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## **THE C-TERMINUS OF CIS DEFINES ITS INTERACTION PATTERN**

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Running Title: SOCS-box function

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### **Summary**

**Proteins of the Suppressors of Cytokine Signaling (SOCS) family are characterised by a conserved modular structure with pre-SH2, SH2 and SOCS-box domains. Several members including CIS, SOCS1 and SOCS3 are rapidly induced upon cytokine receptor activation and function in a negative feedback loop, attenuating signalling at the receptor level. We used a recently developed mammalian two-hybrid system (MAPPIT) to analyse SOCS protein interaction patterns in intact cells, allowing direct comparison with biological function. We find that, besides the SH2 domain, the C-terminal part of the CIS SOCS-box is required for functional interaction with the cytokine receptor motifs examined, but not with the N-terminal Death Domain of the TLR adaptor MyD88. Mutagenesis revealed that one single tyrosine at position 253 is a critical binding determinant. Much in contrast, substrate binding by the highly related SOCS2 protein, and also by SOCS1 and SOCS3, does not require their SOCS-box.**

**KEY WORDS: MAPPIT, Signal transduction, SOCS-box, MyD88, Cytokine receptor**

## Introduction

A wide spectrum of  $\alpha$ -helical bundle cytokines contributes to growth, differentiation and survival of hematopoietic cells. Examples include the colony-stimulating factors (CSFs), erythropoietin (Epo) and several interleukins (ILs) such as IL-5. More recently, also leptin, a hormone-like member of this family, was shown to promote proliferation of hematopoietic progenitors [1-3]. All these cytokines activate the highly conserved JAK/STAT signalling pathway upon receptor binding. Signalling via these receptors is under tight control including negative feedback by rapidly induced SOCS proteins. CIS was the founding member of the SOCS protein family that consists of 8 members: SOCS1-7 and CIS [4,5]. All SOCS proteins comprise an SH2-domain responsible for association with phosphotyrosine motifs, an N-terminal preSH2-domain and a C-terminal SOCS-box [5]. They can modulate receptor activation and signalling via at least three distinct mechanisms.

CIS can inhibit Epo and growth hormone (GH) signalling by competition for STAT5 docking sites at the receptor level [6-8]. Consistent with this, CIS suppresses Epo-induced cell proliferation and promotes apoptosis of erythroid progenitor cells [9,10]. Phenotypes of CIS transgenic mice and of STAT5a and/or b knockout mice show clear similarities, lending further support for CIS as a specific negative feedback regulator of STAT5-mediated cytokine signalling [4,6,7,11]. Direct interference with STAT5 recruitment is also suggested for SOCS2-mediated inhibition of GH action [12,13]. Interestingly, SOCS2 shows a dual effect on GH signalling. Mice lacking SOCS2 and SOCS2 transgenic mice both exhibit increased growth [12,14,15]. This may be explained through direct binding and functional interference between SOCS proteins [16,17].

CIS-dependent receptor degradation was reported for the EpoR and GHR [6,18]. The SOCS-box of SOCS proteins can interact with the Elongins B and C [19], which form a complex with proteins of the Cullin and Rbx families. This assembly is a E3 ubiquitin ligase complex that is responsible for specific targeting of associated proteins for poly-ubiquitination [20]. This way, several SOCS proteins can inhibit signalling by marking associated signalling components for proteasomal degradation.

SOCS1 and -3 carry a KIR (kinase inhibitory region) domain in their N-terminal region that acts as a pseudo-substrate for direct inhibition of JAK kinase activity. SOCS1 interacts

directly with the phosphorylated activation loop of JAK2 via its SH2 domain [21] while SOCS3 shows only weak affinity for JAK2 and is thought to bind to the receptor in close proximity of the kinase [22]. This is exemplified for SOCS3 that was recently identified as a potent inhibitor of LR signalling involved in regulation of energy balance. SOCS3 haplo-insufficient mice or neural cell-specific SOCS3 knockout mice show augmented leptin sensitivity in the hypothalamus associated with a remarkable attenuation of diet-induced obesity, suggesting a key role for SOCS3 in leptin resistance [23,24].

SOCS proteins are also involved in regulating JAK-STAT independent pathways such as insulin and TNF- $\alpha$  signalling [25-27]. Also, triggering of Toll-like receptors (TLRs), key players in innate immunity, leads to the induction of CIS, SOCS1 and SOCS3 [28-30]. Evidence linking SOCS proteins to TLR signalling arose from the analysis of SOCS1-deficient mice that show enhanced sensitivity to LPS-induced sepsis [31,32] and from SOCS1-deficient mice that lack endotoxin tolerance. Moreover, macrophages lacking SOCS1 produce increased levels of nitric oxide and pro-inflammatory cytokines in response to LPS. Conversely, SOCS1 over-expression in macrophages suppresses LPS-induced NF- $\kappa$ B activation. Based on these data, SOCS1 was prompted as a negative regulator of TLR signalling, although no direct target of SOCS1 could be identified. Recently, two groups reported that SOCS1 has an indirect inhibitory effect on TLR signalling, targeting the secondary type I IFN signalling pathway and not the main NF- $\kappa$ B pathway [30,33].

In this paper we examine the binding modalities of SOCS proteins in more detail. We demonstrate that the SOCS-box of CIS, and more particularly its C-terminal tyrosine, is essential for interaction with recruitment motifs in the EpoR and LR and for its inhibitory role on STAT5 activation. In contrast, the SOCS-box is not required for SOCS2 receptor interaction or for signalling inhibition by SOCS1 and SOCS3. Furthermore, we identified the universal TLR adaptor MyD88 as a target for CIS. This interaction is SOCS-box-independent, indicating a different binding modus compared to the cytokine receptors.

## Experimental Procedures

### Constructs

Generation of the mutant murine LRs by mutagenesis and cloning in the pMET7 expression vector was published elsewhere [34]. EpoR-bait constructs containing 2 extra leucines in the transmembrane were described before [35]. The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter was used as previously described [36]. The pGL3-beta-casein-luciferase reporter consisting of 5 repeats of the STAT5-responsive motif of the  $\beta$ -casein promoter was a gift from Dr. Ivo Touw. Generation of the prey constructs pMG2-CIS and pMG2-SOCS2 was described before [35].

CISd5, CISdbox (aa 1-221) , CISOY249F and CISOY253F prey constructs were generated by site directed mutagenesis on pMG1-CIS using following primer pairs:

CISd5:

5'-GACTACCTCCGACAGTGATATCTCCAACCTCTGATCTAG-3' and

5' CTAGATCAGAGTTGGAGATATCACTGTCTGGAGGTAGTC-3',

CISdbox:

5'-GTGCGCAGGAGCAGTTGATATCGCTTACAACATCTGTG-3' and

5'-CACAGATGTTGTAAGCGATATCAACTGCTCCTGCGCAC-3',

CISOY249F:

5'-GGCGTATGGCCGACTTCTTAAGACAGTACCCCTTCC-3' and

5'-GGAAGGGGTACTGTCTTAAGAAGTCGGCCATACGCC-3',

CISOY253F:

5'-GACTACCTCCGACAGTTCCCCTTCCAGCTGTGATCTAGAGAAAAACCTCC-3' and

5'-GGAGGTTTTTCTCTAGATCACAGCTGGAAGGGGAAGTGTCTGGAGGTAGTC-3',

The CIS mutants were then transferred to the pMG2 vector and the pMET7-FLAG expression vector by EcoRI-XbaI cloning.

