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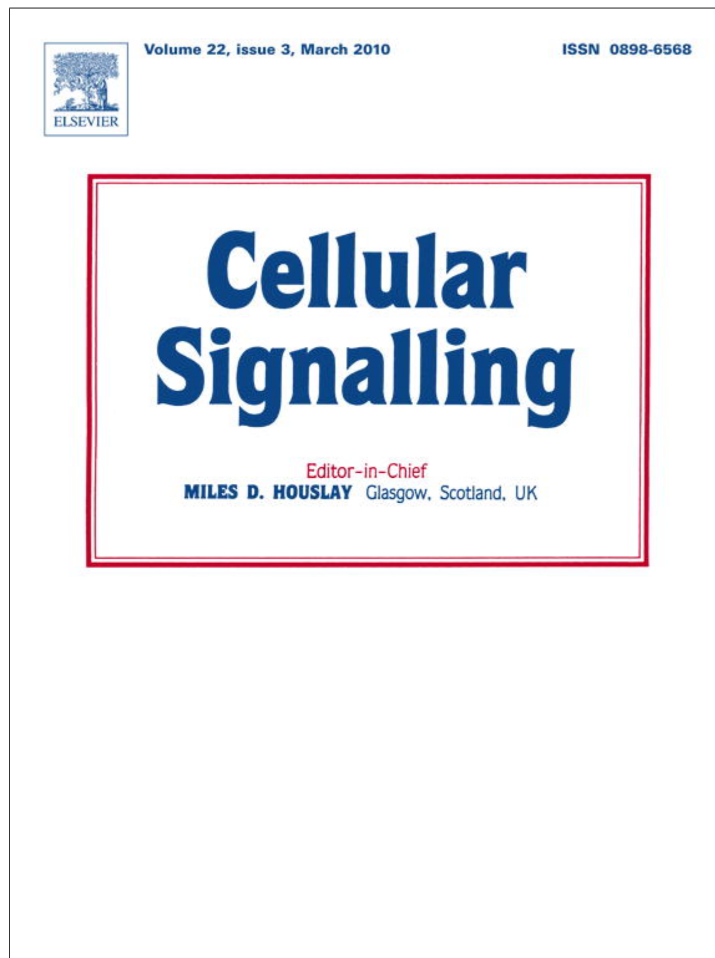

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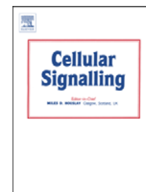

A non-catalytic function of the Src family tyrosine kinases controls prolactin-induced Jak2 signaling

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ABSTRACT

The cytokine prolactin (PRL) plays important roles in the proliferation and differentiation of the mammary gland and it has been implicated in tumorigenesis. The prolactin receptor (PRLR) is devoid of catalytic activity and its mitogenic response is controlled by cytoplasmic tyrosine kinases of the Src (SFK) and Jak families. How PRLR uses these kinases for signaling is not well understood. Previous studies indicated that PRLR-induced Jak2 activation does not require SFK catalytic activity in favor of separate signaling operating on this cellular response. Here we show that, nevertheless, PRLR requires Src-SH2 and -SH3 domains for Jak2 signaling. In W53 lymphoid cells, conditional expression of two c-Src non-catalytic mutants, either SrcK295M/Y527F or SrcΔK, whose SH3 and SH2 domains are exposed, controls Jak2/Stat5 activation by recruiting Jak2, avoiding its activation by endogenous active SFK. In contrast, the kinase inactive SrcK295M mutant, with inaccessible SH3 and SH2 domains, does not. Furthermore, all three mutants attenuate PRLR-induced Akt and p70S6K activation. Accordingly, PRLR-induced Jak2/Stat5 signaling is inhibited in MCF7 breast cancer cells by Src depletion, expression of SrcK295M/Y527F or active Src harboring an inactive SH2 (SrcR175L) or SH3 domain (SrcW118A). Finally, Jak2/Stat5 pathway is also reduced in *Src*$^{-/-}$ mice mammary glands. We thus conclude that, in addition to Akt and p70S6K, SFK regulate PRLR-induced Jak2 signaling through a kinase-independent mechanism.

