# A region encompassing the FERM domain of Jak1 is necessary for binding to the cytokine receptor gp130

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**Abstract The terminal portion of the Janus kinases (Jaks) contains a divergent FERM (Four-point-one, Ezrin, Radixin, Moesin) homology domain comprising 19 conserved hydrophobic regions. To determine the role of this domain in governing recruitment of Jak1, but not Jak3, to the gp130 subunit of the interleukin-6 family of cytokine receptors, the interaction of three Jak1/Jak3 chimeras with gp130 was investigated. Chimeras 1, 2 and 3 (Jak1 FERM regions 1–19, 1–18 and 1–8/Jak3, respectively) were all enzymically active. Chimeras 1 and 2 interacted with the cytoplasmic domain of gp130, although less efficiently than Jak1. Only chimera 2, however, restored gp130 signalling in Jak1-negative cells. The data are consistent with recruitment of Jak1 to gp130 through the Jak1 FERM domain, but also emphasise the likely requirement for precise Jak/receptor orientation to sustain function.** 

*Key words:* Jak kinase; FERM domain; gp130; Cytokine receptor

## 1. Introduction

Janus kinases (Jaks) are a family of cytoplasmic tyrosine kinases involved in cytokine receptor signalling. There are four known mammalian Jaks: Jak1, Jak2 and Tyk2, which are ubiquitously expressed, and Jak3, which is restricted to cells of haematopoietic origin. Jaks are essential for signalling in response to numerous cytokines and growth factors. They are non-covalently associated with cytokine receptors and become activated upon cytokine-induced receptor aggregation. Activated Jaks phosphorylate tyrosine residues in the cytoplasmic tail of the receptors, leading to recruitment and activation of signal transducers and activators of transcription (STATs) and other signalling molecules (reviewed in [1]).

The specificity of Jak/receptor interactions is likely critical for cytokine signalling. The Jaks are not equivalent. Different Jaks are associated with the various cytokine receptors, and can play distinct roles in individual receptor complexes. For example, Jaks 1 and 2 and Tyk2 are all activated in response to interleukin (IL)-6, but efficient signalling is dependent on Jak1 [2]. In contrast, Jak2 is the sole Jak activated in response to erythropoietin (Epo) [3] and triggers the activation of Jak1 in the interferon (IFN)-γ receptor [4]. Clearly, any real understanding of cytokine signalling will require adequate definition of the structure/function relationships of Jak/receptor complexes. The Jaks are known to interact with the cytoplasmic, membrane proximal, domains of cytokine receptors and, in the case of the class I receptors including the gp130 subunit of the IL-6 family receptors, with Box1 and -2 motifs within these domains [5,6]. Thus, for the cytokine receptors some of the molecular determinants governing Jak recruitment have been identified. Much less, however, is known about the nature of the interacting Jak domain(s), particularly for Jak1. The Jaks contain seven regions showing high sequence homology – the Jak homology domains (JH) 1–7 [7]. The most C-terminal JH region (JH1) is a classical tyrosine kinase domain, whereas the adjacent JH2 kinase-like domain lacks catalytic activity, but has been implicated in regulating the kinase activity of the JH1 domain [8,9]. Jak deletion and chimera analyses have established the importance of Jak N-termini in interaction with the receptors. In general, for Jak2, Jak3 and Tyk2, elements within the most N-terminal region encompassing JH6 and JH7 are essential for binding to the receptor, but substantially larger fragments are required to mediate functional binding [6,10–14]. Accordingly, despite substantial progress, there is no uniform structural model for Jak/receptor interaction, and the further definition of such interactions has been hampered until very recently by the lack of structural information on the N-terminal portion of the Jaks. Sequence analysis has, however, now identified, within the N-terminal region of the Jaks, homology with the band four-point-one, ezrin, radixin, moesin (FERM) domain (also referred to as band 4.1/JEF domain, for 4.1, Jak, ERM and FAK) [15]. This domain was first identified in the N-termini of these cytoskeletal proteins and is involved in targeting them to the plasma membrane. The FERM domains are approximately 400 amino acids in length and contain 19 conserved hydrophobic regions. The X-ray crystal structures of the moesin and radixin FERM domains were recently solved [16,17] and revealed a clover-shaped structure composed of three compact subdomains (termed F1, F2, F3 in moesin) that show similarity to ubiquitin (F1), acyl-CoA binding protein (F2) and a phosphotyrosine binding and pleckstrin homology domains (F3).

