# Chapter 7
# Role of Src Family Kinases in Prolactin Signaling

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**Abstract** Prolactin (PRL) is a polypeptide hormone/cytokine mainly synthesized by the lactotrophic cells of the adenohypophysis. In addition to the best-known role in mammary gland development and the functional differentiation of its epithelium, PRL is involved in regulation of multiple physiological processes in higher organisms contributing to their homeostasis. PRL has been also associated with pathology, including breast cancer. Therefore, it is relevant to determine the molecular mechanisms by which PRL controls cellular functions. Here, we analyze the role of Src family kinases (SFKs) in the intracellular signaling pathways controlled by PRL in several model systems. The data show that SFKs are essential components in transmitting signals upon PRL receptor stimulation, as they control activation of Jak2/Stat5 and other routes that regulate PRL cellular responses.

**Abbreviations**

| | |
|---|---|
| BaF-3 cells | Mouse pro-B lymphocytes |
| BrdU | 5-bromo-2'-deoxyuridine |
| CEF | Chick embryo fibroblasts |
| CSK | Carboxyl-terminus Src kinase |
| EGF | Epidermal growth factor |
| EGFR, ErbB1 | Epidermal growth factor receptor |
| Erk1/2 | Extracellular signal-regulated kinase 1/2 |
| Fak | Focal adhesion kinase |
| FERM domain | F for 4.1 protein, E for erzin, R for radixin, and M for moesin |
| FGF | Fibroblast growth factor |

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| | |
|---|---|
| FKHRL1, FOXO 3 | Forkhead box O3 transcription factor |
| GH | Growth hormone |
| Gab2 | Grb2-associated-binding protein 2 |
| Grb2 | Growth factor receptor-bound protein 2 |
| GSK3ß | Glycogen synthase kinase 3beta |
| IEG | Immediate early genes |
| IL-3 | Interleukin 3 |
| IGF-IR | Insulin-like growth factor I receptor |
| IP | Immunoprecipitation |
| Jak | Janus family kinases |
| JH domain | Jak homology domain |
| Mek1/2 | Mitogen-activated protein kinase 1/2 |
| NDF | Neuregulin differentiation factor |
| PDGF | Platelet-derived growth factor |
| PRL | Prolactin |
| PRLR | Prolactin receptor |
| PI3K | Phosphoinositide 3-kinase |
| RCAS A or B | Replication-competent avian retroviral vectors derived from RSV |
| RSV | Rous Sarcoma Virus |
| SFKs | Src family kinases |
| SH3 domain | Src homology domain 3 |
| SH2 domain | Src homology domain 2 |
| Shp2 | Tyrosine phosphatase containing two SH2 domains |
| Stat | Signal transducers and activators of transcription |
| WB | Western blot |
| W53 cells | BaF-3 cells expressing the long form of the PRLR from rat ovary |

## 7.1 Introduction

The landmark in prolactin (PRL) molecular signaling was the cloning and characterization of prolactin receptors (PRLR) in the late 1980s [51]. The PRLR is a member of the type I cytokine receptor family, which also includes receptors for growth hormone, interleukins-2–7, erythropoietin, granulocyte colony stimulating factor, granulocyte–macrophage colony stimulating factor, etc. [9, 40, 96]. Although several isoforms of PRLR have been described, the short, intermediate, and long forms are the most common in biological systems. The short and the long forms, generated by alternative RNA splicing, differ in length and sequence of the cytoplasmic domain. The intermediate form of the receptor, found in the rat thymoma cell line Nb2, is generated from the long form through a partial in-frame deletion of 198 amino acids of the cytoplasmic domain [15].

PRLR isoforms do not exhibit intrinsic enzymatic activity. However, in NB2-11C cells it was promptly shown that PRL induced tyrosine phosphorylation by association and activation of a 120 kDa protein kinase [81], subsequently identified as Jak2 in Nb2, in mouse mammary explants [17] and in BaF-3 cells (mouse pro-B lymphocytes) expressing the long form of PRLR [24]. Soon after, it was found that the mammary gland transcription factor MGF/Stat5, a central component of the lactogenic hormone-signaling pathway, was phosphorylated and activated by Jak2 upon PRL stimulation [34]. In this context, Jak2 conditional knockout mice showed the essential role of this kinase for proliferation, differentiation of alveolar cells, and maintenance of lactation [93]. All these data doubtlessly established the role of Jak2 for PRL signaling.

In the early 1990s, collaborating with my colleague J.P. García-Ruiz, who found that a 60 kDa protein had been phosphorylated upon PRL stimulation of hepatocytes from lactating rats, we established association and activation of the proto-oncogenic c-Src (pp60-c-Src) to PRLR [11]. Similarly, PRL caused activation of Fyn, another member of the Src family kinases (SFKs), in Nb2 cells [20].

Since these first observations several groups have worked to learn about the role of Src kinases in PRL signaling. Here we revise these data, but first, we describe some information about structure and function of Jak and Src family of tyrosine kinases.

## 7.2 The Janus Protein Tyrosine Kinase Family

The Jak family ("Janus kinase" or "Just another kinase", Jak) [98, 99] contains four members: Jak1, Jak2, Jak3, and Tyk2 that exhibit a high degree of homology. Mammalian expression of Jak1, Jak2, and Tyk2 is ubiquitous whereas Jak3 expression is predominantly hematopoietic [44]. Jak family proteins are located at the plasma membrane where they interact with the cytokine receptor [39].

The Jak family has a size between 120 and 140 kDa corresponding to about 1100 amino acids. Its members have a conserved structure in insects, birds, and mammals and display seven Jak homology domains (JH) (Fig. 7.1). The main characteristic of this family is the presence, at the extreme C-terminus, of a tyrosine kinase catalytic domain (JH1) followed by an inactive pseudo-kinase domain (JH2), which is involved in the regulation of enzyme activity. Indeed, it suppresses basal kinase activity and allows stimulation upon binding of the ligand to the receptor [83]. At the N-terminus, the JH3, JH4, and JH5 domains possess homology with SH2 domains

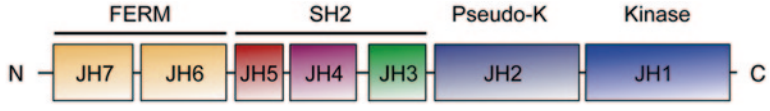



**Fig. 7.1** The Janus protein tyrosine kinase family (Jak): structural domains.

(SH2-like) and through them may interact with proteins containing phosphorylated tyrosine residues. The JH6 and JH7 domains are homologous to the FERM domain, found in molecules as Band 4.1, radixin, myosin, etc. The FERM domain has 300 amino acids in length. In addition regulating the kinase activity by interacting with the JH1 domain [41, 107], FERM is responsible for the association of Jak2 with Box I of PRLR [41, 56]. This interaction is not dependent on ligand binding to the receptor [6].

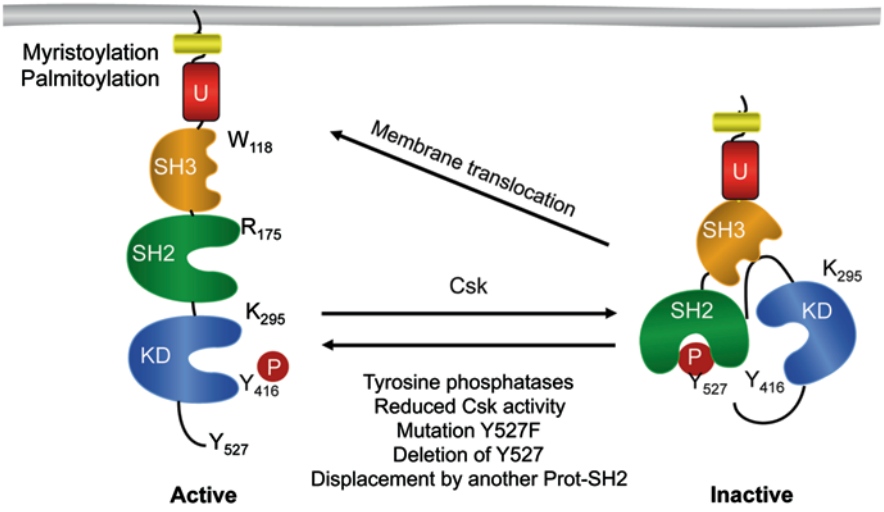
Jak2 is associated and activated by a large number of cytokine receptors [17, 43, 88], and it is considered the major effector for PRLR signaling [76, 81, 82, 86, 100]. Therefore, it is not surprising that conditional knockout mice for Jak2 and PRLR show similarities in their mammary gland phenotypes [92].

Activation of Jak2 by PRLR (Fig. 7.4) occurs by dimerization and the conformational change of the receptor after the ligand binding. This causes the juxtaposition of two Jak2 molecules and their autophosphorylation/transphosphorylation on tyrosine residues Y1007 and Y1008, which are located in the activation loop of the kinase domain [28, 61]. Besides the Y1007 and Y1008, there are other autophosphorylation residues that regulate Jak2 catalytic activity, among them, Y813, between JH1 and JH2 domains, which allows binding of the SH2-Bß adapter molecule. In addition, Y221 in the FERM domain positively modulates Jak2, while Y570 [7, 27, 53], and S523 phosphorylation, between JH3 and JH2 domains, negatively regulate Jak2 enzymatic activity [46, 64].

## 7.3 The Src Family of Tyrosine Kinases

Identification and characterization of Src have been associated with several milestones on molecular and cellular biology. It started more than a century ago when Peyton Rous described “a transmissible avian neoplasm” [79] and is still continuing today. The efforts of many scientists to decipher the structure and function of the Rous Sarcoma Virus (RSV) have given rise to multiple biological concepts, including retrovirus, oncogene, proto-oncogene, Src homology domains SH2 and SH3, later found in many other proteins involved in cellular signaling [74, 91], tyrosine protein kinases, protein tyrosine phosphorylation/dephosphorylation, etc. that have changed our understanding of cell biology as well as oncology [62].

Src is the prototype member of the SFKs, a family of non-receptor tyrosine kinases consisting of nine members (Src, Yes, Fyn, Fgr, Lyn, Hck, Lck, Yrk, and Blk), four of them Src, Yes, Yrk, and Fyn are ubiquitously expressed [91]. The structure of SFKs is modular (Fig. 7.2). At the amino-terminus it contains signals for interactions with fatty acid (myristic and palmitic acids), which allow them to associate with the cellular membrane. The first 40–60 amino acids constitute the unique domain (U, also named as SH4), which is the most dissimilar region among family members, therefore providing specificity. The Src homology domain 3 (SH3) allows intra/intermolecular interactions with proline-rich sequences. The Src




| c-Src mutants | Kinase activity | SH3 | SH2 |
|---|---|---|---|
| SrcK$^-$ : K295M ($ATP_{BS}$) | Inactive | Inactive/Active | Inactive/active |
| SrcDN : K295M/Y527F | Inactive | active | active |
| SrcΔK : Δ A259-L533 | Inactive | active | active |
| Src-SH3$^-$ : W118A | Active | Inactive | Active |
| Src-SH2$^-$ : R175L | Active | Active | Inactive |

**Fig. 7.2** Structure and functionality of the SFKs. The SFKs have a modular structure; the nine members have at amino-terminus sequence an acylation signal, allowing their association with plasma membrane, which is followed by the unique domain (U or SH4), the SH3, SH2, the linker or bridge, the kinase domain (KD, SH1), and a regulatory sequence. The indicated residues, which positions referred to chicken nomenclature, are essential to maintain functionality of the Src family members. The K295 is required for binding to ATP ($ATP_{BS}$) and auto-phosphorylation of Y416 induces maximal enzymatic activity. Phosphorylation of Y527 by CSK facilitates its intramolecular interaction with the SH2 domain. This inactive conformation, stabilized by the contact of the proline residue of the linker/bridge, prevents functionality of the SH2, SH3, and KD. This restrained structure can be released by several mechanisms causing Y527 to be unphosphorylated: displacement by another SH2-containing protein with higher affinity for the p-Y527, mutation of Y57F or deletion of this carboxyl-terminus tail, as occurs in the oncogenic form of SFKs. Furthermore, mutations of W118A and R175L cause functional inactivation of SH3 and SH2 domains, respectively, provoking constitutive activation of KD.

homology domain 2 (SH2) binds to tyrosine phosphorylated proteins. The linker, a connecting sequence between the SH2 and the catalytic domain, contains a proline residue. The catalytic domain (KD or SH1) includes the amino-terminus lobe, which contains the K295 (chicken nomenclature) residue required for ATP-binding and the carboxyl-terminus activation loop. In contrast to the viral proteins (v-Src), the proto-oncogenic (c-Src) has a carboxyl-terminus regulatory tail containing a tyrosine residue (Fig. 7.2) [74, 91].

This conserved modular structure among family members suggests a common regulatory mechanism. Interestingly, tyrosine phosphorylation/dephosphorylation was found to control conversion between inactive and active conformation of SFKs. In this context, Src was identified as a phosphorylated protein of 60 kDa (pp60-c-Src) with tyrosine kinase activity [91]. Two tyrosine residues Y416 and 527 (chicken nomenclature, used from now on) were identified. The Y416 resides in the activation loop of the kinase domain and its autophosphorylation fully activates enzymatic activity. The Y527 is at the carboxyl-terminus regulatory tail that when phosphorylated by the CSK (carboxyl-terminus Src kinase) interacts intramolecularly with the SH2 domain. At the same time, the SH3 domain binds the linker containing a proline residue. Together, these intramolecular interactions induce a closed conformation of the SFKs and restrain their catalytic activity. Under this condition not only the kinase activity is blocked but also the adapter SH2 and SH3 domains are occupied and, therefore, cannot interact with other cellular proteins to form signaling complexes [91] (Fig. 7.2).

The SFKs members can be activated through the interaction of a variety of receptors for growth factors, cytokines, steroid hormones, etc. [16, 26, 31, 65, 68, 74, 87, 91] and transmit intracellular signals upon receptor activations. Also, integrins activate the Fak (focal adhesion kinase)/Src complex, which in turn regulates adhesion, migration, invasion, etc. [66]. Therefore, we can conclude that the SFKs are implicated in the regulation of many cellular signal transduction pathways that control division, motility, adhesion, migration, angiogenesis, survival, differentiation, etc. [29, 35, 91, 102]. It is then not surprising that deregulation of their expression and/or activity is associated with a variety of tumors [35, 90, 97, 104]. However, there is no evidence for Src kinase mutations associated with overexpression and/or hyperactivation of these proto-oncogenes in cancer. It is the action of CSK by phosphorylating the carboxyl-terminus tyrosine residue Y527 and inactivating SFKs [71], or the counteracting protein tyrosine phosphatases de-phosphorylating Y527 and activating SFKs [13, 70], the major players of this game.

## 7.4 Interaction of Prolactin Receptor and SFKs

The first published report about activation of SFKs by PRL described the interaction of PRLR with Fyn in Nb2 cells. In these cells, which express the three major isoforms of the PRLR (short, intermediate, and long forms), Fyn is constitutively associated with all isoforms of PRLR. PRL stimulation of Nb2 causes activation of Fyn. Furthermore, PRLR dimerization appears to be a requisite for Fyn activation [20]. In early 1990s, my colleague J.P. García-Ruiz (Centro de Biología Molecular Severo Ochoa (CBMSO), Madrid) observed a protein of 60 kDa tyrosine-phosphorylated in PRL-stimulated hepatocytes from lactating rat, and in collaboration with her we identified this protein as c-Src (pp60-c-Src). In this biological model, PRL increases association of c-Src to PRLR and its activation [11]. The short form of PRLR is predominant in rat liver [67].

As shown that PRLR interacts with Src and Jak kinases, a question was raised as to what was the interrelationship between these tyrosine kinases and PRLR. Using chick embryo fibroblasts (CEF) that do not express PRLR and the retroviral expression vectors RCAS with different envelope proteins (RCAS-A and RCAS-B) [42], PRLR long isoform from rat ovary [72], Jak2, and Src mutants were expressed in these cells [30].

As a common feature of the cytokine receptor superfamily, the PRLR isoforms contain a proline-rich sequence (PPVPGP) within the cytoplasmic juxtamembrane region named Box I, which is required for Jak2 association and activation by PRL. Therefore, mutations or deletions of these proline residues within Box I prevent binding of Jak2 and receptor functionality [22, 24, 56, 75]. In this context, while PRL stimulation of the wild-type receptor in CEF induces phosphorylation of the receptor, Jak2, and Src, expression of a PRLR mutant with all proline residues of Box I substituted by alanine residues (AAVAGA, $PRLR_{4P\text{-}A}$) inhibits tyrosine phosphorylation of the PRLR and association/activation of Jak2, but does not block Src stimulation. However, expression of Jak2 with the kinase-domain deleted (Jak2ΔK) together with the wild-type PRLR prevents receptor phosphorylation but not SFKs activation, indicating that Jak2 phosphorylates PRLR. Furthermore, a c-Src kinase-defective mutant ($SrcK^{-}$), containing the K295M point mutation, which avoids interaction of SFKs catalytic domain with ATP, does not alter PRLR tyrosine phosphorylation upon PRL stimulation [30].

To further determine the SFKs requirement for interaction with the PRLR, CEF were infected with RCAS-A comprising the wild-type long isoform of the receptor, and RCAS-B, containing different c-Src mutants with deletion of either myristoylation signal (precluding association to the plasma membrane), SH3 (c-SrcΔSH3), SH2 (c-SrcΔSH2), or the kinase domain (c-SrcΔK). The results show that the SH2 or the SH3 domains of Src are not required for interaction with the PRLR. However, the non-myristoylation mutant abrogates PRLR/c-Src interaction, indicating that location of c-Src at the plasma membrane is essential (Fig. 7.3)

These data indicate that the long form of PRLR interacts with both c-Src and Jak2. Then, PRL activates both kinases, but it is Jak2 that phosphorylates the receptor [30] (Fig. 7.4). Depending on the biological system and the physiological conditions, the consequences of these activations could induce proliferation, survival, or differentiation responses. Other receptors also activate SFKs and Jak2, among them receptors for GH, erythropoietin, thrombopoietin, interleukins, etc. [45, 48, 54, 80, 92, 101].

We can conclude that the three major isoforms of PRLR are able to interact and activate SFKs members in different cellular models [1, 2, 11, 18, 20, 30, 31, 33, 87, 77]. However, the short form of PRLR is not able to induce gene transcription of milk proteins [12, 21, 57, 85].

Phosphorylation of PRLR by Jak2 has multiple biological consequences. Thus, phosphorylated tyrosine residues at the carboxyl-terminus region serve as docking sites for Stat5 association and subsequent phosphorylation by Jak2. Indeed, substitution of the last tyrosine residue for phenylalanine (Y/F) results in abrogation of PRL induction of Jak2/Stat5/ß-casein [3, 55, 76].

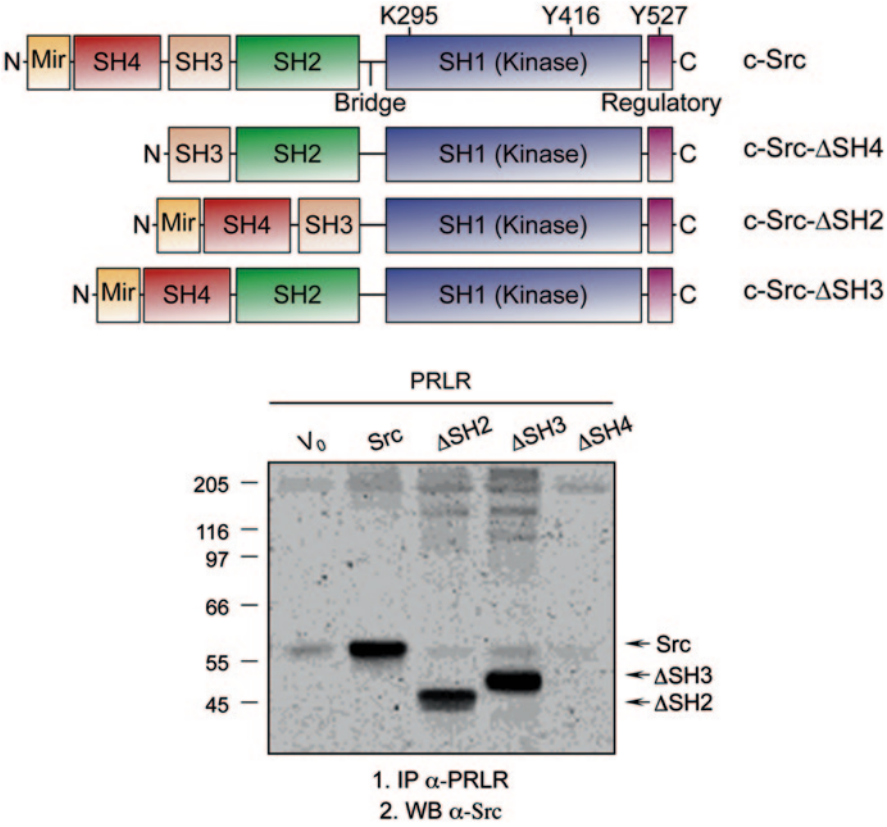



**Fig. 7.3** Interaction between PRLR and SFKs. Chicken embryo fibroblasts (*CEF*) were retrovirally infected to express the long isoform of the PRLR from rat ovary and different forms of c-Src. The association of PRLR and c-Src forms was determined by immunoprecipitation (*IP*) of PRLR followed by western blot (*WB*) detection of Src.

## 7.5 Regulation of Jak2 by SFKs

The association of Src kinases and Jak2 with PRLR and their activation by PRL-induced receptor dimerization brings about the question of whether or not there is a relationship between these two tyrosine kinases.

Interestingly, PP1, a SFKs catalytic activity inhibitor, does not alter PRL-induced PRLR tyrosine phosphorylation and Jak2/Stat5 activation in W53 cells, indicating that SFKs enzymatic activity is not involved in PRL stimulation of the PRLR/Jak2/Stat5 pathway [31]. Similarly, PP1 does not inhibit Jak2 autophosphorylation in T47D [1]. In this context, another SFKs inhibitor, SU6656, also fails to inhibit PRL activation of Jak2 in T47D [2]. As shown in CEF [30], in W53 cells tetracycline-conditional (Tet-On system) expression of $SrcK^{-}$ does not block PRL activation of the Jak2/Stat5 pathway. However, the dominant negative c-Src mutant (SrcDN,

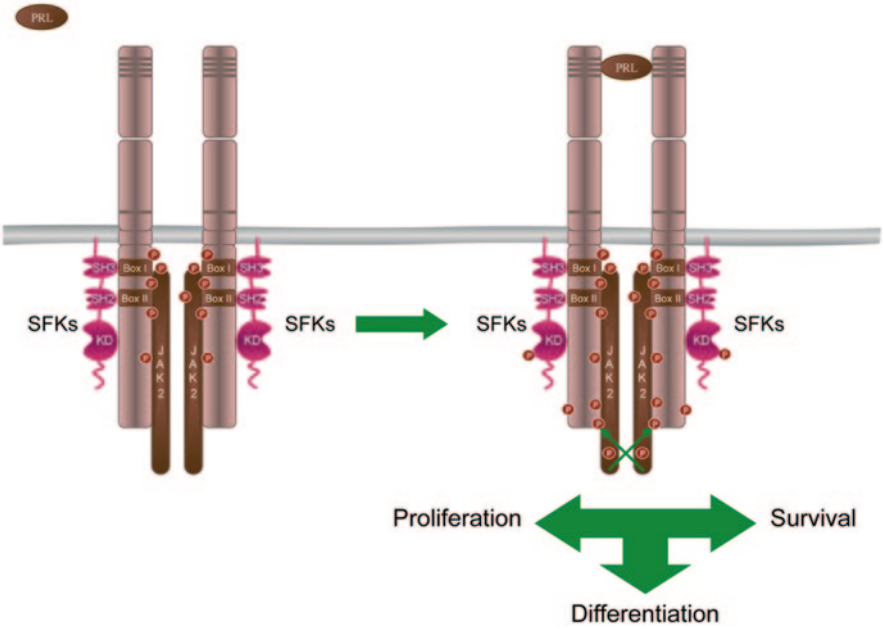



**Fig. 7.4** PRLR dimerization and Jak2 and SFKs activation. Src kinases and Jak2 are associated with PRLR. Upon cellular stimulation with PRL, receptor dimerization takes place and Src kinases and Jak2 activate. Jak2 phosphorylates PRLR under the control of Src kinases. Subsequently, several signaling pathways induce cellular responses that, depending on physiological conditions, could be proliferation, differentiation, survival, etc.

c-Src-K295M/Y527F), devoid of enzymatic activity but with functional SH2 and SH3 domains, inhibits PRL activation of Jak2/Stat5, suggesting that these docking regions of SFKs may control this event. Consistently, expression of SrcΔK, which only contains functional SH2 and SH3 domains, blocks PRL stimulation of the Jak2/Stat5 pathway. However, conditional expression of Jak2ΔK in W53 cells does alter PRL activation of SFKs [33]. Furthermore, in MCF7 cells, conditional expression of SrcDN also abrogates Jak2/Stat5 activation. Moreover, functional inactivation of either the SH2 domain (c-Src-SH2$^{-}$, c-Src-R175L) or the SH3 domain (c-Src-SH3$^{-}$, c-Src-W118A), which consequently have constitutive kinase activity, inhibits Jak2/Stat5 pathway. Together, these results support the conclusion that the function of the SH2 and SH3 adapter domains, independently of the catalytic activity of SFKs controls the Jak2/Stat5 pathway. Besides, constitutive suppression of c-Src in MCF7 cells by means of a specific shRNA significantly represses PRL activation of Jak2/Stat5 pathway. Also, *src*$^{-/-}$ mice appear to have normal mammary gland development during pregnancy but fail the secretory activation. These animals show normal levels of PRL but reduced expression of PRLR at postpartum with diminished activation of Stat5 [95]. In extracts from the mammary gland isolated from *src*$^{-/-}$ mice at lactation, Jak2/Stat5 is impaired as compared to controls from *src*$^{+/+}$ mice [33]. In this context, in mammary epithelial cells isolated from mid-pregnant *Jak2*$^{-/-}$ and

*Cdkn2a*$^{-/-}$ mice PRL induces Src/Fak/Erk1-2 activation [84]. Moreover, stimulation of NE1 and A431 cells by EGF or NDF activates the Jak2/Stat5 pathway, which is blocked by selective inhibitors of SFKs catalytic activity PP1 and CGP77675, as well as by expression of SrcDN. These authors propose that EGF binds EGFR (ErbB1) inducing receptor dimerization and autophosphorylation, which in turn recruits Src that phosphorylates and activates the receptor preassociated Jak2 and Stat5 [73]. CGP77675 directly inhibits Jak2 activity in in vitro kinase assays (unpublished data).

## 7.6 Role of Shp2 in SFKs Regulation of Jak2 Activation

We have been unable to detect direct interaction between SFKs and Jak2 by co-immunoprecipitation assays, which suggests the existence of another interconnecting molecule. In this context, the tyrosine phosphatase Shp2 has been implicated in PDGF-stimulated SFKs activation in fibroblasts [94, 104] and in Jak2 activation by PRL and IL-3 [10, 103]. Shp2 contains two amino-terminus SH2 domains followed by the catalytic domain, and carboxyl-terminuses Y542 and Y580, separated by a proline-rich sequence [69] (Fig. 7.5a). Crystallographic studies of C-terminus-truncated Shp2 demonstrated that its basal catalytic activity is inhibited because of interaction between N-SH2 and phosphatase domains [38]. A role of Y542 and Y580 phosphorylation in the regulation of the phosphatase activity has been suggested. Thus, substitution of these residues by non-hydrolysable phosphonyl amino acids stimulates phosphatase activity, which in combination with mutations of the SH2 domains indicates that the interaction between phosphorylated Y542 and N-SH2 domain releases basal inhibition [59]. Moreover, phosphatase activity is required for all actions of Shp2, including signaling cascades stimulated by growth factors, cell adhesion molecules, and cytokines [3, 69, 78]. However, it remains to be determined if Jak2 and SFKs can regulate Shp2 activity through Y542 and Y580 phosphorylation. This event occurs in sequential fashion [5] inducing conformational changes that promote Shp2 functions [59, 60, 69]. Indeed, conditional expression of Shp2-Y542F-Y580 in W53 cells prevents PRL-induction of Shp2-Y580 phosphorylation (Fig. 7.5b). SFKs functionalities play a role in PRL stimulation of Shp2 phosphorylation. While conditional expression of SrcK$^{-}$, SrcDN, or SrcΔK mutant in W53 cells shows Shp2 colocalized with all c-Src interfering mutants at the plasma membrane (Garcia-Martinez, unpublished data), its phosphorylation is blocked by SrcDN and SrcΔK but not by SrcK$^{-}$ (Fig. 7.6a). Interestingly, the endogenously expressed Src family members Fyn and Lyn, which are the only ones activated by PRL in W53 cells [31], interact with unphosphorylated Shp2 (Garcia-Martinez, unpublished data). Nonetheless, the Fyn-Shp2 and Lyn-Shp2 complexes are reduced upon induction of SrcDN and SrcΔK, presumably because overexpression of Src docking domains facilitates SFKs-Shp2 association (Fig. 7.7a). In this context, interaction of c-Src-SH3 domain and Shp2-proline-rich sequence, demonstrated by pull-down experiments [94], is responsible for stabilization of the active form of

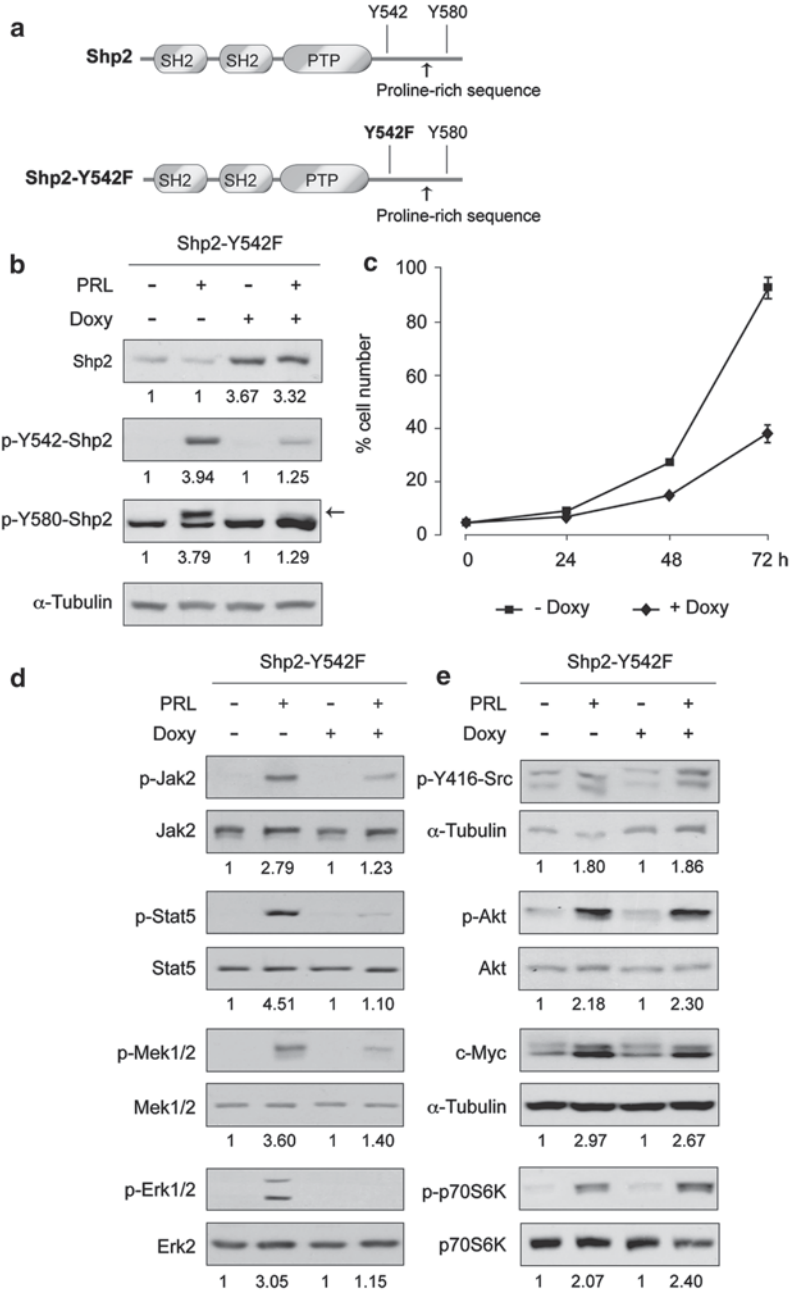



**Fig. 7.5** Role of Y542-Shp2 phosphorylation on PRL stimulation of cell proliferation and signaling. **a** Schematic structure of Shp2 and Shp2-Y542F-Y580 mutant. **b** Exponentially growing cultures of W53 ($5 \times 10^5$ cells/ml) with conditional expression (Tet-On system) of Shp2-Y542F-Y580 were transferred overnight to PRL-depleted media in the absence or presence of Doxy (2 μg/ml).