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1. Introduction

Prolactin (PRL) is a cytokine that mediates many physiological functions [1,2] including growth and differentiation of the mammary gland [3] and regulation of the immune system [4]. Elevated levels of PRL have been associated with diseases of the immune system and with prostate and breast cancer [5–7]. PRL receptor (PRLR) is a member of the type I cytokine receptor subfamily. It is devoid of intrinsic enzymatic activity, but is constitutively associated with the cytoplasmic tyrosine kinase Jak2 and the ones of the Src family (SFK). Binding of PRL induces dimerization of the receptor and subsequent activation of Jak2 and SFK [8–17]. Activation of Jak2 leads to tyrosine phosphorylation of the receptor, thus further propagating intracellular signaling [18,19]. Consistent with its mandatory role in this cellular process, *jak2* knockout in mice induces a phenotype that closely resembles the one of *PRLR*$^{-/-}$ [20]. Additional results obtained from conditional *jak2* depletion confirm its crucial role for PRL-induced mammary gland development. Interestingly, PRL still activated c-Src in the absence of Jak2 [21] suggesting that this tyrosine kinase does not regulate SFK activities. Consistently, expression of PRLR4P/A variant [PRLR with mutations in Box I (PPVPGP/AAVAGA)], which exhibited a defect in Jak2 binding and activation [22], is able to stimulate c-Src [13].

SFK are composed of a N-terminal sequence with lipid binding sites important for plasma membrane localization, followed by a SH3 and a SH2 domain involved in protein–protein interactions, a catalytic domain and a short C-terminus with a conserved tyrosine residue (Y527 in chicken Src) that, when phosphorylated, inhibits kinase activity. At the molecular level, SFK regulation relies on intramolecular contacts of the SH2 domain with pY527 and the SH3 domain with a linker sequence flanked between the SH2 and the catalytic domain that keeps the kinase in a closed and inactive

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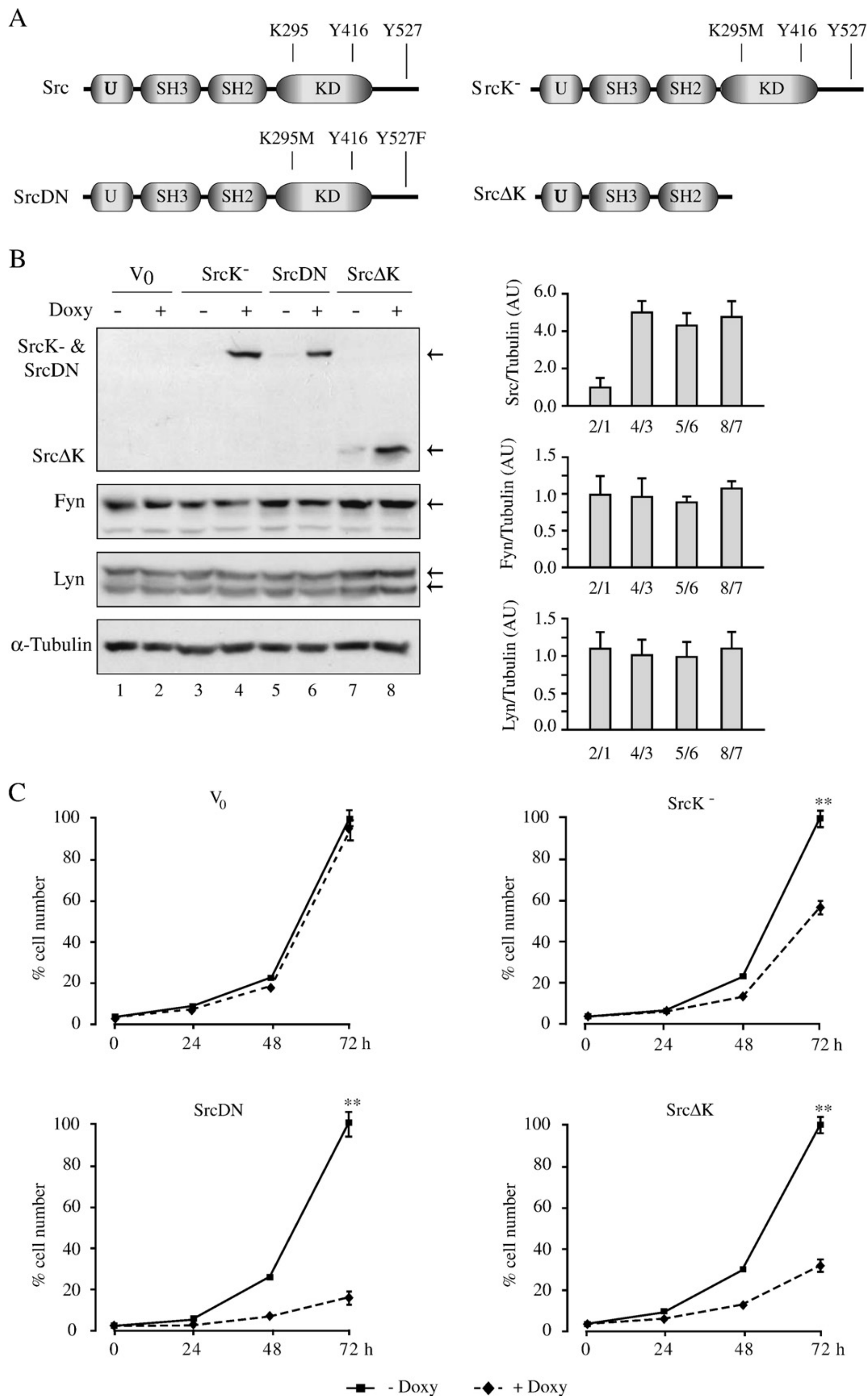