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*Abbreviations:* EpoR, erythropoietin receptor; FERM, four-point-one, ezrin, radixin, moesin; IFN, interferon; IL, interleukin; Jak, Janus kinase; STAT, signal transducer and activator of transcription

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Here, we were concerned to ask if the predicted Jak1 FERM domain defines a functional unit with respect to Jak/receptor interaction. To this end, Jak1/Jak3 chimeras were constructed to determine whether the substitution of the FERM domain of Jak1 would confer gp130 binding upon Jak3.

## 2. Materials and methods

### 2.1. Cell culture and DNA transfections

Jak1-negative mutant U4C cells were derived from HT1080 fibrosarcoma cells, which do not express Jak3, as described previously [12,18]. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum and 400 μg/ml neomycin (G418, Gibco BRL). Transfections of the various expression constructs (10 μg total DNA per 10 cm culture dish) were carried out using Superfect (Qiagen), according to manufacturer's instructions.

### 2.2. Plasmids and generation of Jak1/Jak3 chimeras

The expression constructs for Jak1 [19], Jak3 [20], Jak1.KE [4], IL-5Rβ/gp130 [21] and erythropoietin receptor (EpoR)/gp130 [22] have been described previously. The Jak1/Jak3 chimeras were generated by fusion PCR [23] with the junctions at the following amino acid positions: Jak1 415/Jak3 363 (chimera 1), Jak1 404/Jak3 353 (chimera 2), Jak1 183/Jak3 169 (chimera 3). All chimeric constructs were subcloned into the pcDNA 3.1 expression vector (Invitrogen) and were checked by automated DNA cycle sequencing.

### 2.3. Cell lysis, (co-) immunoprecipitation and Western blotting

Immuno- and co-immunoprecipitations were performed 24 h after transfection. For immunoprecipitations and electrophoresis mobility shift assay (EMSA; see below), cells were lysed in 0.5% NP40, 50 mM Tris (pH 8.0), 10% glycerol, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin, and 1 μg/ml leupeptin. For co-immunoprecipitations, cells were lysed in 1% Brij 97, 20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 100 U/ml aprotinin, and 1 μg/ml leupeptin. Lysates were cleared by centrifugation and were subsequently incubated with appropriate antibodies (16–18 h at 4°C) and protein A–Sepharose (1 h at 4°C), washed three times in lysis buffer