SOCS2Y149F was generated using the previously described pMG1-SOCS2 as template [36] and the primer set

5'-GCAGAATTCACCCTGCGGTGCCTGGAGCC-3' and

5'-CGCTGCGGCCGCTTATACCTGGAATTTGAATTCTTCCAAGTAATC-3'

and was first cloned in the pMG1 vector as an EcoRI-NotI fragment, and then transferred to the pMG2 vector using EcoRI-XbaI. SOCS2Y190F and SOCS2dbbox (aa 1-159) were amplified from the pEF-FLAG-I/mSOCS2 construct (gift from Dr. Starr) using the

5'-GCAGAATTCACCCTGCGGTGCCTGGAGCC-3' and the

5'-

GGTCGTCTAGAGCGGCCGCTTATACCTGGAATTTATATTCTTCCAAGAAATCTTTTAG  
TCTTGTTG-3' and

5'-GCTGGGCGGCCGCTTATGATGTATACAGAGGTTTGG-3' primers respectively, and were also cloned in the pMG2 vector. SOCS2 was transferred from the pMG2 vector to the pMET7-FLAG expression vector as an EcoRI-XbaI or EcoRI-NotI fragment.

The pMET7-FLAG-SOCS3 expression vector was described elsewhere [37]. SOCS3dbbox was amplified from the pMET7-FLAG-SOCS3 template using primers

5'-GCGAGATCTCAGAATTCGTCACCCACAGCAAGTTTCC-3' and

5'-CGCTTCTAGATTAGTTGGAGGAGAGAGGTCGG-3' allowing EcoRI-XbaI based cloning in the pMet7-FLAG vector.

The pMet7-FLAGmSOCS1 and pMet7-FLAGmSOCS1dbbox constructs were generated by amplifying SOCS1 and SOCS1dbbox from the pEF-FLAG-I/mSOCS1 construct (gift from Dr. Starr) with the

5'-CCAGCGAATTCATGGCGCGCCAGGACTACAAGGAC-3' and

5'-GGTCGTCTAGATCAGATCTGGAAGGGGAAGGAAC-3' or

5'-GGTCGTCTAGATCAGCGGCGCTGGCGCAGCGGGGCCCCCAAC-3' primer sets, respectively and EcoRI-XbaI cloning in the pMet7-FLAG vector.

EpoR cDNA was amplified from TF1-derived using primers

5'-CGGGGTACCATGGACCACCTCGGGGCGTCC-3' and

5'-CGCTCTAGACTAAGAGCAAGCCACATAGC-3' and was cloned in the pSVsport vector by KpnI-XbaI cloning. The pECE-STAT5B expression vector was a gift from Dr. Becker.

Mouse full-length MyD88 was amplified using the

5'-GCGCGAGCTCAATGTCTGCGGGAGACCCCCGCG-3' and

5'-GCGTGCGGCCGCTCAGGGCAGGGACAAAGCC-3' primer pair on a pCAGGSE-mMyD88 expression vector (gift from Dr. Beyaert). After SacI/NotI digestion, the fragment was cloned in the pCEL(2L) vector, which was described earlier [35]. This resulted in the mMyD88

bait construct. The mMyD88(N) and the mMyD88TIR bait vectors were made in an analogous manner using primer pairs

5'-GCGCGAGCTCAATGTCTGCGGGAGACCCCG-3' /

5'-GCTCGCGGCCGCTTACGTTTGTCTAGGGGGTC-3'

and

5'-GCGCGAGCTCAATGCCGGAACCTTTTCGATGCC-3' /

5'-GCTCGCGGCCGCTCAGGGCAGGGACAAAGCC-3' respectively.

The pCAGGSE-mMyD88(N) and pCAGGSEmMyD88 DD expression vectors were generated by amplification using oligo pairs

5'-GGCAAAGAATTGAATTCCACCATGGGTGCGC-3' /

5'-GCGCCTCGAGTCAAAGTTCCGGCGTTTGTCTAGGGGGTC-3' and

5'-GGCAAAGAATTGAATTCCACCATGGGTGCGC-3' /

5'-GCGCCTCGAGTCAGACAGACGCGCCAGAGCGCCCCTGCC-3' respectively on pCAGGSE-mMyD88, followed by a EcoRI/XhoI digestion and ligation in the pCAGGSE vector.

Generation of the pMET7TAP2 construct was described elsewhere [17]. CIS, CISdbox, CISY249F and CISY253F were introduced in a TAP2 construct via EcoRI-KpnI cloning from the respective pMG2 constructs.

An overview of the bait constructs used in this study is given in Table 1.

### **Cell culture, transfection and reporter assays**

Cell culture conditions, transfection procedures and luciferase assays for Hek293T cells were previously described [37]. For a typical luciferase experiment,  $4 \times 10^5$  cells were seeded in 6-well plates 24 hours before transfecting them overnight with the desired constructs together with the luciferase reporter gene. Cells were left untreated (negative control NC) or were stimulated overnight with 100 ng/ml leptin or 3.3 ng/ml Epo and luciferase activity of the transfected cells was measured by chemiluminescence.

### **Co-immunoprecipitation**

Approximately  $2 \times 10^6$  Hek293T cells were transfected with different combinations of mMyD88-E, mMyD88(N), mMyD88 DD, mCIS-FLAG, mCISY253F and mCISdBox. Cleared lysates (modified RIPA lysis buffer: 200mM NaCl, 50mM Tris-HCl pH8, 0,05% SDS, 2mM EDTA, 1% NP40, 0,5% DOC, Complete™ Protease Inhibitor Cocktail (Roche)) were incubated

with 4 µg/ml anti-FLAG mouse monoclonal antibody (Sigma) and protein G-sepharose (Amersham Biosciences). After immunoprecipitation, SDS-PAGE and Western Blotting, interactions were detected using anti-E-Tag antibody (Amersham Biosciences) and anti-mouse-HRP (horseradish peroxidase) (Amersham Biosciences).

### **Phosphopeptide affinity chromatography**

The phosphopeptide affinity chromatography procedure was as previously described [37].

### **Electrophoretic mobility shift assay (EMSA)**

Hek293T cells transiently transfected with the desired constructs were starved for 4 hours in serum-free medium and subsequently stimulated with 5 ng/ml Epo for 15 min or were left untreated. Protein concentrations of the nuclear extracts were measured with the BioRad protein assay. Double-stranded oligonucleotides based on the  $\beta$ -casein promoter (sense: CAGATTTCTAGGAATTC; antisense: GGATTTGAATTCC TAGAAATC) were labelled by filling in 5' protruding ends with Klenow enzyme, using [ $\alpha$ -32P]dATP (3,000 Ci/mmol; 10 mCi/ml). This probe binds STAT5 homodimers. Nuclear extracts (5 µg of protein) were incubated with about 10 fmol (20,000 cpm) of probe in gel-shift incubation buffer (10 mM HEPES pH 7.8, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 5% glycerol, 5mM DTT, 2 mM pefablock SC, 1 mg/ml BSA, 0.1 mg/ml poly (dI-dC)) for 10 min at room temperature (RT). The supershifting anti-STAT5 antibodies were incubated with the nuclear extracts for 10 min at RT before addition of the radio-labelled  $\beta$ -casein probe. The protein-DNA complexes were separated on a 4.5%-(w/v)-polyacrylamide gel containing 7.5 % glycerol in 0.5-fold TBE at 20V/cm for 90 min. Gels were fixed in water/methanol/acetic acid (80:10:10, by vol.) for 30 min, dried and autoradiographed.

