present can help to find the E2 partner(s) in SOCS3-mediated CSF3R ubiquitination. Another approach would be to systematically knock-down all E2 conjugating enzymes in a CSF3R ubiquitination assay. However a knock-down approach may fail if there are multiple E2s involved. For instance, depletion of the anaphase promoting complex (APC)-specific E2 UBE2C did not completely stabilize APC substrates leading to the discovery of another APC-interacting E2, UBE2S [65]

7. Ubiquitin dependent forward routing of the CSF3R

Besides controlling endocytic trafficking, ubiquitination of receptors may also be an important determinant for receptor stability in the biosynthetic pathway. Membrane receptors undergo a complex process of post-translational modifications and maturation before delivery to the plasma membrane. The low levels of CSF3R at the plasma membrane compared with the large intracellular pool implicate a tightly controlled regulation the amount of receptors at the plasma membrane [66]. In Chapter 2, we report that the juxtamembrane K632 negatively and uniquely controls the CSF3R membrane expression. Previously, WD40 repeat and SOCS box containing proteins (WSB)-2 was found to interact with the C-terminal part of the CSF3R ligand independently and reduce steady-state CSF3R expression on the plasma membrane [67]. It was confirmed that that the action of WSB2 on receptor membrane expression dependent on the presence of lysines. Recently, WSB2 was also found to bind to the membrane-proximal cytoplasmic region of the IL21R, and similar to CSF3R negatively controlled the receptor expression [68].

β-Trcp is another E3 ligase containing a WD40 repeat, which thereby could potentially also bind to the C-terminal part of the CSF3R. β-Trcp is known to bind to other activated cytokine receptors and has been implicated in endocytic routing of these receptors [69, 70]. However, we were unable to show an interaction of the CSF3R with β-Trcp using co-immunoprecipitation.

8. Deubiquitinating enzymes involved in CSF3R routing

The attachment of ubiquitin moieties to lysine residues is reversed by the action of DUBs, hence it is likely that deubiquitination also plays an important role in endocytosis and signaling of receptors. Two endosomal DUBs, AMSH and UBPY, are involved in routing of activated receptors. Both are known to bind to the ESCRT-0 subunit STAM1 and to ESCRT-III [71-74]. We show that both AMSH and UBPY deubiquitinate CSF3R but do not increase the stability of the CSF3R (Chapter 4). In fact, AMSH even strongly reduced the steady-state CSF3R levels independent of receptor activation and internalization. A family of DUBs comprising DUB1, DUB1A, DUB2 and DUB2A, was previously found to be induced by different cytokines [75-78]. One family member, *DUB2A* was specifically upregulated in

response to CSF3 stimulation in myeloid cells. Why expression of *DUB2A* but not other DUBs was induced by CSF3 is still unknown. The promotor regions of the different DUBs are highly homologous. Studying the accessibility of the promotor region in these hematopoietic cells may reveal why expression of DUB2A, but not the other DUB family members, is specifically induced by CSF3. DUB2 is highly identical to DUB2A and overexpression reduced the ubiquitination status of the CSF3R and enhances CSF3R stability by promoting its accumulation in early endosomes and sustaining STAT5 signaling. DUB2 was previously shown to counteract the E3 ligase Cbl in ubiquitination and degradation of the common cytokine receptor γc which is involved in lymphocyte proliferation and survival [79]. The mode of action of the different DUBs in the endosomal pathway and the intracellular location where they deubiquitinate CSF3R are shown schematically in Figure 3.

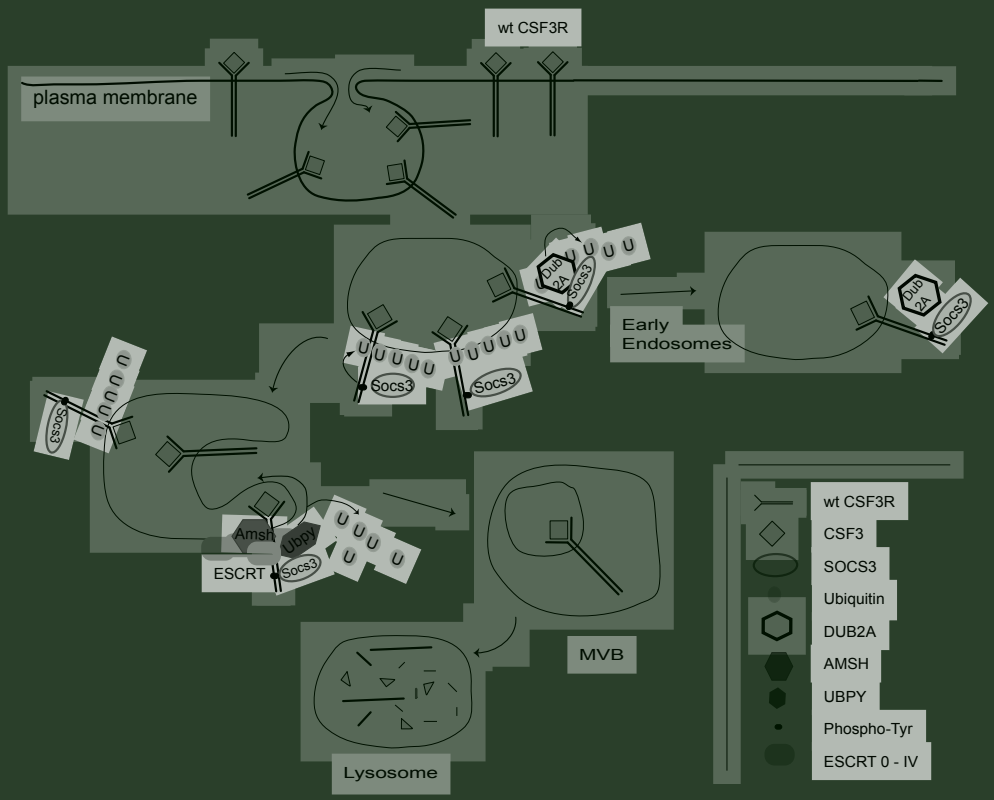


Figure 3: Schematic representation of the location of DUB2A, AMSH and UBPY in activated CSF3R trafficking.

At this point, we cannot exclude that DUB2 acts indirectly on the CSF3R through deubiquitination of CSF3R-associated partners or members of the endocytotic machinery thereby leading indirectly to its deubiquitination and block in lysosomal trafficking. This is not without precedent because DUB1, a highly related family member, was shown to be involved in deubiquitinating dynein heavy chain which is involved in routing [80]. In conclusion, SOCS3

and DUB2A, both induced by CSF3 stimulation in hematopoietic cells, act in a negative and positive feedback-loop to regulate the CSF3R routing and signaling. The balanced expression of these proteins critically determines CSF3R localization and signaling fate.