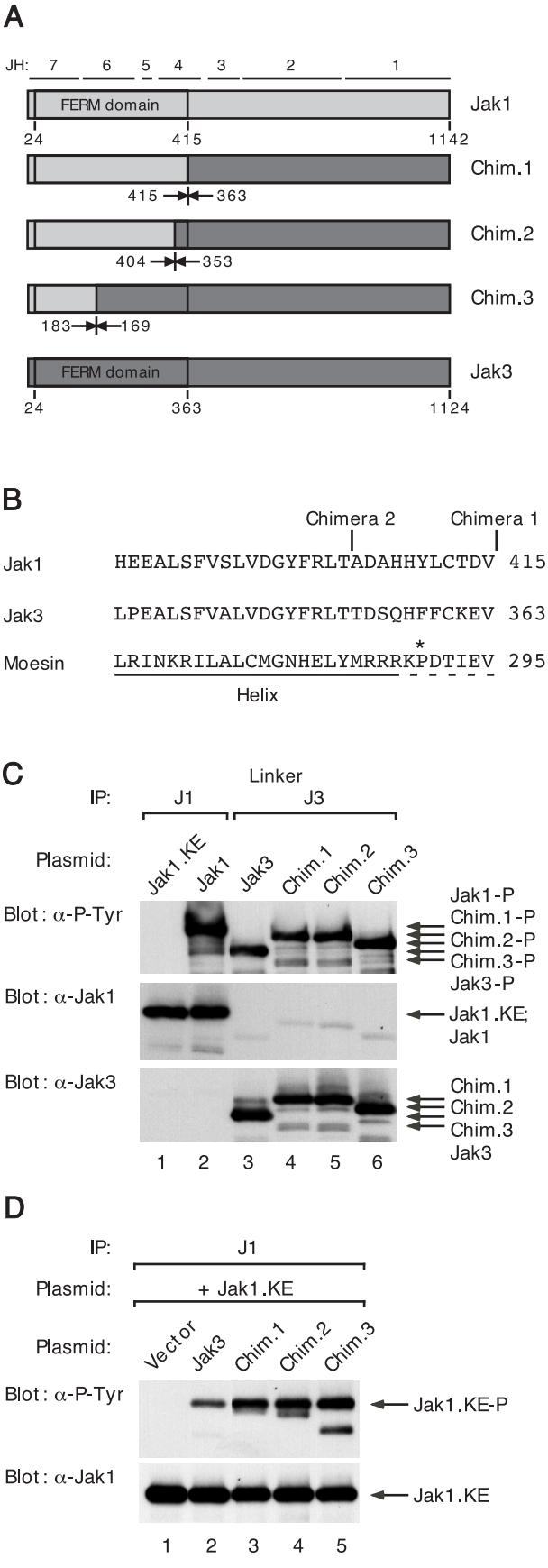


Fig. 1. Expression and catalytic activity of Jak1/Jak3 chimeras. A: Schematic diagram of the Jak1/Jak3 chimeras. JH domains 7–1 are indicated (top). Junctions between Jak1 and 3 are indicated by the arrowheads; numbers to the left and right of the arrowheads represent the amino acid positions in Jak1 and 3, respectively. B: Alignment of the C-terminal amino acid sequences of the FERM domains of Jak1 (P23458) Jak3 (P52333) and moesin (P26038). The Jak1/Jak3 junctions of chimeras 1 and 2 are indicated. The positions of the α-helix and initial residues of the linker are based on the X-ray crystal structure of the moesin FERM domain [16]. The asterisk indicates the last residue of the crystalised moesin FERM domain fragment. C: U4C cells which lack both Jak1 and Jak3 were transiently transfected with the indicated expression plasmids. After 24 h, whole cell lysates were prepared and immunoprecipitated with either anti-Jak1 (J1) or anti-Jak3 (J3) antibodies as indicated (IP). The Jak3 antibody was raised against the C-terminus of Jak3 and was suitable for both immunoprecipitation and detection of the Jak1/Jak3 chimeras. Blots were probed with anti-phosphotyrosine antibodies (α-P-Tyr; top panel), and examined for Jak1.KE and Jak1 protein (middle panel) and Jak3 and chimera protein (bottom panel) by reprobing blots with Jak1 or Jak3 antibodies as indicated. D: U4C cells were co-transfected with the kinase-dead Jak1.KE mutant and empty vector (pcDNA 3.1) or expression plasmids encoding wild-type Jak3 or Jak1/Jak3 chimeras. After 24 h, whole cell lysates were prepared and immunoprecipitated with anti-Jak1 (J1). Blots were probed with phosphotyrosine antibodies (α-P-Tyr; upper panel) and examined for Jak1.KE protein (lower panel). Arrows indicate the various Jaks and chimeras. Results are representative of four independent experiments.

and electrophoresed on 6.5% polyacrylamide–SDS gels. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore), and Western blot analyses were performed as described previously [24]. The following antibodies were used: polyclonal antibodies against Jak1 (HR 785), Jak3 (C21) and IL-5Rβ (N20)(all from Santa Cruz Biotech.), and monoclonal anti-phosphotyrosine antibodies PY20 (Affiniti) and 4G10 (Upstate Biotechnology). Following enhanced chemoluminescence and autoradiography, membranes were stripped in 62.5 mM Tris (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol, and then reprobed as required.