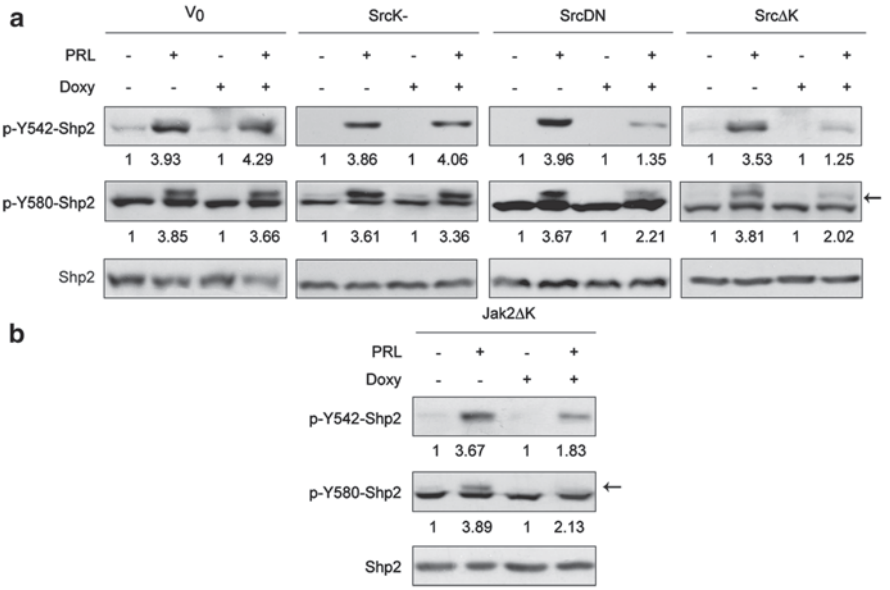



**Fig. 7.6** Role of SFKs and Jak2 kinases on PRL stimulation of Shp2 phosphorylation. **a** Exponentially growing cultures of W53 ($5 \times 10^5$ cells/ml) with conditional expression (Tet-On system) of SrcK$^-$, SrcDN and SrcΔK or empty vector $V_0$ were treated as in Fig. 7.5b. Protein extracts (20 μg) were used to determine p-Y542- and p-Y580-Shp2 by WB with specific phospho-antibodies. Membranes were then reblotted with anti-Shp2 as a loading control. **b** Extracts (20 μg) from exponentially growing cultures ($5 \times 10^5$ cells/ml) of cells with conditional expression of Jak2ΔK were treated as in (**a**) and used to determine the specific phosphorylation sites of Shp2. The results represent one of three independent experiments.

SFKs [37]. Furthermore, SrcDN, SrcΔK, as well as Jak2ΔK and Shp2-Y542F-Y580 reduce Shp2-PRLR complex (Fig. 7.7b). This finding is likely due to inhibition of tyrosine phosphorylation of Shp2 by these interfering mutants (Figs. 7.5b and 7.6), which induce a closed conformation with inaccessible SH2 domains, as previously shown [59, 60, 69]. In addition, these mutants inhibit phosphorylation of PRLR, to which the carboxyl-terminus Shp2 binds [3]. In turn, Shp2 appears to be

Cells were then stimulated with PRL (100 ng/ml, 15 min). Protein extracts (20 μg) were used to determine Shp2, p-Y542-, and p-Y580-Shp2 by WB. The arrow indicates the p-Y580-Shp2 band. The lower band is, according to the company New England Biolabs, nonspecific. **c** To determine proliferation, cells were plated at 3 × 104 cells/ml in complete medium containing 5 ng/ml of PRL, and cultured in the absence or presence of Doxy (2 μg/ml) for 24, 48, and 72 h. Cells were collected by centrifugation, incubated with trypan blue, and viable cells were counted. The percentage of cell growth was calculated considering the number of control cells at 72 h as 100 %. The results shown represent the average ± SD of three independent experiments carried out in triplicate**. d** Detection of p-Jak2, p-Stat5, p-Mek1/2, and p-Erk1/2 by WB was carried out as above. **e** WB detection of p-Y416-c-Src, p-Akt, Myc, and p-p70S6K was carried out as in panel (**b**). The quantifications were normalized by the values of their loading controls and referred to conditions-PRL, considered as 1. The results represent one of three independent experiments.

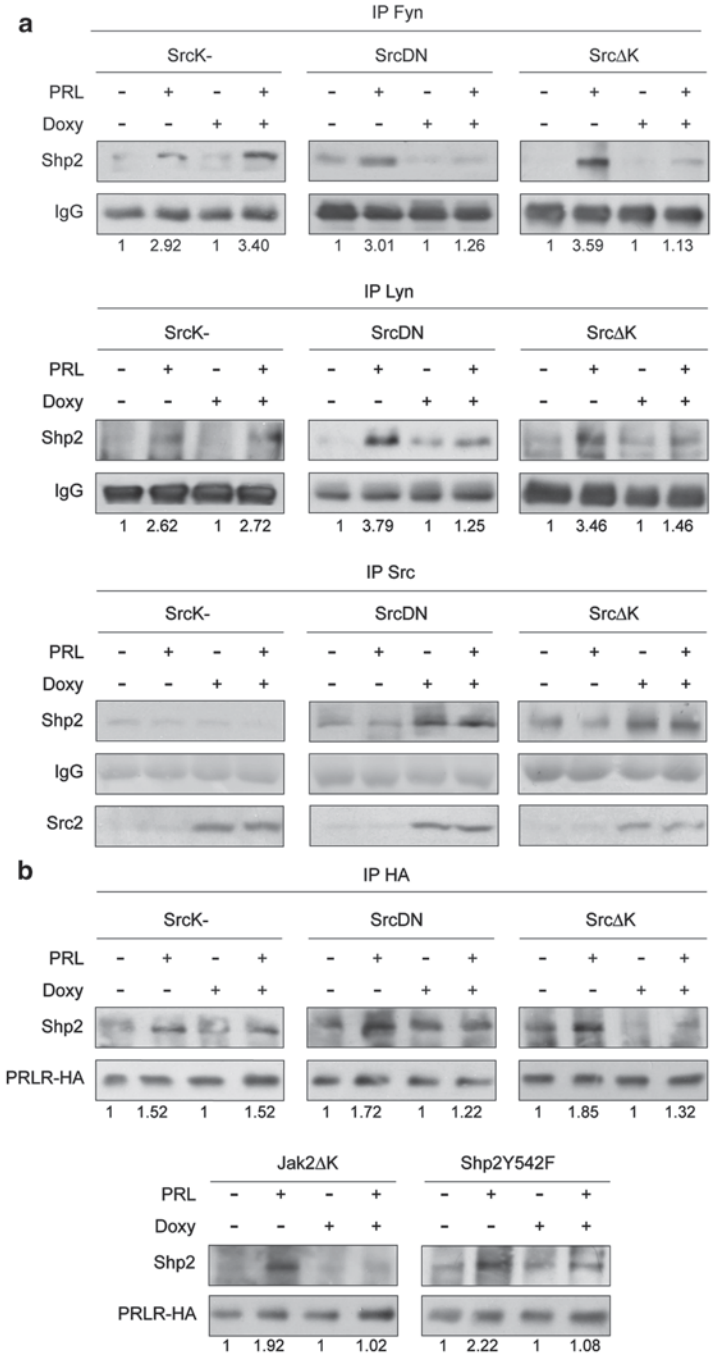



**Fig. 7.7** Interaction of Shp2 with SFKs and PRLR. Exponentially growing cultures of W53 ($5 \times 10^5$ cells/ml) were treated as in Fig. 7.5b. **a**, **b** Clear cell lysates were used to immunoprecipitate Fyn, Lyn, or c-Src mutants (**a**) or the PRLR HA-tagged (**b**). Immune-complexes were used to

involved in Jak2 activation, as Shp2-Y542F-Y580 blocks PRL activation of Jak2/Stat5 (Fig. 7.5d). In this context, Shp2 by dephosphorylating Jak2 Y1007 impedes SOCS-1 association and ubiquitination, which ultimately leads to Jak2 stabilization [4]. This reciprocal modulation between Shp2 and Jak2/Stat5 is also observed in mice with selective suppression of Shp2 in the mammary gland, where PRL stimulation of PRLR-Jak2/Stat5 complex and the subsequent activation of Jak2/Stat5 are impaired [50]. Similarly, Shp2 is required for physical association/activation of Stat5a and milk protein gene transcription in PRL-stimulated HC11, a nontumorigenic mouse mammary epithelial cell line [8], and in GH-treated T47D [19]. In addition, in MCF7 cells, expression of SrcDN or c-Src depletion inhibits PRL-induced Jak2 and Shp2 phosphorylation (Fig. 7.8) [33]. Interestingly, in this breast cancer cell line SFKs catalytic activity appears to be involved in PRL regulation of Shp2 association and dephosphorylation of IGF-IR, which blocks its internalization maintaining its activation [18]. In contrast, suppression of Shp2 in T47D does not prevent PRL activation of Jak2/Stat5 [49]. Furthermore, we cannot exclude the possibility that Shp2 may have different functions while interacting with either SFKs or Jak2, since the proline-rich sequence and the tyrosine-phosphorylated residues could also serve as docking as shown for Grb2 [5] and Gab2 [3].

Together, these findings indicate that SFK-scaffold functions regulate Shp2 activation in PRL-stimulated W53 and MCF7 cells. Considering these results and those published by others we propose a model in which PRL causes receptor dimerization and activation of Jak2, Fyn, and Lyn. These activated SFKs bind to unphosphorylated Shp2. At the same time, Jak2 phosphorylates PRLR, which favors Shp2–SFKs complex dissociation due to the interaction of SH2 domains of Shp2 with the phosphorylated PRLR. In turn, Jak2 sequentially phosphorylates Shp2 at Y542 and Y580, as proposed [58, 63]. Alternatively, another kinase may be involved in Shp2 phosphorylation. The phosphorylated/activated Shp2 then controls Jak2 activation and also serves as docking site for Grb2 and Gab2 [3, 5] (Fig. 7.9).

