RESEARCH ARTICLE

Oncogenic deletion mutants of gp130 signal from intracellular compartments

Dirk Schmidt-Arras1,*, Miryam Müller1, Marija Stevanovic1, Silke Horn1, Antje Schütt1, Juri Bergmann2, Ruven Wilkens1, Annett Lickert1 and Stefan Rose-John1

ABSTRACT

Interleukin 6 (IL-6) and, hence, activation of the IL-6 receptor signalling subunit glycoprotein 130 (gp130; also known as interleukin-6 receptor subunit β, IL6ST), has been linked to inflammation and tumour formation. Recently, deletion mutations in gp130 have been identified in inflammatory hepatocellular adenoma. The mutations clustered around one IL-6-binding epitope and rendered gp130 constitutively active in a ligand-independent manner. Here, we show that gp130 deletion mutants, but not wild-type gp130, localise predominantly to intracellular compartments, notably the endoplasmic reticulum (ER) and early endosomes. One of the most frequent mutants, gp130 Y186-Y190del (gp130 ΔYY), is retained in the ER quality control system because of its association with the chaperone calnexin. Furthermore, we can show that gp130 ΔYY induces downstream signalling from both ER and endosomes, and that both signals contribute to ligand-independent cell proliferation. We also demonstrate that the endosomal localisation of gp130 ΔYY is crucial for fully fledged STAT3 activation. Aberrant signalling from intracellular compartments might explain the tumorigenic potential of naturally occurring somatic mutations of gp130.

KEY WORDS: Glycoprotein 130, gp130, Interleukin 6, IL-6, Endoplasmic reticulum, ER, Endosome, Inflammatory hepatocellular adenoma, IHCA

INTRODUCTION

Interleukin 6 (IL-6) is a pleiotropic cytokine involved in inflammation, regeneration and tumour formation (Grivennikov and Karin, 2008; Scheller et al., 2011). Activation of downstream signals by IL-6 is preceded by the formation of an IL-6 receptor complex: initially IL-6 binds to the IL-6 receptor (IL-6R), which is followed by the binding of the IL-6–IL-6R complex to the signal transducing subunit glycoprotein 130 (gp130; also known as interleukin-6 receptor subunit β, IL6ST). Induction of the IL-6 receptor complex leads to intracellular activation of JAK family kinases, mainly JAK1, JAK2 and TYK2, with JAK1 being the essential kinase (Heinrich et al., 2003). Subsequently, JAK kinases phosphorylate gp130, at multiple tyrosine residues, and STAT family proteins, like STAT3 and STAT1. Binding of the protein tyrosine phosphatase SHP-2 to phosphorylated Tyr-759 on gp130 induces activation of the Ras–mitogen-activated protein kinase (MAPK) pathway (Heinrich et al., 2003). Activation of STAT3 rapidly leads to the transcriptional upregulation of SOCS3. SOCS3 in turn competes with SHP-2 for the binding to phosphorylated Tyr-759, inactivates JAK kinases and hence diminishes signalling through the STAT3 and MAPK pathways.

In hepatocytes, activation of gp130 results in the induction of acute-phase proteins like serum amyloid A (SAA) and C-reactive protein (CRP). Inflammatory hepatocellular adenoma (IHCA) is a rare benign liver tumour affecting mostly women and is frequently associated with obesity and alcohol abuse (Bioulac-Sage et al., 2007). It is characterised by the presence of lymphocytic infiltrates, increased hepatocytic production of acute-phase proteins and persistent STAT3 phosphorylation (Rebouissou et al., 2009). Whereas in 8% of IHCA cases this can be linked to activating STAT3 mutations (Pilati et al., 2011), the most frequently occurring mutations (60% of IHCA cases) are small deletion mutations within the gp130-encoding IL6ST gene (Rebouissou et al., 2009). Surprisingly, gp130 deletion mutants are constitutively active, leading to persistent STAT3 activation and subsequent upregulation of acute-phase proteins, but also its negative-feedback regulator SOCS3, when transfected into the hepatocytic cell line Hep3B (Rebouissou et al., 2009). Recently, it has been shown that gp130 activation is regulated by the connectivity of two gp130 extracellular domains involved in contacting the cytokine IL-6. The mutations detected by Rebouissou et al. are thought to induce conformational changes within this region of the protein, leading to constitutive activation (Schütt et al., 2013).

Transmembrane proteins are synthesized into the endoplasmic reticulum (ER) and co-translationally glycosylated at asparagine residues. The glycan structure is transferred en bloc, and predominantly consists of mannose residues and is therefore termed the high-mannose structure. Terminal glucose residues are added by a glucosyltransferase and removed by glucosidases. Monoglucosylated proteins enter the ER quality control system and are bound to the chaperones calnexin or calreticulin, which exhibit lectin functions. The proteins are then subjected to a cycle of de- and re-glycosylation. Properly folded proteins are recognised by the ER-associated degradation (ERAD), a proteasome-mediated process (Brodsky and Skach, 2011). Misfolded proteins are subjected to ER- associated degradation (ERAD), a proteasome-mediated process (Brodsky, 2012).

Received 3 March 2013; Accepted 15 October 2013

1Christian-Albrechts-University, Institute of Biochemistry, 24118 Kiel, Germany.
2University Hospital, Department of General, Thoracic and Transplantation Surgery, 24105 Kiel, Germany.

*Author for correspondence (darras@biochem.uni-kiel.de)
the underlying mechanisms in more detail. We observed that gp130 ΔYY localises to the ER and early endosomes. ER retention of gp130 ΔYY was linked to prolonged association with the ER quality control component calnexin. We can furthermore demonstrate that gp130 ΔYY emits signals from both intracellular pools that contribute to ligand-independent cell proliferation and that induction of downstream signals is differentially regulated on a subcellular level.

RESULTS

gp130 ΔYY localises to the ER and early endosomes

While analysing the signalling mechanisms of the oncogenic deletion mutant gp130 Y186-Y190del (gp130 ΔYY) we realised that EYFP–gp130 ΔYY was predominantly detectable as a 160 kDa form, whereas EYFP-fused wild-type gp130 is predominantly present as a 180 kDa form (Fig. 1A). It has been previously shown that wild-type (WT) gp130 is synthesised as a 130 kDa polypeptide, which matures to a 150 kDa form owing to altered glycosylation (Albino et al., 1983; Gerhardt et al., 1994). In order to find out whether the size differences between gp130 ΔYY and gp130 WT are due to differences in glycosylation, we performed endoglycosidase digestion. Whereas PNGase F shifted both bands to a single one at around 130 kDa, representing unglycosylated EYFP-fused gp130, EndoH only shifted the lower 160 kDa form to the unglycosylated 130 kDa form (Fig. 1B). These data indicate that the lower, 160 kDa form represents the high-mannose immature form of gp130, whereas the upper 180 kDa form is the mature, complex glycosylated form, presumably present at the cell surface.