9. (A)AAGUGC seed-containing miRNAs act on CSF3R MAPK signaling by targeting SQSTM1

MicroRNAs (miRNAs) are small noncoding RNAs that bind to the 3'-untranslated region of target mRNAs [81]. In Chapter 6 we reported that (A)AAGUGC-seed containing miRNAs affect CSF3 dependent ERK signaling and CSF3R stability by targeting SQSTM1. We observed SQSTM1 binding to the activated CSF3R complex when the CSF3R resides in late endosomes. Studies with SQSTM1 mutants should be used to elucidate the SQSTM1 binding specificities to the CSF3R complex. SQSTM1 could be binding to the CSF3R with its ubiquitin associated (UBA) domain because binding of SQSTM1 to the K5R mutant was severely reduced. However this may also be due to a block in routing of this receptor beyond the early endosomes, if SQSTM1 anchors to the receptor mainly at the late endosome. SQSTM1 is known to interact with the TrkA receptor via its UBA domain and to be involved in its internalization and trafficking. It was described that SQTM1 serves as a shuttling protein for interaction of ubiquitinated TrkA with the proteasome [82]. SQSTM1 is implicated in targeting proteins for autophagosomal degradation [83], however we did not detect CSF3R in LC3-positive autophagosome.

We showed enhanced P-ERK levels by CSF3R activation when SQSTM1 expression was silenced which could be due to two different mechanisms that are not mutually exclusive. Several studies have shown SQSTM1 dependent regulation of MAPK activity by inhibition and direct sequestration of ERKs [84, 85]. Recent data have also shown that the MEK-ERK pathway is anchored to the late endosomes [86]. Defective routing of the CSF3R from late endosome to lysosome could therefore result in prolonged ERK signaling. MiRNA dependent regulation of receptor signaling provides the cell with an additional mechanism to regulate signaling output. For instance, miRNAs have been implicated in control of toll-like receptor signalling, by targeting components of their signal transduction pathway or by direct regulation of receptor expression [87, 88].

10. Perspective

The CSF3R is important for neutrophil development and several mutations and polymorphisms in the *CSF3R* gene have been reported in myeloid disorders such as severe congenital neutropenia (SCN) [89-93]. These receptor mutations were found to perturb CSF3R signaling, which determines the balance between proliferation and differentiation of myeloid progenitors to mature neutrophils. Lack of lysosomal sorting of the d715 CSF3R mutant due to lack in internalization or ubiquitination also leads to prolonged CSF3 signaling and hyperproliferation [94, 95]. Cell signaling and endocytosis were previously seen as distinct

processes but are now recognized to be bi-directionally linked [21]. Tight regulation of signal duration and receptor routing is therefore crucial in determining the functional fate of cells. Activation of the CSF3R occurs at the plasma membrane but signaling continues when the receptor reaches the early endosome and the late endosome. Inactivation of different signaling molecules occurs at several time points when the receptor resides in different compartments. For example down regulation of PI3K pathway occurs mostly at the plasma membrane while ERK signaling even continues when the receptor is in late endosomes [37]. By using different live cell imaging techniques such as total internal reflection fluorescence microscope (TIRFM) with fluorescently labeled signaling molecules, the dynamics of CSF3R signaling can now be studied in further detail and be quantified. This will further clarify the relationship between CSF3R endocytosis and the duration and intensity of different signaling mechanisms in time and space.

As the role of positive and negative regulatory proteins or protein complexes in controlling CSF3R signaling and routing is becoming more clear, different strategies to correct responses of cells that have deregulated CSF3R routing and signaling can be envisaged. For example, since DUB2A opposes the effects of SOCS3 on CSF3R ubiquitination, it is now worthwhile to investigate whether alteration in DUBs are linked with CSF3R deregulated signaling in human myeloid malignancies.

REFERENCES

1. O'Sullivan, L.A., et al., *Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease*. Mol Immunol, 2007. **44**(10): p. 2497-506.
2. Kovarik, P., et al., *Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression*. Embo J, 2001. **20**(1-2): p. 91-100.
3. Soldaini, E., et al., *DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites*. Mol Cell Biol, 2000. **20**(1): p. 389-401.
4. Hawkins, R.D. and B. Ren, *Genome-wide location analysis: insights on transcriptional regulation*. Hum Mol Genet, 2006. **15 Spec No 1**: p. R1-7.
5. He, K., et al., *Janus kinase 2 determinants for growth hormone receptor association, surface assembly, and signaling*. Mol Endocrinol, 2003. **17**(11): p. 2211-27.
6. Huang, L.J., S.N. Constantinescu, and H.F. Lodish, *The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor*. Mol Cell, 2001. **8**(6): p. 1327-38.
7. Royer, Y., et al., *Janus kinases affect thrombopoietin receptor cell surface localization and stability*. J Biol Chem, 2005. **280**(29): p. 27251-61.
8. Radtke, S., et al., *Novel role of Janus kinase 1 in the regulation of oncostatin M receptor surface expression*. J Biol Chem, 2002. **277**(13): p. 11297-305.
9. Barge, R.M., et al., *Tryptophan 650 of human granulocyte colony-stimulating factor (G-CSF) receptor, implicated in the activation of JAK2, is also required for G-CSF-mediated activation of signaling complexes of the p21ras route*. Blood, 1996. **87**(6): p. 2148-53.
10. Freedman, M.H. and B.P. Alter, *Risk of myelodysplastic syndrome and acute myeloid leukemia in congenital neutropenias*. Semin Hematol, 2002. **39**(2): p. 128-33.
11. Knoops, L., et al., *JAK kinases overexpression promotes in vitro cell transformation*. Oncogene, 2008. **27**(11): p. 1511-9.
12. Baxter, E.J., et al., *Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders*. Lancet, 2005. **365**(9464): p. 1054-61.
13. James, C., et al., *A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera*. Nature, 2005. **434**(7037): p. 1144-8.
14. James, C., et al., *A JAK2 mutation in myeloproliferative disorders: pathogenesis and therapeutic and scientific prospects*. Trends Mol Med, 2005. **11**(12): p. 546-54.
15. Vainchenker, W. and S.N. Constantinescu, *A unique activating mutation in JAK2 (V617F) is at the origin of polycythemia vera and allows a new classification of myeloproliferative diseases*. Hematology Am Soc Hematol Educ Program, 2005: p. 195-200.
16. Lu, X., et al., *Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation*. Proc Natl Acad Sci U S A, 2005. **102**(52): p. 18962-7.
17. Lu, X., L.J. Huang, and H.F. Lodish, *Dimerization by a cytokine receptor is necessary for constitutive activation of JAK2V617F*. J Biol Chem, 2008. **283**(9): p. 5258-66.
18. Steensma, D.P., et al., *JAK2 V617F is a rare finding in de novo acute myeloid leukemia, but STAT3 activation is common and remains unexplained*. Leukemia, 2006. **20**(6): p. 971-8.
19. Shen, Y.M., et al., *[Detection of JAK2V617F mutation and its clinical significance in 80 patients with M2 acute myelogenous leukemia]*. Zhonghua Zhong Liu Za Zhi, 2009. **31**(5): p. 366-70.

