SUPPLEMENTAL MATERIAL AND METHODS

Construction of Jun-gp130 (L-gp130), Fos-gp130, Δcys-Fos-gp130 and Jun-WSX-1 expression plasmids

Standard cloning procedures were performed as described (Sambrook *et al.*, 1989). The Jun-gp130 (also known as L-gp130) was described previously (Stuhlmann-Laeisz *et al.*, 2006). The Fos-gp130 fusion receptor has the following assembly: gp130 signal peptide (MLTLQTWLVQALFIFLTTESTG), Flag sequence (DYKDDDDK), linker (for Fos-gp130:ELCGG, for Δcys-Fos-gp130: ELGGG), human Fos protein fragment (LTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAAY) (O'Shea *et al.*, 1989) and a N-terminally truncated gp130-protein (15 aa of the extracellular domain, transmembrane domain and cytoplamic domain – amino acids 606-918). In brief, we used pQE30-Fos, which encodes for a codon optimized Fos protein (Geneart, Regensburg, Germany), as a template for PCR to amplify the Fos-coding sequence (5´primer: 5´-3´: GAATTGTGCGGCGGCTTAACTGATACACTCCAA [Fos-gp130] or 5´primer: 5´-3´: GAATTGGGCGGCGGCTTAACTGATACACTCCAA [Δcys-Fos-gp130] and 3´primer: 5´-3´: AGTCGAATTCAGCTGCCAGGATGAACTC [both]). The coding sequence of the gp130 signal peptide, FLAG tag and linker was amplified by PCR, using the plasmid pBSK-Jun-gp130 as a template (5´primer: 5´-3´: GATCCTCGAGTCTAGACCCCGCAAG and 3´primer 5´-3´: GCCGCCGCACAATTCTTTATC [Fos-gp130] or GCCGCCGCCCAATTCTTTATC [Δcys-Fos-gp130]). Both PCR products were combined in subsequent PCR using the 5´ and 3´ primers of the initial PCRs. The purified PCR product was digested with *Xho*I and *Eco*RI and subcloned into pBSK-gp130(ΔN-term). This vector was obtained by digestion of pBSK-Jun-gp130 with *Xho*I and *Eco*RI. The resulting plasmids were named pBSK-Fos-gp130 or pBSK-Δcys-Fos-gp130.

The Jun-WSX-1 receptor has the following assembly: gp130 signal peptide (MLTLQTWLVQALFIFLTTESTG), Flag sequence (DYKDDDDK), linker (for Jun-gp130:ELCGG), human Jun protein fragment (RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMN) (O'Shea *et al.*, 1989) and a N-terminally truncated WSX-1-protein (15 aa of the extracellular domain, transmembrane domain and cytoplamic domain – amino acids 500-623). In brief, the truncated murine WSX-1 coding sequence was amplified by PCR using cDNA from Ba/F3-cells as a template (5´primer 5´-3´: GACTGAATTCTCACTTCACCTACCAGATAATAGG and 3´primer 5´-3´: GACTGGATCCTCAGACTAGAAGGCCCAGCTCCTC). The purified PCR product was

digested with *Eco*RI and *Bam*HI and subcloned in the vector pBSK-Jun, which was obtained by digestion of pBSK-Jun-gp130 with *Eco*RI and *Bam*HI. The resulting plasmid was named pBSK-Jun-WSX-1.

All constructs were subcloned into pMOWS (Ketteler *et al.*, 2002). In brief, the vector was digested with *Xag*I and blunt ends were generated by Klenow fragment reaction. The inserts were digested with *Xho*I and blunt ends were generated by Klenow fragment reaction. In a second digestion step, vector and inserts were digested with *Bam*HI, purified and subcloned. The resulting plasmids were named pMOWS-Jun-gp130 (pMOWS-L-gp130), pMOWS-Fos-gp130, pMOWS-Δcys-Fos-gp130 and pMOWS-Jun-WSX-1.

The constructs Fos-gp130 and Δcys-Fos-gp130 were subcloned into p409. In brief, the plasmid p409 was digested with *Sal*I and *Not*I. The cDNAs coding for Fos-gp130 and Δcys-Fos-gp130 were digested with *Xho*I and *Not*I, purified and subcloned. The resulting Plasmids were named p409-Fos-gp130 and p409Δcys-Fos-gp130.

The cDNA coding for Δcys-Fos-gp130 was additionally subcloned into pEYFP-gp130 (Tenhumberg *et al*., 2006). In brief, the plasmid pEYFP-gp130 was digested with *Xho*I and *Xmi*I as well as the cDNA coding for Δcys-Fos-gp130. Both were purified and subcloned. The resulting Plasmid was named pEYFP-Δcys-Fos-gp130.

Detection of fusion receptors by RT-PCR

For detection of fusion receptor gene transcription of retrovirally transduced Ba/F3-gp130 cells RNA was isolated, reverse transcribed and PCR was performed like described above.

The following primer combinations were used: IL-15-gp130 (5´IL-15-primer, 5´-3´: CCACCATGGACAGCAAAG and 3´gp130-primer, 5´-3´: TCACTGAGGCATGTAGCC), IL-15-WSX-1 (5´IL-15-primer and 3´WSX-1-primer, 5´-3´: TCAGACTAGAAGGCCCAG), IL-15-LIFR (5´IL-15-primer and 3´LIFR-primer, 5´-3´: CCACCATGGACAGCAAAG), IL-15-OSMR (5´IL-15-primer and 3´OSMR-primer, 5´-3´: TTAGCAGTAGTGTTCACC), , IL-15Rα-sushi-gp130 (5´IL-15Rα-sushi-primer, 5´-3´: CCACCATGTTGACGTTGC and 3´gp130-primer), IL-15Rα-sushi-WSX-1 (5´IL-15Rα-sushi-primer and 3´WSX-1-primer) and IL-15Rα-sushi-GPL (5´IL-15Rα-sushi-primer and 3´GPL-primer, 5´-3´: GGATCCTTAGACTTCTCCCTTGG)

Proliferation assay

The proliferation assay was performed like described above. Different amounts of recombinant IL-15 and soluble IL-15Rα sushi domain were added as indicated.

Co-culture experiments of Ba/F-gp130-GFP cells with Ba/F-gp130 cells stably transduced with fusion receptor genes

Ba/F3-gp130 cells expressing GFP (2 x 10^5 cells) were co-cultured in a 12-well plate with Ba/F3-gp130 cells expressing either IL-15-gp130, IL-15-WSX-1 + sushi-gp130 or Fos-gp130 (1 x 10^4 cells) at a ratio of 20:1. After 24 h, 48 h, 72 h and 96 h the green fluorescent cells were quantified by flow cytometry.

***Expression of IL-15 in* E.coli**

The coding sequence of human IL-15 was cloned into pET30 expression vector using *Bam*HI and *Hin*dIII restriction sites. The obtained plasmid was transformed in *E.coli* SG13009. Protein expression was performed in LB-Media at 37°C, for induction 1 mM Isopropyl-β-D-thiogalactopyranosid was added at an OD_{600} of 0.5-0.8. Bacteria were harvested 3 h after IPTG addition, re-suspended in 20 mM sodium phosphate buffer pH 7.4, containing 0.5% Tween 20 and 1 mM EDTA. Afterwards the inclusion bodies were purified by repetitive sonification (two times in 20 mM sodium phosphate buffer pH 7.4, 0.5% Tween 20 and three times in 20 mM sodium phosphate buffer pH 7.4). IL-15 protein was solubilised in 50 mM Tris pH 8.6, 8 M urea, 20 mM imidazole, 100 mM dithiothreitol, and stepwise dialyzed against 50 mM Tris pH 7.1 containing 2 M urea, 2 mM reduced glutathione and 0.2 mM oxidised glutathione and 50 mM Tris pH 7.0, respectively. After refolding, protein solution was centrifuged at 13,000 rpm for 30 minutes at 4°C to remove insoluble impurities. The soluble part was analyzed in the same buffer via size exclusion chromatography on a Superdex75 column (GE Healthcare) and by CD-spectroscopy to verify correct folding.