### **TAP2 purification and mass spectrometry**

Hek293T cells were transfected with the appropriate TAP2 constructs. The TAP2 purification procedure was followed as described previously [17]. Proteins were visualised on a polyacrylamide gel by silver staining, or for mass-spectrometry analysis with Sypro Ruby protein gel stain according to the manufacturers' instructions (Molecular probes). Proteins of interest were excised, prepared for mass spectrometry and applied for nano-LC-MS/MS analysis on an Ultimate (Dionex, Amsterdam, The Netherlands) in-line connected to an Esquire HCT ion trap (Bruker Daltonics, Bremen, Germany).

### **Modeling method**



Molecular models were built for the CIS-elonginB-elonginC complex, using the crystal structure of the SOCS2-elonginB-elonginC complex as a template [38]. The sequences of CIS and SOCS2 were automatically aligned using the sequence alignment editor of moe (chemical computing group). Using this alignment, 150 models were built for the CIS-elonginB-elonginC complex in MODELLER version 8.1 [39], 10 models with the best DOPE and molpdf scores were selected, and evaluated.

## Results

### Design of MAPPIT experiments

We previously reported a new two-hybrid method to study protein-protein interactions in intact mammalian cells, termed MAPPIT (MAMmalian Protein-Protein Interaction Trap) [36]. Briefly, a bait protein is C-terminally linked to a chimeric EpoR/LR that is deficient in STAT3 recruitment, whilst a prey protein is attached to the string of 4 functional STAT3 recruitment sites of gp130. Association of bait and prey leads to STAT3 activation and subsequent activation of the STAT3-responsive rat Pancreatitis Associated Protein I (rPAPI) promoter-luciferase reporter. To examine interactions with the EpoR, we used its intracellular receptor tyrosine motifs as baits. We also analysed interactions with the LR itself by mutating the STAT3 recruiting Y1138 to phenylalanine. One or both of the two membrane-proximal tyrosines at positions Y985 and Y1077 were mutated to phenylalanine to examine tyrosine-specific interactions. These LR mutants were termed LR(YFF) and LR(FYF). MAPPIT configurations used in this manuscript are shown in Figure 1A.

### The C-terminus of CIS but not of SOCS2 is required for receptor binding

We recently showed interaction of CIS and SOCS2 with the EpoR and the LR. CIS and SOCS2 both interact with Y402 and to a lesser extent also with Y344 and the double Y430/Y432 motifs of the EpoR, and with the Y1077 position in the LR. Although both are highly related, only CIS associated with Y985 of the LR and only SOCS2 with the pY480 motif of the EpoR [17,35]. Here we examine the binding modus of CIS and SOCS2 in more detail. It is well established that interaction of SOCS proteins with their receptor targets depends on their SH2 domains [40,41]. Figure 1B shows a diagrammatic presentation of the structure of SOCS proteins. For MAPPIT use, CIS and SOCS2 preys were generated by N-terminal fusion with part of gp130. Figure 2A shows the critical requirement of the CIS SOCSbox for CISprey binding to the EpoRY402 motif in a MAPPIT experiment. C-terminal deletion of the entire SOCS-box resulted in complete loss of MAPPIT signalling. Detailed mapping showed that deletion of the 5 C-terminal aa and even a single Y253F mutation leads to impaired MAPPIT signalling (Figure 2A). In contrast, no effect was observed upon replacing the second conserved tyrosine at position 294 with phenylalanine. We next performed a similar analysis for SOCS2. Here, deletion of the

entire SOCS-box, or Y to F mutation of both conserved tyrosines did not significantly affect signalling (Figure 2B). Very similar observations were obtained in MAPPIT experiments for the EpoR Y430/Y432 motif (Figure 2C). In figure 2D we further expand this dataset to the LR Y985 and Y1077 positions, demonstrating that these findings are not limited to the EpoR system.

CIS binding to the EpoR pY402 motif was also evaluated by phosphopeptide affinity chromatography. Figure 2E clearly shows loss of CIS binding by deletion of the SOCSbox or by introduction of the single Y253F mutation. In contrast to the MAPPIT dataset, complete loss of binding is also observed with the Y249F mutant. This may be explained by a lowered binding affinity so that the interaction with the EpoR pY402 motif is still detected with MAPPIT but not with peptide affinity chromatography. In line with such assumption, MAPPIT detects interactions without the need for any purification step.

### **No role for the SOCS1 and SOCS3 SOCS-box for receptor binding**

We also examined the role of the SOCS-box for the inhibitory function of SOCS1 and SOCS3. In this assay system, with clear inhibition of EpoR and LR signal transduction by co-expression of SOCS1 and SOCS3, deletion of the complete SOCS-box of SOCS1 or SOCS3 did not significantly alter the inhibitory effect (Figure 3A-B). We conclude that the SOCS-box of SOCS1 and 3, in analogy with SOCS2, does not contain critical determinants involved in substrate binding. This highlights the unique new property of the CIS SOCS-box, which we next evaluated in more detail.

### **Critical role of Y253 in CIS function**

We first investigated the functional implications of the CIS SOCS-box mutations. CIS operates in a classical negative feedback loop on EpoR signalling: it is rapidly and strongly induced by activated STAT5 upon EpoR activation, subsequently binds to the Y402 site in the EpoR, and there inhibits STAT5 activation. We first looked at the effect of CIS mutations on STAT5-dependent  $\beta$ -casein reporter activity using the EpoR Y402 bait. Wild-type CIS clearly abrogated reporter induction as expected. In contrast, co-expression of mutant proteins with C-terminal deletion of the SOCS-box, or with the Y253F mutation was unable to impair reporter induction. The CIS Y249F mutant inhibited signalling to a similar extent as wild type CIS protein (Figure 4A). Expression of all CIS variants was verified via Western blot (not shown).

Confirmation was obtained using electrophoretic mobility shift assays. A  $^{32}\text{P}$  labelled probe corresponding to a  $\beta$ -casein STAT5 binding site was used to visualise bound STAT5 complexes. Whilst wild-type CIS clearly suppressed the formation of nuclear STAT5-DNA complexes, deletion of the complete SOCS-box, as well as the Y253F mutant, resulted in loss of inhibition (figure 4B). Again, the Y249F mutant behaved as wild-type CIS. Supershift with anti-FLAG antibody confirmed the presence of STAT5B in the complexes (not shown).

Reporter assays were also performed on the wild-type EpoR. Although the effects were less pronounced, the tendencies clearly corresponded to what we observed for the EpoR bait construct (figure 4C). This weaker effect is most likely explained by the incomplete overlap of STAT5 and CIS binding sites [6,35].