### 2.4. EMSA

24 h after transfection, cells were stimulated for 20 min with Epo (100 U/ml; a generous gift from Dr B. Hilger, Roche Diagnostics GmbH), and lysed as described above. EMSAs were performed, as described previously [12], using a double-stranded $^{32}$P-end-labelled oligonucleotide containing the mutated SIE site from the c-fos promoter (5'-GTCGACATTTCCCGTAAATC-3') [25].

## 3. Results

Three Jak1/Jak3 chimeras were constructed (Fig. 1A). Chimera 1 contains the entire Jak1 FERM domain, including all 19 conserved hydrophobic regions. For chimeras 2 and 3, the junctions between Jak1 and Jak3 were made within the stretches of highly conserved residues in FERM regions 18 and 8, respectively. C-terminal sequences of the Jak1 and Jak3 FERM domains, including the Jak1/Jak3 junctions of chimeras 1 and 2, are shown in Fig. 1B. The rational for chimera 3 reflects the original suggestion that the FERM domain consists of two duplicated modules, the more N-terminal of which encompasses FERM regions 1–8 [26]. The more recently available crystal structures indicate, however, that the FERM domain is composed of three structural subdomains with no evidence for a duplication [16,17]. Accordingly, here, chimera 3 serves mainly as a negative control.

### 3.1. Expression and catalytic activity of Jak1/Jak3 chimeras

The Jak1/Jak3 chimeras, wild-type Jak1 and 3 and a kinase-negative Jak1, Jak1.KE, were independently expressed in U4C cells which lack both Jak1 and Jak3 (Section 2). Western blot analyses of Jak protein (Fig. 1C, lower panels) and Jak tyrosine phosphorylation (Fig. 1C, upper panel) indicated that Jaks 1 and 3 and the chimeras were comparably expressed and similarly enzymically active. Transiently expressed Jaks constitutively autophosphorylate [27]. Consistent with this, the transfected Jaks 1 and 3 and all three chimeras were comparably phosphorylated (Fig. 1C, upper panel). The Jak1.KE was, however, phosphorylated only when co-transfected with Jak3 or the chimeras (Fig. 1D). Furthermore, all three chimeras induced weak constitutive STAT5 DNA-binding activity (data not shown). Thus, all three chimeras were similarly expressed and have full auto- and 'trans'-kinase activity.

### 3.2. Binding of Jak1/Jak3 chimeras to the cytoplasmic domain of gp130

To assess the ability of the Jak1/Jak3 chimeras to interact constitutively with gp130, co-immunoprecipitation experiments were performed. The chimeric receptor IL-5Rβ/gp130, comprising the extracellular part of IL-5Rβ and the transmembrane and intracellular tail of gp130 (successfully employed previously in this type of experiment [28]), was used again here. U4C cells were transiently co-transfected with this receptor and the various Jaks and chimeras. The latter were immunoprecipitated with appropriate Jak antibodies, and co-