Fig. 1. Effect of c-Src mutants on cell proliferation. (A) Schematic structure of c-Src and SrcK^{-} (K295M), SrcDN (K295M/Y527F) and SrcΔK mutants. (B) Exponentially growing cultures of control cells (containing the empty pTet-Splice, V_0) or cells with inducible expression of SrcK^{-}, SrcDN or SrcΔK in complete medium were incubated in absence or presence of doxycycline (Doxy, 2 μg/ml, 24 h). Expression of c-Src mutants, Fyn and Lyn were determined by WB using anti-c-Src mAb 327, anti-Fyn and anti-Lyn. Membrane was reblotted with anti-α-tubulin for loading control. Arrows mark position of detected proteins. Values were normalized to levels of a-tubulin and quantified determining the ratio of +/− PRL stimulation for each cell line. (C) Cells were plated at 3×10^4 cells/ml 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 by centrifugation, incubated with Trypan blue and viable cells were counted as described in Materials and methods. 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. Columns, means; bars, standard error; *$p\leq0.05$ ($n=3$); **$p\leq0.01$ ($n=3$).

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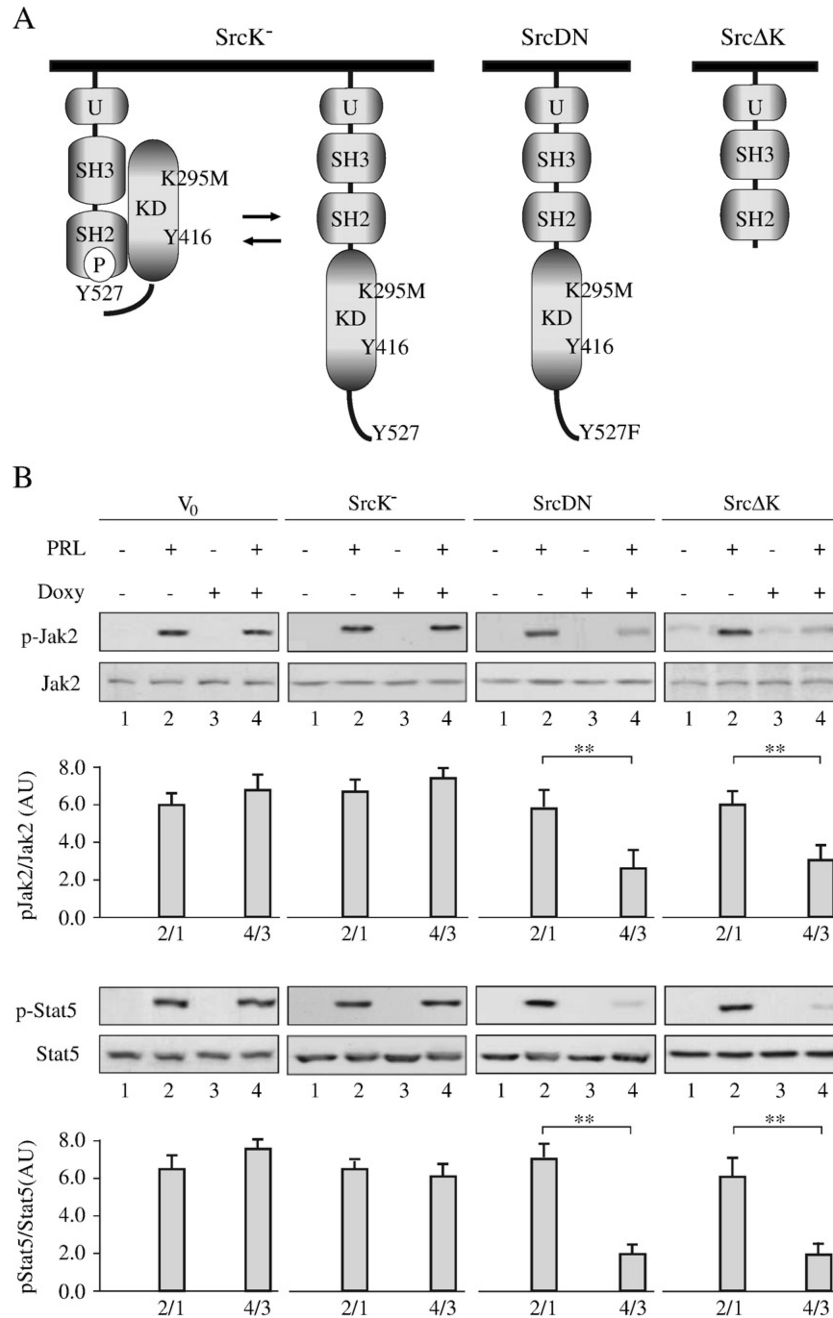