## 7.7 Role of SFKs in PRL Induction of Cell Proliferation

PRL can induce survival, proliferation, or differentiation depending on the cell type, the physiological condition, and the stimuli. Cell survival as a result of PRL-induced SFKs activation has been observed in a variety of cell systems, including W53, MCF7, T47D, etc.[1, 2, 23, 36].

Several pieces of evidence support the role of SFKs in mammary gland development and lactogenesis. In HC11, expression of SrcK$^-$ inhibits ß-casein production

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detect Shp2, c-Src mutants, or PRLR by WB. Detection of immunoglobulins and anti-HA from immunoprecipitation served as loading controls. Values were normalized as in Fig. 7.5. The results represent one of three independent experiments.

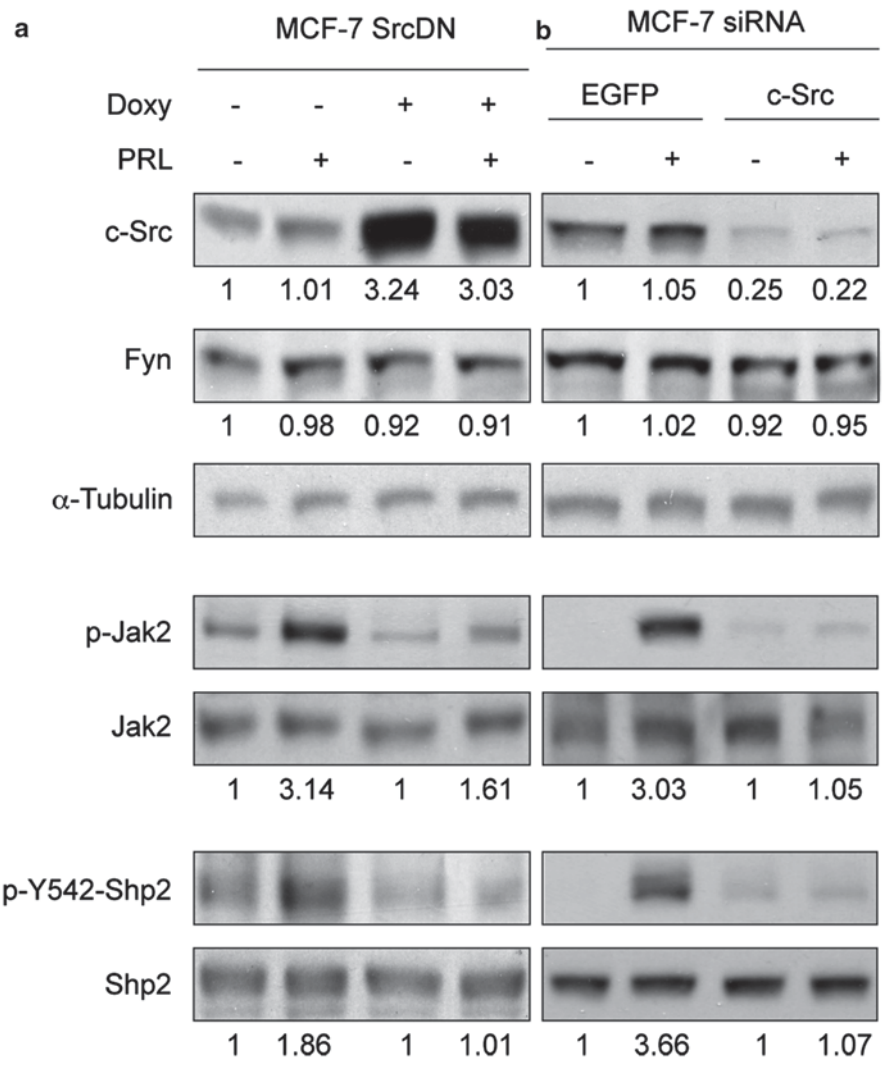


**Fig. 7.8** Role of c-Src in PRL stimulation of Jak2 and Shp2-Y542 phosphorylation in MCF7. **a**, **b** Exponentially growing cell cultures of MCF7-Tet-On-SrcDN (**a**) and of c-Src-siRNA-MCF7 and EGFP-siRNA-MCF7, as a control (**b**), were transferred for 48 h to serum-free media for starvation. The MCF7-Tet-On-SrcDN cultures were maintained during this time in absence or presence of Doxy (2 μg/ml). Cell cultures were then stimulated with PRL (100 ng/ml, 15 min). Protein extracts (20 μg) were used to determine the levels of c-Src, Fyn, and Jak2 and Shp2-Y542 phosphorylation by WB. Membranes were reblotted with α-tubulin, anti-Jak2, and anti-Shp2 for loading controls. The quantified values were normalized to their loading controls and referred to condition-PRL, considered as 1.

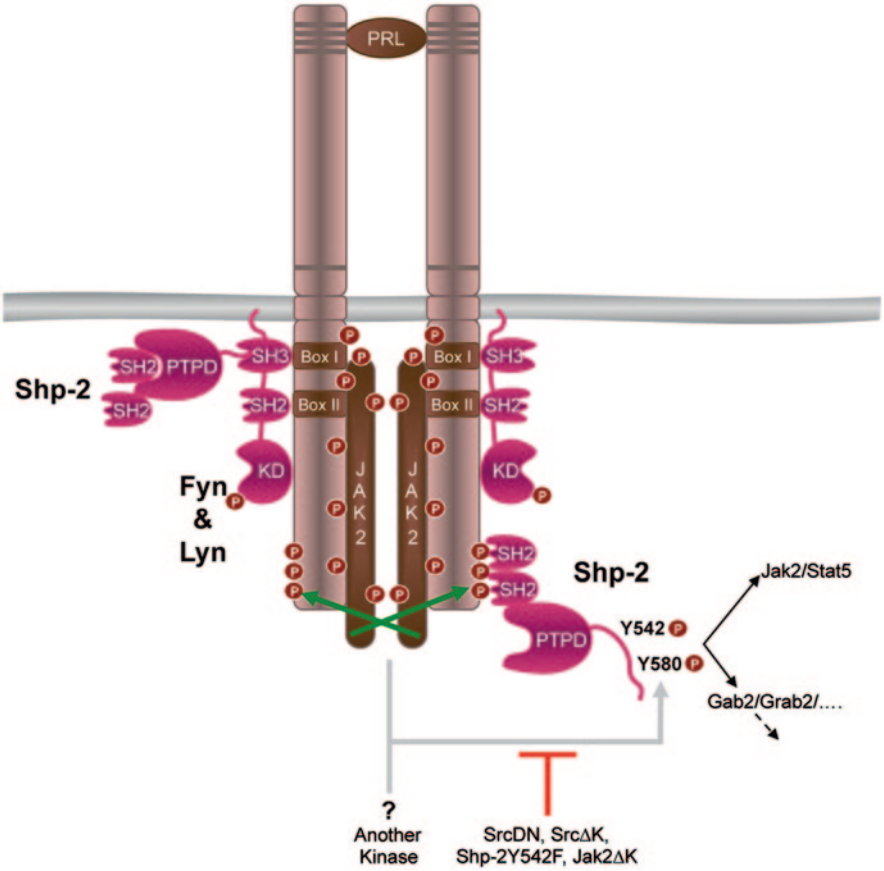


**Fig. 7.9** Complex interaction between SFKs, Jak2, and Shp2. The interaction PRL-PRLR causes activation of Fyn, Lyn and Jak2 and phosphorylation of PRLR. Shp2 associates through its proline-rich region with the SH3-SFKs. Upon tyrosine phosphorylation of PRLR, Shp2 forms a complex with the receptor via its carboxyl-terminus SH2, facilitating Y542 and Y580 phosphorylation by Jak2 or another kinase. These phosphorylations in Shp2 eliminate its restricted conformation. Shp2 then positively regulates Jak2/Stat5 and serves as docking molecule to form complexes with Gab2, Grab2, etc., diversifying signaling pathways.

induced by the lactogenic complex (PRL, insulin, hydrocortisone) [89]; we have also observed this effect by conditional expression of SrcDN (unpublished results). However, overexpression of viral Src blocks mammary gland epithelial cell differentiation, inhibiting ß-casein gene expression [47]. Deletion of c-Src in mice causes defects in development and lactation [32, 52, 95] and prevents PRL activation of Jak2/Stat5 in mammary gland explants [33]. In pancreatic ß-cells, inhibition of SFKs catalytic activity by PP2 completely abolishes the increase of intracellular [$Ca^{2+}$] and insulin secretion induced by ovine PRL [106].

SFKs are also required for PRL stimulation of cell proliferation. Multiple pieces of evidence support this premise. In hepatocytes from lactating rats, PRL activates c-Src

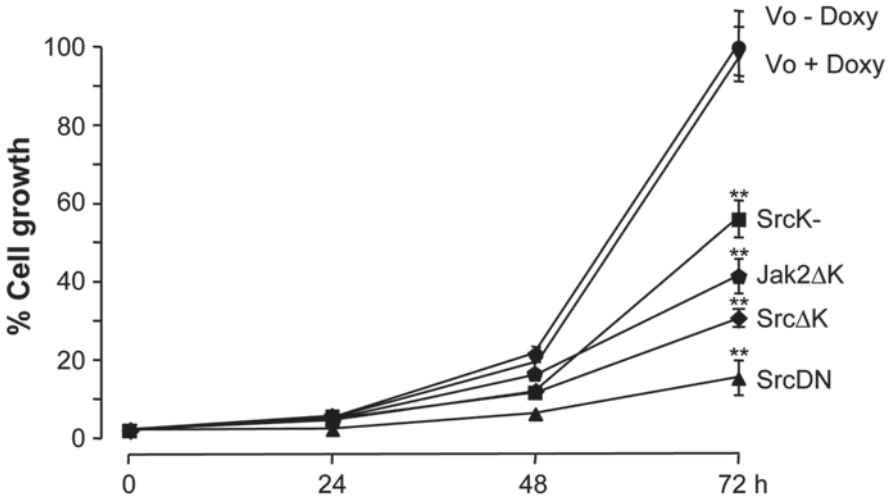


**Fig. 7.10.** Role of Src kinases and Jak2 in PRL stimulation of cell proliferation. W53 cells conditionally expressing c-Src mutants or Jak2ΔK were plated in complete medium containing 5 ng/ml of PRL, and cultured with or without Doxy (2 μg/ml) for 24, 48, and 72 h. Cells were collected, incubated with trypan blue and viable cells were counted. The percentage of cell growth was calculated considering the number of control cells (Vo, empty vector) at 72 h as 100 %. The results represent the average ± SD of three independent experiments carried out in triplicate. $^{**}p \leq 0.01$ ($n=3$). [33].

and induces expression of c-*fos* and c-*jun* [11]; in Nb2 cells PRL stimulation of Fyn matches proliferation [20]. Different and complementary approaches, such us the use of inhibitors of the SFKs catalytic activity or conditional expression of c-Src mutants, later confirmed the involvement of SFKs in cell proliferation control by PRL.