### **CIS interaction with MyD88 does not depend on its SOCS-box**

SOCS proteins are also rapidly induced after Toll-like receptor stimulation. However, no interaction partner of the SOCS proteins in TLR signalling has been described so far. A possible target of SOCS proteins in TLR signalling is the adaptor protein MyD88, which is used by most TLRs. To investigate this possibility, we performed MAPPIT analysis using MyD88 as bait and the CISprey. As shown in Figure 5A, we observed clear induction of luciferase activity implying interaction between CIS and MyD88. To investigate the role of the SOCS-box of CIS in this interaction, we next analysed the effect of the abovementioned CIS mutants. Much in contrast to association of CIS with the EpoR and LR, interaction between MyD88 and CIS was not affected by any CIS mutation, including deletion of its entire SOCS-box. These data were confirmed by co-immunoprecipitation (figure 5B). Here, we transiently co-expressed E-tagged MyD88 (MyD88-E) and FLAG-tagged CIS (CIS-FLAG), CIS Y253F (CISY253F-FLAG) or CIS lacking its SOCS-box (CISdBox-FLAG). In every case, MyD88-E was co-immunoprecipitated using an anti-FLAG antibody.

MyD88 consists of two interaction domains: a C-terminal 'TIR' domain and a N-terminal 'Death Domain', linked together by a short intermediate domain. To examine the role of either domain in CIS binding, we created MAPPIT baits containing the N-terminal part of MyD88 encompassing the Death Domain and the intermediate domain, or the C-terminal TIR domain. MAPPIT analysis clearly shows that only the N-terminal part of MyD88 interacts with CIS (figure 5C). Again, co-immunoprecipitation studies confirmed these findings (figure 5D).

Together, we here clearly document a role for the MyD88 Death Domain in CIS recruitment, and show that this interaction solely depends on the CIS SH2 domain.

### **C-terminal mutations in CIS do not affect Elongin B/C and cullin5 recruitment**

We used a variant of the Tandem Affinity Purification (TAP) method developed by Puig and co-workers [42] (S.E., unpublished results) to analyse protein complexes of CIS and its mutants. Cullin5 and Elongins B and C were identified as interacting partners of CIS. While deletion of the complete SOCS-box of CIS abrogates their association, mutation of the tyrosines at position 249 or 253 to phenylalanine did not significantly influence Cullin5 or Elongin B and C binding (Figure 6A).

### **Modeling of the SOCS-box of CIS**

We used the crystal structure of the SOCS2-elongin C-elongin B complex [38] to build a molecular model for CIS, bound to Elongin B and C (Figure 6C). This model, together with sequence alignment with other SOCS proteins (Figure 6B) showed that residues involved in Elongin C binding are very conserved. As for SOCS2, the C-terminal residues of CIS, mutated in this work are not part of its actual Elongin C-binding site and mutation of the C-terminus of CIS is thus not predicted to directly affect Elongin C binding. As in SOCS2, the C-terminus of CIS is buried in the interface between the SH2 domain and the SOCS-Box domain. The hydroxyl group of the completely buried Y253 hydrogen bonds to the buried C-terminal carboxyl group. The C-terminus of CIS or SOCS2 is not able to make direct contact with a phosphopeptide substrate bound to the SH2 domain (Figure 6C).

## Discussion

SOCS proteins typically consist of a phosphotyrosine binding SH2 domain, a C-terminal SOCS-box involved in proteasome recruitment and a pre-SH2 domain that only in the case of SOCS1 and SOCS3 contains a JAK kinase blocking KIR domain. Association of SOCS proteins with their target substrates is believed to occur solely via their SH2 domain. We here took a closer look at the binding modus of SOCS proteins using the MAPPIT approach, combined with biochemical and functional analyses.

A key finding is that the SOCS-box of CIS is essential for association with recruitment motifs in cytokine receptors including the EpoR and LR. Deletion of the entire SOCS-box completely abrogated binding, and more detailed mutagenesis analysis revealed the critical role of the single C-terminal Y253 residue. These findings were confirmed by peptide affinity chromatography using the phosphorylated or non-phosphorylated EpoRY402 motif. Furthermore, reporter assays and EMSA experiments extended these findings to functional activation of STAT5. Indirect effects of the mutations on the structural integrity of CIS could be ruled out since clear SOCS-box-independent interaction was observed with the unrelated MyD88 protein as bait. Association of the Y249F CIS mutant with the EpoR pY402 motif could not be demonstrated by peptide affinity chromatography, suggesting Y249 might serve a similar role as Y253. However, MAPPIT experiments showed indisputable association of this CIS mutant with the same EpoRY402 motif. Only this latter interaction was functionally confirmed by the clear inhibitory effect seen in EpoRY402-dependent STAT5 recruitment and activation. The Y249F mutant thus only modestly reduced binding affinity compared to wild-type CIS. This reduced binding affinity of CISY249F completely abolished binding with the EpoR pY402 motif in a peptide affinity chromatography experiment, much in contrast with MAPPIT. The MAPPIT technique therefore reveals itself as a sensitive tool for the identification of weaker, but functionally highly relevant protein interactions.

In line with our findings that the SOCS-box of CIS is essential for EpoR association, Ketteler et al. previously reported that the SOCS-box of CIS is essential for the apoptotic effect of CIS on erythroid progenitor cells. Seemingly contradictory to our observations they also found that the SOCS-box of CIS was not required for inhibition of EpoR-induced proliferative responses [10]. However, this anti-proliferative effect may be due to CIS interference with

intermediate signalling molecules coupling to the cell cycle. CIS can indeed associate with downstream effector molecules in a SOCS-box independent modus as we showed for MyD88.

This critical role of the CIS SOCS-box in substrate binding may be a unique feature of CIS, and was not seen for the highly related SOCS2 protein, or for SOCS1 and -3. The corresponding mutation of the conserved tyrosine in the SOCS-box of SOCS2 or even deletion of its entire SOCS-box did not show any significant effect on receptor association. Likewise, the inhibitory effect of SOCS1 and SOCS3 on cytokine receptor signalling was hardly affected by removal of the SOCS-box. Previously, the SOCS-box of SOCS1 was also reported dispensable for LIF, IL-6 and GHR signalling inhibition but not for G-CSF signal transduction [40,41,43,44]. In vivo deletion of the entire SOCS-box of SOCS1 however leads to partial loss of SOCS1 function [45], most likely reflecting its role in Elongin B/C binding, thus establishing an E3 ubiquitin ligation complex leading to proteasomal degradation of associated receptor complexes.

Using MAPPIT, we could also demonstrate the association of CIS with the universal TLR adaptor MyD88. This interaction was confirmed by co-immunoprecipitation. In contrast to the data described above, mutation of the conserved C-terminal tyrosine at position 253 to phenylalanine or deletion of the complete SOCS-box of CIS had no effect at all on MyD88 binding. Further analysis of this association revealed a critical role for the Death Domain of MyD88 in CIS binding. More studies are required to elucidate the functional consequences of this interaction. We also observed interaction of other members of the SOCS protein family with MyD88 and its splicing variant lacking the intermediate domain (P.U., unpublished results), and structural and functional analyses of these interactions are ongoing. Interestingly, our results imply differential modulation by CIS of signalling via cytokine receptors and Toll-like receptors.