Fig. 2. Role of SFK on PRL activation of Jak2/Stat5. (A) Schematic conformations of c-Src mutants. (B) Exponentially growing cultures (5×10^5 cells/ml) were washed with RPMI-1640 and transferred overnight to PRL-depleted media in the absence or presence of Doxy (2 µg/ml). Cells were then stimulated with PRL (100 ng/ml, 15 min). Protein extracts (20 µg) were used to determine PRL stimulation of Jak2 and Stat5 by WB with anti-phospho-specific antibodies. Membranes were reblotted with specific antibodies recognizing Jak2 and Stat5 for loading controls. The results represent one of three independent experiments carried out in triplicate. The degree of Jak2 and Stat5 activation were normalized by the values of their loading controls and quantified by determining the ratio of +/− PRL stimulation for each cell line. Columns, means; bars, standard error; $*p\le0.05$ ($n=3$); $**p\le0.01$ ($n=3$).

conformation. Opening this ternary structure by any means is predicted to induce kinase activation. Interestingly, the inactive conformation additionally restrains the interactions of SH3 and SH2 with specific interactors important for signaling [23]. While the majority of SFK functions depend on their catalytic activity [24,25], several studies pointed to additional non-catalytic/adaptor functions that rely on the ability of the SH2 and SH3 domains to generate protein-signaling complexes [26–28].

The importance of Src in PRLR signaling has been illustrated with the *Src*$^{-/-}$ mice that exhibit a delay in ductal outgrowth and reduction in the number of "terminal end buds" of the mammary gland [29]. These mice also have alterations in alveolar cell organization and secretory functions, resulting in lactation failure, which is consistent with changes in PRLR signaling [30]. In accordance with this function, we reported that in W53 lymphoid cells (BaF-3 cells expressing the long form of PRLR from rat liver [31]), SFK play important roles in PRLR mitogenic signaling, implicating both Akt/Myc and p70S6 kinase-dependent pathways. However, inhibition of SFK catalytic activity, either by expressing the kinase inactive SrcK295M mutant (SrcK$^-$) or by treating cells with the pharmacological inhibitors PP1/PP2 did not affect Jak2 activation [14,15,17]. This allowed us to suggest that SFK and Jak2 initiate

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separate signaling cascades for mitogenesis. Surprisingly, we observed that the kinase inactive mutant K295M/Y527F (SrcDN), where tyrosine 527 has been replaced by phenylalanine, inhibits PRL-dependent proliferation more efficiently than SrcK⁻. While SH3 and SH2 could be engaged in intramolecular interactions in SrcK⁻, Y527F mutation additionally stabilizes SrcDN in an open conformation, thus