SUPPLEMENTAL RESULTS

Specific Jun/Fos chimeric gp130-type receptor heterodimerization was not achieved due to unexpected homodimerization of the Fos chimeric gp130 receptor chimeras

IL-6 binding to membrane-associated or soluble IL-6 receptor induces homodimerization of gp130 (Scheller *et al.*, 2006). We have substituted the entire extracellular portion of gp130 by the 40-amino acid Fos leucine zipper sequence of the transcription factor Fos and the entire extracellular portion of WSX-1 by the 39-amino acid Jun leucine zipper sequence of the transcription factor Jun (O'Shea *et al.*, 1989) as schematically illustrated in Supplemental Figure 1A. To ease immunochemical detection of the chimeric proteins, a FLAG epitope tag was placed immediately NH_2-terminal of the leucine zipper sequence. At the junction between the FLAG and leucine zipper, a short glycine linker was introduced to enhance flexibility of the protein which may facilitate subsequent heterodimer formation. The transmembrane and cytoplasmic domains of the gp130 receptor and the WSX-1 receptor were left intact. The resulting chimeric proteins were named Fos-gp130 and Jun-WSX-1 (Supplemental Figure 1A). Previously we have shown that a fusion protein without the leucine zipper, but containing the signal peptide, FLAG epitope tag, transmembrane and cytoplasmic domain of gp130 receptor (Δ-gp130) was not active on its own (Stuhlmann-Laeisz *et al.*, 2006).

To stabilize the leucine zipper mediated heterodimerization of gp130 and WSX-1, we engineered a cysteine residue between the leucine zipper and the FLAG epitope tag sequence in the Fos-gp130 and Jun-WSX-1 protein. Moreover, we constructed the chimeric protein Δcys-Fos-gp130 in which the cysteine residue was replaced by a glycine residue to prevent unwanted stabilization of a Fos-gp130 homodimer by a disulfide-bridge.

First, we asked whether the chimeric receptors Fos-gp130, Δcys-Fos-gp130 and Jun-WSX-1 were able to induce ligand independent cell growth in Ba/F3-gp130 cells. Ba/F3-gp130 cells grow in the presence of Hyper-IL-6, a fusion protein of IL-6 and the soluble IL-6R connected by a flexible linker (Fischer *et al.*, 1997). We selected Ba/F3-gp130 cells as a cellular system to assess the long-term activity of the chimeric heterodimeric receptor complexes, because these cells were successfully used to show ligand independent and constitutive activation of the homodimeric Jun-gp130 receptor fusion proteins (L-gp130) (Stuhlmann-Laeisz *et al.*, 2006). Ba/F3-gp130 cells were retrovirally transduced with the cDNA encoding Fos-gp130, Δcys-Fos-gp130 and Jun-WSX-1. In addition we used the previously described cell line Ba/F3-gp130-L-gp130 (Stuhlmann-Laeisz *et al.*, 2006). After selection, cells expressing Fos-

gp130, Δcys-Fos-gp130, L-gp130 or Jun-WSX-1 were grown in medium lacking Hyper-IL-6. Surprisingly, the Ba/F3-gp130-Fos-gp130 cells and Ba/F3-gp130-Δcys-Fos-gp130 cells proliferated in the absence of Hyper-IL-6, indicating that the Fos peptide alone was able to mediate homodimerization of gp130. On the other hand, Ba/F3-gp130-Jun-WSX-1 cells were not able to grow cytokine independently (Supplemental Figure 1B). We would like to note, that the cell lines expressing L-gp130, Fos-gp130 and Δcys-Fos-gp130 were generated independently, therefore it is very difficult to compare the proliferation rates on a quantitative rather than a qualitative level.

Since Fos-gp130 contained a single cystein residue in its extra-cellular portion, we were interested if the cysteins of two Fos-gp130 molecules were forming a disulphide bond. Therefore, we performed an anti-Flag-tag Western blot analysis of cell lysates containing Fos-gp130 or Δcys-Fos-gp130 cells under reducing and non-reducing conditions. As depicted in Supplemental Figure 1C, monomeric Fos-gp130 could be detected under reducing conditions and a dimeric form of Fos-gp130 under non-reducing conditions. Importantly dimeric Δcys-Fos-gp130 was not detected under non-reducing conditions. We conclude that Fos-gp130 homodimers are stablized by intermolecular disulphide bridges.

To demonstrate the physical interaction of two Δcys-Fos-gp130 proteins, we performed co-immunoprecipitation experiments using lysates containing Δcys-Fos-gp130 and Δcys-Fos-gp130-EYFP. We used either single or double transfected cells (Δcys-Fos-gp130 or Δcys-Fos-gp130+Δcys-Fos-gp130-EYFP) and used anti-GFP antibodies for the co-immunoprecipitation of Δcys-Fos-gp130. As indicated in the left panel of Supplemental Figure 1D, Δcys-Fos-gp130 was not detected after anti-GFP-immunoprecipitation in single transfected cells, indicating that Δcys-Fos-gp130 does not unspecifically interact with anti-GFP-antibodies and protein G agarose. However, Δcys-Fos-gp130 could be specifically co-immunoprecipitated with Δcys-Fos-gp130-GFP as indicated on the right panel of Supplemental Figure 1D. We conclude that Fos-gp130 homodimers can form even in the absence of stabilizing intermolecular disulphide bridges. As a control we used cell lysates from single and double transfected cells.