It has been shown that high-mannose glycan structures are mainly present in the ER. In order to analyse localisation of gp130, we took advantage of the pre-B cell line Ba/F3 which is the only available cell line lacking endogenous expression of gp130 (Gearing et al., 1994). We initially transduced Ba/F3 cells with expression constructs for Myc-tagged gp130 WT and gp130 ΔYY and analysed surface localisation by flow cytometry using anti-gp130 antibodies. Whereas wild-type gp130 was readily detectable at the cell surface, only a minor fraction of gp130 ΔYY could be detected at the cell surface (Fig. 1C). To guard against unequal expression levels we permeabilised cells prior to

---

**Fig. 1.** gp130 ΔYY is intracellularly retained. (A) HepG2 cells were transiently transfected with expression constructs for EYFP-tagged gp130 WT or gp130 ΔYY. Cells were lysed and gp130 variants were isolated using anti-GFP antibodies and analysed by SDS-PAGE and anti-gp130 immunoblotting. (B) HepG2 cells were transiently transfected with expression constructs for EYFP-tagged gp130 WT or gp130 ΔYY. Cells were lysed and gp130 variants were isolated using anti-GFP antibodies. Immunoprecipitates were subsequently subjected to EndoH or PNGase F digestion or mock-treated, as indicated, and subsequently analysed by SDS-PAGE and anti-gp130 immunoblotting. CG, complex glycosylated; HM, high mannose; DG, deglycosylated. (C) FACS analysis of Ba/F3 cells stably transduced with Myc-tagged gp130 variants. Cells were stained using anti-gp130 (B-P4) antibody, followed by DyLight649-labelled anti-mouse antibody and analysed using a BD FACSCanto. White plots represent isotype controls; grey plots are anti-gp130-antibody-bound cells. (D) FACS analysis of Ba/F3 cells stably transduced with Myc-tagged gp130 variants. Cells were permeabilised prior to staining with anti-gp130 (B-P4) antibody, followed by staining with DyLight649-labelled anti-mouse antibody and analysed using a BD FACSCanto. While plots represent isotype controls; grey plots are anti-gp130-antibody-bound cells.

342
anti-gp130 staining and FACS analysis. After permeabilisation we detected gp130 signals for both, gp130 WT, as well as gp130 ΔYY-transduced Ba/F3 cells, indicating that gp130 ΔYY was predominantly localised in an intracellular compartment (Fig. 1D).

In order to determine the subcellular localisation of gp130 variants, we performed confocal microscopy on HepG2 cells transiently transfected with EYFP-tagged wild-type gp130 or gp130 ΔYY along with ECFP- or mCherry-tagged subcellular marker proteins. Wild-type gp130 was detectable at the plasma membrane and partially in intracellular vesicular structures that did not overlap with signals from an ECFP-tagged ER marker (Fig. 2A). However, in stark contrast, gp130 ΔYY colocalised almost entirely with the ER marker signal and was barely detectable at the plasma membrane, in accordance with our FACS data (Fig. 2B). These data indicate that a major fraction of gp130 ΔYY is retained in the ER.

The trafficking of secreted cargo proteins starts with their packaging into vesicles emerging at defined sites of the ER, also termed transitional ER (tER). In order to find out whether ER retention of gp130 ΔYY is due to a defect of ER vesicle transport to the Golgi, we co-expressed EYFP-tagged gp130 ΔYY with Sec16–mCherry (Witte et al., 2011), a marker of the transitional ER (Connerly et al., 2005; Inumaa et al., 2007; Witte et al., 2011) However, we did not observe any colocalisation, indicating that ER retention of gp130 ΔYY occurs before the packaging into ER transport vesicles (Fig. 2C). Furthermore, when we analysed HepG2 cells transfected with EYFP–gp130 ΔYY or EYFP–gp130 WT and an ECFP-fused Golgi marker, a minor fraction of gp130 ΔYY could be detected in the Golgi compartment (Fig. 2E), whereas gp130 WT was absent from the Golgi (Fig. 2D). These data indicate that gp130 ΔYY is able to exit the ER (Fig. 2D).

During our confocal microscopy analysis of EYFP–gp130 ΔYY, we realised that in particular z-stacks, a fraction of gp130 ΔYY was localised in vesicular structures that did not colocalise with the co-expressed ER marker (supplementary material Fig. S2A). We assumed that these vesicular structures might represent an endosomal compartment. Therefore, we co-transfected HepG2 cells with expression constructs for EYFP–gp130 variants and mCherry-fused Rab5a (Taylor et al., 2011), a marker for early endosomes, and analysed the cells by confocal microscopy. As expected, vesicles positive for EYFP–gp130 ΔYY colocalised extensively with Rab5a-positive signals (Fig. 2F). In cells expressing wild-type gp130, we also detected EYFP-positive vesicles that colocalised with Rab5a-positive compartments (Fig. 2G). However, the number and size of those vesicles was significantly smaller when compared to cells expressing gp130 ΔYY. We could furthermore detect gp130 ΔYY partially in Lamp1-positive vesicles, corresponding to lysosomes, whereas wild-type gp130 was absent from lysosomes (supplementary material Fig. S2B,C).

Taken together, these data indicate that constitutively active gp130 deletion mutants are predominantly localised to the ER and to early endosomes.

gp130 ΔYY is retained by the ER quality control system

We further sought to analyse the underlying mechanisms of the intracellular localisation of gp130 ΔYY. In order to determine whether prolonged localisation of gp130 ΔYY in the ER is the result of impaired protein folding or protein maturation, we analysed the maturation properties of gp130 ΔYY in a pulse-chase experiment. HepG2 cells were transiently transfected with either EYFP-tagged gp130 WT or gp130 ΔYY. Cells were subsequently labelled with 35S-cysteine and methionine for 20 minutes and chased in non-radiolabeled medium. Cells were lysed at the indicated time points and gp130 was isolated by anti-GFP immunoprecipitation. Incorporation of radioactive amino acids was analysed by SDS-PAGE and fluorography. Whereas wild-type gp130 matured within 2 hours from the high-mannose to the complex glycosylated form, gp130 ΔYY did not mature completely within the observation time and a substantial portion of gp130 ΔYY migrated at the position of the high-mannose form of gp130 (Fig. 3A). We furthermore noticed that the intensity of the gp130 WT signal decreased after 2 hours, whereas the signal strength for gp130 ΔYY was unaltered (Fig. 3A), suggesting that the protein stability of gp130 ΔYY could be increased.