20. Shide, K., et al., *Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F*. Leukemia, 2008. **22**(1): p. 87-95.
21. Sorkin, A. and M. von Zastrow, *Endocytosis and signalling: intertwining molecular networks*. Nat Rev Mol Cell Biol, 2009. **10**(9): p. 609-22.
22. Zwang, Y. and Y. Yarden, *Systems biology of growth factor-induced receptor endocytosis*. Traffic, 2009. **10**(4): p. 349-63.
23. Grant, B.D. and J.G. Donaldson, *Pathways and mechanisms of endocytic recycling*. Nat Rev Mol Cell Biol, 2009. **10**(9): p. 597-608.
24. Doherty, G.J. and H.T. McMahon, *Mechanisms of endocytosis*. Annu Rev Biochem, 2009. **78**: p. 857-902.
25. Sorkin, A. and L.K. Goh, *Endocytosis and intracellular trafficking of ErbBs*. Exp Cell Res, 2009. **315**(4): p. 683-96.
26. Hofmann, K. and L. Falquet, *A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems*. Trends Biochem Sci, 2001. **26**(6): p. 347-50.
27. Kazazic, M., et al., *Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits*. Traffic, 2009. **10**(2): p. 235-45.
28. Levkowitz, G., et al., *c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor*. Genes Dev, 1998. **12**(23): p. 3663-74.
29. Umebayashi, K., H. Stenmark, and T. Yoshimori, *Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation*. Mol Biol Cell, 2008. **19**(8): p. 3454-62.
30. Grovdal, L.M., et al., *Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation*. Exp Cell Res, 2004. **300**(2): p. 388-95.
31. Huang, F., L.K. Goh, and A. Sorkin, *EGF receptor ubiquitination is not necessary for its internalization*. Proc Natl Acad Sci U S A, 2007. **104**(43): p. 16904-9.
32. Grishin, A., et al., *Involvement of Shc and Cbl-PI 3-kinase in Lyn-dependent proliferative signaling pathways for G-CSF*. Oncogene, 2000. **19**(1): p. 97-105.
33. Wang, L., et al., *Repression of c-Cbl leads to enhanced G-CSF Jak-STAT signaling without increased cell proliferation*. Oncogene, 2002. **21**(34): p. 5346-55.
34. Naka, T., et al., *Structure and function of a new STAT-induced STAT inhibitor*. Nature, 1997. **387**(6636): p. 924-9.
35. Martens, N., et al., *Suppressor of cytokine signaling 7 inhibits prolactin, growth hormone, and leptin signaling by interacting with STAT5 or STAT3 and attenuating their nuclear translocation*. J Biol Chem, 2005. **280**(14): p. 13817-23.
36. Tomic, S., N. Chughtai, and S. Ali, *SOCS-1, -2, -3: selective targets and functions downstream of the prolactin receptor*. Mol Cell Endocrinol, 1999. **158**(1-2): p. 45-54.
37. Irandoust, M.I., et al., *Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor*. EMBO J, 2007. **26**(7): p. 1782-93.
38. Ram, P.A. and D.J. Waxman, *SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms*. J Biol Chem, 1999. **274**(50): p. 35553-61.
39. Fujimoto, M. and T. Naka, *Regulation of cytokine signaling by SOCS family molecules*. Trends Immunol, 2003. **24**(12): p. 659-66.
40. Kile, B.T., et al., *The SOCS box: a tale of destruction and degradation*. Trends Biochem Sci, 2002. **27**(5): p. 235-41.

41. Kamura, T., et al., *The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families*. Genes Dev, 1998. **12**(24): p. 3872-81.
42. Kamura, T., et al., *VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases*. Genes Dev, 2004. **18**(24): p. 3055-65.
43. Verdier, F., et al., *Proteasomes regulate erythropoietin receptor and signal transducer and activator of transcription 5 (STAT5) activation. Possible involvement of the ubiquitinated Cis protein*. J Biol Chem, 1998. **273**(43): p. 28185-90.
44. Landsman, T. and D.J. Waxman, *Role of the cytokine-induced SH2 domain-containing protein CIS in growth hormone receptor internalization*. J Biol Chem, 2005. **280**(45): p. 37471-80.
45. van Kerkhof, P., et al., *Endocytosis and degradation of the growth hormone receptor are proteasome-dependent*. J Biol Chem, 2000. **275**(3): p. 1575-80.
46. Slagsvold, T., et al., *Endosomal and non-endosomal functions of ESCRT proteins*. Trends Cell Biol, 2006. **16**(6): p. 317-26.
47. Hirano, S., et al., *Structural basis of ubiquitin recognition by mammalian Eap45 GLUE domain*. Nat Struct Mol Biol, 2006. **13**(11): p. 1031-2.
48. Woelk, T., et al., *The ubiquitination code: a signalling problem*. Cell Div, 2007. **2**: p. 11.
49. Ye, Y. and M. Rape, *Building ubiquitin chains: E2 enzymes at work*. Nat Rev Mol Cell Biol, 2009. **10**(11): p. 755-64.
50. Geetha, T. and M.W. Wooten, *TrkA receptor endolysosomal degradation is both ubiquitin and proteasome dependent*. Traffic, 2008. **9**(7): p. 1146-56.
51. Walrafen, P., et al., *Both proteasomes and lysosomes degrade the activated erythropoietin receptor*. Blood, 2005. **105**(2): p. 600-8.
52. Saur, S.J., et al., *Ubiquitination and degradation of the thrombopoietin receptor c-Mpl*. Blood, 2010. **115**(6): p. 1254-63.
53. Pickart, C.M. and M.J. Eddins, *Ubiquitin: structures, functions, mechanisms*. Biochim Biophys Acta, 2004. **1695**(1-3): p. 55-72.
54. Wenzel, D.M., K.E. Stoll, and R.E. Klevit, *E2s: structurally economical and functionally replete*. Biochem J, 2010. **433**(1): p. 31-42.
55. Deng, L., et al., *Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain*. Cell, 2000. **103**(2): p. 351-61.
56. VanDemark, A.P., et al., *Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer*. Cell, 2001. **105**(6): p. 711-20.
57. Haldeman, M.T., et al., *Structure and function of ubiquitin conjugating enzyme E2-25K: the tail is a core-dependent activity element*. Biochemistry, 1997. **36**(34): p. 10526-37.
58. Jin, L., et al., *Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex*. Cell, 2008. **133**(4): p. 653-65.
59. Petroski, M.D., et al., *Substrate modification with lysine 63-linked ubiquitin chains through the UBC13-UEV1A ubiquitin-conjugating enzyme*. J Biol Chem, 2007. **282**(41): p. 29936-45.
60. Eddins, M.J., et al., *Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation*. Nat Struct Mol Biol, 2006. **13**(10): p. 915-20.
61. Das, R., et al., *Allosteric activation of E2-RING finger-mediated ubiquitylation by a structurally defined specific E2-binding region of gp78*. Mol Cell, 2009. **34**(6): p. 674-85.
62. Markson, G., et al., *Analysis of the human E2 ubiquitin conjugating enzyme protein interaction network*. Genome Res, 2009. **19**(10): p. 1905-11.