These results were complemented by analysis of the Δcys-Fos-gp130 receptor phosphorylation. To demonstrate that Δcys-Fos-gp130 is phosphorylated at tyrosine residues after forced dimerization via the Fos peptide, a immunoprecipitation assay with COS-7 lysate containing Δcys-Fos-gp130 was performed. The lysate was incubated with anti-gp130 specific antibodies and Δcys-Fos-gp130 was precipitated. Western blot analysis with an anti-

phospho-tyrosine specific antibody revealed that precipitated Δcys-Fos-gp130 was phosphorylated at tyrosine residues (Supplemental Figure 1E). Furthermore the phosphorylation of STAT3 in the retrovirally transduced Ba/F3-gp130 cells was analyzed by Western blot.

Ba/F3-gp130-Fos-gp130 cells and Ba/F3-gp130-Δcys-Fos-gp130 as well as Ba/F3-gp130-L-gp130 cells but not Ba/F3-gp130-Jun-WSX-1 cells showed cytokine independent phosphorylation of STAT3 (Supplemental Figure 1F). Our results indicated that the Jun/Fos heterodimerization strategy will not exclusively lead to gp130 heterodimerization with other members of this receptor family. Even though it was reported that Fos leucine zippers form very unstable homodimers (O'Shea *et al.*, 1989), the Fos mediated homodimerization of Fos-gp130 receptor chimeras unexpectedly was strong enough to induce ligand independent growth in Ba/F3-gp130-Fos-gp130 cells.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Attempt to establish Jun-Fos heterodimeric chimeric receptors failed upon Fos homodimerization of Fos-gp130 and Δcys-Fos-gp130. (A) Scheme of the gp130 and WSX-1 receptors with the extracellular domains comprising immunglobulin-like domain (Ig, light green), cytokine binding domain (CBD, dark green), three fibronectin-like domains (FNIII, green), transmembrane domain (TM) and cytoplasmic domain (CD, black line). Dimerization is induced after binding of IL-27. Gp130 and WSX-1 were truncated 15 amino acids above the transmembrane domain and replaced by the leucine zipper region of the human Jun or Fos gene and optional stabilized with an additional cysteine. (B) Functionality of gp130 chimeric receptor proteins in stable transduced Ba/F3-gp130 cell lines. Equal numbers of Ba/F3-gp130 cells stably transduced with Jun-WSX-1, L-gp130, Fos-gp130 or Δcys-Fos-gp130 were cultured for 3 days in the absence of Hyper-IL-6. As a control Ba/F3-gp130 were cultured in the presence or absence of 10 ng/ml Hyper-IL-6. Proliferation was measured as indicated in material and methods. (C) Detection of Fos-gp130 homodimers under non-reducing conditions. COS-7 cells were transiently transfected with p409-YFP, p409-Fos-gp130 or p409-Δcys-Fos-gp130. 48 h after transfection, cells were lyzed and 50 µg of protein lysates were separated by SDS-PAGE under reducing or non-reducing conditions. Proteins were transferred onto PVDF membrane and were detected with anti-FLAG-tag mAbs and visualized by ECL detection. (D) Detection of Δcys-Fos-gp130 homodimers by co-immunoprecipitation. COS-7 cells were transiently transfected with p409-Δcys-Fos-gp130 or p409-Δcys-Fos-gp130 and pEYFP-Δcys-Fos-gp130. 48 h after transfection cells were lyzed and Δcys-Fos-gp130-EYFP was immunoprecipitated with anti-GFP mAbs. As a control lysates were incubated only with protein G agarose. Input, immunoprecipitation supernatant and protein G agarose control supernatant were separated by SDS-PAGE. Proteins were transferred onto PVDF membrane and were detected with anti-GFP Abs and visualized by ECL detection. The membrane was stripped, probed with anti-gp130 Abs and proteins were visualized by ECL detection. (E) Tyrosine-phosphorylation of Δcys-Fos-gp130. COS-7 cells were transiently transfected with p409-Δcys-Fos-gp130 and starved for the last 20 h before lysis. 48 h after transfection, COS-7 cells were lyzed and Δcys-Fos-gp130 was immunoprecipitated with anti-gp130 Abs. Immunoprecipitation supernatant was separated by SDS-PAGE. Proteins were transferred onto PVDF membrane and were detected with an anti-phospho-tyrosine (PY) mAbs and visualized by ECL detection. To confirm immunoprecipitation of Δcys-Fos-gp130 the membrane was stripped, probed with anti-FLAG-tag mAbs and Δcys-Fos-gp130 was visualized by ECL detection.