We have previously seen that ER retention of oncogenic receptor tyrosine kinases was causally linked to its kinase activity and hence to its autophosphorylation status (Schmidt-Arras et al., 2005). We therefore hypothesized that ER retention of gp130 ΔYY might be conferred by its constitutive phosphorylation status. It has been shown that binding of JAK kinases to gp130 is mediated by a region called Box1, comprising amino acids Ile651 to Ser659 (Thiel et al., 1998). Furthermore Trp652, within the Box1, has been shown to be crucial for JAK activation (Haan et al., 2002). We therefore generated expression constructs for EYFP–gp130 ΔYY either devoid of Box1 or with a mutated Trp652. We analysed expression of the mutants gp130 ΔYY I651-S659del (gp130 ΔYY ΔBox1) and gp130 ΔYY W652A in HepG2 cells by anti-gp130 immunoblotting. Although we did not detect a change in receptor phosphorylation for gp130 ΔYY W652A, it was absent in the case of gp130 ΔYY ΔBox1. However, neither of the mutants displayed an overt increase in maturation, as revealed by the intensity ratio of the 180 kDa to the 160 kDa band (Fig. 3B). We furthermore did not detect a change in subcellular localisation of gp130 ΔYY when transfected HepG2 cells were treated for 3 hours with the JAK kinase inhibitor P6 (supplementary material Fig. S3).

We have recently shown that single point-mutations of gp130 lead to its constitutive activation (Schüt et al., 2013). We anticipated that if ER retention is linked to gp130 activity or phosphorylation status, these mutants should be also retained in the ER. We therefore co-transfected HepG2 cells with expression constructs for the constitutively active mutants EYFP–gp130 V252G or EYFP–gp130 C172S, along with a ECFP-fused ER marker. For both mutants, we could detect localisation to the plasma membrane and to vesicular structures, but very little colocalisation with the ER marker, indicating that these constitutively active mutants are not retained within the ER (Fig. 3C). These data are in accordance with our previous finding that these mutants are detectable at the cell surface by flow cytometry (Schüt et al., 2013). Taken together, these results indicate that deficiency in maturation of gp130 ΔYY is not mediated by its phosphorylation status or activation of downstream signalling pathways.

We therefore hypothesised that a folding defect in its extracellular domain might be the reason for the impaired maturation of gp130 ΔYY. Secreted proteins with improper folding display prolonged contact with proteins of the ER quality control system (Brodsky and Skach, 2011). Possessing lectin-function, it binds to monoglucosylated proteins, allowing for proper protein folding (Ihara et al., 1999; Vassilakos et al., 1996; Ware et al.,
Fig. 2. gp130 ΔYY localises to the ER and early endosomes. (A–G) HepG2 cells were seeded in ibidi μ-dishes and subsequently transfected with expression constructs for EYFP-tagged gp130 variants along with the indicated subcellular marker proteins. Live cells were then analysed using an Olympus Fluoview 1000 confocal laser scanning microscope. Please note that the ECFP channel has been false coloured in red to allow for a better contrast in merged images. White arrows indicate plasma membrane-localisation. Green arrows indicate gp130 ΔYY-containing intracellular compartments not colocalising with the indicated marker protein. Yellow arrows indicate colocalisation with the indicated marker protein.
We therefore anticipated that impaired maturation of gp130ΔYY was causally linked to calnexin association. We performed anti-GFP immunoprecipitations from HepG2 cells transfected either with EYFP–gp130 WT or EYFP–gp130ΔYY and analysed calnexin association by anti-calnexin immunoblotting. Association of gp130ΔYY with calnexin was increased as compared to wild-type gp130 (Fig. 4A).

In order to find out whether calnexin mediates the maturation deficiency of gp130ΔYY, we stably transduced mouse embryonic fibroblasts (MEFs) derived from wild-type (WT) or calnexin deficient (CNXΔYY/ΔYY) mice with retroviral expression constructs for Myc-tagged gp130 wild-type or gp130ΔYY. We analysed gp130 cell surface localisation by flow cytometry using anti-gp130 antibodies. As observed in Ba/F3 cells, wild-type gp130 was readily detectable on the surface of WT MEFs, whereas gp130ΔYY surface localisation was significantly reduced in these cells (Fig. 4B, histograms in light grey). However, when we permeabilised cells prior to anti-gp130 staining, we detected a significant shift in fluorescence for gp130ΔYY (Fig. 4C, histograms in light grey). These data indicate that gp130ΔYY is also retained in the ER in MEFs. Interestingly, whereas gp130 WT was present as a unique fraction, gp130ΔYY could be detected as two distinct populations in permeabilised wild-type MEFs. We assume that the fraction with the lower gp130 signal represents the ER pool of gp130ΔYY, as permeabilisation of the ER membrane might be less efficient. Surprisingly, in calnexin-deficient MEFs, gp130ΔYY was detectable at the cell surface to the same extent as wild-type gp130 (Fig. 4B, histograms in dark grey). Permeabilisation prior to anti-gp130 staining resulted only in a modest shift in fluorescence in CNXΔYY/ΔYY MEFs for both gp130 WT and gp130ΔYY (Fig. 4C, histograms in dark grey). Surprisingly, when analysed by SDS-PAGE and immunoblotting, both wild-type and mutant gp130 were predominantly detectable as the 130 kDa form (Fig. 4D, upper panel). We believe that this is the result of incomplete deglucosylation, which, in consequence, impairs further glycan modification. We furthermore noticed that gp130ΔYY-induced STAT3 activation was strongly reduced when expressed in CNXΔYY/ΔYY MEFs as compared to when expressed in wild-type MEFs (Fig. 4D, lower panel). It is possible that the
altered glycosylation of gp130 ΔYY in CNX−/− MEFs accounts for the observed reduced gp130 activation. Taken together, these data clearly show that calnexin contributes to the intracellular retention of gp130 ΔYY and that presence of calnexin seems to coincide with activation of STAT3.