63. van Wijk, S.J., et al., *A comprehensive framework of E2-RING E3 interactions of the human ubiquitin-proteasome system*. Mol Syst Biol, 2009. **5**: p. 295.

64. Huang, D.T., et al., *E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification*. Mol Cell, 2009. **33**(4): p. 483-95.

65. Williamson, A., et al., *Identification of a physiological E2 module for the human anaphase-promoting complex*. Proc Natl Acad Sci U S A, 2009. **106**(43): p. 18213-8.

66. Aarts, L.H., et al., *Receptor activation and 2 distinct COOH-terminal motifs control G-CSF receptor distribution and internalization kinetics*. Blood, 2004. **103**(2): p. 571-9.

67. Erkeland, S.J., et al., *Novel role of WD40 and SOCS box protein-2 in steady-state distribution of granulocyte colony-stimulating factor receptor and G-CSF-controlled proliferation and differentiation signaling*. Oncogene, 2007. **26**(14): p. 1985-94.

68. Nara, H., et al., *Regulation of interleukin-21 receptor expression and its signal transduction by WSB-2*. Biochem Biophys Res Commun, 2010. **392**(2): p. 171-7.

69. Li, Y., et al., *Negative regulation of prolactin receptor stability and signaling mediated by SCF(beta-TrCP) E3 ubiquitin ligase*. Mol Cell Biol, 2004. **24**(9): p. 4038-48.

70. Meyer, L., et al., *beta-Trcp mediates ubiquitination and degradation of the erythropoietin receptor and controls cell proliferation*. Blood, 2007. **109**(12): p. 5215-22.

71. Agromayor, M. and J. Martin-Serrano, *Interaction of AMSH with ESCRT-III and deubiquitination of endosomal cargo*. J Biol Chem, 2006. **281**(32): p. 23083-91.

72. Kaneko, T., et al., *Structural insight into modest binding of a non-PXXP ligand to the signal transducing adaptor molecule-2 Src homology 3 domain*. J Biol Chem, 2003. **278**(48): p. 48162-8.

73. Kato, M., K. Miyazawa, and N. Kitamura, *A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP*. J Biol Chem, 2000. **275**(48): p. 37481-7.

74. Row, P.E., et al., *The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation*. J Biol Chem, 2007. **282**(42): p. 30929-37.

75. Baek, K.H., et al., *DUB-1A, a novel deubiquitinating enzyme subfamily member, is polyubiquitinated and cytokine-inducible in B-lymphocytes*. J Biol Chem, 2004. **279**(4): p. 2368-76.

76. Baek, K.H., et al., *DUB-2A, a new member of the DUB subfamily of hematopoietic deubiquitinating enzymes*. Blood, 2001. **98**(3): p. 636-42.

77. Zhu, Y., et al., *DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes*. J Biol Chem, 1997. **272**(1): p. 51-7.

78. Zhu, Y., et al., *The murine DUB-1 gene is specifically induced by the betac subunit of interleukin-3 receptor*. Mol Cell Biol, 1996. **16**(9): p. 4808-17.

79. Gesbert, F., V. Malarde, and A. Dautry-Varsat, *Ubiquitination of the common cytokine receptor gammac and regulation of expression by an ubiquitination/deubiquitination machinery*. Biochem Biophys Res Commun, 2005. **334**(2): p. 474-80.

80. Lee, M.Y., et al., *DUB-1, a fate determinant of dynein heavy chain in B-lymphocytes, is regulated by the ubiquitin-proteasome pathway*. J Cell Biochem, 2008. **105**(6): p. 1420-9.

81. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions*. Cell, 2009. **136**(2): p. 215-33.

82. Geetha, T., et al., *p62 serves as a shuttling factor for TrkA interaction with the proteasome*. Biochem Biophys Res Commun, 2008. **374**(1): p. 33-7.

83. Kirkin, V., et al., *A role for ubiquitin in selective autophagy*. Mol Cell, 2009. **34**(3): p. 259-69.

84. Lee, S.J., et al., *A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis*. EMBO Rep, 2010. **11**(3): p. 226-32.

85. Moscat, J., M.T. Diaz-Meco, and M.W. Wooten, *Signal integration and diversification through the p62 scaffold protein*. Trends Biochem Sci, 2007. **32**(2): p. 95-100.

86. Nada, S., et al., *The novel lipid raft adaptor p18 controls endosome dynamics by anchoring the MEK-ERK pathway to late endosomes*. Embo J, 2009. **28**(5): p. 477-89.

87. O'Neill, L.A., F.J. Sheedy, and C.E. McCoy, *MicroRNAs: the fine-tuners of Toll-like receptor signalling*. Nat Rev Immunol, 2011.

88. Giles, K.M., et al., *MicroRNA regulation of growth factor receptor signaling in human cancer cells*. Methods Mol Biol, 2011. **676**: p. 147-63.

89. Dong, F., et al., *Mutations in the granulocyte colony-stimulating factor receptor gene in patients with severe congenital neutropenia*. Leukemia, 1997. **11**(1): p. 120-5.

90. Dong, F., et al., *A point mutation in the granulocyte colony-stimulating factor receptor (G-CSF-R) gene in a case of acute myeloid leukemia results in the overexpression of a novel G-CSF-R isoform*. Blood, 1995. **85**(4): p. 902-11.

91. Wolfler, A., et al., *A functional single-nucleotide polymorphism of the G-CSF receptor gene predisposes individuals to high-risk myelodysplastic syndrome*. Blood, 2005. **105**(9): p. 3731-6.

92. Ward, A.C., et al., *Novel point mutation in the extracellular domain of the granulocyte colony-stimulating factor (G-CSF) receptor in a case of severe congenital neutropenia hyporesponsive to G-CSF treatment*. J Exp Med, 1999. **190**(4): p. 497-507.

93. Awaya, N., et al., *Novel variant isoform of G-CSF receptor involved in induction of proliferation of FDCP-2 cells: relevance to the pathogenesis of myelodysplastic syndrome*. J Cell Physiol, 2002. **191**(3): p. 327-35.

94. van de Geijn, G.J., et al., *G-CSF receptor truncations found in SCN/AML relieve SOCS3-controlled inhibition of STAT5 but leave suppression of STAT3 intact*. Blood, 2004. **104**(3): p. 667-74.

95. McLemore, M.L., J. Poursine-Laurent, and D.C. Link, *Increased granulocyte colony-stimulating factor responsiveness but normal resting granulopoiesis in mice carrying a targeted granulocyte colony-stimulating factor receptor mutation derived from a patient with severe congenital neutropenia*. J Clin Invest, 1998. **102**(3): p. 483-92.

96. Prchal, J.F. and A.A. Axelrad, *Letter: Bone-marrow responses in polycythemia vera*. N Engl J Med, 1974. **290**(24): p. 1382.