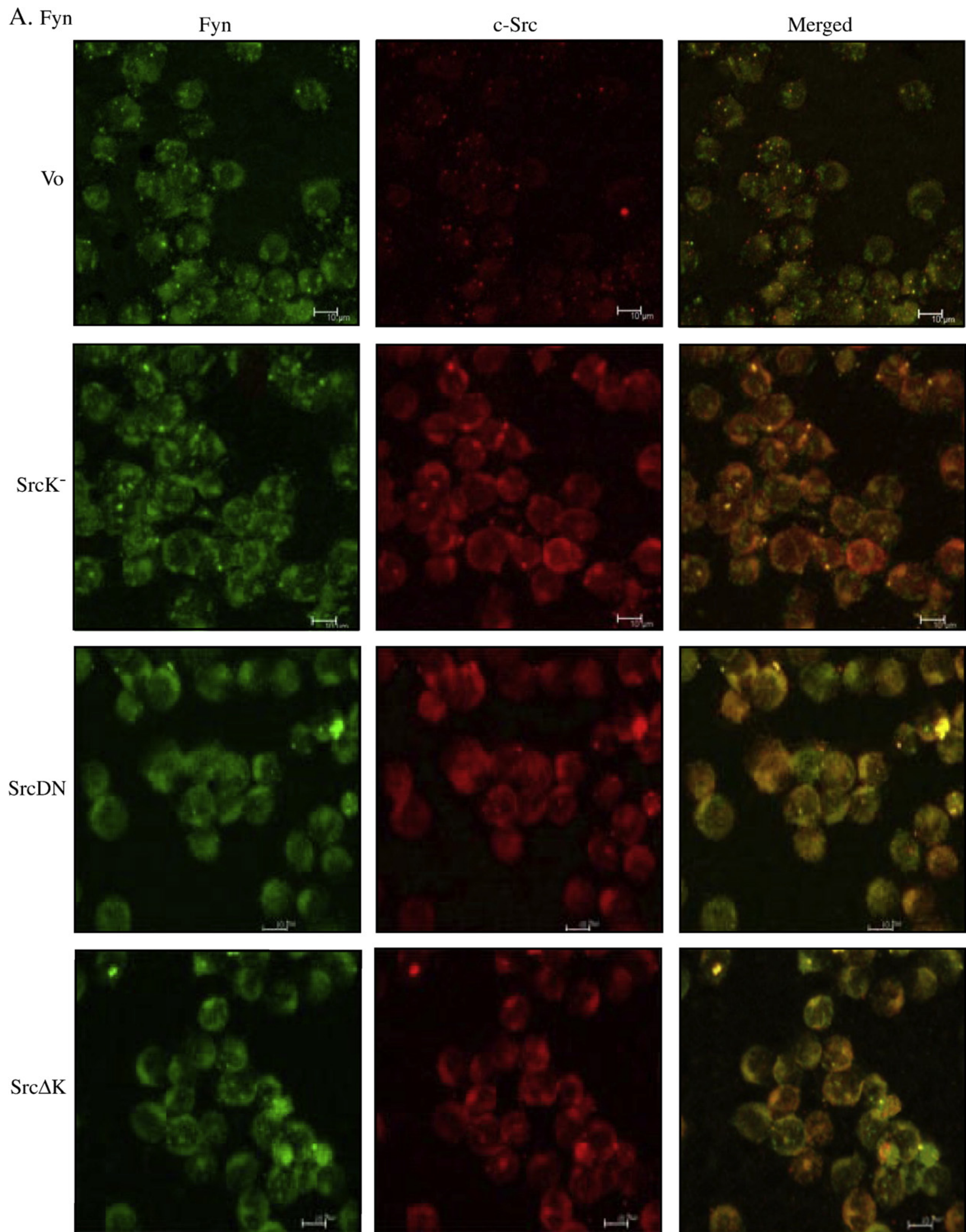

Fig. 3. Co-localization of Fyn and Lyn with c-Src mutants. Confocal microscopy analysis of Fyn (A), Lyn (B) and distribution of c-Src mutants in W53 TET-ON-SrcK⁻, -SrcDN and -SrcΔK cells. Exponentially growing cultures of W53 TET-ON-SrcK⁻, -SrcDN and -SrcΔK were treated overnight with 2 µg/ml Doxy, while W53 TET-ON-Vo cells were not treated with Doxy. All cells were processed for immunofluorescence with rabbit anti-Fyn, rabbit anti-Lyn and mouse mAb-c-Src327 antibodies plus goat anti-rabbit Alexa-488-conjugated antibody or goat anti-mouse Alexa-647-conjugated antibody. Photographs were taken from Alexa 488 signal (left column), Alexa 647 signal (middle column), and merged (right column). Scale bar 10 µm. Results are representative of three separate experiments.