A crystal structure for SOCS2 in a complex with elongin C and B was recently determined [38]. A molecular model was built for the CIS-elonginC-elonginB complex in order to get structural insight into the role of the CIS C-terminus on substrate recognition. In the SOCS2 structure and the CIS model, the C-terminus is buried in the interface between the SH2 domain and the SOCS-box excluding the possibility that this C-terminus could make direct contact with a phosphopeptide substrate bound to the SH2 domain (figure 6C). In both CIS and SOCS2, the hydroxyl group of the last tyrosine hydrogen bonds to the buried C-terminal carboxyl group. The Y253F mutation in CIS can thus be expected to influence the protein structure or folding: removing the tyrosine hydroxyl group may render burial of the C-terminus

energetically unfavorable. The tyrosine at position 253 of CIS may therefore play a structural role. One can speculate that this affects stability of the packing between SOCS-box and SH2 domain, but how this might affect binding to a phosphopeptide substrate remains unclear. Allosteric effects on the substrate binding pocket cannot be excluded. However, the direct environment of the C-termini in the CIS model and SOCS structure are very similar, hinting that the same phenomenon would be expected for the Y194F mutation in SOCS2, while this mutation has no effect on interaction with its phosphopeptide substrates.

Tyrosine phosphorylation of SOCS proteins has been reported before. Cacalano et al. showed Epo-induced phosphorylation of the two conserved tyrosines in the SOCS-box of SOCS3, including Y221 that corresponds to the Y253 position in CIS. Interestingly, the C-terminal pY221 allowed binding and functional coupling to the Ras signalling pathway on the one hand while both phosphorylated tyrosines, situated centrally and C-terminally in the SOCS-box, were involved in abrogation of Elongin C interaction [46,47]. Intriguingly, as in CIS and SOCS2, Y221 in SOCS3 is also predicted (not shown) to be buried in the interface between the SH2-domain and the SOCS-box, and its hydroxyl group hydrogen bonds to the buried C-terminal carboxyl group. It is therefore likely that phosphorylation of Y221 in SOCS3, and possibly Y253 in CIS, requires changes in the conformation of the C-terminus. One possibility is that burial of the C-terminus as seen in the SOCS2 crystal structure depends on binding of the Elongin complex. In the absence of Elongin binding, the C-terminal tyrosine motifs may be accessible for phosphorylation. In this structural modus, phosphorylation-dependent interactions may occur with signaling molecules or with accessory proteins that facilitate interactions with (a subset of) substrates. Mutating Y253 in CIS may then prevent the phosphorylation-driven structural change required for downstream interactions. Deletion of the 4 C-terminal aa in the CISprey, eliminating the putative phosphorylation context, leads to impaired MAPKIT signaling, adding evidence to this phosphorylation hypothesis (not shown). Of note, it seems unlikely that disturbed Elongin binding causes the substrate binding defects in the CIS mutants: TAP2 purification of wild type CIS and of its C-terminal tyrosine mutants showed clear association with Elongins B and C and with Cullin 5, which was completely lost when the entire SOCS-box of CIS was deleted. Thus, Elongin C binding does not involve the C-terminus of CIS and has no role in the effect of mutating Y253.



The role of the C-terminus of CIS in substrate binding is remarkably similar to the role of the C-terminal helix of the Von Hippel-Lindau (VHL) tumour suppressor protein. The VHL protein is part of a VHL/E3 ligase complex involved in ubiquitination of transcription factors like hypoxia inducible factor (HIF)-1 $\alpha$ , targeting them for degradation. The VHL/E3 ligase complex binds and ubiquitinylates two oxygen-dependent degradation domains (HIF-ODDD). The crystal structure of VHL protein, bound to a hydroxylated HIF-1  $\alpha$  peptide, and to the Elongins C and B has been determined (figure 6D) [48]. This revealed that the SOCS-box of VHL is followed by a C-terminal helix which is not directly involved in binding to the hydroxylated HIF-1 $\alpha$  peptide. Like the C-terminus of CIS, this helix tightly interacts with the SH2 domain. Lewis et al. recently reported that this C-terminal helix is critical for ubiquitination of HIF-1 $\alpha$ . Deletion of this C-terminal peptide impaired VHL binding and ubiquitinylation of the C-terminal HIF-ODDD, while ubiquitinylation and degradation of the N-terminal HIF-ODDD is hardly affected [49]. The role of the C-terminal peptide in VHL thus shows some striking parallels with the CIS C-terminus. As for CIS, deletion of the VHL C-terminus specifically affects certain functions/interactions, leaving other functions unaltered. It was suggested that deletion of the C-terminus might affect VHL substrate binding by secondary folding effects.

In brief, we have shown that the SOCS-box of CIS is essential for interaction with target cytokine receptors but not with the universal TLR adaptor MyD88. It appears that the biological role of the SOCS-box is more complex than simple recruitment of a ubiquitin-ligation complex, and is also involved in (regulated) substrate binding. Depending on the type of SOCS protein, this may include receptor recruitment motifs, alternative signalling pathways, and other SOCS proteins [16,17]. The precise underlying controls that are involved in these diverse functions of the SOCS-box remain to be clarified.

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**Table 1 as referred in Experimental Procedures**

1. Extracellular EpoR - Intracellular LRF3 (Y985F-Y1077F-Y1138F) bait constructs	
	<i>Bait protein</i>
Mock bait	No bait
EpoRY402 bait	Y402 motif of the EpoR
EpoRY430/Y432 bait	Y430/Y402 motif of the EpoR
mMyD88 bait	MyD88
mMyD88(N) bait	N-terminal part of MyD88 (Death Domain and intermediate domain)
mMyD88(TIR) bait	C-terminal TIR domain of MyD88
2. Extracellular LR – Intracellular LR bait constructs	
LR(F3)	LR (Y985F-Y1077F-Y1138F)
LR(YYF)	LR (Y1138F)
LR(YFF)	LR (Y1077F-Y1138F)
LR(FYF)	LR (Y985F-Y1138F)
3. Non-MAPPIT receptor constructs	
EpoR	WT EpoR
LR(FFY)	LR (Y985F-Y1077F)
LR(YFY)	LR (Y1077F)

**Table 1:** overview of the bait constructs used in this study



## Figure legends

### **Fig. 1. MAPPIT.**

**A.** MAPPIT, a cytokine receptor-based two-hybrid method, is displayed in the left panel, with the various receptor motifs used in this study. The right panel shows a variant of the MAPPIT technique using the STAT3 signalling-deficient LR as bait. Both MAPPIT methods are described in more detail in the results section.

**B,** Schematic structure of SOCS proteins. Conserved tyrosines in the SOCS-box are indicated with a black box.

### **Fig. 2. The CIS C-terminus is critical for interaction with EpoR motifs.**

**A.** Interaction of CISprey mutants with EpoRY402. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY402 bait, various mutants of the pMG2-CIS prey construct and with the pXP2d2-rPAP1-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. All preys were also tested for interaction with a “mock bait” lacking an EpoR tyrosine motif, and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS and s.d. of triplicate measurements are plotted.

**B.** Interaction of SOCS2prey mutants with EpoRY402. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY402 bait, various mutants of the pMG2-SOCS2 prey construct and with the pXP2d2-rPAP1-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. All preys were also tested for interaction with a “mock bait” lacking the EpoR motif, and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS + s.d.

**C.** Interaction of CIS/SOCS2prey mutants with EpoRY430/Y432. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY430/Y432 bait, various mutants of the pMG2-CIS and pMG2-SOCS2 prey constructs and with the pXP2d2-rPAP1-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours.

Luciferase activities were measured in triplicates. All preys were also tested for interaction with a “mock bait” lacking the EpoR motif, and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS+ s.d.