The inhibitors of SFKs catalytic activity PP1 and herbimycin A abrogate [$^3$H]-thymidine incorporation in PRL-stimulated W53. Furthermore, BrdU pulse-label experiments show that in these cells PP1 blocks cell cycle in G1, indicating that the tyrosine kinase activity of SFKs is required for the G1/S transition [30]. PP1 and PP2 also reduce cell proliferation of MCF7 and T47D induced by PRL [1, 23], while SU6656, another SFKs inhibitor, blocks PRL signaling pathways required for MCF7 and T47D proliferation [2, 36].

Furthermore, transient expression of PRLR together with Csk or with c-SrcK$^-$ in BaF-3 cells partially prevents PRL-induced [$^3$H]-thymidine incorporation. SrcDN and Jak2ΔK inhibit more effectively PRL-induced cell proliferation than Csk or c-SrcK$^-$ [31]. Supporting these observations, conditional expression of c-SrcK$^-$, c-SrcDN, c-SrcΔK, or Jak2ΔK in W53 cells causes analogous results [33] (Fig. 7.10). The strongest inhibition of cell proliferation observed with SrcDN could be explained by the fact that, in addition to being a kinase-dead mutant, it also has an open conformation that exposes its SH2 and SH3 domains. Indeed, SrcΔK that only expresses fully active SH2 and SH3 causes similar effect. These findings suggest the involvement of SH2 and/or SH3 domains of SFKs in the control of PRL-induced cell proliferation. In addition, these results also indirectly support the dependency

of Jak2 activation on the SFKs, as the inhibitory effect of Jak2ΔK is similar to that obtained by SrcDN or SrcΔK. Moreover, SrcDN blocks PRL activation of Jak2 in W53 and MCF7 cells [33].

## 7.8 Role of SFKs-depending Signaling Pathways in the Regulation of IEG Expression

Cell cycle progression through the G1 phase is a prerequisite for DNA replication (S phase) and cell division. The immediate early genes (IEG) expression is required for G1/S transition. Since inhibition of SFKs blocks W53 cell cycle at G1 [31], it is important to study the signaling pathways controlling IEG expression to understand the involvement of SFKs in the control of cell proliferation by PRL.

In hepatocytes from lactating rats, PRL activates c-Src and induces expression of c-*fos* and c-*jun* [11]. PRL stimulation of W53, MCF7, and T47D proliferation induces expression of IEG c-*fos*, c-*jun*, and cyclin D1, which in turn controls c-Myc expression. The cellular levels of these essential factors are controlled, at least in part, by catalytic activity of SFKs, as their expression is reduced by PP1/PP2/SU6656 [1, 2, 23, 31].

Also, conditional expression of SrcK$^{-}$, SrcDN, and Src-ΔK in W53 cells significantly reduces c-Myc expression [33]. Induction of c-*myc* expression by PRL depends on the SFKs/PI3K/Akt pathway. Upon phosphorylation/activation, Akt translocates to the nucleus where it phosphorylates FKHRL1 and induces nuclear export of this transcription factor that represses c-Myc transcription. Akt also phosphorylates and inactivates GSK3ß, which in turn abrogates Myc phosphorylation and its subsequent degradation. Consequently, these mechanisms stabilize c-Myc expression. Interestingly, while in W53 cells one can delineate a SFKs/Akt/FKHRL1 and GSK3ß pathway for the control of Myc expression, which is not altered by conditional expression of Jak2ΔK [23, 33], studies in mammary epithelial cells isolated from conditional Jak2$^{-/-}$ mice indicate that Jak2 controls Akt activation and cyclin D1 expression [84].

Expression of c-*fos* in W53 cells does not require the SFKs/PI3K dependent pathway, as it is not affected by LY294002. Instead, it is regulated by Mek1/2-Erk1/2 pathway as PRL induction of c-*fos* is abrogated by PD184352, an inhibitor of Mek1/2 activity [23]. Since Jak2ΔK prevents Mek1/2-Erk1/2 activation and SFKs regulates Jak2, the SFKs/Jak2/Mek1/2-Ekr1/2 pathway appears to control c-*fos* expression [33]. Furthermore, Shp2 association with SFKs, tyrosine phosphorylated PRLR, and Jak2 ends in Shp2 phosphorylation at Y542 and Y580. Phosphorylated Y542-Shp2 in response to FGF or PDGF serves as docking site for Grb2 that is required for Ekr1/2 activation [5]. Then Shp2 may be mediated through its interaction with Grb2 in PRL induction of c-*fos*.

Analyses of the signaling cascades that control IEG expression below SFKs show that PRL induction of cell proliferation involves different mechanisms depending

on the cell type. Thus, in MCF7 and T47D induction of c-Myc and cyclin D1 by PRL requires Src activation of the PI3K and Mek1/2-Erk1/2 pathways, as shown by inhibition of these pathways by PP1, LY294002, and PD184352, respectively. The control of PI3K and the Mek1/2-Erk1/2 pathways by SFKs appears to be independent one from each other, as inhibition of PI3K by LY294002 does not alter activation of the Mek1/2-Erk1/2 and vice versa [1]. Also, in MCF7, PRL induction of AP1 is controlled by c-Src, Jak2, PI3K, and PKC through the control of Erk1/2 activation. However, in this study there is no hierarchical analysis among these pathways for Erk1/2-AP1 activation, with Jak2 seeming to be the major mediator [36]. Moreover, inhibition of SFKs enzymatic activity by SU6656 blocks PRL stimulation of Fak-Y925, PI3K/Akt, Mek1/2-Erk1/2 in MCF7 and T47D without affecting Jak2 phosphorylation [2], which is consistent with previous results obtained with PP1/PP2 [1].

## 7.9 Concluding Remarks

The data obtained in several biological models undoubtedly demonstrate the fundamental role of the SFKs and Jak2 for PRL signaling. These tyrosine kinases are preassociated with the three major isoforms of the PRLR and are activated upon receptor dimerization induced by ligand binding. Within the receptor juxtamembrane region there is a proline-rich sequence named Box I, which is present in most of the cytokine receptor superfamily. Multiple and undisputable evidence show that this is the domain where Jak2 binds to the receptor. It is also clear that Jak2 is mainly responsible for the tyrosine phosphorylation of the PRLR. As to where and how the receptor interacts with the SFKs there are no complete answers. Mutations at the proline residues of Box I of PRLR, which prevent binding and activation of Jak2, and consequently receptor phosphorylation, do not affect PRLR binding and activation of SFKs. Moreover, catalytic inactivating mutations of Jak2 reproduce the same effect. These data imply that the SFKs SH2 or SH3 domains may not be required for interaction with the PRLR. Indeed, deletion of c-Src SH2 or SH3 domains does not preclude binding to PRLR. Furthermore, the catalytic activity and the kinase domain are also not needed. However, it requires membrane location of c-Src. In this context, it will be important to precisely determine the structural requirements of the PRLR and the SFKs to interact with each other.

While SFKs and Jak2 appear to be needed for many of the biological actions of PRL, several pieces of evidence support the fact that inhibition of the enzymatic activity of one of them does not affect the other in PRL signaling. However, suppression of c-Src alters PRL activation of Jak2. Furthermore, the adapter domains SH2 and/or SH3 seem to be necessary for Jak2 activation. While both SFKs and Jak2 bind and are activated by PRL, there is no clear evidence for direct interaction between them. Considering the results obtained by several groups and our own data, we propose that the tyrosine phosphatase Shp2 is the nexus between both enzymes,

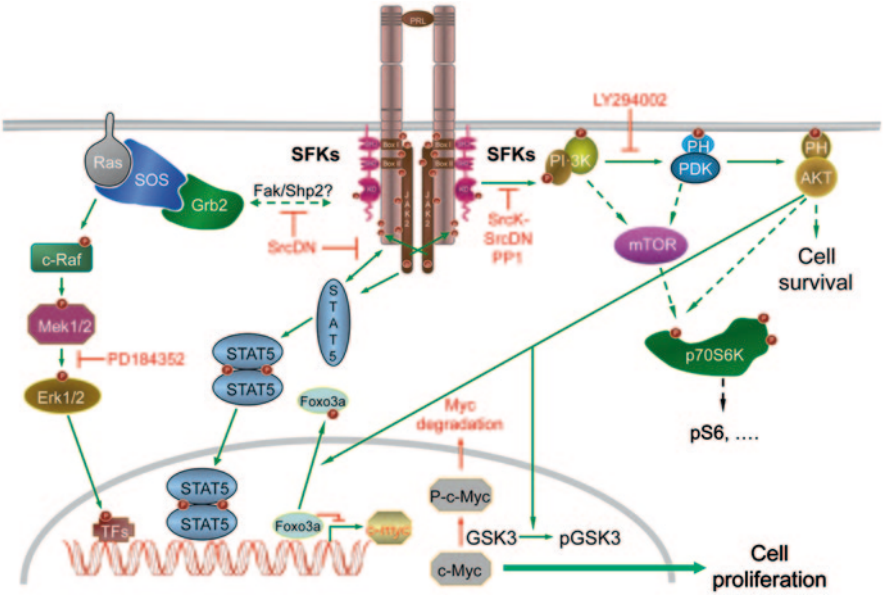


**Fig. 7.11** Role of SFKs in PRL cellular signaling. PRL-induced PRLR dimerization causes activation of SFKs and Jak2, which in turns phosphorylates PRLR, in addition to the regulation of Shp2, which is involved in the control of Jak2 activation by SFKs (see Fig. 7.9). SFKs, through its tyrosine kinase activity and the adapter domains SH2 and SH3, regulate several signaling pathways, as deciphered by conditional expression of c-Src, Fak, Jak2, Shp2, Akt, and FKHRL1, as well as by the use of selective inhibitors of SFKs, IP3K, Mek1/2, and GSK3ß. The results obtained in several cell types [1, 11, 23, 30, 31, 33] allows us to delineate this incomplete schema, which describes some of the signaling cascades that control PRL signaling through the SFKs.

playing a role in the regulation of Jak2 by SFKs and subsequent regulation of some signaling pathways.

Integration of the results obtained in our multiple experimental systems led us to propose this complex signaling model for PRL (Fig. 7.11).

A variety of cytokines and growth factors through activation of their membrane receptors activate the SFKs, which in turn act as connecting networks controlling multiple signaling pathways eliciting different cellular responses depending on the biological model and the physiological conditions.