SUMMARY

Experiments reported in this thesis have specifically dealt with the mechanisms that regulate intracellular trafficking of the CSF3R and the implications on signaling. Chapter 1 gives an overview of current knowledge in routing and ligand-dependent signal transduction of the CSF3R. There are several negative regulatory pathways responsible for switching-off CSF3R signaling. CSF3R internalization and lysosomal routing is one of the major negative feedback mechanisms described to ensure proper signal duration.

Chapter 2 focuses on steady-state CSF3R membrane expression. It is shown that two mechanisms controlling forward routing lead to increased cell surface expression and stability of the CSF3R. The first involves the juxtamembrane K632, which controls the distribution of the receptor presumably by ubiquitin controlled degradation; the second involves JAK proteins. Notably, the effect of JAKs is not exerted by masking motifs essential for receptor internalization or by diminishing the level of K632 ubiquitination. Importantly, it is shown in this chapter that higher JAK levels in combination with routing defective receptors result in ligand-independent signaling and cell survival, cellular features associated with leukemic transformation in myeloproliferative disorders.

Experiments presented in Chapters 3 and 4 deal with different aspects of CSF3R routing. In Chapter 3 it is shown that positioning of K632, which is ubiquitinated by SOCS3 after CSF3 activation, is flexible, but that membrane-proximal localization is indispensable for directing lysosomal routing. Specifically, neither fusion of the juxtamembrane domain nor ubiquitin to the C-terminus of CSF3R mutant in which all the lysines were mutated to arginines could restore lysosomal routing. These findings argue against the existence of a linear motif, in which exact positioning of a lysine residue is critical, for ubiquitin-mediated lysosomal routing. On the other hand, positioning of a lysine in proximity of the plasma membrane was found to be essential, suggesting that in addition to lysine ubiquitination, interactions with membrane-associated components are needed to trigger lysosomal routing. Ubiquitination of the severe congenital neutropenia-derived d715-CSF3R mutant, which fails to internalize and lacks the SOCS3 recruitment site (Y729), was severely reduced. Direct fusion of ubiquitin to d715-CSF3R mutant restored internalization but failed to guide this mutant to lysosomes. This shows a marked distinction between Ub-mediated internalization which is insensitive to the Ub positioning and Ub-mediated receptor routing which is dependent on the juxtamembrane localization of Ub.

In Chapter 4, the role of deubiquitinating enzymes belonging to the ESCRT machinery, UBPY and AMSH and of a family of DUBs upregulated by cytokines in hematopoietic cells was investigated. All these deubiquitinating enzymes caused decreased ubiquitination of activated CSF3Rs in an overexpression setting. However, DUB2A, which was specifically induced by CSF3 in hematopoietic cells, profoundly enhanced the stability of the CSF3R by accumulation of the receptor in early endosomes. These data fit in a model in which SOCS3

is upregulated by CSF3 and ubiquitinates K632, which is crucial for lysosomal degradation of the CSF3R. Subsequently, DUB2A expression is induced, which counterbalances the E3 ligase activity of SOCS3.

In Chapter 5, an unbiased mass-spectrometry approach was used in an attempt to discover key players involved in CSF3R signaling and routing. One of the identified proteins was sequestosome 1 (SQSTM1), which' role in relation to the CSF3R is described in Chapter 6. SQSTM1 contains an ubiquitin binding domain and binds to the CSF3R predominantly in the late endosomal compartment. Knock-down of SQSTM1 resulted in enhanced CSF3R stability, which presumably results from ineffective CSF3R trafficking from the late endosomal compartment towards lysosomes. Subsequently, enhanced ERK signaling induced by CSF3 was observed when SQSTM1 expression was silenced. ERK signaling is active when the CSF3R is in late endosomes. Defective routing of the CSF3R to lysosomal degradation is therefore a plausible explanation for the enhanced ERK activation.

NEDERLANDSE SAMENVATTING

De beschreven experimenten in dit proefschrift hebben vooral betrekking op het ontrafelen van de mechanismen die intracellulaire routing van de CSF3R reguleren en de implicaties van receptor routing voor de signaalfunctie.

In *Hoofdstuk 1* wordt een overzicht gegeven van de huidige kennis op het gebied van routing en ligandafhankelijke signaaltransductie van de CSF3R. Er zijn een aantal negatief regulerende mechanismen betrokken bij het beëindigen van CSF3R signalering. Internalisatie en lysosomale routing van de CSF3R zijn belangrijke negatieve feedback mechanismen, welke zorgen voor een gecontroleerde duur en intensiteit van intracellulaire signalering.

In *Hoofdstuk 2* zijn experimenten beschreven die gericht zijn op "steady-state" membraan expressie van de CSF3R. Er worden twee mechanismen getoond die de voorwaartse routing van de receptor beïnvloeden en daarbij leiden tot een verhoogde CSF3R membraanexpressie en stabiliteit. Het eerste is afhankelijk van de lysine (K)632, die in de directe nabijheid van het membraan gepositioneerd is, en de receptor membraan distributie waarschijnlijk beïnvloedt door ubiquitine afhankelijke degradatie. Bij het tweede mechanisme zijn JAK eiwitten betrokken. Het precieze mechanisme waarmee JAKs CSF3R membraan expressie beïnvloeden zal nog opgehelderd moeten worden. Het effect van JAKs wordt in elk geval niet bewerkstelligd door het ontoegankelijk maken van motieven die essentieel zijn voor receptor internalisatie of door het verminderen van K632 ubiquitinering. Hogere JAK niveaus in combinatie met de expressie van routingdefectieve CSF3 receptoren resulteerde in ligandonafhankelijke signalering en celoverleving, kenmerken die geassocieerd zijn met leukemische transformatie in myeloproliferatieve aandoeningen.

De *Hoofdstukken 3 en 4* behandelen verschillende aspecten van CSF3R routing. In *Hoofdstuk 3* wordt getoond dat de positie van K632, die geubiquitineerd wordt door SOCS3 na CSF3R activering, enigszins flexibel is, maar dat de membraan-proximale locatie onmisbaar is voor het sturen van lysosomale routing. Noch fusie van het juxtamembraan domein, noch van ubiquitine aan de C-terminus van de K5R CSF3R mutant, waarin de lysines vervangen zijn door arginines, kon de lysosomale routing herstellen. Deze bevindingen pleiten tegen het bestaan van een lineair motief, waarin de exacte positie van een lysine bepalend is voor ubiquitine-gemedieerde lysosomale routing. Daarentegen bleek de positionering van de lysine in de nabijheid van het plasmamembraan essentieel, wat suggereert dat naast lysine ubiquitinering, interacties met membraangeassocieerde componenten nodig zijn om lysosomale routing te bewerkstelligen. Ubiquitinering van de d715-CSF3R mutant, die niet internaliseert en de SOCS3 bindingsplaats (Y729) mist, was drastisch verminderd. Directe fusie van ubiquitine aan de d715-CSF3R mutant herstelde internalisatie, maar leidde niet tot routing van deze receptor mutant naar lysosomen. Dit laat een belangrijk verschil zien tussen ubiquitine-gemedieerde internalisatie waarbij positionering niet van belang is en ubiquitine-gemedieerde receptor routing welke wel afhankelijk is van de juxtamembraan lokalisatie van ubiquitine.

In *Hoofdstuk 4* is onderzocht of deubiquitineringsenzymen UBPY en AMSH, die binden aan het ESCRT complex, en DUB1, DUB1A, DUB2 en DUB2A, die opgereguleerd worden

door cytokines in hematopoietische cellen, een rol spelen bij de regulatie van CSF3R routing en signalering. Overexpressie van deze enzymen resulteerde in verminderde ubiquitinering van geactiveerde CSF3 receptoren. DUB2A werd echter als enige specifiek geïnduceerd door CSF3 in hematopoietische cellen en verhoogde de stabiliteit van de CSF3R door accumulatie van de receptor in vroege endosomen. Op basis van deze data wordt een model gepostuleerd waarin SOCS3 na CSF3 stimulatie wordt opgereguleerd en K632 ubiquitineert, wat cruciaal is voor lysosomale CSF3R degradatie ("negatieve feedback loop"). Vervolgens wordt DUB2A opgereguleerd, welke de E3 ligase activiteit van SOCS3 tegenwerkt en daardoor CSF3R degradatie en signaalremming voorkomt ("positieve feedback loop").

In *Hoofdstuk 5* is massaspectrometrie gebruikt om eiwitten betrokken bij CSF3R signalering en routing te identificeren. Eén van de gevonden eiwitten is sequestosome 1 (SQSTM1), waarvan de rol in relatie tot de CSF3R beschreven wordt in *Hoofdstuk 6*. SQSTM1 bevat een domein dat ubiquitine kan binden. Getoond wordt dat SQSTM1 aan de CSF3R bindt wanneer deze zich in het late endosomale compartiment bevindt. Knockdown van SQSTM1 resulteerde in verhoogde CSF3R stabiliteit, waarschijnlijk door ineffectieve CSF3R routing van het late endosomale compartiment naar het lysosoom. Tevens werd gevonden dat CSF3-geïnduceerde ERK signalering verhoogd is wanneer SQSTM1 expressie wordt geblokkeerd. ERK signalering is nog actief wanneer de CSF3R zich bevindt in late endosomen. Verminderde routing van de CSF3R naar lysosomen is daarom een aannemelijk verklaring voor de verhoogde ERK activatie.

ABBREVIATIONS

7-AAD	7-amino-actinomycin D
AML	acute myeloid leukemia
AMSH	associated molecule with the SH3 domain of STAM
AP	adaptor protein complex
APC	anaphasepromoting complex
Bio-CSF3	biotinylated granulocyte colony-stimulating factor 3
BC	bar-coded
BM	bone marrow
β-Trcp	β-transducin repeat-containing protein
CCP	clathrin coated pit
CCV	clathrin coated vesicle
CDKN	cyclin-dependent kinase inhibitor
CEBPA	CCAAT/enhancer-binding protein alpha
CFU	colony-forming unit
CHMP	charged multivesicular body protein
CHX	cycloheximide
CIS	cytokine inducible SH2-containing protein
CLSM	confocal laser scanning microscopy
CSF	colony-stimulating factor
CSF3R	granulocyte colony-stimulating factor 3 receptor
DMEM	Dulbecco's modified Eagle's medium
DOR	δ-opioid peptide receptor
DOX	doxycycline
DUB	deubiquitinating enzyme
EEA1	early endosome antigen 1
ECS	elongin-cullin-socs box
EGFR	epidermal growth factor receptor
EndoH	endoglycosidase H
Epo	erythropoietin
EpoR	erythropoietin receptor
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
ERAD	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
ESCRT	endosomal sorting complex required for transport
ET	essential thrombocythemia
EV	empty vector

FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FERM	Band 4.1, Ezrin, Radixin, Moesin
FLT3	fms-related tyrosine kinase 3
GFP	green fluorescent protein
GGA	golgi-associated γ-adaptin ear homology domain Arf (ADP-ribosylation factor)-interacting protein
HEK	human embryonic kidney
HGNC	HUGO Gene Nomenclature Committee
HSC	hematopoietic stem cells
IFNAR1	interferon receptor-α1
Ig	immunoglobulin
IL	interleukin
IMF	idiopathic myelofibrosis
IP	immunoprecipitation
IRES	internal ribosome entry site
JAK	janus kinase
JAMM	JAB1, MPN, MOV34 metalloenzymes
JNK	c-Jun N-terminal kinase
KIR	kinase inhibitory region
MAPK	mitogen-activated protein kinase
MβCD	methyl-β-cyclodextrin
miRNA	micro-ribonucleic acid
MJD	Machado Joseph Disease domain proteins
MFI	mean fluorescence intensity
MIT	microtubule interacting and transport
MSCV	murine stem cell virus
MVB	multivesicular body
NET	neutrophil extracellular traps
NGFR	nerve growth factor receptor
OSMR	oncostatin M receptor
OTU	ovarian tumour proteins
PBC	peripheral blood cells
PE	phycoerythrin
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
PP2	protein phosphatase 2
PtdIns3P	phosphatidylinositol 3-phosphate
PV	polycythemia vera

qPCR	quantitative polymerase chain reaction
RI	ribonuclease inhibitor
RT-PCR	real-time polymerase chain reaction
SCF	stem cell factor
SCN	severe congenital neutropenia
SD	standard deviation
SE	standard error
SEM	standard error of mean
SH	src homology
SILAC	stable isotope labeling with amino acids in cell culture
SOCS	suppressor of cytokine signaling
SQSTM1	sequestosome 1
SS	steady-state
STAM	signal transducing adaptor molecule
STAT	signal transducer and activator of transcription
TIRFM	total internal reflection fluorescence microscope
TGN	trans-golgi network
TM	transmembrane
Tpo	thrombopoietin
TpoR	thrombopoietin receptor
TRAF	tumor necrosis factor receptor-associated factor
TrkA	neurotrophic tyrosine kinase receptor A
TYK2	tyrosine kinase 2
Ub	ubiquitin
UBPY	ubiquitin isopeptidase Y
UCH	ubiquitin C-terminal hydrolases
USP	ubiquitin-specific peptidase
UTR	untranslated region
WSB	WD40 repeat and SOCS box containing proteins
WT	wild-type
YWHA	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

DANKWOORD

Het is zover! De voltooiing van dit proefschrift is het resultaat van vier en een half jaar met heel veel plezier werken op de afdeling hematologie binnen het Erasmus MC. Ik wil hierbij alle mensen bedanken, die direct of indirect een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Door jullie bijdrage en samenwerking is de tijd voorbij gevlogen! Graag wil ik enkele mensen in het bijzonder bedanken, beginnend bij mijn promotor.

Beste **Ivo**, jij hebt mij de gelegenheid geboden om te werken aan het CSF3R onderzoeksproject. Ik heb het als bijzonder prettig ervaren dat je altijd bereid was om samen met mij de laatste resultaten te bespreken. Ik bewonder je enorme hoeveelheid kennis en wil je bedanken voor het snel becommentariëren van alle door mij aangeleverde manuscripten. Daarnaast zal ik nooit vergeten dat ik dankzij jou mijn Batavus fiets wél heb teruggekregen!

De leden van mijn kleine commissie, prof. dr. **Ruud Delwel**, prof. dr. **Rudi Hendriks** en dr. **Marieke von Lindern,** wil ik bedanken voor het beoordelen van mijn manuscript. Ik wil me kort richten op een voor mij bijzonder lid van deze kleine commissie. Beste **Marieke**, ik heb genoten van jouw vele verhalen, luisterend oor en je adviezen. Bij het geven van presentaties probeer ik nu niet meer vragend over te komen, maar of ik daar altijd in slaag blijft de vraag. Dat jij als hoofd van de afdeling hematopoiese op Sanquin nog zelf cellen kweekt en experimenten doet, maakt jou voor mij de ultieme wetenschapper. Hartelijk dank voor jouw bijdrage aan mijn ontwikkeling tijdens het promotietraject!
Prof. dr. **Iris Behrmann** thank you for being an opponent in my committee. It was always a pleasure to meet you at the Recepteur network meetings. Dr. **Bert van der Reijden** en dr. **Erik Wiemer**, ik ervaar het als een waar genoegen u als opponent in de grote commissie te hebben.

Dear **Karishma**, we have both worked for over four years on studying different signaling mechanisms of the CSF3 receptor. You were always prepared to lend a helping hand or to discuss results, I enjoyed working together. Outside work you spoiled me lots of times with your nice Indian cooking, I hope to enjoy your cooking in the future and I would like to thank you for your friendship as well. Personally it is a great honor that you will stand next to me as paranymph during the defense of this thesis.
Dear **Tanja**, you are the microscope queen, at least that is my humble opinion. It was great working with you! I enjoyed our conversations especially whenever things, research related, were not working out as expected. When I think of little miss sunshine, I will think of you. I wish you all the best in making your decisions for the future. I am delighted that you are my paranymph.

Beste **Renée**, mijn dank is groot voor je hulp met statistiek, maar ook voor al je inbreng en commentaar op mijn werk tijdens de werkbesprekingen. Ook heb ik genoten van jouw organisatietalent van bezigheden buiten het werk om. Ik ben inmiddels tot de conclusie gekomen dat Zumba minder geschikt is voor ons blanken, wat niet weg neemt dat het een geweldige ervaring was om dit samen te proberen. Het doet mij veel deugd dat je naast me zal staan als paranimf tijdens de verdediging van dit proefschrift.

Ik heb goede herinneringen aan alle evenementen die vanuit de afdeling hematologie zijn georganiseerd. De skitrips naar Winterberg en Frankrijk waren geweldig en waren tevens mijn kennismaking met wintersport. Met veel plezier zal ik terug denken aan de bierclubs, stapavonden, pokeravonden en etentjes waar onder andere **Andrzej, Arturo, Erdogan, Eric V, Helen, Irene, Joyce, Lucila, Marshall, Menno, Merel, Monique, Onno, Rasti, Simone, Stefan, Su Ming** en **Tamara** deze gelegenheden tot een succes maakten.

Beste **Stefan**, ik hoop dat je mijn laatste stelling hebt gelezen want deze is geïnspireerd op onze samenwerking. Ik ben namelijk van mening dat samenwerken wetenschappelijk onderzoek naar een hoger niveau brengt. De grootte van jouw netwerk, je enthousiasme en het feit dat je altijd klaar staat voor discussie overtuigt mij bij voorbaat dat ik van jou artikelen van hoge kwaliteit kan verwachten in de toekomst. Ik ben er erg trots op dat onze samenwerking heeft geresulteerd in een mooie publicatie in Blood.
Dear **Albert**, thanks for all your input and fruitful discussions that we had about our scientific work and social life. It was very nice to have you as roommate during my PhD. I learned a lot from you.
Dear **Rasti**, it was nice playing tennis together, your spinning service is great! I think that we were the best team ever. Furthermore, I enjoyed all the conversations and drinks we had. Even and perhaps especially the strong drinks you got from Slovakia. Good luck finishing your thesis.
Dear **Arturo**, thanks for all the Spanish lessons. You were a teacher full of Spanish temperament and I am sorry that I was not a very good student. The visit to Madrid was fabulous, thank you for showing us around.
Beste **Onno** als mijn vaste vraagbaak in het Touw-team wil ik je graag bedanken. Je hebt me vaak bijgestaan op het lab en ook geholpen wanneer ik iets met een computer programma wilde en er even niet uit kwam. Het was leuk je als buurman op het lab te hebben en buiten de werkuren hebben we gezellige uurtjes in de kroeg doorgebracht.
Beste **Carola,** je hebt hard meegewerkt om de DUB2A paper tot een goed einde te brengen. Met jouw enthousiasme en inzet ben je deskundig geworden in Western blot en qPCR. Bedankt voor jouw bijdrage aan dit proefschrift.
Beste **Jo,** ik wil je succes wensen met de laatste loodjes voor het schrijven van jouw proefschrift. Neem je als afleiding zo nu en dan een heavenly hazel? Ik hoop dat je dan net als ik

met plezier terug kijkt op onze gezamenlijke pauzes waarin wij tussen alle experimenten door gezellig deze lekkere koffie nuttigden.

Ook de overige leden, oud-leden en studenten van de Touw/von Lindern groep **Astrid, Andrzej**, **Bart**, **Godfrey, Hanna, Judith**, **Jurgen**, **Jurjen**, **Mahban**, **Marijke, Marleen**, **Paulette** en **Sylvana** wil ik erg bedanken voor de gezelligheid en de hulp.

Corine, bedankt voor je inzet. Natuurlijk ervaar ik het ook als jammer dat de high-troughput screening voor UBE2's anders heeft uitgepakt dan verwacht. Ook wil ik **Sanne** en **Lisanne** bedanken voor de bijdragen tijdens hun stage.

Beste **Fatima**, tijdens mijn eerste jaar kwam ik bij je zitten op de kopkamer en we bleken vlak bij elkaar te wonen. We hebben veel lol gehad tijdens onze sportieve Kick en Fun uurtjes. Nu ik toch niet voor die postdoc baan bij je in Turkije ben gegaan, zal ik dat proberen te compenseren door wat vaker vakantie bij jou te komen vieren.
Beste **Sophie**, ondanks dat we geheel ander soort onderzoek hebben verricht liep ons traject redelijk gelijk op. Na veel over onze volgende carrièrestap en mogelijkheden te hebben gepraat, hebben we nu allebei een leuke baan gevonden. Ik wens je veel succes met je verdere carrière.
Egied, bedankt dat je de layout van mijn boekje hebt willen verzorgen. Ik ben onder de indruk van je artistieke werk. Graag wil ik **Ans, Jan** en **Sevilay** bedanken voor alle ondersteuning, hulp en gezelligheid. Tevens wil ik **Eric B**, bedanken voor zijn input. **Diana** thanks for the nice times and good luck with your PhD. **Jeroen Demmers** wil ik bedanken voor het mogelijk maken en het uitvoeren van de massaspectrometrie.