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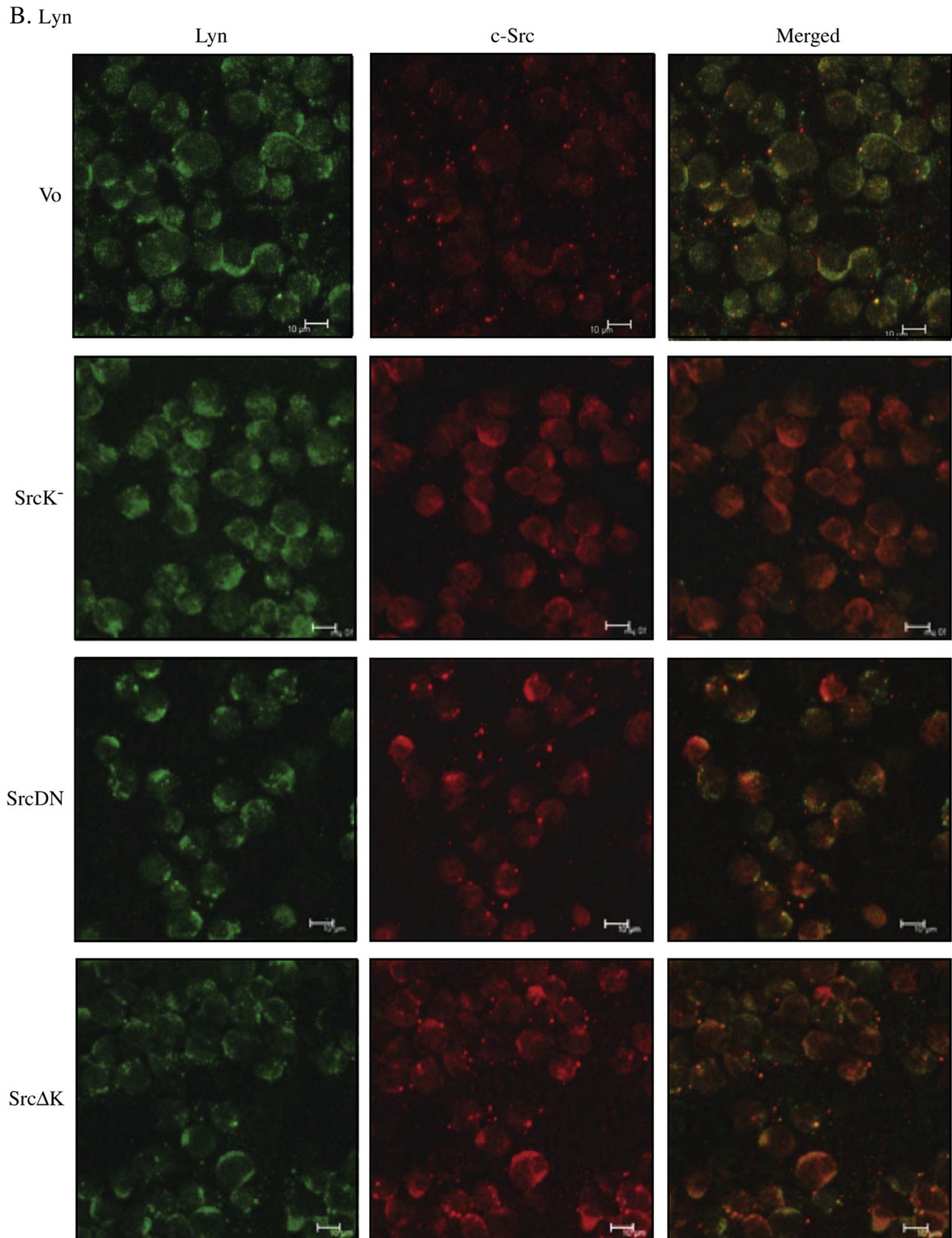

Fig. 3 (*continued*).

exposing SH3 and SH2 domains for binding with signaling partners. We thus raised the hypothesis that SFK could have additional functions in PRLR signaling, implicating an SH2/SH3 adaptor-dependent mechanism. Here we present evidences supporting the existence of such mechanism operating in PRLR-induced Jak2 signaling.

2. Materials and methods

2.1. Reagents and antibodies

RPMI-1640, DMEM and FBS were from Invitrogen (Carlsbad, CA). Tet System Approved FBS was from Clontech (Palo Alto, CA) Ovine