**D.** Interaction of CIS/SOCS2prey mutants with the LR(YFF) and LR(FYF). Hek293T cells were transiently co-transfected with plasmids encoding different LR tyrosine mutants, various mutants of the pMG2-CIS or pMG2-SOCS2 prey constructs and with the pXP2d2-rPAP1-luci reporter. The transfected cells were either stimulated for 24 hours with leptin or were left untreated (NS). Luciferase measurements were performed in triplicate. All preys were also tested for interaction with the LR lacking intracellular tyrosines and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS+ s.d.

**E.** Peptide affinity chromatography. Hek293T cells were transfected with various mutants of CIS or SOCS2. The lysates were incubated with the (phospho-)tyrosine peptides corresponding to the Y402 motif of the EpoR. Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody.

**Fig. 3. The CIS C-terminus of SOCS1 and SOCS3 is not essential for signalling inhibition.**

**A-B.** Inhibition of EpoR and LR signalling by SOCS1/SOCS3 mutants. Hek293T cells were transiently co-transfected with plasmids encoding different LR tyrosine mutants or EpoR, various mutants of SOCS1 and SOCS3 and with the pXP2d2-rPAP1-luci reporter. The LR tyrosine mutants are used to minimize interference of other inhibitors. The transfected cells were either stimulated for 24 h with ligand or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as ratio stimulated/NS+ s.d.

**Fig. 4. Critical role of CIS C-terminus in blocking STAT5 activation.**

**A.** STAT5 reporter assay using the EpoRY402 motif. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY402 bait, various mutants of FLAG-tagged CIS and with the pGL3-5xbeta-casein-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. Data are expressed as ratio stimulated/NS + s.d.

**B,** EMSA using the EpoRY402 motif. Hek293 Flp-In cells were transiently co-transfected with plasmids encoding the EpoR Y402 bait, various FLAG-tagged CIS mutants and STAT5B. Nuclear lysates were incubated with <sup>32</sup>P labelled probe corresponding to a β-casein STAT5 binding site to reveal active STAT5 complexes.

**C,** STAT5 reporter assay using the WT-EpoR. Hek293T cells were transiently co-transfected with plasmids encoding the EpoR, various mutants of FLAG-tagged CIS and with the pGL3-5xbeta-casein-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. Data are expressed as ratio stimulated/NS + s.d.

**Fig. 5. The SOCS-box of CIS is not critical for interaction with MyD88.**

**A,** Interaction of MyD88 and CIS prey constructs. Hek293T cells were transiently co-transfected with the MAPPIT mock bait or the mMyD88 bait plasmid (0.1 µg), various CIS or CIS-mutant prey (0.5 µg) constructs and with the pXP2d2-rPAP1-luci reporter (0.2 µg). MyD88 prey was used as a positive control. The transfected cells were either stimulated for 24 h with Epo or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as ratio stimulated/NS + s.d.

**B,** Co-immunoprecipitation analysis. Hek293T cells were transiently co-transfected with combinations of mMyD88-E, mCIS-FLAG, mCISY253F and mCISdBox. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E.

**C,** Role for the N-terminal domain of MyD88 in CIS binding. Hek293T cells were transiently co-transfected with the MAPPIT mock bait, the mMyD88(N) bait or the mMyD88TIR bait vectors (0.1 µg), various CIS or CIS-mutant prey (0.5 µg) constructs and with the pXP2d2-rPAP1-luci reporter (0.2 µg). The MyD88 prey construct was used as a positive control. The

transfected cells were either stimulated for 24 h with Epo or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as ratio stimulated/NS + s.d.

**D.** Co-immunoprecipitation analysis. Hek293T cells were transiently co-transfected with combinations of mMyD88(N)-E, and mCIS-FLAG. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E.

**Fig. 6. Role of the CIS C-terminus in ElonginB/C association.**

**A.** TAP2 purification using CIS mutants. Hek293T cells were transiently transfected with various mutants of the pMet7TAP2-CIS construct. Cell lysates were purified by the TAP2 method and were then loaded on 14% polyacrylamide gel. After silver-staining the protein bands indicated with an arrow were analysed by mass spectrometry and identified as the annotated protein.

**B.** Sequence alignment of SOCS-boxes. SOCS-box sequences of murine SOCS1,2 and 3 and CIS were aligned using the t\_coffee algorithm. An arrow indicates the Y253 positions. Increasing grey shading corresponds with increasing % identity.

**C.** Homology model of mouse CIS, in complex with Elongin C and B. The CIS SOCS-box is shown in black. The position of the phosphopeptide substrate in the model is indicated by a sulphate ion, copied from the SOCS2 template structure, that mimics the phosphate group of the phosphopeptide substrate. Y253 hydrogen bonds with the C-terminal carboxyl group.

**D.** Crystal structure of VHL protein (1LQB), bound to its hydroxylated HIF-1 $\alpha$  substrate (black), and to Elongins C and B. The pVHL SOCS-box and the hydroxyproline binding domain are in black. The extra C-terminal helix (dark grey) is indicated.

Figure 1:

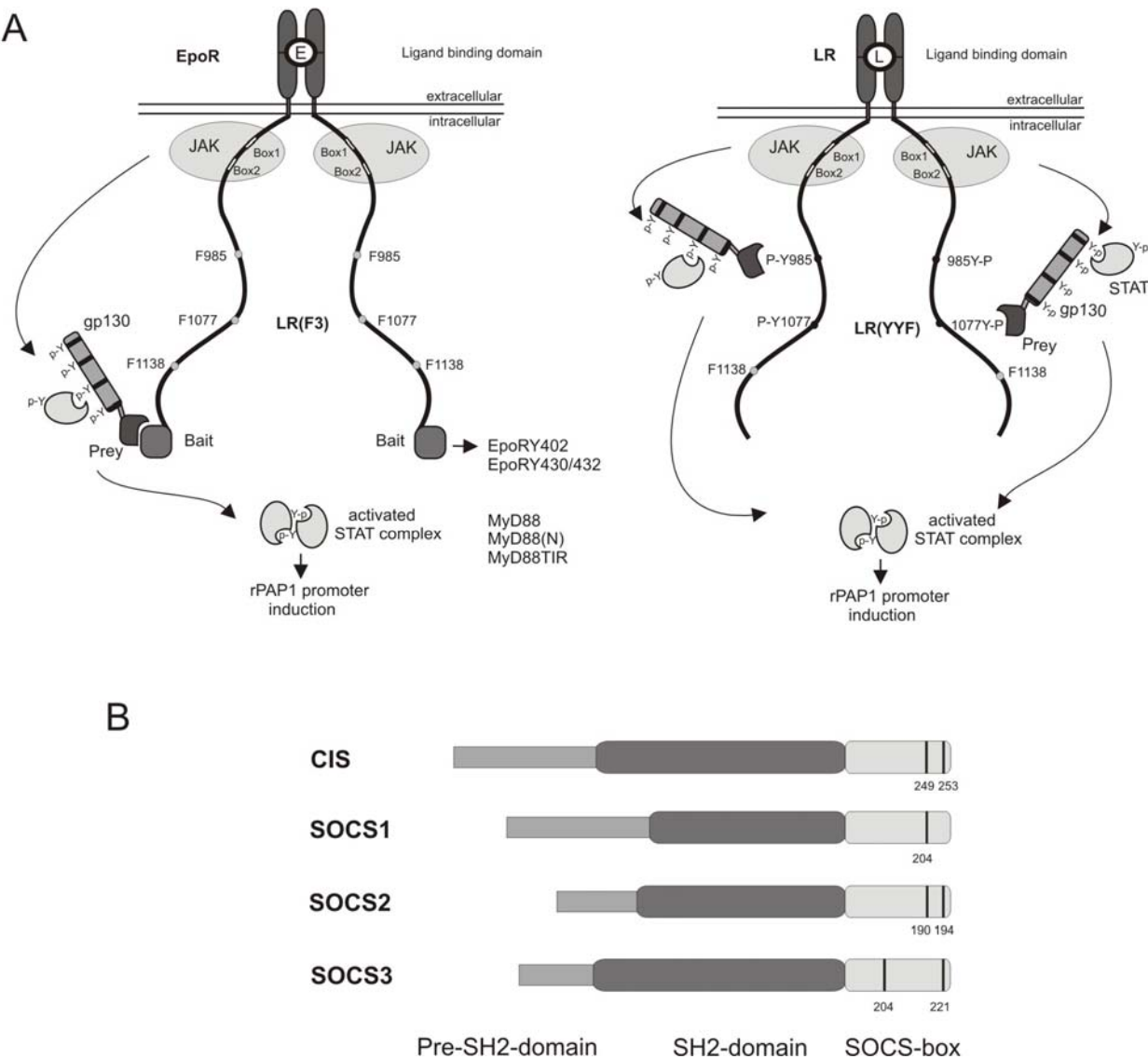


Figure 2:

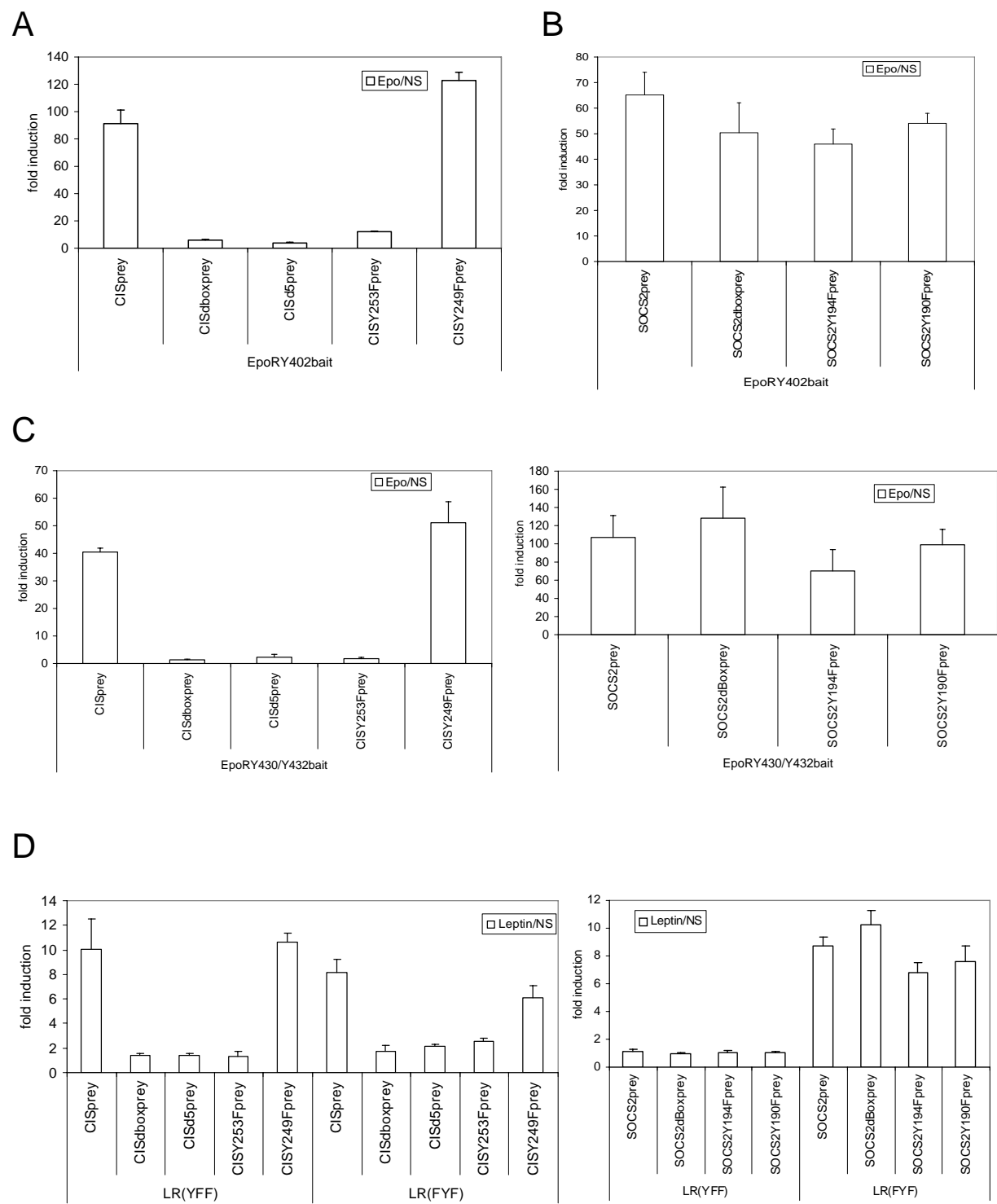
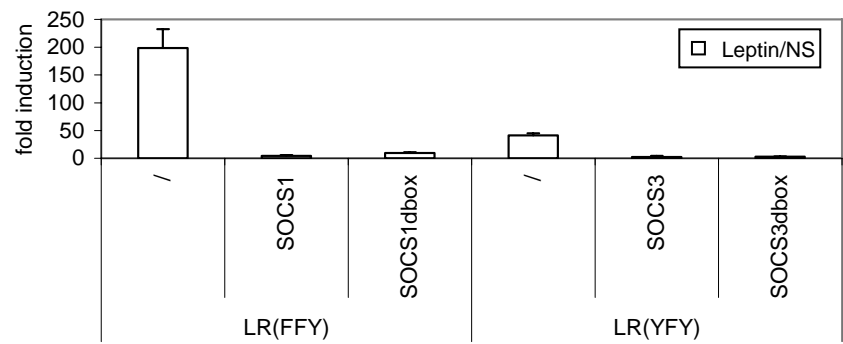


Figure 3:

A



B

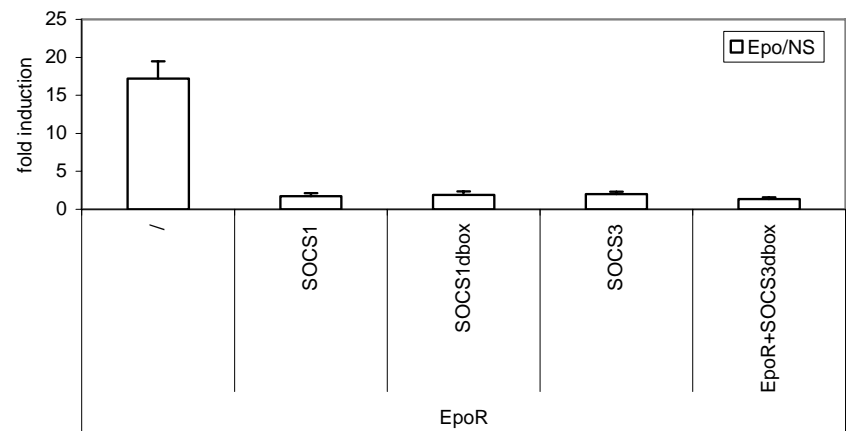


Figure 4:

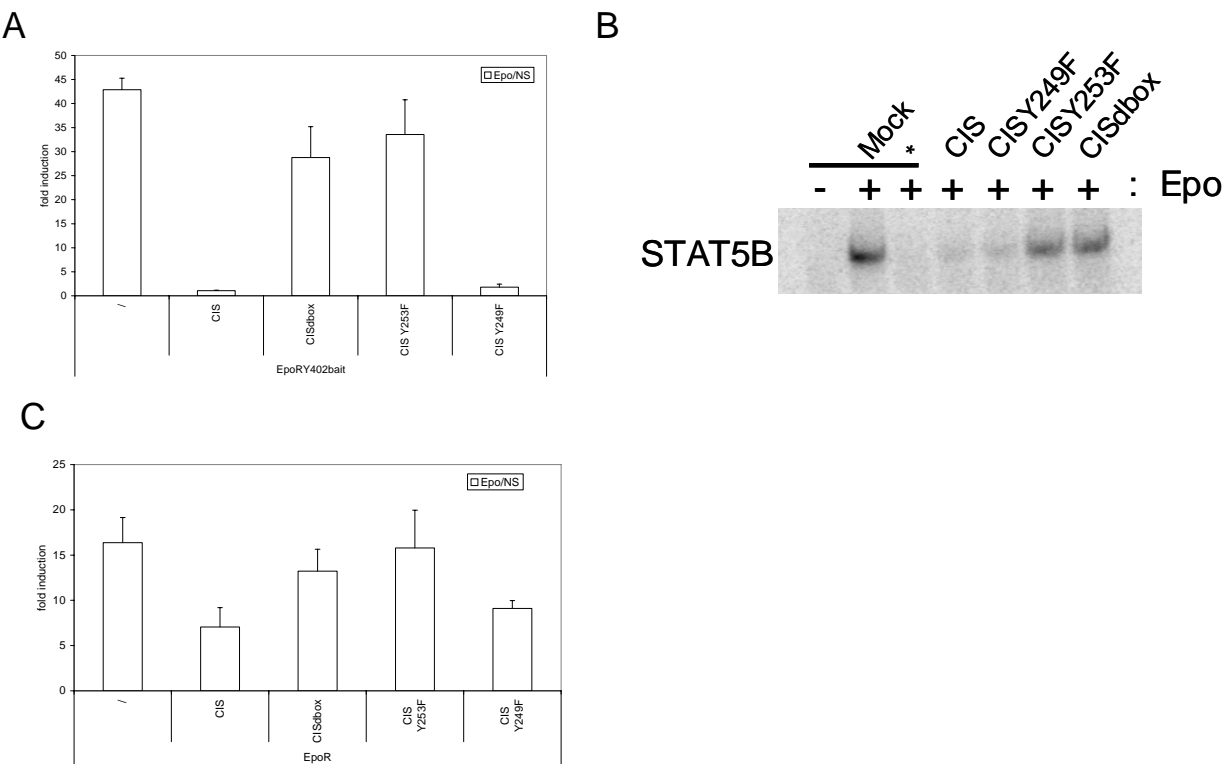




Figure 5:

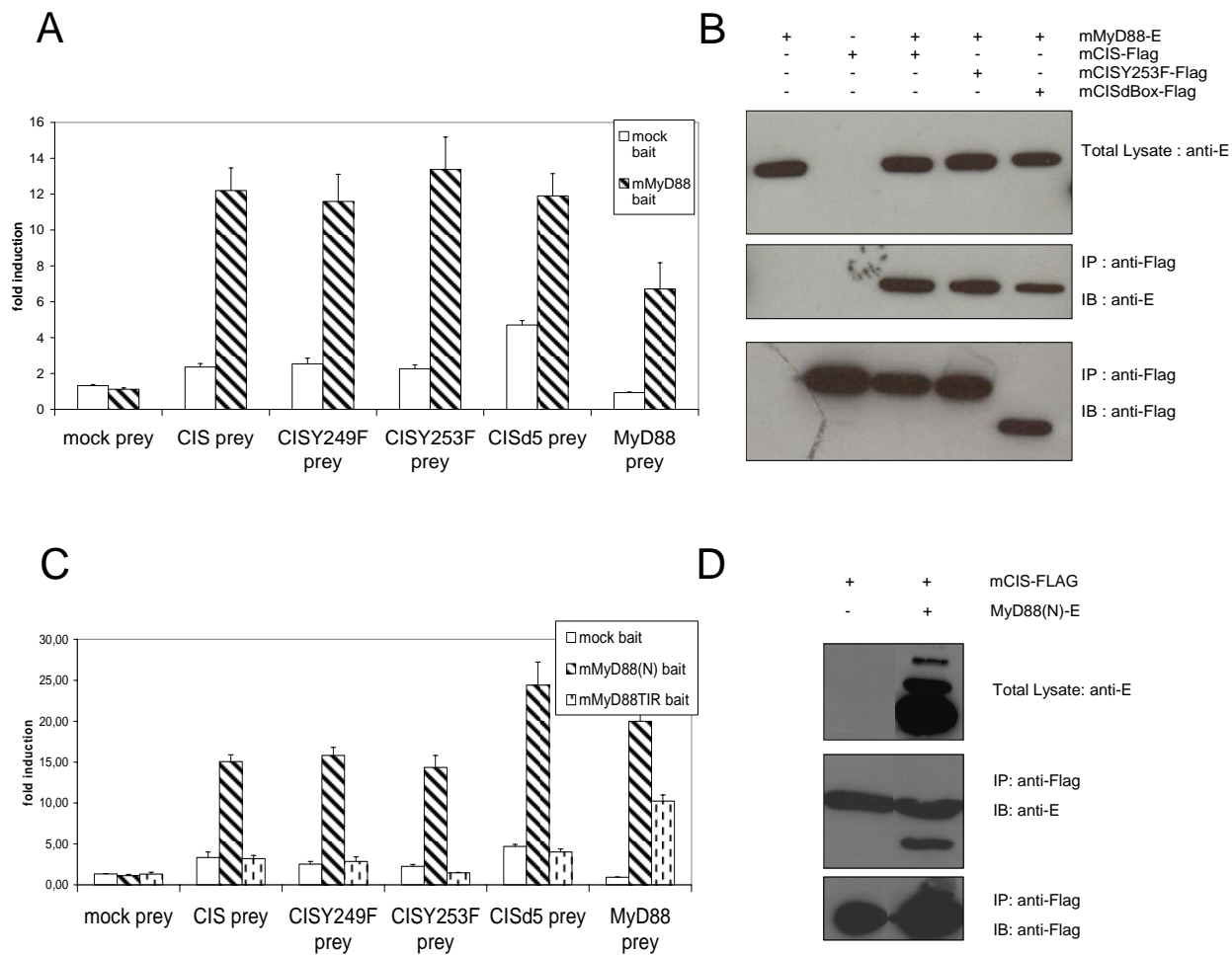


Figure 6:

