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# JANUS KINASES PROMOTE CELL SURFACE EXPRESSION AND PROVOKE AUTONOMOUS SIGNALLING FROM ROUTING DEFECTIVE G-CSF RECEPTORS

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**Running title:** JAK-controlled G-CSF receptor trafficking

**Abbreviations:** AML, acute myeloid leukaemia; CHX, cycloheximide; CSF3R, colony-stimulating factor 3 receptor; EpoR, erythropoietin receptor; ER, endoplasmic reticulum; ESCRT, endosomal sorting required for transport; GF, growth factor; GGA, Golgi-localized  $\gamma$ -ear containing; IL-3, interleukin 3; IFNAR, interferon  $\alpha$  receptor; JAK, janus tyrosine kinase; MFI, mean fluorescence intensity; OSMR, oncostatin M receptor; SH2, src homology 2; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; TpoR, thrombopoietin receptor.

## SYNOPSIS

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

## INTRODUCTION

The granulocyte colony-stimulating factor receptor (G-CSFR, now referred to as 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 Janus tyrosine kinases (JAKs) become docking sites for Src homology 2 (SH2)-containing signalling proteins such as signal transducer and activator of transcription (STAT)-3, the adapter proteins SHC and GRB2, and suppressor of cytokine signalling (SOCS)-3 [2]. The cytoplasmic domain of CSF3R further comprises a dileucine-based motif crucial for internalization [3], and five conserved lysine (K) residues. We have recently shown that one of these lysines, the juxtamembrane K632, is the major determinant for ligand-induced lysosomal routing of the activated CSF3R, a process that involves SOCS3-mediated ubiquitination of K632 [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 G-CSF-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 acute myeloid leukaemia (AML) [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 Y729, essential for SOCS3-induced ubiquitination of K632 [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 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 [7]. Similarly, binding of JAK2 or TYK2 to the thrombopoietin receptor (TpoR) augmented membrane expression and stability of its mature (EndoH resistant) form [8, 9]. Studies on the interferon receptor- $\alpha 1$  (IFNAR1) revealed that binding of TYK2 attenuates degradation and increases cell surface expression by masking dileucine- and tyrosine-based internalization motifs present in the JAK binding domain of the receptor [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 erythropoietin receptor (EpoR), where JAK2 binding does not affect receptor endocytosis, but enhances the appropriate folding of the EpoR protein in the ER, leading to more efficient Golgi processing and enhanced forward routing of mature protein to the cell surface [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 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 from 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% CO<sub>2</sub>. 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% CO<sub>2</sub>. 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 NaN<sub>3</sub>) for 1 hr at 4°C. After washing, cells were incubated at 37°C for 0, 30, 60, 120 and 240 min. Next, cells were stained with secondary antibody goat anti-mouse-PE in PSA for 1 hr at 4°C and, after a final

wash, analyzed by flow cytometry using a FACS Calibur (Becton-Dickinson Biosciences). Mean fluorescence intensity of histograms was taken as a measure of the average CSF3R expression.

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

### **Receptor protein stability assay**

Phoenix E cells were transiently transfected with CSF3R constructs in pBabe with or without co-transfecting murine JAK2 using calcium phosphate precipitation. Two days after transfection, cells were incubated for 3 hrs with 50 µg/ml cycloheximide (CHX) to block protein synthesis or left untreated. Ba/F3 cells stably transduced with CSF3R and JAK vectors were treated with 50 µg/ml CHX for 5 hrs. Cells were washed with cold PBS and resuspended in lysis buffer containing 20 mM Tris-HCl pH8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% v/v NP-40, 10% v/v Glycerol, 50 µg/ml aprotinin, 1 mM pefablock and 2 mM Na3VO4. Lysates were put on ice for 30 min, centrifuged for 15 min. at 14,000 rpm to remove insoluble proteins. CSF3R was pulled down from lysates by adding magnetic protein G-beads (Dynabeads, Invitrogen) pre-incubated with CSF3R o/n at 4°C. Beads were incubated in Laemmli buffer for 5 min. at 95°C and then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting as described previously [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 G-CSF (Bio-G) prepared using a biotin labeling kit according to manufacturers protocol (Roche Molecular Biochemicals, Mannheim, Germany) for 1 hr at 37°C. Cells were washed twice with cold PBS and lysed. CSF3R was pulled down using streptavidin coated magnetic beads (Dynabeads, Invitrogen). Beads were washed and resuspended in Laemmli buffer, incubated for 5 min. at 95°C and subjected to Western blotting as described [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 BioG for 30 min. at 37°C. Cells were then washed twice with cold PBS and resuspended in lysis buffer. For total CSF3R levels cells were first lysed and subsequently 1:50 BioG was added o/n at 4°C. CSF3R was pulled down using streptavidin-coated magnetic beads. For EndoH treatment (New England Biolabs, Ipswich, United States), beads were washed and resuspended in 50µl 1x GD denaturation buffer and boiled for 10 min. at 100°C. After adding 10x G5 reaction buffer, the supernatant was divided into two portions, to one of which EndoH was added and to the other H<sub>2</sub>O 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 [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 (Fig. 1A and 1B). Parental Ba/F3 cells and the JAK-overexpressing cells were then retrovirally transduced to express wt CSF3R and cell membrane expression was determined by flow cytometry. JAK1, JAK2 or TYK2 transduced cells showed a 2- to 4-fold higher level of CSF3R expression on the cell surface compared to parental control cells (Fig. 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 (Fig. 1D). All cell lines showed comparable cell surface expression of CSF3R, implicating that JAK1, JAK2 and TYK2 are redundant in controlling steady-state cell CSF3R expression (Fig. 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 and performed pull down experiments on cells pre-treated with biotinylated G-CSF. CSF3R-W650R was severely hampered in both JAK2 binding and activation (Fig. 2A, left panel and Figure 2B). The two CSF3R bands visualized in Figure 2A represent glycosylation variants of the mature receptor form that are insensitive to Endo H treatment (Figure 2A, right panel). 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 (Fig. 2C). Notably, membrane expression of CSF3R-W650R in parental Ba/F3 cells was equal to wt CSF3R, suggesting that JAK binding is not required for CSF3R expression as such (Fig. 2C).

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

One of the mechanisms implicated in the JAK-mediated effects on cell surface expression of cytokine receptors is that motifs involved in receptor endocytosis and lysosomal degradation are masked by steric interference [7, 10, 11]. We previously demonstrated that ubiquitination of lysines in the intracellular domain of CSF3R is important for ligand-induced lysosomal targeting but not for receptor internalization [4]. To determine whether receptor lysines are involved in ligand-independent (constitutive) endocytosis, we studied membrane expression of a receptor mutant in which all 5 cytoplasmic lysines were replaced by arginines (mutant K5R). Myeloid 32D cells stably expressing K5R showed higher membrane expression levels compared to wt CSF3R (Fig. 3A), a result that was confirmed by confocal microscopy in 32D cells (Fig. 3B, upper panel) and in HeLa cells (Fig. 3B, lower panel). However, kinetics of ligand independent internalization of wt and K5R CSF3R were similar, indicating that lysines are not critical in this process (Fig. 4A). Taken together, these results suggest that lysines 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 lysines in CSF3R stability. To this end, Ba/F3 cells stably expressing wt or K5R CSF3R were incubated for 3-6 hrs with cycloheximide (CHX) to inhibit de novo protein synthesis. While degradation of wt CSF3R was observed after inhibition of protein synthesis, K5R CSF3R was found to be stable (Fig. 4B). The lysosome inhibitor bafilomycin prevented degradation of wt CSF3R, suggesting that steady state receptor levels are regulated via lysosomal degradation and that lysines are important for this mechanism.

Because the lysines 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 K632 is the major inhibitory determinant for constitutive CSF3R cell surface expression (Fig. 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 Fig. 5A, 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 CSF3R-d715 and K5R (Fig. 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, K632 remains the most prominent lysine involved in the downregulation of CSF3R surface expression (Fig. 5C, compare to Fig. 4C).

### JAKs do not inhibit ubiquitination of K632 in ligand-activated CSF3R

From the above studies, K632 emerges as a major negative determinant in constitutive CSF3R expression. Upon ligand activation of the CSF3R-JAK complex K632 is ubiquitinated, which is essential for G-CSF-induced lysosomal routing of CSF3R [4]. Because JAKs are activated in this setting, we wondered whether increased JAK levels may interfere with ubiquitination of K632 to attenuate ligand-induced lysosomal degradation. As shown in Fig. 6A, ubiquitination of K632, the only cytoplasmic lysine retained in mKA, increased proportionally with elevated CSF3R levels in JAK1, JAK2 or TYK2-overexpressing cells relative to parental cell controls. Together, these data indicate that neither in forward nor retrograde routing of CSF3R JAKs interfere with the function of K632.

### JAKs enhance protein stability of CSF3R

To determine if JAKs influence CSF3R protein stability, as e.g., shown for the TpoR [8], Ba/F3 parental cells and JAK transfectants expressing wt CSF3R were cultured in the presence or absence of CHX for 5 hrs to block protein synthesis. 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 (Fig. 6B and data not shown). In contrast, both the mature and immature forms of K5R were

stabilized (Fig. 6B). These data indicate that JAKs protect both the mature and immature form of CSF3R, provided that receptor lysine-mediated degradation is prevented.

### **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 cooperate 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) (Fig. 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 (Fig. 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 ( $IC_{50} = 1\text{nM}$ ), MEK inhibitor U0126 and Akt inhibitor IV completely abolished cell survival. Blocking of PI-3 kinase, acting upstream of PKB/Akt, by LY294002 also severely diminished survival as did inhibition of Src activity by tyrosine kinase inhibitor PP2. This result indicates that multiple pathways contribute to the growth-factor independent cell survival of the routing defective K5R CSF3R mutant. In contrast, inhibition of the stress-associated p38 MAPK [25] by SB203580, resulted in significantly increased survival. Because both STAT3 and STAT5 have been implicated as important signalling molecules for G-CSF-induced proliferation and survival [26, 27], we investigated whether these STATs are constitutively activated in JAK2-overexpressing Ba/F3 cells harbouring CSF3R K5R or d715. A prominent ligand independent activation of STAT3, but not STAT5, was observed in JAK2-overexpressing Ba/F3 cells transduced with mutants K5R or d715, but not with wt CSF3R (Fig. 7D).

## **DISCUSSION**

We investigated the role of JAK proteins in controlling CSF3R expression and intracellular routing. Specifically, we addressed to what extent JAKs interfere with the internalization and lysosomal routing machinery, linked to the dileucine-based internalization and conserved cytoplasmic lysines, respectively. Major observations were: (1) increased JAKs significantly elevated CSF3R membrane expression and protein levels, (2) JAKs did not exert this effect by masking motifs essential for internalization, as previously demonstrated for IFNAR1, or by reducing the ubiquitination of the lysosomal routing determinant K632 of CSF3R and (3) simultaneous perturbation of internalization or lysosomal routing and JAK-mediated increase in forward routing resulted in ligand-independent activation of CSF3R complexes.

Enhanced cell surface expression after JAK overexpression has been reported for a number of cytokine receptors [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 to 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 CSF3R-W650R might still suffice to promote cell surface routing.

Among the 5 conserved lysines in the cytoplasmic tail of CSF3R, the juxtamembrane residue K632, in addition to being a major determinant for ligand-induced lysosomal routing [4], also appeared to be uniquely involved in the control of constitutive CSF3R cell surface expression. Although lysines have been reported to regulate ligand independent internalization of the short isoform of the leptin receptor



[30], we could not detect any role of lysines in internalization of the CSF3R. Hence, loss of K632 rather leads to enhanced cell surface expression by redirection of receptors in the biosynthetic pathway from the endoplasmic reticulum-lysosomal degradative route towards the plasma membrane. Major players involved in intracellular trafficking of surface membrane proteins are the endosomal sorting required for transport (ESCRT) and Golgi-localized  $\gamma$ -ear containing (GGA) protein complexes, respectively [31]. Because key proteins within these complexes, such as Hrs and GGA, interact with ubiquitinated lysines in cargo proteins but simultaneously require interaction with phosphoinositides to increase affinity [32, 33], this might explain why membrane proximal positioning of lysine K632 is imperative for lysosomal routing of CSF3R. This is supported by recent experiments showing that relocation of K632 to a more membrane distant position disrupted its role in routing despite the fact that ubiquitination was unaffected (Wölfler et al., submitted for publication). How JAKs control basal cell surface expression of CSF3R remains to be resolved. Because JAKs bind with their N-terminal FERM domain to the juxtamembrane domain of CSF3R that encompasses K632, a feasible explanation was that JAK overexpression would mask the function of this lysine, e.g., by preventing its ubiquitination or by hindering the binding of effector proteins. This hypothesis can now be discarded because increased JAK levels did not prevent ubiquitination of K632 and elevated cell surface levels of mutants K632R and K5R at as efficiently as the wt CSF3R. Most likely, JAKs associate with the CSF3R already early in the biosynthetic pathway and help to protect CSF3R from misfolding and degradation, similar to what has been proposed for the EpoR [12].

Abnormal responses to haematopoietic growth factors, including G-CSF, 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 strongly 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 lysines in CSF3R or proteins of the ESCRT machinery. Notably, this is not without precedent, since it was demonstrated earlier that expression of the deubiquitinating enzyme DUB-2 increases survival of haematopoietic cells following cytokine withdrawal [36].

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## FIGURE LEGENDS

**Figure 1:** JAK1, JAK2 and TYK2 augment cell surface expression of CSF3R. *A.* JAK overexpression in Ba/F3 cells assessed by flow cytometry using CD2- or CD4-FITC antibodies. Control is Ba/F3 cells without antibody staining. *B.* Western blot analysis showing JAK levels in parental Ba/F3 cells and JAK transfectants. 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. 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,  $\gamma$ 2A 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. Control is 2C4 cells without antibody staining.

**Figure 2:** JAK-induced surface expression of CSF3R depends on a tryptophan residue (W650) that is critical for JAK binding. *A.* Left panel: JAK2 overexpressing Ba/F3 cells with the indicated CSF3R variants were stimulated with biotinylated G-CSF (Bio-G) for 30 min. Receptor complexes were isolated using streptavidin pull down and analyzed by Western blotting for the presence of CSF3R, JAK2 or phospho-JAK2. For loading control, the blot was restained for actin. A representative blot of three independent experiments is shown. Right panel: Lack of EndoH sensitivity of BioG-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 panel) and phospho-JAK2 (right panel) binding to wt CSF3R, mutant K5R and W650R. Data are expressed as mean  $\pm$  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)  $\pm$  SEM. \* $P$  < 0.05 compared to parental Ba/F3 cells expressing wt CSF3R.

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

**Figure 4:** Lysines are important for steady state CSF3R cell surface expression but are not involved in constitutive internalization. *A.* 32D cells stably expressing wt or K5R CSF3R were labelled with CSF3R antibody and were allowed to internalize during 4 hrs in the absence of ligand. CSF3Rs remaining at the cell surface were labelled with goat anti-mouse-PE and analyzed by flow cytometry. Data from three independent experiments are expressed as MFI  $\pm$  SEM relative to  $t=0$ , which was set at 100%. *B.* Protein synthesis in Ba/F3 cells stably expressing wt or K5R was blocked by CHX added to the medium for 0, 3 and 6 hrs. The amount of CSF3R in total cells lysates was visualized on Western blot using CSF3R specific antibody (lanes 1-3). Addition of the lysosomal inhibitor bafilomycin shows that 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  $\pm$  SEM of 4-7 clones relative to wt receptor. \* $P < 0.05$  compared to wt receptor. \*\*\* $P < 0.001$  mKA compared to 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 G-CSF (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  $\pm$  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  $\pm$  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 to wt CSF3R. \*\*\* $P < 0.001$  mKA compared to K5R CSF3R.

**Figure 6:** JAKs control CSF3R expression in the biosynthetic pathway irrespective of receptor ubiquitination. *A.* Ubiquitination status of the lysine at position 632 of membrane CSF3R determined in Ba/F3 parental and JAK overexpressing cells. Cells were stimulated with Bio-G for 30 min. in the presence of bafilomycin, where after CSF3R was pulled down with streptavidin-coated magnetic beads, eluted and subjected to Western blot analysis using anti-ubiquitin antibody P4D1. Vertical line (left) indicates size variation of receptor-ubiquitin complexes. Blots were restained with anti-CSF3R to determine CSF3R loading. As an input control, an immunoblot with whole cell lysate was stained for actin. *B.* Stability of wt and K5R CSF3R transiently expressed with or without JAK2 in Phoenix E cells. Cells were left untreated or treated with CHX for 3 hrs to block protein synthesis. CSF3R was immunoprecipitated using CSF3R antibodies and subjected to western blotting using the same antibodies. Quantification of mature and immature forms of CSF3R is shown in the right 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. 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 IL3 and transferred to medium without growth-factors. *A.* total cell numbers and *B.* the percentage of living cells based on 7-AAD staining were assessed at the indicated time points. Representative graphs of three independent experiments are shown. *C.* Effects of various kinase inhibitors on cell survival. See main text for details on the specificity of different compounds. DMSO: solvent control. Representative graphs of three independent experiments are shown. *D.* Cell lysates were made after removal of growth factor (GF) at the indicated time points (hrs) and subjected to Western blot analysis with antibodies against pSTAT3, pSTAT5 and actin for loading control.



Figure 1

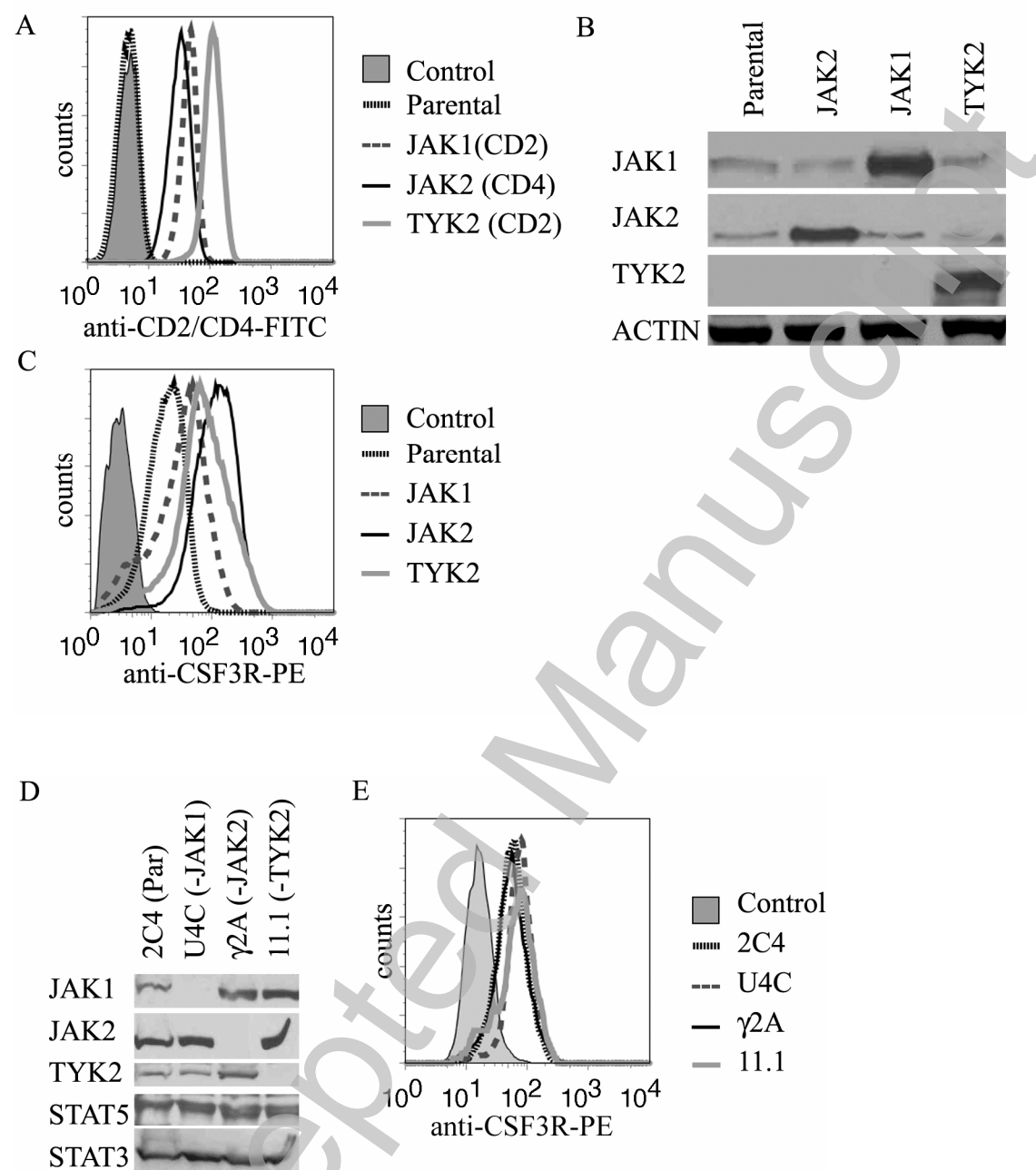


Figure 2

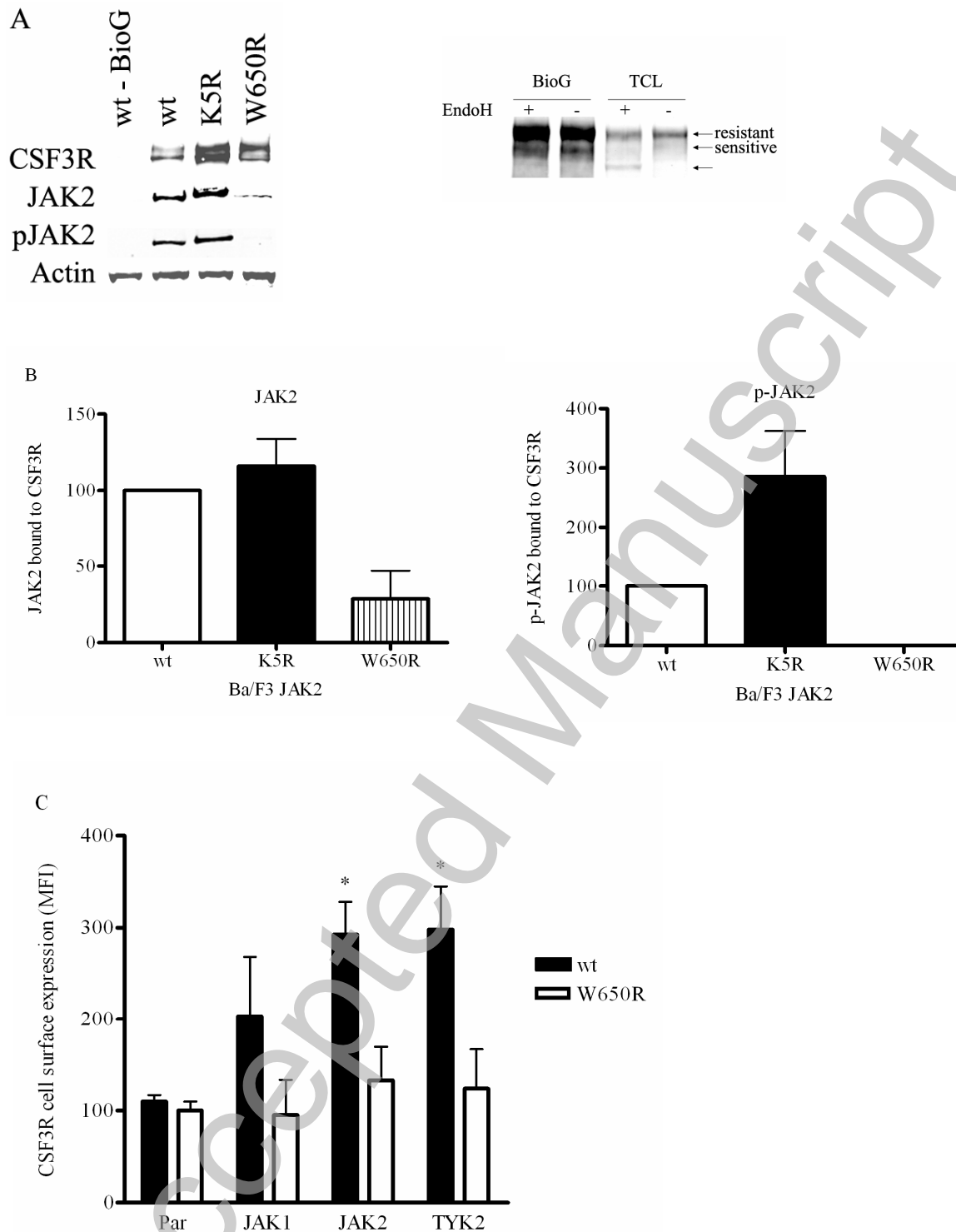


Figure 3

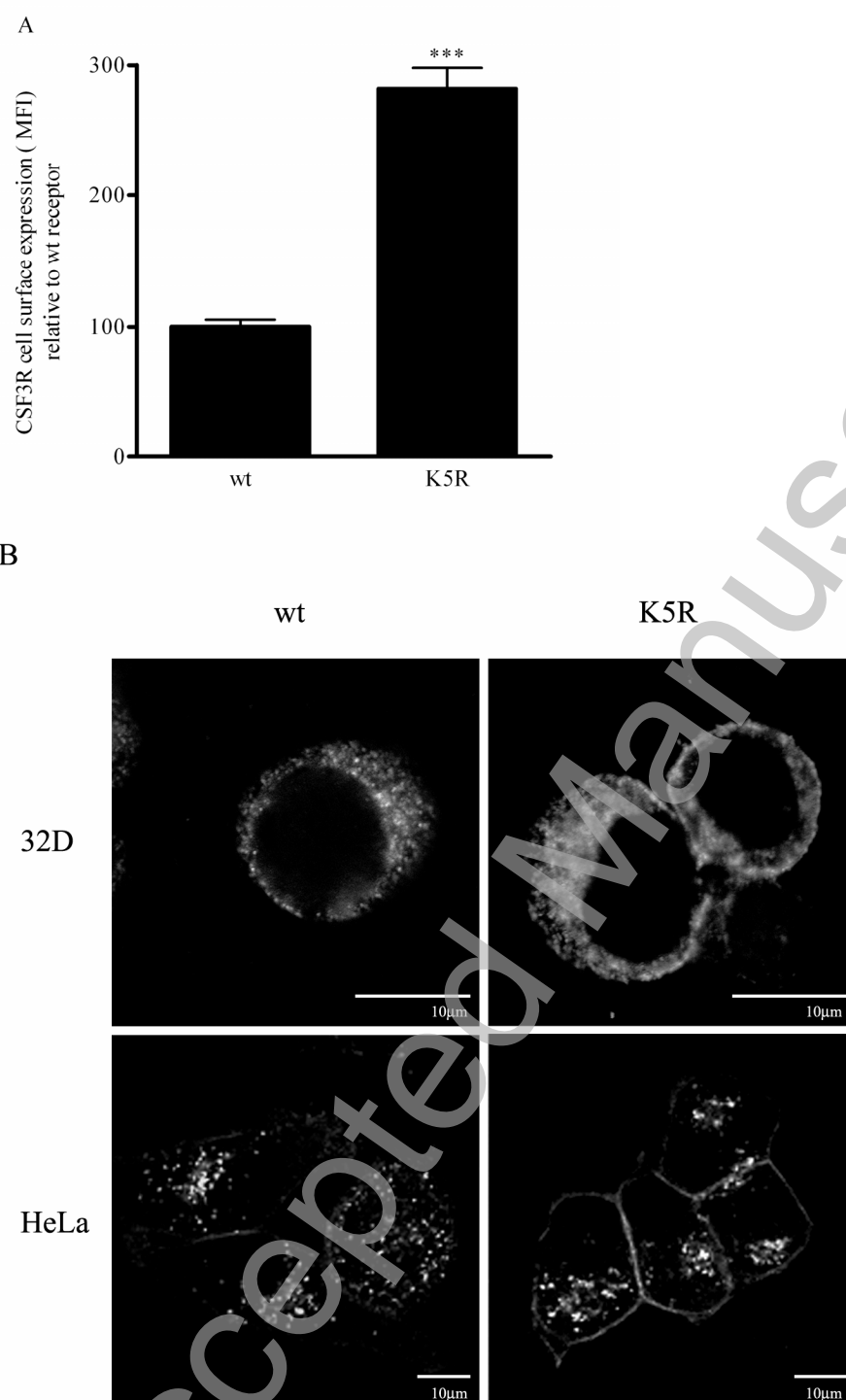


Figure 4

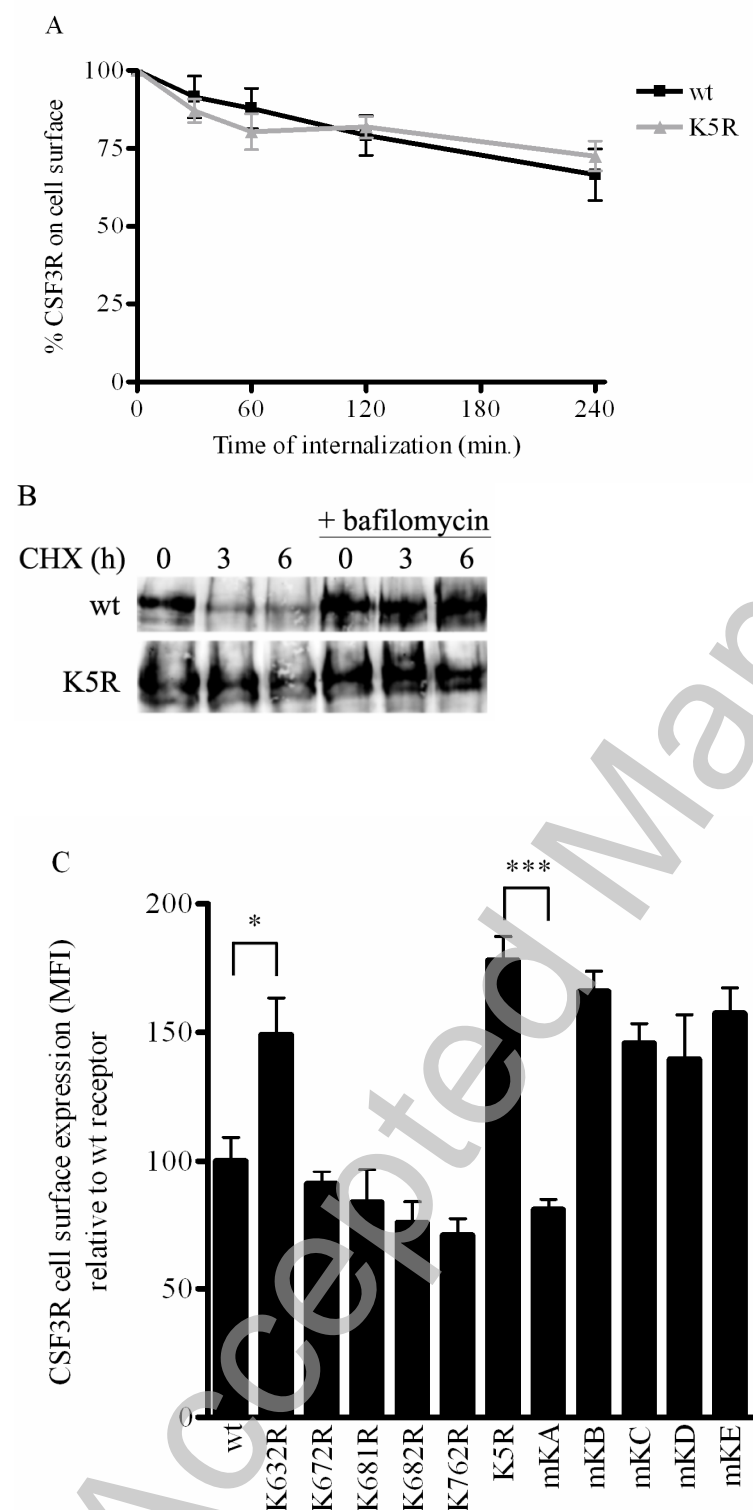
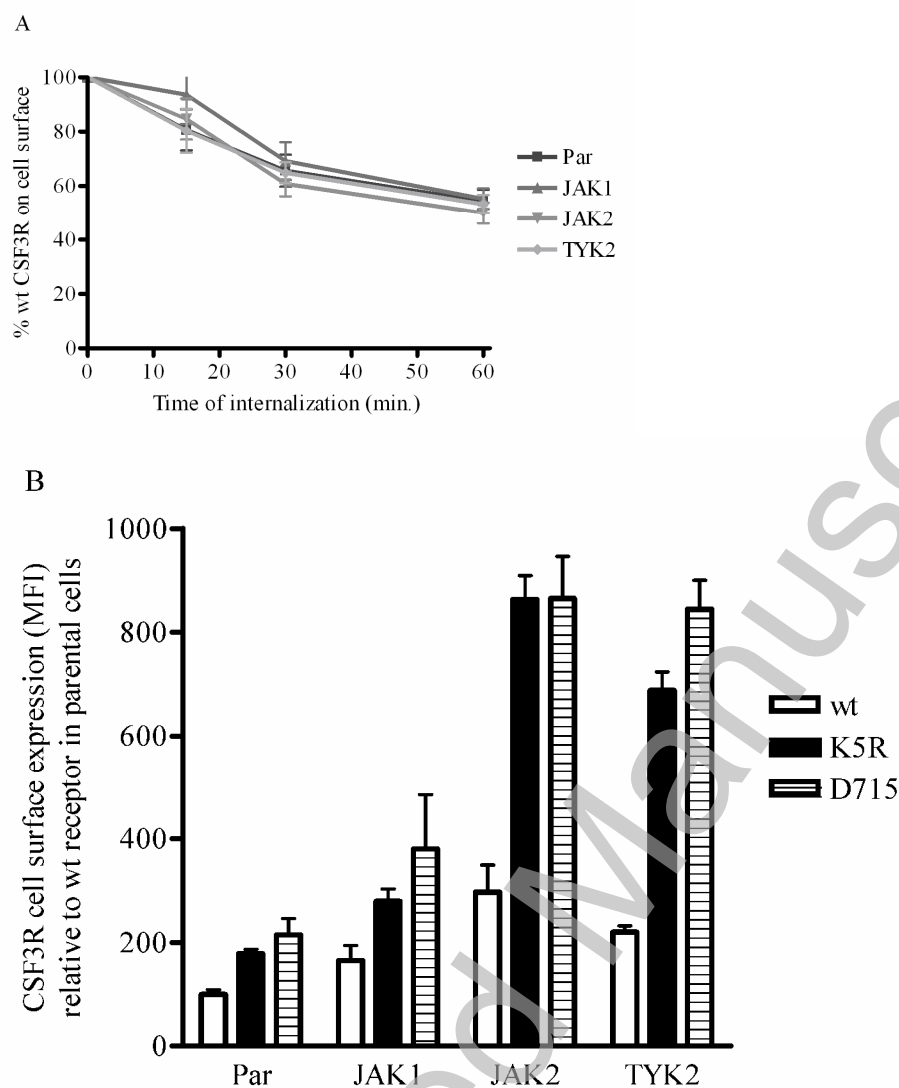


Figure 5





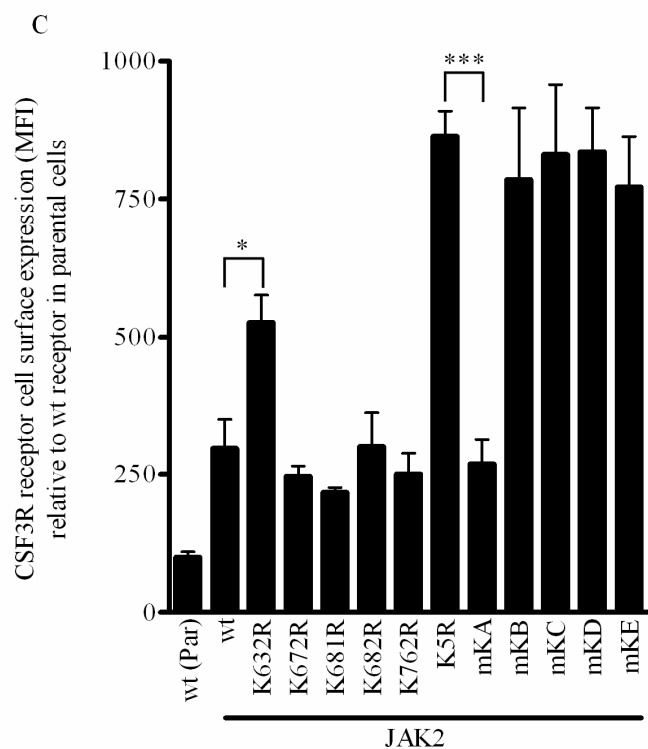
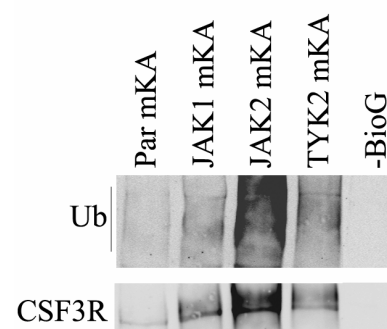


Figure 6

A



B

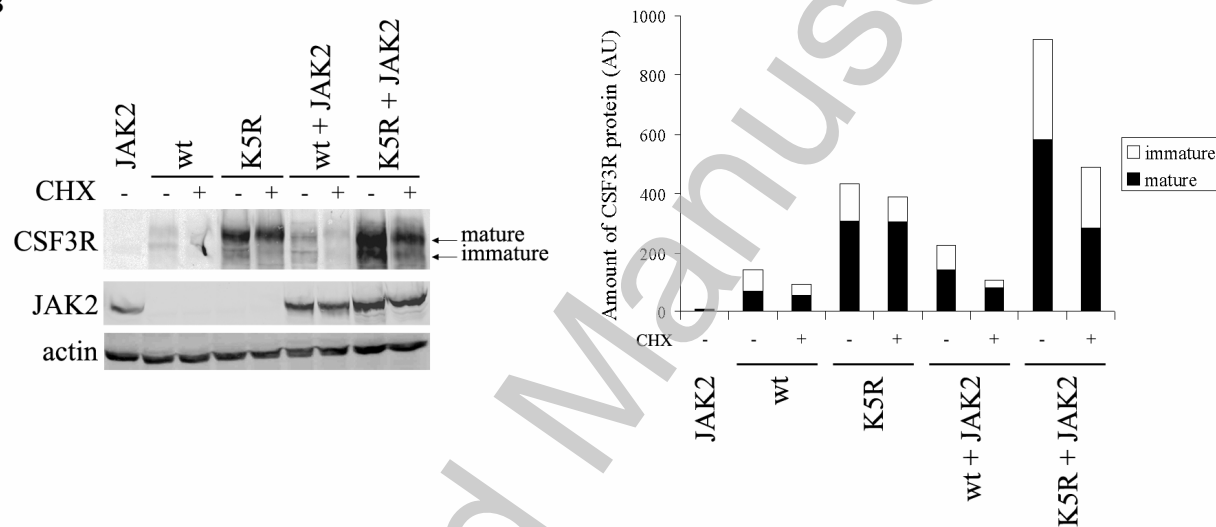
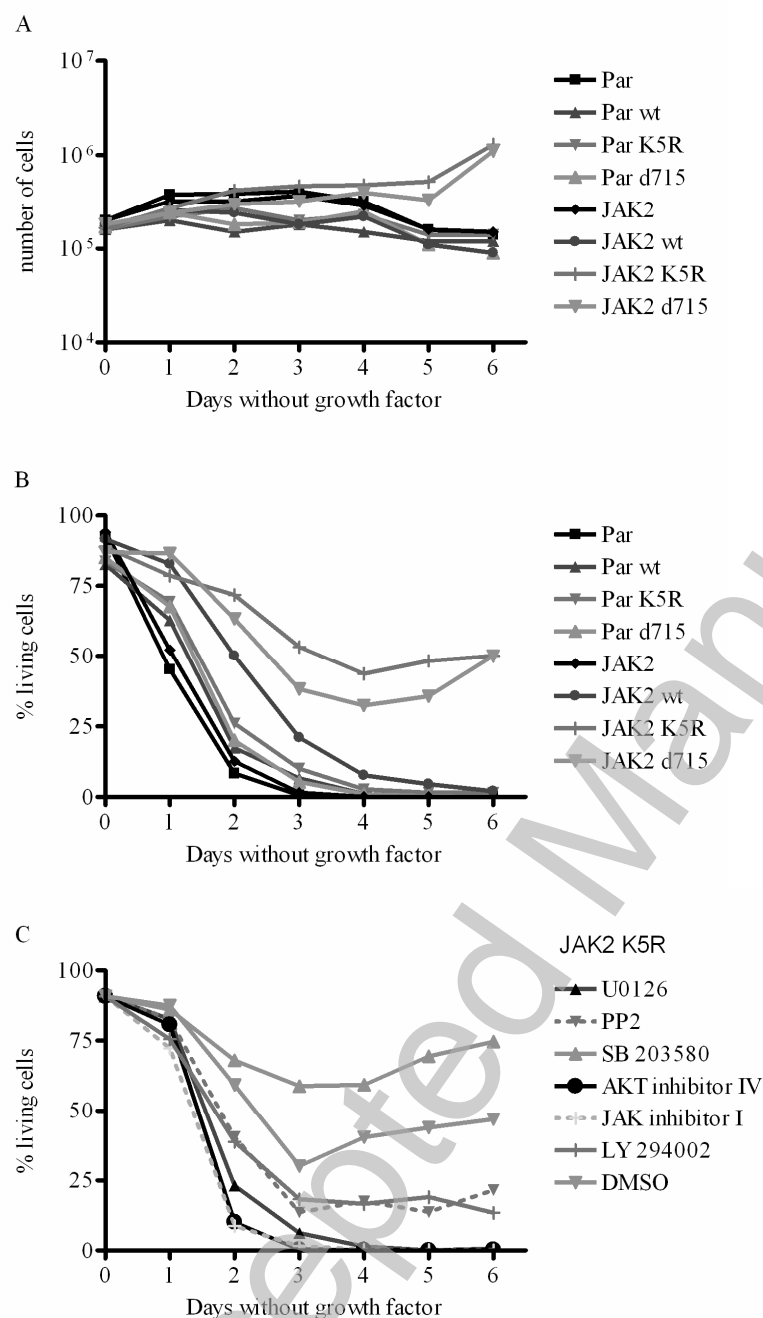
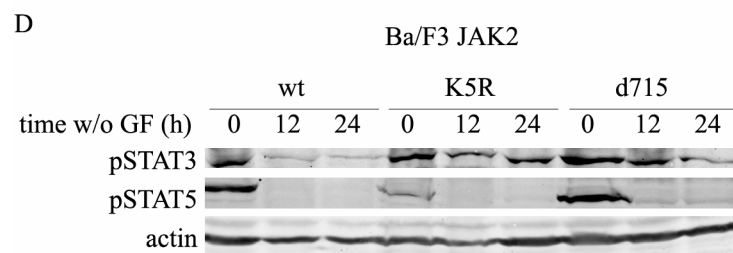


Figure 7





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