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PRL (PRL, NIDDK-oPRL-20, 31 IU/mg) was from the NHPP (NIDDK, Bethesda, MD). BCA protein assay reagent was from Pierce (Rockford, IL). Anti-p70S6K was from G. Thomas (Genomic Research Institute, University of Cincinnati). Mouse monoclonal antibody to c-Src 327 was a gift from J.S. Brugge (Harvard University). Antibodies against Erk2 (C-14), Stat 5b (C-17), Akt1/2 (H-136), HA (Y-11), Jak2 (C-20 and HR-758), Fyn (FYN3), Src (Src2) and c-Myc (C-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pMek1/2, pErk1/2 (pp42/44), pp70S6K, and pAkt (S473) were from New England Biolabs (Beverly, MA). Anti-α-tubulin, BSA, Triton X-100, Trypan Blue and Doxycycline (Doxy) were from Sigma-Aldrich (St Louis, MS). Anti-p-c-Src (Y416), anti-p-Jak2 (Y1007 and Y1008), and horseradish peroxidase-conjugated secondary antibodies were from Biosource International (Camarillo, CA). ECL was from GE Healthcare (Buckinghamshire, UK).

2.2. Generation of cell lines

To generate W53 TET-ON cell lines with inducible expression of SrcK^{-} (K295M), SrcDN (K295M/Y527F), SrcΔK, Jak2ΔK (Fresno Vara et al., 2001), BaF-3-rt-Tet cells [32] were co-transfected by electroporation (960 μF, 300 V) with 20 μg of empty pTet-Splice (V_o, control)