Prolonged localisation of misfolded proteins within the ER induces an ER stress response, also termed the unfolded protein response (UPR). Whereas we could detect XBP-1 splicing (XBP-1s), a major hallmark of ER stress (Hetz and Glimcher, 2009), in tunicamycin-treated HepG2 cells, XBP-1 splicing was absent in cells overexpressing gp130 ΔYY (Fig. 4E).

These data indicate that the UPR seems not to be activated upon retention of gp130 ΔYY within the ER. In order to prevent ER stress, cells are able to subject proteins that are unable to fold properly to a process called ER-associated degradation (ERAD), resulting in proteasomal degradation (Brodsky, 2012). We detected no significant accumulation of gp130 WT or gp130 ΔYY, when we treated transfected HepG2 cells with the proteasome inhibitor Bortezomib (Meister et al., 2007), suggesting that gp130 ΔYY is not subjected to proteasomal degradation and hence ERAD (supplementary material Fig. S4).

**gp130 ΔYY emits signals from the ER**

In order to determine whether gp130 ΔYY is able to induce signal transduction from within an ER compartment, we sought to artificially entrap gp130 ΔYY completely in the ER. We therefore employed a recently described streptavidin-based system (Boncompain et al., 2012), which also allows the release of ER-retained proteins by the addition of exogenous biotin (Fig. 5A). Accordingly, we inserted the streptavidin-binding peptide (SBP) at the N-terminus and termed the construct SBP–gp130–EYFP (supplementary material Fig. S1C). We transfected HeLa cells with expression constructs for SBP–gp130 WT–EYFP and SBP–gp130 ΔYY–EYFP. Cells were stimulated with 40 μM tunicamycin. Cells were lysed and analysed by SDS-PAGE and immunoblotting. If indicated, gp130 was isolated by anti-Myc immunoprecipitation prior to SDS-PAGE. Phosphorylated STAT3 (P-STAT3) and STAT3 signals were quantified by densitometry and the ratio P-STAT3:STAT3 is given below the P-STAT3 panel. (E) ER retention of gp130 ΔYY does not induce an unfolded protein response. HepG2 cells were transiently transfected with gp130 WT or gp130 ΔYY. If indicated, untransfected cells were treated with 5 μg/ml tunicamycin. Cells were lysed and splicing of XBP-1 mRNA was assessed by RT-PCR and analysed on a 2.5% agarose gel.
D-biotin and lysed at the indicated time points. Gp130 was isolated by anti-GFP immunoprecipitation and maturation was followed by the increase of apparent molecular mass as judged by migration on immunoblots. We observed a shift in molecular mass as early as 30 minutes after stimulation with D-biotin for both SBP–gp130 WT–EYFP and SBP–gp130 ΔYY–EYFP (Fig. 5B). Interestingly, the increase in molecular mass reached a maximum after 60 minutes, suggesting that after 30 minutes SBP-gp130 EYFP variants are still subjected to glycan modification within the Golgi.

Phosphorylation of STAT3 was absent in cells transfected with SBP–gp130 WT–EYFP and did not increase significantly upon addition of biotin. However, we detected a weak, but significant, STAT3 phosphorylation in cells transfected with SBP–gp130 ΔYY in the absence of biotin, suggesting that gp130 ΔYY was active in the ER. When we transfected SBP–gp130 ΔYY–EYFP into HEK293T cells, we could indeed detect a slight, but significant, receptor phosphorylation (supplementary material Fig. S5). In the presence of biotin, phosphorylation of STAT3...
Fig. 6. See next page for legend.
strongly increased over time, reaching a maximum of activation after 60 minutes. This was also reflected by an increased SBP–gp130 \( \Delta YY \)–EYFP receptor phosphorylation in HEK293T cells (supplementary material Fig. S5). Taken together, these data indicate that gp130 \( \Delta YY \) is active in the ER, whereas fully fledged receptor activation is achieved outside the ER.

Next, we wanted to investigate whether gp130 \( \Delta YY \) signals emitted from the ER contribute to ligand-independent cell proliferation. We took advantage of the previously described Ba/F3-gp130 cell line. Ba/F3 cells that had been stably transducted with expression constructs for EYFP-tagged gp130 \( \Delta YY \) were treated with 100 nM P6 for 1 hour. Live cells were subsequently analysed using an Olympus Fluoview 1000 confocal laser scanning microscope. Representative images for untreated and treated with 100 nM P6 gp130 \( \Delta YY \) cells are shown in Fig. 6C; supplementary material Fig. S6). Plasma membrane localisation of gp130 \( \Delta YY \) was detected when cells were treated with dynasore (Fig. 6B, C). Surprisingly, when we analysed cells that were treated with 100 nM P6 gp130 \( \Delta YY \) cells were stimulated for 10 minutes with 100 ng/ml Hyper-IL-6, this was also reflected by an increased SBP–gp130 \( \Delta YY \)–EYFP receptor phosphorylation in HEK293T cells (supplementary material Fig. S5). Taken together, these data indicate that gp130 \( \Delta YY \) is active in the ER, whereas fully fledged receptor activation is achieved outside the ER.

Endosomal localisation of gp130 \( \Delta YY \) is crucial for downstream signalling

As indicated above, a substantial amount of gp130 \( \Delta YY \) also localised to early endosomes. We therefore sought to investigate the impact of endocytosis on gp130 \( \Delta YY \) signalling. We transfected HepG2 cells with expression constructs for EYFP-fused wild-type gp130 or gp130 \( \Delta YY \) and blocked internalisation by the use of increasing concentrations of the dynamin inhibitor dynasore. Using confocal microscopy we observed a significant concentration-dependent decrease in endosomal localisation of gp130 \( \Delta YY \) as measured by colocalisation with Rab5a-positive vesicles (Fig. 6C; supplementary material Fig. S6). Plasma membrane localisation of gp130 \( \Delta YY \) was detectable when cells were treated with dynasore (Fig. 6B; supplementary material Fig. S6), but not in untreated cells (Fig. 6A; supplementary material Fig. S6), indicating that plasma membrane localisation of gp130 \( \Delta YY \) precedes its transport to early endosomes. However, a large fraction of gp130 \( \Delta YY \) still colocalised with Rab5a in dynasore-treated cells, indicating that blockade of internalisation was incomplete (Fig. 6B).