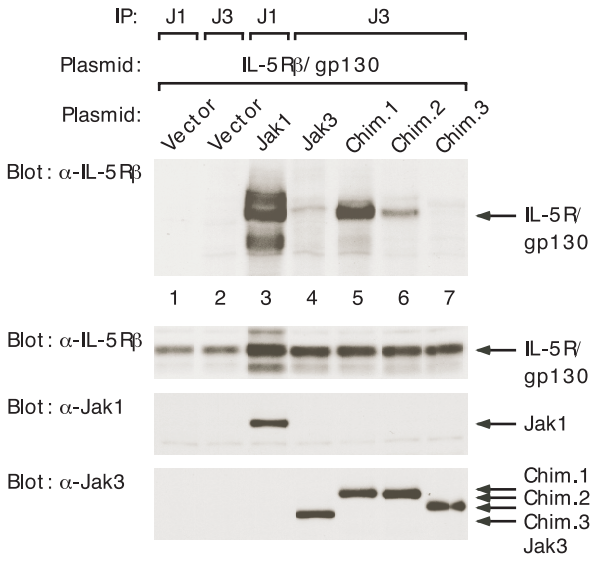


Fig. 2. Binding of Jak1/Jak3 chimeras to the cytoplasmic domain of gp130. U4C cells were transiently co-transfected with the chimeric receptor construct IL-5Rβ/gp130 (encoding the ectodomain of IL-5Rβ and the transmembrane and intracellular regions of gp130) and either empty vector (pcDNA 3.1) or Jak1, Jak3, or chimera 1, 2 or 3. After 24 h, whole cell lysates were prepared and immunoprecipitated with either anti-Jak1 (J1) or anti-Jak3 (J3) as indicated (IP). Blots were probed with antibodies directed against the ectodomain of IL-5Rβ (upper panel). In parallel, samples of the whole cell lysates were electrophoresed, blotted, and examined for protein expression (lower panels). Arrows indicate the chimeric receptor IL-5Rβ/gp130 and the Jaks and Jak1/Jak3 chimeras. Results are representative of four independent experiments.

precipitation of IL-5Rβ/gp130 was examined by probing the blots with an appropriate antibody to IL-5Rβ. As expected, the IL-5Rβ/gp130 receptor co-precipitated with Jak1 but not Jak3 (Fig. 2, upper panel, lanes 3 and 4). Chimera 1, which contains the entire Jak1 FERM domain, interacted with the intracellular gp130 domain, comparably to Jak1 (Fig. 2, lane 5). Chimera 2, in which the last 11 C-terminal amino acids of the Jak1 FERM domain were replaced with the corresponding Jak3 amino acids (Fig. 1B), also interacted, but with much less efficiently (Fig. 2, lane 6). No binding was observed for chimera 3 (Fig. 2, lane 7). Expression from the various constructs was similar as assessed by Western blot analyses of whole cell lysates (Fig. 2, lower panels). It would appear, therefore, that a region corresponding to the FERM domain of Jak1 can confer efficient binding to the cytoplasmic domain of gp130 upon Jak3.

### 3.3. Jak1/Jak3 chimera 2 sustains gp130-mediated signalling in Jak1-negative cells

Next, we determined whether the Jak1/Jak3 chimeras could restore Jak1-dependent signalling in response to ligand in Jak1-negative cells. For a ligand-mediated response, the IL-5Rβ/gp130 receptor requires co-expression of the complementary IL-5 receptor subunit, IL-5Rα/gp130 [21]. Accordingly for simplicity, here, use of the homodimeric erythropoietin/gp130 receptor chimera (EpoR/gp130: EpoR external domain/gp130 transmembrane and internal domain [22]) was preferred. As expected, in Jak1-negative U4C cells co-transfection of Jak1 with the EpoR/gp130 chimera restored Epo-mediated activation of STAT1 (Fig. 3, lanes 2 and 4, and [2]).

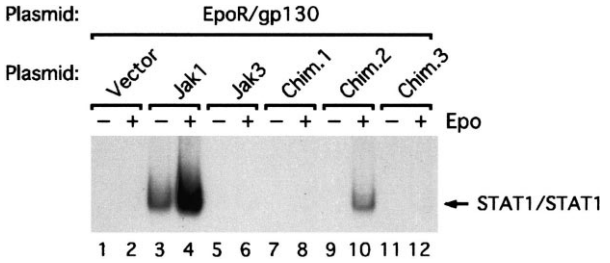


Fig. 3. Jak1/Jak3 chimera 2 sustains gp130-mediated signalling in Jak1-negative cells. U4C cells were co-transfected with the chimeric receptor construct EpoR/gp130 (encoding the ectodomain of EpoR and the transmembrane and intracellular regions of gp130) and either empty vector (pcDNA 3.1) or Jak3 or chimera 1, 2 or 3. After 24 h, cells were treated with Epo for 20 min where indicated, and whole cell extracts were prepared and analysed for STAT1 homodimers (arrow) by EMSA using the SIE probe. Results are representative of four independent experiments.

Consistent with the co-precipitation data (Fig. 2), neither Jak3 nor chimera 3 rescued STAT1 activation (Fig. 3, lanes 6 and 12). Despite good binding to the IL-5Rβ/gp130 (Fig. 2), chimera 1 could not substitute for Jak1 with respect to signalling through the EpoR/gp130 chimera (Fig. 3, lane 8). More positively, chimera 2, which also bound the IL-5Rβ/gp130 receptor subunit, although less efficiently (Fig. 2), did restore STAT1 activation (Fig. 3, lane 10). Thus, it would appear that ligation of Jak1/Jak3 within the highly conserved FERM region 18, although detrimental to receptor binding, has better conserved functionality in chimera 2.