Mijn nieuwe collega's van het **KCHL HagaZiekenhuis** wil ik bedanken voor het feit dat ik me zeer welkom voel. Ik zie ernaar uit de komende jaren met jullie samen te werken.

Lieve **Oma**, **Gerrit, (schoon)familie** en **vrienden**, bedankt dat jullie al deze jaren interesse in de voortgang van mijn onderzoek hebben getoond. Tevens bedankt voor jullie begrip dat ik niet altijd tijd had om af te spreken. In deze categorie wil ik Martine bedanken voor het ontwerpen van de voorkant van dit proefschrift. Ik vind de omslag erg mooi geworden. Graag wil ik ook **Jan-Willem** en **Nuray** bedanken. Ik ben blij jullie te hebben leren kennen tijdens mijn stage bij Solvay. Nuray veel succes met het afronden van jouw proefschrift en Jan-Willem bedankt voor je hulp.

Lieve **pa** en **ma**, zonder jullie steun en support zou ik dit nooit bereikt hebben. Ik ben dankbaar dat jullie mij de mogelijkheid hebben gegeven om naar Amsterdam te verhuizen om

daar te gaan studeren. We zijn een klein gezin maar vergeet nooit: samen kunnen we alles aan. Dit proefschrift draag ik vanuit mijn hart aan jullie op!

Helaas voor allen die hierboven reeds genoemd zijn, mijn grootste dank gaat uit naar **Joost**. Altijd als ik gefrustreerd raakte door bijvoorbeeld onverwachte experimentele uitkomsten, wist je me weer op te beuren, te motiveren en alles in perspectief te plaatsen. Je bent zelfs een uitstekende hulp gebleken als mijn persoonlijke weekend Casy operator! Bedankt voor alles wat je voor me doet. Ik hoop ooit alle tijd die ik niet samen met jou heb kunnen besteden, wegens het werk nodig voor dit proefschrift, goed te kunnen maken. Hoe? Misschien moeten we het daar maar eens over hebben!

Annemarie

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 22 september 1982 te Almelo. Zij behaalde haar gymnasium diploma in 2000 aan het christelijk lyceum Het Noordik, te Almelo. Vervolgens heeft zij haar bachelor farmaceutische wetenschappen in 2003 en master scheikunde met als afstudeerrichting biochemie/ biomoleculaire wetenschappen in 2005 behaald aan de Vrije Universiteit (VU) in Amsterdam. Tijdens haar masteropleiding heeft zij zes maanden onderzoek gedaan naar de functie van de moleculaire chaperone Cdc37p bij het reguleren van MAPK pathways onder leiding van Dr. M.H. Siderius aan de VU. Daarna heeft zij nog een stage van zes maanden gelopen bij Solvay pharmaceuticals waarin de rol van chemokines in astrocyt-geïnduceerde monocyt migratie werd bestudeerd onder begeleiding van Dr. E. Ronken and Dr. A. Vallès. Hierdoor was haar interesse gewekt in wetenschappelijk onderzoek en besloot zij te promoveren. In januari 2006 is zij begonnen als promovenda in de groep van Prof. dr. I.P. Touw op de afdeling hematologie van het Erasmus MC. Het werk dat voor dit promotietraject is uitgevoerd, staat beschreven in het proefschrift dat nu voor u ligt. In december 2010 is zij begonnen aan haar opleiding tot klinisch chemicus in het HagaZiekenhuis in Den Haag onder supervisie van Dr. P.F.H. Franck.

PUBLICATIONS

CSF3R signaling: An update. Karishma K. Palande, **Annemarie Meenhuis** and Ivo P. Touw. *Manuscript in preparation.*

MiR-17/20/93/106 promote hematopoietic cell expansion by targeting Sequestosome 1-regulated pathways in mice. **Annemarie Meenhuis**, Peter van Veelen, Iris J. van den Berge, Su M. Sun, Erdogan Taskesen, Patrick Stern, Arnoud H. de Ru, Arjan J. van Adrichem, Jeroen Demmers, Mojca Jongen-Lavrencic, Bob Löwenberg, Ivo P. Touw, Phillip A. Sharp and Stefan J. Erkeland. *Blood, in press.*

The deubiquitinating enzyme DUB2A enhances CSF3 signaling by attenuating lysosomal routing of CSF3 receptor. **Annemarie Meenhuis**, Carola Verwijmeren, Onno Roovers and Ivo P. Touw. *Biochem. J. (2011) 434, 343–351.*

Site-Specific ubiquitination determines lysosomal sorting and signal attenuation of the granulocyte colony-stimulating factor receptor. Albert Wölfler, Mahban Irandoust, **Annemarie Meenhuis**, Judith Gits, Onno Roovers and Ivo P. Touw. *Traffic (2009) 10, 1168–1179.*

Janus kinases promote cell-surface expression and provoke autonomous signalling from routing-defective G-CSF receptors. **Annemarie Meenhuis**, Mahban Irandoust, Albert Wölfler, Onno Roovers, Marijke Valkhof and Ivo P. Touw. *Biochem. J. (2009) 417, 737–746.*

Cdc37p is involved in osmoadaptation and controls high osmolarity-induced cross-talk via the MAP kinase Kss1p. Yang XX, Hawle P, Bebelman JP, **Meenhuis A**, Siderius M, van der Vies SM. *FEMS Yeast Res. (2007) 6, 796-807.*

PHD PORTFOLIO

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Courses		
- Annual Course Molecular Medicine (MM)	2006	1.8
- Basic and Translational Oncology (MM)	2006	1.8
- In vivo Imaging: "From Molecule to Organism" (MM)	2007	1.8
- SPSS introduction course (MM)	2010	0.6
Workshops and Seminars		
- Workshop Browsing Genes and Genomes with Ensembl (MM)	2007	0.5
- Workshop on Bioinformatics Analysis, Tools and Services (MM)	2008	0.5
- Erasmus Hematology lectures	2006-2010	2.5
Oral presentations		
- 9 Hematology Presentations	2006-2010	4.5
- 3 Journal Club Presentations	2006-2010	1.5
- Ghent Recepteur Conference	2009	0.5
- Rotterdam Recepteur Conference	2009	0.5
- 4th Dutch Hematology Congress	2010	0.5
- 14th Molecular Medicine Day	2010	0.5
Poster presentations (3x)	2006-2008	2
(Inter)national conferences		
- Seven symposia and congresses	2006-2010	7.3
2.Teaching activities		
Lecturing: Hemapoiesis Course for 1st year Medicine Students	2007	0.5
Other:		
- Supervising HLO student (28 weeks)	2008	7
- Organizing Invited Speaker Lunch Hematology	2007-2008	1
- Organizing GOL-days (MM)	2007	2
Total		37.3