When analysed by immunoblotting, the distribution of the 180 kDa and the 160 kDa bands was unaltered in both wild-type gp130 and gp130 \( \Delta YY \) (Fig. 6D). Surprisingly, when we analysed cells that were treated with 100 \( \mu \)M dynasore, by immunoblotting, we detected a significant reduction of STAT3 phosphorylation (by \( \sim 50\% \)) in both gp130 \( \Delta YY \) cells and Hyper-IL-6-stimulated gp130 WT cells. STAT3 activation was absent in untreated and dynasore-treated cells with unstimulated gp130 WT (supplementary material Fig. S7). These data suggest that activation of STAT3 pathways by gp130 does not occur at the plasma membrane, but at early endosomes. Interestingly, phosphorylation of ERK1/2 was significantly increased (approximately twofold) when cells were treated with dynasore, indicating that activation of the MAPK cascade takes place at the plasma membrane and is shut down at early endosomes (Fig. 6D; supplementary material Fig. S7).
Internalisation of wild-type gp130 has been linked to the intracellular di-leucine motif at positions 786 and 787 (Dittrich et al., 1996). Therefore, we constructed internalisation deficient gp130 ΔYY by mutating the di-leucine motif. Internalisation-deficient gp130 ΔYY mutants L786A, L787A (gp130 ΔYY LLAA) were transfected into HepG2 cells along with mCherry-fused Rab5a and cells were analysed by confocal microscopy. As has been reported previously for wild-type gp130 LLLAA (Dittrich et al., 1996), gp130 ΔYY LLAA was now present at the plasma membrane (Fig. 6E, white arrow). However, internalisation was not completely impaired, as we still could detect some localisation of gp130 ΔYY LLAA to Rab5a-positive vesicles (Fig. 6E, yellow arrows). Receptor phosphorylation and concomitant STAT3 phosphorylation was reduced in cells transfected with gp130 ΔYY LLLAA as compared to gp130 ΔYY (Fig. 6F), but not completely abrogated. Activation of STAT3 by gp130 ΔYY LLAA could be completely inhibited by the addition of dynasore (supplementary material Fig. S7), indicating that STAT3 was activated by the fraction of gp130 ΔYY LLAA localising to early endosomes. These findings are in line with the above-described experiments employing dynamin inhibition. Taken together, our data support the notion that activation of the MAPK cascade by gp130 ΔYY LLAA is crucial for its transforming capability, we assessed the proliferation of Ba/F3-gp130 cells stably transfected with Myc-tagged gp130 ΔYY. We have previously shown that Ba/F3-gp130/gp130 ΔYY cells are independent of growth factor (Schütz et al., 2013; Sommer et al., 2012). Proliferation of Ba/F3-gp130/ gp130 ΔYY cells treated with 50 μM dynasore for 24 hours was significantly reduced (by 50%) (Fig. 6G), but not completely abolished. In order to guard against unspecific toxicity, we also treated Ba/F3 cells stably transfected with the cytoplasmic fusion protein TEL-PDGFβR (Uecker et al., 2010; Jousset et al., 1997), assuming that TEL-PDGFβR-driven proliferation is independent of endocytosis. As expected, proliferation of Ba/F3-TEL- PDGFβR cells was unaffected by 50 μM dynasore, excluding a toxic effect of dynasore at this concentration. These results demonstrate that endocytosis of gp130 ΔYY contributes to ligand-independent cell proliferation.

Taken together, our data indicate that an endosomal localisation of constitutively active gp130 deletion mutants is crucial for STAT3 activation and necessary for the induction of cell proliferation.

**Negative-feedback regulation by SOCS3 is not impaired in gp130 ΔYY**

Activation of gp130 is followed by the induction of a negative-feedback regulation. One major negative regulator of gp130 is the suppressor of cytokine signalling 3 (SOCS3). It has been shown that SOCS3 is upregulated by STAT3 to negatively regulate JAK kinase activity and to compete with SHP-2 for binding to phosphorylated Tyr759 of gp130 (Heinrich et al., 2003). Interestingly, SOCS3 is upregulated in cells expressing gp130 deletion mutants (Rebouissou et al., 2009). We sought to analyse whether localisation of gp130 ΔYY to intracellular compartments results in insensitivity to SOCS3 inhibition. We therefore transfected HEK293T cells with expression constructs for EYFP-tagged gp130 WT or gp130 ΔYY along with Myc-tagged SOCS3. If indicated, cells were stimulated with Hyper-IL-6 prior to cell lysis. Cell lysates were analysed by SDS-PAGE and immunoblotting. Overexpression of SOCS3 impaired STAT3 phosphorylation in both gp130 ΔYY cells and ligand-stimulated gp130 WT cells (Fig. 6H). Interestingly, phosphorylation of ERK1/2 was reduced in cells co-expressing gp130 ΔYY and SOCS3, but not completely impaired (Fig. 6H). In the light of the above findings, these data suggest a selective binding of SOCS3 to endosomally localised gp130 ΔYY. Next, we sought to analyse whether endogenous levels of SOCS3 were able to regulate gp130 ΔYY-induced STAT3 phosphorylation. Therefore we mutated the SOCS3 binding site Tyr759 (Schmitz et al., 2000) in gp130 ΔYY and anticipated an increased STAT3 phosphorylation in the absence of SOCS3 binding. When transfected into HEK293T cells, gp130 ΔYY Y759F displayed a substantial increase in STAT3 phosphorylation as compared to gp130 ΔYY (Fig. 6I). These data indicate that SOCS3 is able to downregulate gp130 ΔYY-induced signals, but is insufficient for a complete shutdown.

**DISCUSSION**

Recently, deletion mutations of the IL-6 receptor subunit gp130 have been identified in benign inflammatory liver adenomas. The deletions differed in length but all of them resulted in a ligand-independent constitutive activation of gp130. When transfected into Hep3B cells gp130 deletion mutants induced constitutive STAT3 activation and upregulation of SOCS3 and the acute-phase-protein CRP, both known targets of STAT3-induced transcriptional regulation (Rebouissou et al., 2009). However, little was known about the activation and regulation of these gp130 deletion mutants. We have very recently shown that the deletions impair an interdomain hydrophobic interaction leading to subtle conformational changes that render gp130 constitutively active (Schütz et al., 2013).