(F) Activation of STAT3 proteins in Ba/F3-gp130 cells. After 6 h serum starvation, Ba/F3-gp130 cells stably transduced with Jun-WSX-1, L-gp130, Fos-gp130 or Δcys-Fos-gp130 were left untreated, whereas untransduced Ba/F3-gp130 were stimulated for 10 min with 10 ng/ml Hyper-IL-6 or were left untreated. Subsequently, cells were lyzed and 50 µg were separated by SDS/PAGE. Proteins were transferred onto PVDF membrane and were detected with anti-phospho-STAT3 mAbs and visualized by ECL detection. The membrane was stripped and probed with anti-STAT3 mAbs and proteins were visualized by ECL detection.

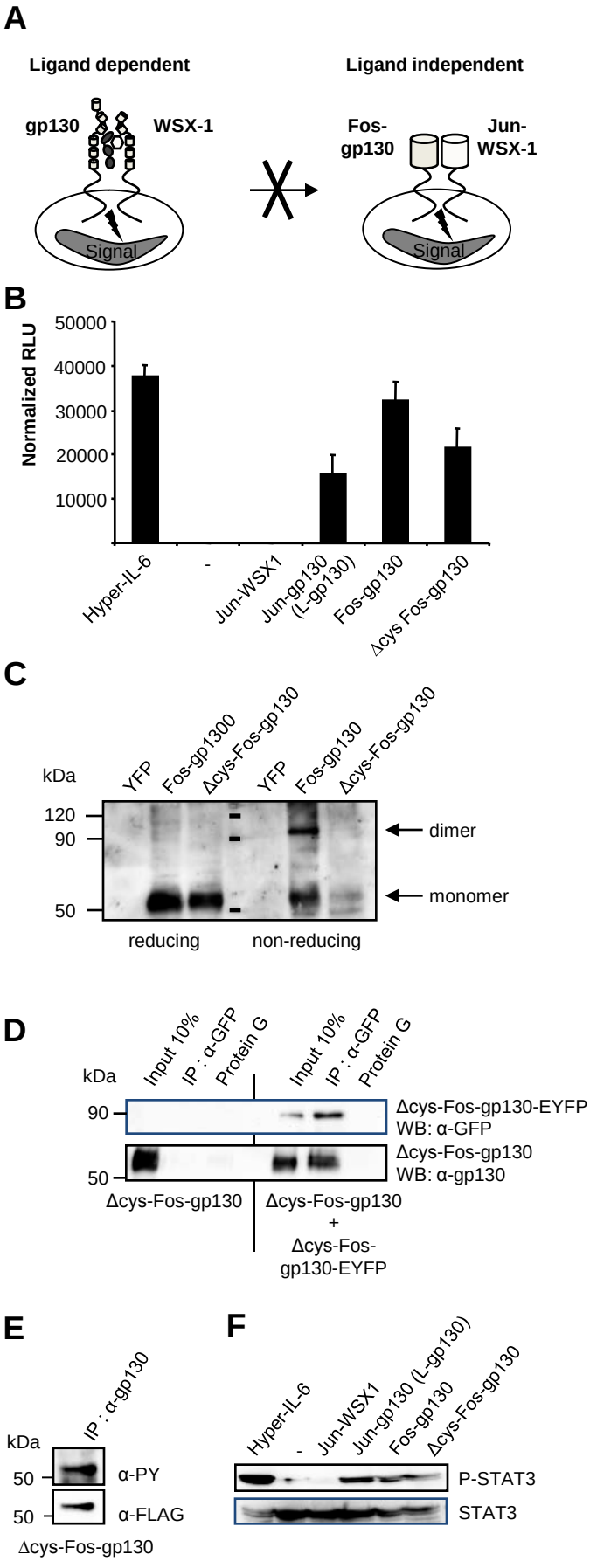
Supplemental Figure 2: Analysis of gp130, WSX-1, LIFR, OSMR and GPL chimeric receptor expression in various stably transfected Ba/F3-gp130 cell lines by RT-PCR.