## 4. Discussion

All three Jak1/Jak3 chimeras were similarly expressed and retained both auto- and trans-kinase activities comparable to that of the parental Jaks 1 and 3 (Fig. 1).

Chimera 2, for which the Jak1 substitution of the Jak3 FERM domain includes all but the last 11 C-terminal amino acids, both bound the cytoplasmic gp130 domain of the IL-5Rβ/gp130 receptor subunit and restored ligand-dependent signalling to STAT1 through the EpoR/gp130 receptor (Figs. 2 and 3). Although the binding and signalling were reduced compared to those observed with Jak1, they were clear and reproducible. It is reasonable to conclude that the region of Jak1 encompassing the FERM domain is sufficient to mediate functional binding to the cytoplasmic domain of gp130. Very weak activation of STAT1 was also observed in response to IFN-γ through the endogenous class II IFN-γ receptor (data not presented). Accordingly, receptor recruitment of the Jak1/Jak3 chimeras is not an artefact of overexpression of the chimeric receptors in the transient co-transfection assays. In addition, it suggests that the FERM domain likely plays a role in the recruitment of Jak1 not only to class I, but also to class II cytokine receptors.

Chimera 1, in which there is substitution of the entire Jak1 for Jak3 FERM domain, bound the IL-5Rβ/gp130 chimera comparably to Jak1 (Fig. 2), but did not signal through the EpoR/gp130 receptor (Fig. 3). In the absence of appropriate antibody, the possibility that this reflects a failure to bind to the latter cannot be excluded, but seems intrinsically unlikely. In fact, there are numerous other examples where binding of a Jak deletion mutant or chimera has not conferred function. For example, a Jak3/Jak1 chimera having all of JH7 and five amino acids of JH6 of Jak3 interacted with the γc receptor subunit, but did not signal, whereas a chimera with the more substantial substitution of both JH6 and JH7 domains was functional [14]. Similar results have been reported for Tyk2 [13]. More importantly, relative to the discrepancy observed here between the two very closely related chimeras 1 and 2, a recent study of Jak2 has emphasised the requirement for very precise orientation of the Jak kinase domains in the EpoR complex for function as distinct from binding [29]. In this respect, it is of interest to note that based on the X-ray crystal structure of the human moesin FERM domain [16], the Jak1/Jak3 junction in chimera 1 is in the linker region that connects the Jak FERM domain to the putative SH2-like domain (Fig. 1B) [15]. Although this junction does not appear to interfere with the functions of the Jak1 FERM- and Jak3 kinase-domains per se, it may be that it alters the positioning of the kinase domain in the receptor complex, impairing the phosphorylation of receptor-associated substrates.

It is also possible, if not likely, that there is differential Jak1/Jak3 substrate specificity for STAT1 and/or gp130. Clearly, any such differentiation cannot be absolute as significant activation of STAT1 was observed with chimera 2, despite relatively poor binding to the receptor. On the other hand, low ligand-independent activation of STAT1 was observed upon overexpression of Jak1, but not the chimeras (Fig. 3, compare lane 3 with lanes 7, 9 and 11). Thus, some degree of substrate specificity may contribute to the limited restoration of function observed.

A recent mutational analysis of the N-terminal portion of Jak1 has identified, in the FERM F1 subdomain, amino acids crucial for the interaction with gp130 and the IFN-γ receptor (C. Haan, H. Is'harc, H. Hermanns, H. Schmitz-Van de Leur, I. Kerr, P. Heinrich, J. Groetzinger, I. Behrmann, submitted). Here, a complementary approach has shown that a region encompassing the Jak1 FERM domain can confer upon Jak3 the ability to bind gp130. These data, together with predictions from the recent crystal structures for the FERM domains of moesin and radixin [16,17], provide a substantial base upon which to build the further analysis of Jak1/receptor/signalling protein interactions, leading ultimately to the full definition of functional Jak/receptor complexes.

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