In the present study, we show that oncogenic gp130 deletion mutants are predominantly localised to intracellular compartments. We selected gp130 Y186-Y190del (gp130 ΔYY), as one of the most frequently occurring mutants, to study the underlying mechanisms in more detail. We made the observation that gp130 ΔYY can be detected intracellularly at two major compartments: the ER and the early endosomes. We identified the ER quality control system and, in particular, the chaperone calnexin to be crucial for STAT3 activation and necessary for the induction of cell proliferation.

In the present study, we show that oncogenic gp130 deletion mutants are predominantly localised to intracellular compartments. We selected gp130 Y186-Y190del (gp130 ΔYY), as one of the most frequently occurring mutants, to study the underlying mechanisms in more detail. We made the observation that gp130 ΔYY can be detected intracellularly at two major compartments: the ER and the early endosomes. We identified the ER quality control system and, in particular, the chaperone calnexin to be crucial for STAT3 activation and necessary for the induction of cell proliferation. We therefore hypothesise that the observed increase in association is due to local folding defects in domain D2 of the extracellular domain of gp130 ΔYY, evoked by the deletion mutation. It has been indeed shown that local folding defects in transmembrane proteins are recognized by the UDP-glucose:glycoprotein glucosyltransferase (UGGT) leading to reglucosylation of the glycan and, in consequence, a prolonged association of the misfolded protein with calnexin (Pearse et al., 2008; Taylor et al., 2004). Biophysical analysis of the recombinant gp130 domains D2–D3 containing a deletion mutation will reveal whether the mutations result in local structural instability. Interestingly, our findings are in contrast to previous reports on oncogenic receptor tyrosine kinases (RTKs). There we have previously shown that ER retention of constitutively active RTKs is linked to their kinase activity and hence autophosphorylation status (Schmidt-Arras et al., 2005). In summary, this demonstrates that the ER is capable of sensing defects in oncogenic transmembrane proteins in two different ways.

Although the impaired maturation and ER retention of gp130 ΔYY was in stark contrast to that of wild-type gp130,
internalisation to early endosomes seemed to have the same underlying mechanisms for both wild-type gp130 and gp130 ΔYY. Interestingly, for both gp130 ΔYY and ligand-stimulated gp130WT, there was a different spatial regulation of downstream signalling pathways. Whereas activation of the Ras–MAPK pathway required plasma membrane localisation, activation of STAT3 seemed to occur predominantly at early endosomes (Fig. 7A,B). This is in accordance with previous reports suggesting a role of endosomes for the activation of STAT3 by IL-6-stimulated wild-type gp130 (Shah et al., 2006; Xu et al., 2007). It is possible that endosomal gp130 ΔYY localisation is required for its capacity for oncogenic transformation. It has been recently demonstrated that, for oncogenic Met mutants, receptor activation alone is not sufficient for oncogenic transformation, but that it is dependent on endosomal localisation of the Met mutants (Joffre et al., 2011). We furthermore could demonstrate that gp130 ΔYY is still subjected to negative regulation by SOCS3. Given the fact that gp130 ΔYY-induced STAT3, but not ERK, activation was completely abolished by SOCS3 overexpression, we hypothesise that regulation of gp130 by SOCS3 occurs at early endosomes (Fig. 7A,B). However, further experiments are needed to clearly corroborate this hypothesis.

Interestingly, we also observed that gp130 ΔYY is able to emit signals from the plasma membrane. We could demonstrate that cell proliferation driven by an activating gp130 point mutant could be blocked by an exogenously added neutralising antibody. However, proliferation driven by gp130 ΔYY was only partially impaired. It is very likely that this is due to other downstream signals than STAT3 activation. Although we have detected STAT3 phosphorylation when gp130 ΔYY was artificially retained in the ER, fully fledged activation of STAT3 occurred outside the ER. Interestingly, this is in contrast to the constitutively active oncogenic RTK, FLT3 ITD (internal tandem duplication). FLT3 ITD-induced STAT3 phosphorylation increased when FLT3 ITD was artificially completely retained in the ER (Schmidt-Arras et al., 2009). This might be linked to the fact that STAT3 activation by gp130 ΔYY is dependent on cytoplasmic tyrosine kinases that might be inefficiently activated at the ER. It is further possible that glycosylation of the extracellular domain has an impact on gp130 ΔYY activation and that complex glycosylation is required to achieve a fully fledged activation of gp130 ΔYY. We furthermore hypothesise that active gp130 ΔYY in the ER is able to induce phosphorylation of unusual substrate proteins that are not present at the plasma membrane or near early endosomes (Fig. 7B).

Interestingly a recent study has suggested that activation of mTOR by wild-type gp130 is independent of receptor phosphorylation and JAK kinase activity (Thiem et al., 2013).

More in-depth studies are needed to decipher the different qualities of downstream signalling pathways evoked from different subcellular compartments. The present study, however, is a strong basis for such future experiments and clearly demonstrates how an oncogenic cytokine receptor ‘profits’ from intracellular control mechanisms to alter its downstream signalling. The presented data are highly relevant for the consideration of therapeutic interventions targeting oncogenic cytokine receptors.

MATERIALS AND METHODS

Materials
Cell culture materials were from PAA laboratories GmbH (Cölbe, Germany). Cysteine- and methionine-free DMEM was from Life Technologies (Darmstadt, Germany). Polyethyleneimine ‘MAX’ (MW 40,000) (PEI) was from Polyscience (Eppelheim, Germany). Phusion™ polymerase, DreamTaq™ polymerase and restriction enzymes were from Fermentas/ThermoFisher (Sankt Leon-Rot, Germany). Antibodies anti-gp130 (C-20, sc-655), anti-gp130 (B-P4, sc-57188), anti-c-Myc (9E10, sc-40) and anti-j-actin (C-4, sc-47778) were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-GFP (7.1 and 13.1, No. 11 814 460 001) was from Roche (Penzberg, Germany), anti-P-STAT3 (D3A7, No. 9154), anti-STAT3 (No. 9139), anti-P-ERK (No. 9101),
anti-ERK (No. 9102), anti-P-Tyr-100 (No. 9411) were from Cell Signalling (Frankfurt, Germany). Dynalight™ 649-labeled anti-mouse (No. 35515) and protein-G–agarose was from Pierce/Thermo Fisher (Bonn, Germany). Purified anti-mouse CD16/CD32 (Mouse BD Fc-block™, No. 553142) was from BD Biosciences (Heidelberg, Germany). The pan-Janus kinase inhibitor Pyridone 6 (P6, No. 420099) was from Calbiochem/Merck (Darmstadt, Germany). Dynasore was from Tocris (Bristol, UK), Tunicamycin was from Applichem (Darmstadt, Germany). [S-35]Met-label (SCIS-103) was from Hartmann Analytik (Braunschweig, Germany). Hyper-IL-6 is a fusion protein between the soluble IL-6 receptor (IL-6R) and IL-6, and has been shown previously to be a potent activator of gp130 (Fischer et al., 1997; Kallen et al., 2000). Hyper-IL-6 was expressed and purified as described previously (Fischer et al., 1997).