Supplemental Figure 3: Unresponsiveness of Ba/F3-gp130 cells to IL-15. (A) Equal numbers of Ba/F3-gp130 cells were cultured for 3 days in the presence of increasing amounts of recombinant IL-15 or recombinant IL-15 and soluble IL-15Rα sushi domain. As a control cells were left untreated or cultured in the presence of Hyper-IL-6 (10 ng/ml). Proliferation was measured as indicated in Material and Methods. (B) Co-culture experiment of Ba/F3-gp130 expressing GFP with Ba/F3-gp130 cells expressing IL-15-gp130, IL-15-WSX-1+sushi-gp130 or Fos-gp130 at a ratio of 20:1. Decrease/death of GFP expressing cells was measured by flow cytometry during a time period of 96 h.

Supplemental Figure 4: Aggregation of recombinant IL-15 and interaction of gp130 chimeric receptors independently of extracellular fused proteins. (A) Size exclusion chromatography on a Superdex75 column of recombinant IL-15 protein (B) Co-immunoprecipitation of Δcys -Fos-gp130-EYFP and sushi-gp130. COS-7 cells were transiently transfected with p409-sushi-gp130 and pEYFP-Δcys-Fos-gp130. 48 h after transfection cells were lyzed and Δcys -Fos-gp130-EYFP was immunoprecipitated with anti-GFP mAbs. As a control lysates were incubated only with protein G agarose. Input, immunoprecipitation supernatant and protein G agarose control supernatant were separated by SDS-PAGE. Proteins were detected with anti-gp130 Abs and visualized by ECL detection. (C) Co-immunoprecipitation of Δcys -Fos-gp130-EYFP and IL-15-gp130. COS-7 cells were transiently transfected with p409-IL-15-gp130 and pEYFP-Δcys-Fos-gp130. 48 h after transfection cells were lyzed and Δcys -Fos-gp130-EYFP was immunoprecipitated with anti-GFP mAbs. As a control lysates were incubated only with protein G agarose. Input, immunoprecipitation supernatant and protein G agarose control supernatant were separated by SDS-PAGE. Proteins were transferred onto PVDF membrane and were detected with anti-gp130 Abs and visualized by ECL detection. (D) Co-immunoprecipitation of sushi-gp130-

EYFP and Δcys -Fos-gp130. The experiment was performed as described in (B). (E) Co-immunoprecipitation of IL-15-gp130-EYFP and Δcys -Fos-gp130. The experiment was performed as described in (C).