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## References


1. Acosta JJ, Munoz RM, Gonzalez L, Subtil-Rodriguez A, Dominguez-Caceres MA, Garcia-Martinez JM, Calcabrini A, Lazaro-Trueba I, Martín-Pérez J (2003) Src mediates PRL-dependent proliferation of T47D and MCF7 cells via the activation of Fak/Erk1/2 and PI3K pathways. Mol Endocrinol 17(11):2268–2282
2. Aksamitiene E, Achanta S, Kolch W, Kholodenko BN, Hoek JB, Kiyatkin A (2011) Prolactin-stimulated activation of ERK1/2 mitogen-activated protein kinases is controlled by PI3-kinase/Rac/PAK signaling pathway in breast c ancer cells. Cell Signal 23 (11):1794–1805
3. Ali S, Ali S (2000) Recruitment of the protein-tyrosine phosphatase SHP-2 to the C-terminal tyrosine of the prolactin receptor and to the adaptor protein Gab2. J Biol Chem 275(50):39073–39080
4. Ali S, Nouhi Z, Chughtai N (2003) SHP-2 Regulates SOCS-1-mediated Janus Kinase-2 Ubiquitination/Degradation downstream of the prolactin receptor. J Biol Chem 278(52):52021–52031
5. Araki T, Nawa H, Neel BG (2003) Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. J Biol Chem 278(43):41677–41684
6. Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, Carter-Su C (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74(2):237–244
7. Argetsinger LS, Kouadio J-LK, Steen H, Stensballe A, Jensen ON, Carter-Su C (2004) Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity. Mol Cell Biol 24(11):4955–4967
8. Ball RK, Friis RR, Schoenenberger CA, Doppler W, Groner B (1988) Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J 7(7):2089–2095
9. Bazan JF (1990) Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci U S A 87(18):6934–6938
10. Berchtold S, Volarevic S, Moriggl R, Mercep M, Groner B (1998) Dominant negative variants of the SHP-2 tyrosine phosphatase inhibit prolactin activation of Jak2 (janus kinase 2) and induction of Stat5 (signal transducer and activator of transcription 5)-dependent transcription. Mol Endocrinol 12(4):556–567
11. Berlanga JJ, Fresno Vara JA, Martín-Pérez J, Garcia Ruiz JP (1995) Prolactin receptor is associated with c-src kinase in rat liver. Mol Endocrinol 9(11):1461–1467
12. Berlanga JJ, Garcia RJ, Perrot AM, Kelly PA, Edery M (1997) The short form of the prolactin (PRL) receptor silences PRL induction of the beta-casein gene promoter. Mol Endocrinol 11(10):1449–1457
13. Berman-Golan D, Granot-Attas S, Elson A (2008) Protein tyrosine phosphatase epsilon and Neu-induced mammary tumorigenesis. Cancer Metastasis Rev 27(2):193–203
14. Bignon C, Daniel N, Belair L, Djiane J (1999) In vitro expression of long and short ovine prolactin receptors: activation of Jak2/STAT5 pathway is not sufficient to account for prolactin signal transduction to the ovine beta-lactoglobulin gene promoter. J Mol Endocrinol 23(2):125–136
15. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19(3):225–268
16. Bromann PA, Korkaya H, Courtneidge SA (2004) The interplay between Src family kinases and receptor tyrosine kinases. Oncogene 23(48):7957–7968
17. Campbell GS, Argetsinger LS, Ihle JN, Kelly PA, Rillema JA, Carter-Su C (1994) Activation of JAK2 tyrosine kinase by prolactin receptors in Nb2 cells and mouse mammary gland explants. Proc Natl Acad Sci U S A 91(12):5232–5236

18. Carver KC, Piazza TM, Schuler LA (2010) Prolactin enhances insulin-like growth factor I receptor phosphorylation by decreasing its association with the tyrosine phosphatase SHP-2 in MCF-7 breast cancer cells. J Biol Chem 285(11):8003–8012
19. Chughtai N, Schimchowitsch S, Lebrun JJ, Ali S (2002) Prolactin Induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta -casein gene promoter in mammary cells. J Biol Chem 277(34):31107–31114
20. Clevenger CV, Medaglia MV (1994) The protein tyrosine kinase P59fyn is associated with prolactin (PRL) receptor and is activated by PRL stimulation of T-lymphocytes. Mol Endocrinol 8(6):674–681
21. Das R, Vonderhaar BK (1995) Transduction of prolactin's (PRL) growth signal through both long and short forms of the PRL receptor. Mol Endocrinol 9(12):1750–1759
22. DaSilva L, Howard OM, Rui H, Kirken RA, Farrar WL (1994) Growth signaling and JAK2 association mediated by membrane-proximal cytoplasmic regions of prolactin receptors. J Biol Chem 269(28):18267–18270
23. Dominguez-Caceres MA, Garcia-Martinez JM, Calcabrini A, Gonzalez L, Porque PG, Leon J, Martin-Perez J (2004) Prolactin induces c-Myc expression and cell survival through activation of Src/Akt pathway in lymphoid cells. Oncogene 23(44):7378–7390
24. Dusanter-Fourt I, Muller O, Ziemiecki A, Mayeux P, Drucker B, Djiane J, Wilks A, Harpur AG, Fischer S, Gisselbrecht S (1994) Identification of JAK protein tyrosine kinases as signaling molecules for prolactin. Functional analysis of prolactin receptor and prolactin- erythropoietin receptor chimera expressed in lymphoid cells. EMBO J 13(11):2583–2591
25. Edery M, Levi Meyrueis C, Paly J, Kelly PA, Djiane J (1994) A limited cytoplasmic region of the prolactin receptor critical for signal transduction. Mol Cell Endocrinol 102(1–2):39–44
26. Faivre EJ, Lange CA (2007) Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Mol Cell Biol 27(2):466–480
27. Feener EP, Rosario F, Dunn SL, Stancheva Z, Myers MG Jr (2004) Tyrosine Phosphorylation of Jak2 in the JH2 domain inhibits cytokine signaling. Mol Cell Biol 24(11):4968–4978
28. Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM, Ihle JN (1997) Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol Cell Biol 17(5):2497–2501
29. Frame MC, Fincham VJ, Carragher NO, Wyke JA (2002) v-Src's hold over actin and cell adhesions. Nat Rev Mol Cell Biol 3(4):233–245
30. Fresno Vara J, Carretero MV, Gerónimo H, Ballmer-Hofer K, Martín-Pérez J (2000) Stimulation of c-Src by prolactin is independent of Jak2. Biochem J 345(Pt 1):17–24
31. Fresno Vara JA, Dominguez Caceres MA, Silva A, Martín-Pérez J (2001) Src family kinases are required for prolactin induction of cell proliferation. Mol Biol Cell 12(7):2171–2183
32. Furth PA, Nakles RE, Millman S, Diaz-Cruz ES, Cabrera MC (2011) Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer. Breast Cancer Res 13(5):220
33. Garcia-Martinez JM, Calcabrini A, Gonzalez L, Martin-Forero E, Agullo-Ortuno MT, Simon V, Watkin H, Anderson SM, Roche S, Martin-Perez J (2010) A non-catalytic function of the Src family tyrosine kinases controls prolactin-induced Jak2 signaling. Cell Signal 22(3):415–426
34. Gouilleux F, Wakao H, Mundt M, Groner B (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J 13(18):4361–4369
35. Guarino M (2010) Src signaling in cancer invasion. J Cell Physiol 223(1):14–26
36. Gutzman JH, Rugowski DE, Schroeder MD, Watters JJ, Schuler LA (2004) Multiple kinase cascades mediate prolactin signals to activating protein-1 in breast cancer cells. Mol Endocrinol 18(12):3064–3075
37. Harrison SC (2003) Variation on an Src-like theme. Cell 112(6):737–740
38. Hof P, Pluskey S, Dhe-Paganon S, Eck MJ, Shoelson SE (1998) Crystal structure of the tyrosine phosphatase SHP-2. Cell 92(4):441–450

39. Hofmann SR, Lam AQ, Frank S, Zhou Y-J, Ramos HL, Kanno Y, Agnello D, Youle RJ, O'Shea JJ (2004) Jak3-independent trafficking of the common {gamma} chain receptor subunit: chaperone function of Jaks revisited. Mol Cell Biol 24(11):5039–5049
40. Horseman ND, Yu-Lee L (1994) Transcriptional regulation by the helix bundle peptide hormones: growth hormone, prolactin, and hematopoietic cytokines. Endocr Rev 15(5):627–649
41. Huang LJ-s, Constantinescu SN, Lodish HF (2001) The N-terminal domain of Janus kinase 2 Is required for Golgi processing and cell surface expression of erythropoietin receptor. Molecular Cell 8(6):1327–1338
42. Hughes SH, Greenhouse JJ, Petropoulos CJ, Sutrave P (1987) Adaptor plasmids simplify the insertion of foreign DNA into helper- independent retroviral vectors. J Virol 61(10):3004–3012
43. Ihle JN (1995) Cytokine receptor signalling. Nature 377(6550):591–594
44. Ihle JN (2001) The Stat family in cytokine signaling. Curr Opin Cell Biol 13(2):211–217
45. Ingley E, Klinken SP (2006) Cross-regulation of JAK and Src kinases. Growth Factors 24(1):89–95
46. Ishida-Takahashi R, Rosario F, Gong Y, Kopp K, Stancheva Z, Chen X, Feener EP, Myers MG Jr (2006) Phosphorylation of Jak2 on Ser523 Inhibits Jak2-dependent leptin receptor signaling. Mol Cell Biol 26(11):4063–4073
47. Jehn B, Costello E, Marti A, Keon N, Deane R, Li F, Friis RR, Burri PH, Martin F, Jaggi R (1992) Overexpression of Mos, Ras, Src, and Fos inhibits mouse mammary epithelial cell differentiation. Mol Cell Biol 12(9):3890–3902
48. Jin H, Lanning NJ, Carter-Su C (2008) JAK2, But Not Src family kinases, is required for STAT, ERK, and Akt signaling in response to growth hormone in preadipocytes and hepatoma cells. Mol Endocrinol 22(8):1825–1841
49. Johnson KJ, Peck AR, Liu C, Tran TH, Utama FE, Sjolund AB, Schaber JD, Witkiewicz AK, Rui H (2010) PTP1B suppresses prolactin activation of Stat5 in breast cancer cells. Am J Pathol 177(6):2971–2983
50. Ke Y, Lesperance J, Zhang EE, Bard-Chapeau EA, Oshima RG, Muller WJ, Feng GS (2006) Conditional deletion of Shp2 in the mammary gland leads to impaired lobulo-alveolar outgrowth and attenuated Stat5 activation. J Biol Chem 281(45):34374–34380
51. Kelly PA, Ali S, Rozakis M, Goujon L, Nagano M, Pellegrini I, Gould D, Djiane J, Edery M, Finidori J, Postel-Vinay MC (1993) The growth hormone/prolactin receptor family. Recent Prog Horm Res 48:123–164
52. Kim H, Laing M, Muller W (2005) c-Src-null mice exhibit defects in normal mammary gland development and ERalpha signaling. Oncogene 24(36):5629–5636
53. Kurzer JH, Argetsinger LS, Zhou Y-J, Kouadio J-LK, O'Shea JJ, Carter-Su C (2004) Tyrosine 813 Is a Site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-B{beta}. Mol Cell Biol 24(10):4557–4570
54. Lannutti BJ, Drachman JG (2004) Lyn tyrosine kinase regulates thrombopoietin-induced proliferation of hematopoietic cell lines and primary megakaryocytic progenitors. Blood 103(10):3736–3743
55. Lebrun JJ, Ali S, Goffin V, Ullrich A, Kelly PA (1995a) A single phosphotyrosine residue of the prolactin receptor is responsible for activation of gene transcription. Proc Natl Acad Sci U S A 92(9):4031–4035
56. Lebrun JJ, Ali S, Ullrich A, Kelly PA (1995b) Proline-rich sequence-mediated Jak2 association to the prolactin receptor is required but not sufficient for signal transduction. J Biol Chem 270(18):10664–10670
57. Lesueur L, Edery M, Ali S, Paly J, Kelly PA, Djiane J (1991) Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein gene transcription. Proc Natl Acad Sci U S A 88(3):824–828
58. Liu CB, Itoh T, Arai K, Watanabe S (1999) Constitutive activation of JAK2 confers murine interleukin-3-independent survival and proliferation of BA/F3 cells. J Biol Chem 274(10):6342–6349

59. Lu W, Gong D, Bar-Sagi D, Cole PA (2001) Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling. Mol Cell 8(4):759–769
60. Lu W, Shen K, Cole PA (2003) Chemical dissection of the effects of tyrosine phosphorylation of SHP-2. Biochemistry 42(18):5461–5468
61. Luo H, Rose P, Barber D, Hanratty WP, Lee S, Roberts TM, D'Andrea AD, Dearolf CR (1997) Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways. Mol Cell Biol 17(3):1562–1571
62. Martin GS (2001) The hunting of the Src. Nat Rev Mol Cell Biol 2(6):467–475
63. Matsumiya T, Imaizumi T, Itaya H, Shibata T, Yoshida H, Sakaki H, Kimura H, Satoh K (2002) Production of growth related oncogene protein-alpha in human umbilical vein endothelial cells stimulated with soluble interleukin-6 receptor-alpha: role of signal transducers, janus kinase 2 and mitogen-activated kinase kinase. Life Sci 70(26):3179–3190
64. Mazurkiewicz-Munoz AM, Argetsinger LS, Kouadio J-LK, Stensballe A, Jensen ON, Cline JM, Carter-Su C (2006) Phosphorylation of JAK2 at Serine 523: a negative regulator of JAK2 that is stimulated by growth hormone and epidermal growth factor. Mol Cell Biol 26(11):4052–4062
65. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Bottero D, Varricchio L, Nanayakkara M, Rotondi A, Auricchio F (2002) Sex steroid hormones act as growth factors. J Steroid Biochem Mol Biol 83(1–5):31–35
66. Mitra SK, Hanson DA, Schlaepfer DD (2005) Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol 6(1):56–68
67. Nagano M, Kelly PA (1994) Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. J Biol Chem 269(18):13337–13345
68. Nagashima T, Maruyama T, Uchida H, Kajitani T, Arase T, Ono M, Oda H, Kagami M, Masuda H, Nishikawa S, Asada H, Yoshimura Y (2008) Activation of SRC kinase and phosphorylation of signal transducer and activator of transcription-5 are required for decidual transformation of human endometrial stromal cells. Endocrinology 149(3):1227–1234
69. Neel BG, Gu H, Pao L (2003) The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci 28(6):284–293
70. Nunes-Xavier CE, Martin-Perez J, Elson A, Pulido R (2013) Protein tyrosine phosphatases as novel targets in breast cancer therapy. Biochim Biophys Acta 1836(2):211–226
71. Okada M (2012) Regulation of the SRC family kinases by Csk. Int J Biol Sci 8(10):1385–1397
72. Okamura H, Zachwieja J, Raguet S, Kelly PA (1989) Characterization and applications of monoclonal antibodies to the prolactin receptor. Endocrinology 124(5):2499–2508
73. Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE (1999) ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J Biol Chem 274(24):17209–17218
74. Parsons SJ, Parsons JT (2004) Src family kinases, key regulators of signal transduction. Oncogene 23(48):7906–7909
75. Pezet A, Buteau H, Kelly PA, Edery M (1997a) The last proline of Box 1 is essential for association with JAK2 and functional activation of the prolactin receptor. Mol Cell Endocrinol 129(2):199–208
76. Pezet A, Ferrag F, Kelly PA, Edery M (1997b) Tyrosine docking sites of the rat prolactin receptor required for association and activation of stat5. J Biol Chem 272(40):25043–25050
77. Piazza TM, Lu JC, Carver KC, Schuler LA (2009) SRC family kinases accelerate prolactin receptor internalization, modulating trafficking and signaling in breast cancer cells. Mol Endocrinol 23(2):202–212
78. Qu C-K, Yu W-M, Azzarelli B, Feng G-S (1999) Genetic evidence that Shp-2 tyrosine phosphatase is a signal enhancer of the epidermal growth factor receptor in mammals. Proc Natl Acad Sci U S A 96(15):8528–8533
79. Rous P (1910) A transmissible avian neoplasm. Sarcoma of the common fowl. Exp Med 12:696–705

80. Rowlinson SW, Yoshizato H, Barclay JL, Brooks AJ, Behncken SN, Kerr LM, Millard K, Palethorpe K, Nielsen K, Clyde-Smith J, Hancock JF, Waters MJ (2008) An agonist-induced conformational change in the growth hormone receptor determines the choice of signalling pathway. Nat Cell Biol 10(6):740–747
81. Rui H, Djeu JY, Evans GA, Kelly PA, Farrar WL (1992) Prolactin receptor triggering. Evidence for rapid tyrosine kinase activation. J Biol Chem 267 (33):24076–24081
82. Rui H, Kirken RA, Farrar WL (1994) Activation of receptor-associated tyrosine kinase JAK2 by prolactin. J Biol Chem 269(7):5364–5368
83. Saharinen P, Takaluoma K, Silvennoinen O (2000) Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. Mol Cell Biol 20 (10):3387–3395
84. Sakamoto K, Creamer BA, Triplett AA, Wagner KU (2007) The Janus kinase 2 (Jak2) is required for expression and nuclear accumulation of Cyclin D1 in proliferating mammary epithelial cells. Mol Endocrinol 21(8):1877–1892
85. Saunier E, Dif F, Kelly PA, Edery M (2003) Targeted expression of the dominant-negative prolactin receptor in the mammary gland of transgenic mice results in impaired lactation. Endocrinology 144(6):2669–2675
86. Shillingford JM, Miyoshi K, Robinson GW, Grimm SL, Rosen JM, Neubauer H, Pfeffer K, Hennighausen L (2002) Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. Mol Endocrinol 16(3):563–570
87. Shupnik MA (2004) Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23(48):7979–7989
88. Silvennoinen O, Witthuhn BA, Quelle FW, Cleveland JL, Yi T, Ihle JN (1993) Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. Proc Natl Acad Sci U S A 90(18):8429–8433
89. Sorensen P, Sheffield LG (1997) Involvement of c-src in beta-casein expression by mammary epithelial cells. Biochem Biophys Res Commun 241(3):710–713
90. Summy JM, Gallick GE (2003) Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev 22(4):337–358
91. Thomas SM, Brugge JS (1997) Cellular functions regulated by Src family kinases. Ann Rev Cell Dev Biol 13:513–609
92. Tilbrook PA, Ingley E, Williams JH, Hibbs ML, Klinken SP (1997) Lyn tyrosine kinase is essential for erythropoietin-induced differentiation of J2E erythroid cells. EMBO J 16(7):1610–1619
93. Wagner K-U, Krempler A, Triplett AA, Qi Y, George NM, Zhu J, Rui H (2004) Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. Mol Cell Biol 24(12):5510–5520
94. Walter AO, Peng ZY, Cartwright CA (1999) The Shp-2 tyrosine phosphatase activates the Src tyrosine kinase by a non-enzymatic mechanism. Oncogene 18(11):1911–1920
95. Watkin HL, Richert MM, Lewis A, Terrell K, McManaman JP, Anderson SM (2008) Lactation failure in Src knockout mice is due to impaired secretory activation. BMC Dev Biol 8(1):6
96. Watowich SS, Wu H, Socolovsky M, Klingmuller U, Constantinescu SN, Lodish HF (1996) Cytokine receptor signal transduction and the control of hematopoietic cell development. Ann Rev Cell Dev Biol 12(91):91–128
97. Wheeler DL, Iida M, Dunn EF (2009) The role of Src in solid tumors. Oncologist 14(7):667–678
98. Wilks AF (1989) Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. PNAS 86(5):1603–1607
99. Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zurcher G, Ziemiecki A (1991) Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. Mol Cell Biol 11(4):2057–2065
100. Xie J, LeBaron MJ, Nevalainen MT, Rui H (2002) Role of tyrosine kinase Jak2 in prolactin-induced differentiation and growth of mammary epithelial cells. J Biol Chem 277(6):14020–14030

101. Xu D, Qu CK (2008) Protein tyrosine phosphatases in the JAK/STAT pathway. Front Biosci 13:4925–4932
102. Yeatman TJ (2004) A renaissance for SRC. Nat Rev Cancer 4(6):470–480
103. Yu WM, Hawley TS, Hawley RG, Qu CK (2003) Catalytic-dependent and -independent roles of SHP-2 tyrosine phosphatase in interleukin-3 signaling. Oncogene 22(38):5995–6004
104. Zhang S, Yu D (2012) Targeting Src family kinases in anti-cancer therapies: turning promise into triumph. Trends Pharmacol Sci 33(3):122–128
105. Zhang SQ, Yang W, Kontaridis MI, Bivona TG, Wen G, Araki T, Luo J, Thompson JA, Schraven BL, Philips MR, Neel BG (2004) Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. Mol Cell 13(3):341–355
106. Zhang F, Zhang Q, Tengholm A, Sjoholm A (2006) Involvement of JAK2 and Src kinase tyrosine phosphorylation in human growth hormone-stimulated increases in cytosolic free Ca2+ and insulin secretion. Am J Physiol Cell Physiol 291(3):C466–475
107. Zhou Y-J, Chen M, Cusack NA, Kimmel LH, Magnuson KS, Boyd JG, Lin W, Roberts JL, Lengi A, Buckley RH (2001) Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for janus kinases. Molecular Cell 8(5):959–969