DNA expression constructs
The retroviral expression constructs pMOWS-myc-gp130 WT, pMOWS-myc-gp130 ΔYY with a C-terminal fusion of the Myc tag to gp130, as well as p409-EYFP-gp130 WT and p409-EYFP-gp130 ΔYY with a C-terminal fusion of EYFP to gp130 have been previously described (Sommer et al., 2012). See also supplementary material Fig. S1 for more details.

Point mutations in either pMOWS-gp130-myc or p409-EYFP-gp130 were generated by site-directed PCR mutagenesis using Phusion polymerase (Fermentas). Please note that for the ease of comparison the position of the mutation is given in analogy to wild-type gp130 and does not consider the deletion of five amino acids in gp130 ΔYY. Primer sequences are available upon request.

Cloning of gp130 variants into the recently published Rush-system was performed as follows. gp130 EYFP cDNA devoid of its proper signal sequence was amplified by PCR and cloned via SphI and XbaI into the previously described Rush-system backbone (Boncompain et al., 2012). See also supplementary material Fig. S1 for further details.

Culture, transfection and transduction of cell lines
Immortalised murine embryonic fibroblasts (MEFs), HEK293T and Phoenix cells were grown as adherent cells in DMEM plus 10% FCS. HepG2 cells were grown in RPMI plus 10% FCS. Ba/F3 cells and stable Ba/F3-myc-gp130 variant cell lines were maintained in RPMI plus 10% FCS and 10 ng/ml IL-3. Ba/F3-TEL-PDGF/B cells were maintained in RPMI plus 10% FCS. The Ba/F3-gp130 cell line and stable Ba/F3-gp130/myc-gp130 variant cell lines were maintained in RPMI plus 10% FCS, supplemented with 10 ng/ml Hyper-IL-6, as previously described (Schütt et al., 2013). Hyper-IL-6 is a fusion protein between the soluble IL-6R and IL-6 and has been shown previously to be a potent activator of gp130 (Fischer et al., 1997; Kallen et al., 2000). HEK293T, Phoenix and HepG2 cells were transduced using polyethyleneimine (1 mg/ml) at a ratio of 1:3 (DNA:PEI).

Retroviral supernatants were generated by transfection of Phoenix cells with different Myc-tagged gp130 variants in the retroviral pMOWS vector. Immortalised MEFs or Ba/F3-based cells were subsequently transduced with retroviral supernatants and selected using 1.5–2 μg/ml puromycin.

Live cell confocal microscopy
For live cell imaging, 2×10^6 HepG2 cells were seeded in a 35-mm glass-bottomed ibidi μ-dish (Martinsried, Germany). Cells were transfected with expression constructs for gp130 variants and an organelle marker if indicated. Cells were starved in RPMI+0.5% FCS overnight before imaging. If indicated, cells were treated for 1 hour with dynasore or for 3 hours with P6 at 37˚C prior to imaging. Confocal microscopy images were taken on an Olympus Fluoview 1000 confocal laser scanning microscope using 60× (NA 1.35) or 100× (NA 1.4) objectives.

In order to quantify colocalisation of Rab5a and gp130 signals in dynasore-treated cells, ten different cells at each dynasore concentration were recorded. Images were thresholded and analysed using the JACoP plugin (Bolte and Cordelieres, 2006) for ImageJ. Colocalisation was determined by the averaged Manders’ coefficient M1, representing the fraction of gp130 ΔYY localising to Rab5a-positive vesicles.

Cells, lysis, immunoprecipitation and immunoblotting
Cells were lysed in RIPA buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS], supplemented with phosphatase and protease inhibitors. For co-immunoprecipitation, cells were lysed in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 0.5% NP-40), supplemented with phosphatase and protease inhibitors. Cell lysates were cleared by centrifugation at 12,000 g for 15 minutes. Subsequently cell lysates were subjected to immunoprecipitation overnight using either anti-Myc (9E10) or anti-GFP (7.1 and 13.1) antibodies and Protein-G–agarose. The beads were washed three times with 500 μl HNGT buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) before boiling in 30 μl 2× Lämmli buffer. Immunoprecipitates were subsequently analysed by 8% SDS-PAGE and immunoblotting using the indicated antibodies. Cell lysates were analysed by 10% SDS-PAGE and immunoblotting using the indicated antibodies.

Metabolic pulse-chase labelling
Pulse–chase analysis was carried out as previously described (Schmidt-Arras et al., 2005). Briefly, HepG2 cells, transiently transfected with expression constructs for EYFP–gp130 WT or gp130 ΔYY were starved for 4 hours in cysteine- and methionine-free DMEM. Cells were labelled for 20 minutes with 200 μCi [S-35]Met-label, containing radiolabelled cysteine and methionine, in cysteine- and methionine-free DMEM. Cells were subsequently chased in DMEM containing 0.1 mM methionine and 0.2 mM cysteine. Cells were lysed at the indicated time points, gp130 was isolated using anti-GFP antibodies and immunoprecipitated gp130 was analysed by SDS-PAGE and fluorography.

MTT cell viability assay
Stably transduced Ba/F3-gp130/Myc-gp130 variant cells and stably transfected Ba/F3-TEL-PDGF/B cells were resuspended in the appropriate growth medium, supplemented with 50 μM of the dynamin inhibitor dynasore or 1 μg/ml B-P4 or control antibody if indicated, and seeded at a density of 5×10^4 cells/ml in 96-well plates in a total volume of 100 μl per well.

As indicated, 24 or 72 hours after seeding, 10 μl of MTT solution (5 mg/ml in PBS) was added to each well and cells were incubated for another 4 hours at 37˚C. 100 μl solubilization buffer (10% SDS in 10 mM HCl) was added and the plate was incubated overnight at 37˚C. Absorption was subsequently measured at 595 nm in an ELISA plate reader (Tekan).

FACS analysis
Stably transduced Ba/F3 cells were washed with PBS prior to staining, and were blocked using Fc-block. Cells were subsequently stained with anti-gp130 (B-P4), followed by DyLight649-labelled anti-mouse and were analysed on a BD FACSCanto flow cytometer (Becton Dickinson). For the staining of intracellular gp130, cells were washed and subsequently resuspended in PBS containing Ca^2+ and Mg^2+. Cells were fixed with 2% 205°C for 3 hours with P6 at 37˚C prior to imaging. Confocal microscopy images were taken on an Olympus Fluoview 1000 confocal laser scanning microscope using 60× (NA 1.35) or 100× (NA 1.4) objectives. In order to quantify colocalisation of Rab5a and gp130 signals in dynasore-treated cells, ten different cells at each dynasore concentration were recorded. Images were thresholded and analysed using the JACoP plugin (Bolte and Cordelieres, 2006) for ImageJ. Colocalisation was determined by the averaged Manders’ coefficient M1, representing the fraction of gp130 ΔYY localising to Rab5a-positive vesicles.

Statistical analysis
Statistical significance was analysed by a two-tailed unpaired Student’s t-test. Student’s t-test and regression analysis were performed using Gnumeric 1.12 software.

Acknowledgements
We thank Jan Sommer (University Düsseldorf, Germany) for providing us expression constructs coding for EYFP-tagged gp130 wild-type and gp130 ΔYY. WT and CNX ^77 MEFs were kindly provided by Marek Michalak (University of Alberta, Canada). The plasmids pECPF-ER and pECFP-GOLGI were a kind gift of Annette Böhm. Ba/F3-TEL-PDGF/B cells were kindly provided by Frank-D. Böhm (Friedrich-Schiller-University, Jena). An expression plasmid encoding for Sec6-mCherry was kindly provided by Anjon Audhya (University of Wisconsin, USA). Rab5a-pmCherry-C1 was kindly provided by Christien Merrifield (MRC)
Centre, Cambridge, UK) via addgene (plasmid number 27679). We are grateful to Gaeille Boncompain and Franck Perez (Institut Curie Paris, France) for providing us the Rush-system. SOCS3 CDNA was kindly provided by Edith Pfitzner (Friedrich-Schiller-University Jena, Germany). Hyper-IL-6 was kindly provided by Inken Lenoren (Christian-Albrechts-University Kiel, Germany). We thank Christine Desel for excellent advice on confocal microscopy techniques. We are grateful to Annette and Frank Pöbner (Friedrich-Schiller-University, Jena) for helpful suggestions and critical reading of the manuscript.

Competing interests
The authors declare no competing interests.

Author contributions
M.M., S.H., A.S., J.B., R.W. and A.L. performed experiments and contributed to writing the manuscript. S.R.-J. interpreted and critically revised data and participated in writing the manuscript. D.S.-A. conceived the study, performed experiments, supervised experimental work and wrote the manuscript.

Funding
This work was supported by the Deutsche Forschungsgemeinschaft (DFG), Bonn [grant number SFB884, project C1 to S.-R.-J.] and by the Cluster of Excellence ‘Inflammation at Interfaces’ to S.-R.-J.

Supplementary material
Supplementary material available online at http://jc.sagepub.com/cgi/content/sumo/130/294/1/DCl

References


Fig. S1. Constructs used in this study. (A) EYFP-gp130 based constructs. The position of the deletion mutation is highlighted as black bar in the gp130 extracellular domain (ECD). Positions of all other secondary mutations within the cytoplasmic region are indicated. (B) myc-gp130 based constructs. (C) SBP-gp130-EYFP construct.
Fig. S2. HepG2 cells were transiently transfected with expression constructs for EYFP-fused gp130 WT or gp130 ΔYY together with either an ECFP-fused ER marker protein (A) or the lysosomal marker protein Lamp1 fused to RFP (B and C). Cells were starved and subsequently analysed by confocal microscopy. Please note that panel A is a different Z-stack of the same cell as in Fig. 2B. White arrows indicated plasma membrane-localisation. Green arrows indicate intracellular gp130 that does not localise to Lamp1-positive vesicles. Red arrows indicate Lamp1-positive vesicles without gp130. Yellow arrows indicate colocalisation of Lamp1 and gp130.
**Fig. S3.** HepG2 cells were transiently transfected with expression constructs for EYFP-gp130 ΔYY and mCherry-fused Rab5a. Cells were starved overnight and treated for 3 hours with 500 nM of the JAK kinase inhibitor P6 prior to analysis by confocal microscopy.

**Fig. S4.** HepG2 cells were transiently transfected with expression constructs for EYFP-gp130 WT or gp130 ΔYY. Cells were treated for 3 hours with the indicated concentrations of the proteasome inhibitor Bortezomib. Cells were subsequently lysed and analysed by SDS-PAGE and immunoblotting.
Fig. S5. HEK293T cells were transiently transfected with expression constructs for SBP-gp130 WT or SBP-gp130 ΔYY EYFP. Cells were starved and subsequently treated with 40 μM D-biotin. Cells were lysed at the indicated time points and SBP-gp130 EYFP variants were isolated using anti-GFP antibodies. Immunoprecipitates were subsequently analysed by SDS-PAGE and immunoblotting. CG: complex glycosylated; HM: highmannose; DG: deglycosylated.
Fig. S6. Representative images of dynasore treated HepG2 cells transiently transfected with EYFP-gp130 ΔYY and mCherry-fused Rab5a. Upper panel shows confocal images. Lower panel shows thresholded images of the respective fluorescent channel, as well as the cytofluorogram. In the cytofluorogram the relative mCherry intensity (y-axis) of a given pixel is plotted as a function of its EYFP intensity (x-axis). Pixels with a high grade of colocalisation cluster around the linear approximation (red line), calculated by linear regression. Image thresholding, cytofluorograms and colocalisation analysis was performed with the JACoP plugin for ImageJ (Bolte and Cordelières, 2006). a.u.: arbitrary units.
Fig. S7. HepG2 cells were transiently transfected with the indicated variants of EYFP-gp130. Cells were starved and subsequently treated for 3 hours with the indicated concentrations of dynasore. Cell lysates were analysed by SDS-PAGE and immunoblotting using the indicated antibodies.