Supplemental Figure 1



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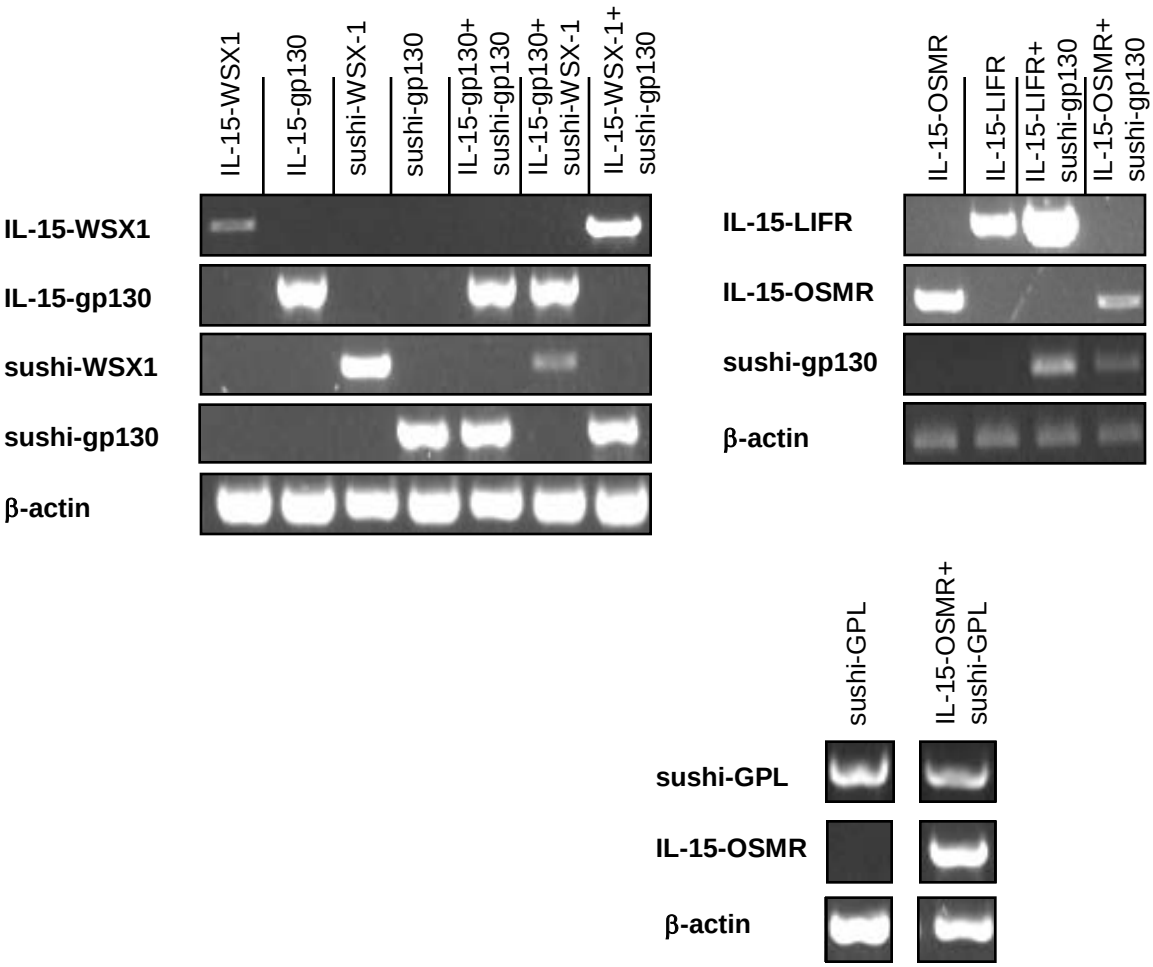

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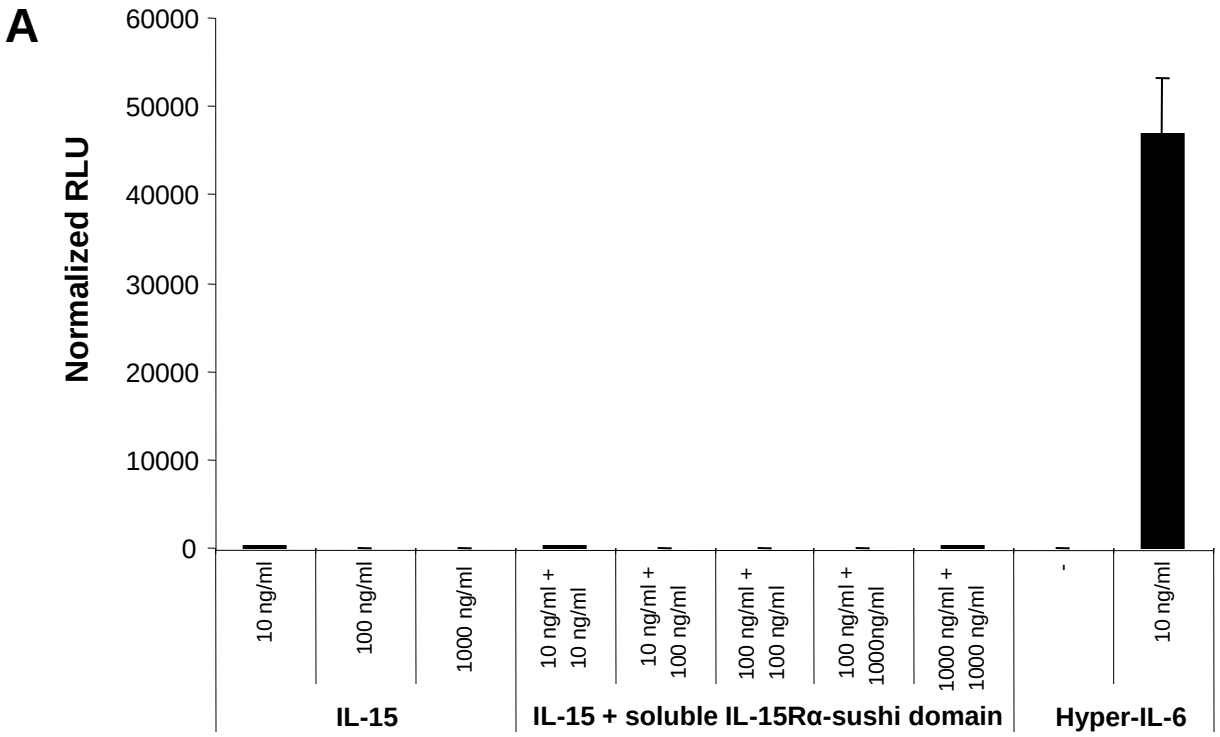

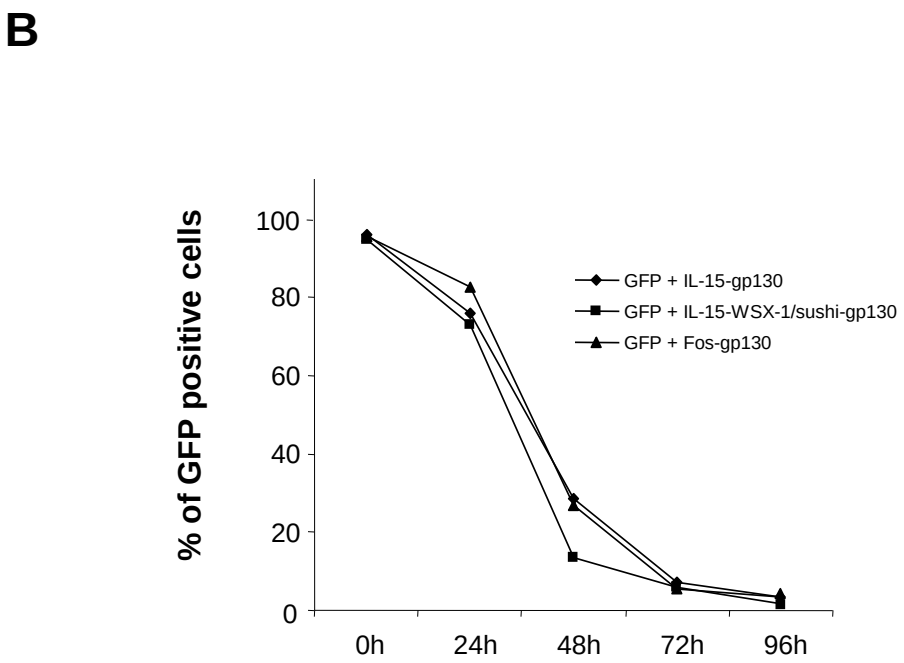

Supplemental Figure 4

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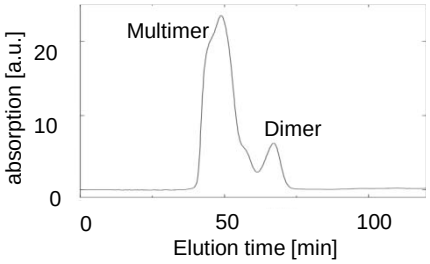

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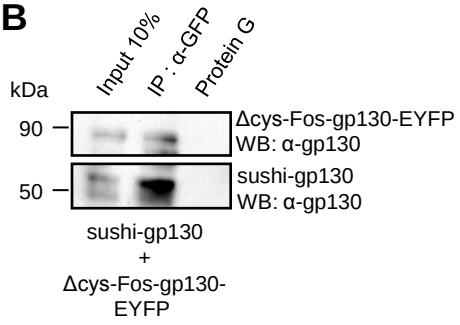

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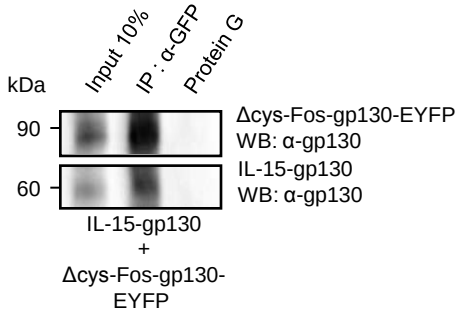

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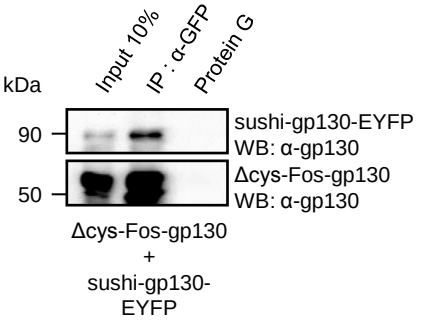

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