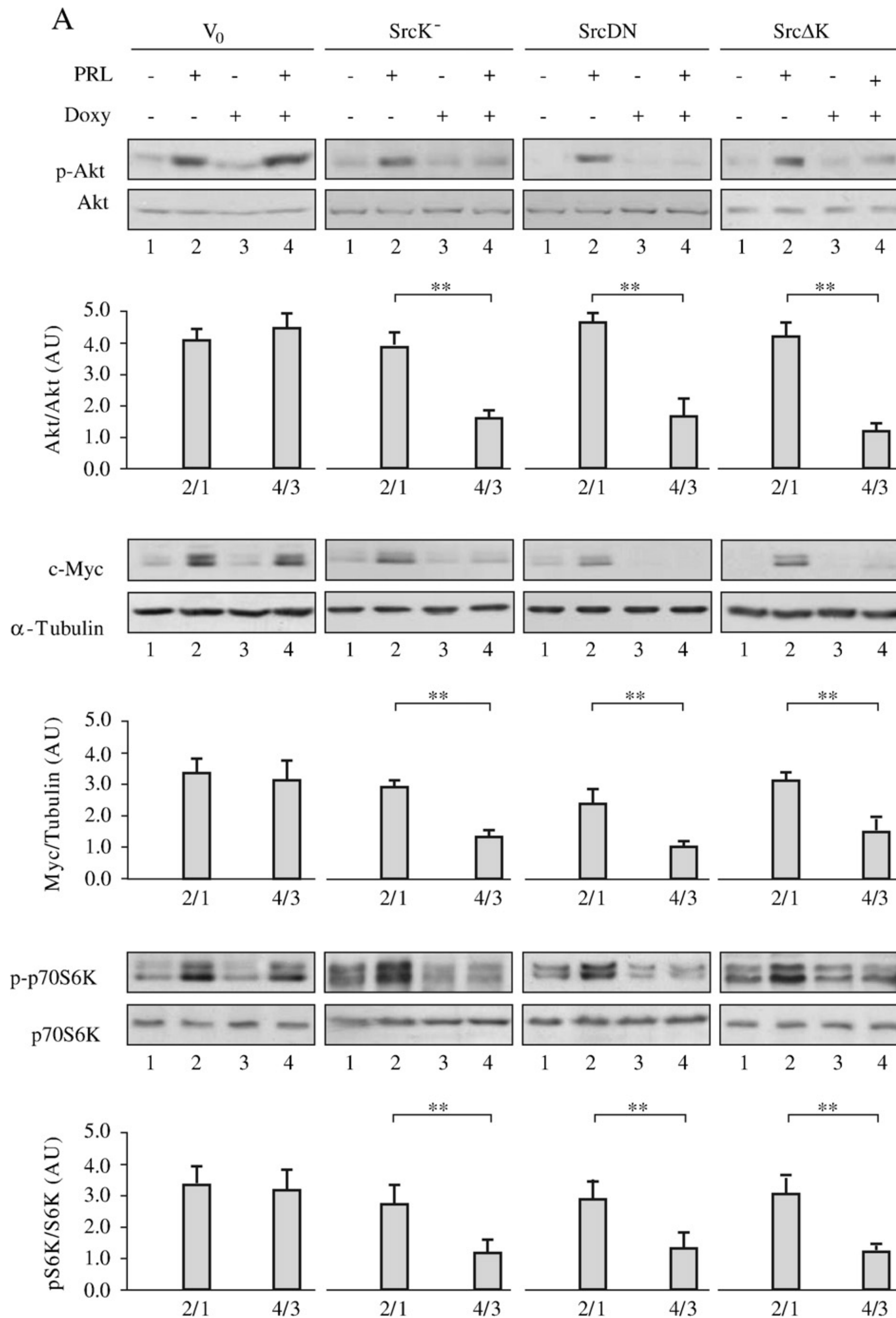

Fig. 4. Effect of c-Src mutants on PRL-mediated Akt, p70S6K, Mek1/2 and Erk1/2 activation and Myc expression. Cell cultures treated as in Fig. 2B were stimulated with PRL (100 ng/ml) for 15 min to detect the kinase activation, and for 60 min for Myc expression. Protein extracts (20 μg) were used to determine: (A) Akt and p70S6K activation and Myc expression; (B) Mek1/2 and Erk1/2 activation, by WB with anti-phospho-specific antibodies and anti-c-Myc antibody. Membranes were reblotted with specific antibodies recognizing Akt, p70S6K, α-tubulin, Merk1/2 or Erk2 for loading controls. The values were normalized and quantified as in Fig. 2B. The results represent one of three independent experiments. Columns, means; bars, standard error; *$p \leq 0.05$ ($n=3$); **$p \leq 0.01$ ($n=3$); ***$p \leq 0.001$ ($n=3$).

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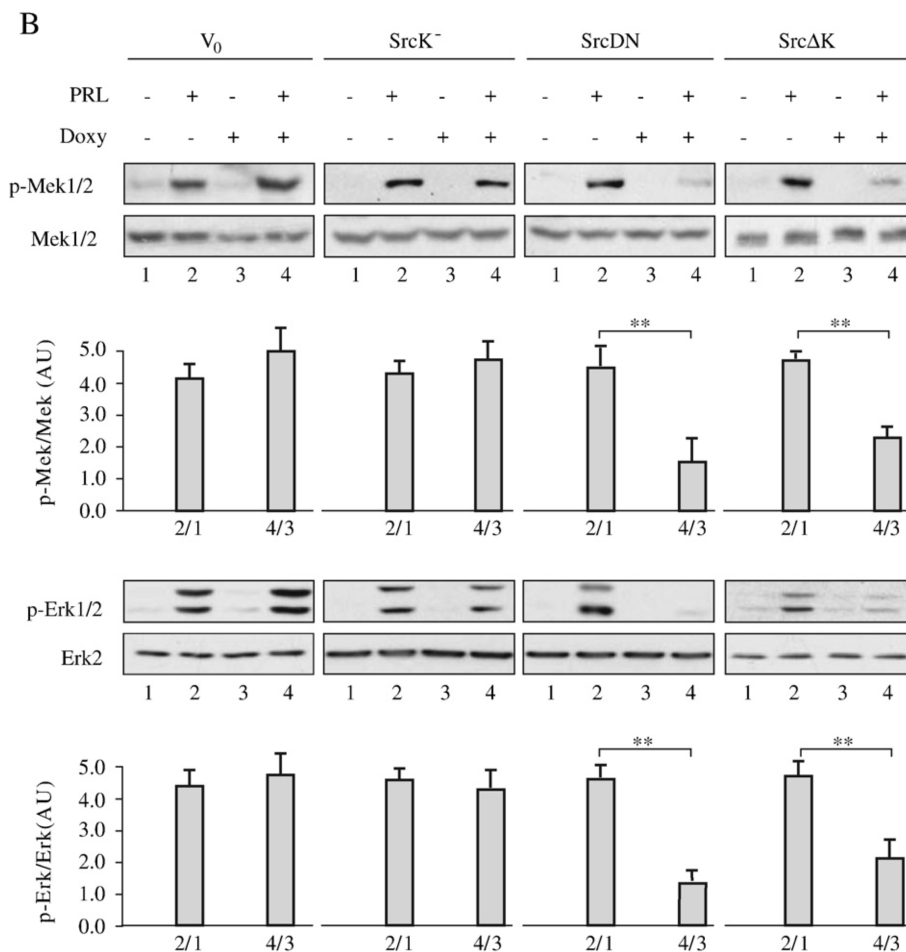

Fig. 4 (*continued*).

or pTet-Splice containing the cDNA of each of the mutants and 4 μg of PRLR-HA [long form of PRLR from rat liver [31]]. SrcΔK was generated by deleting kinase and C-terminal domains (residues 251–533) from chicken c-Src cDNA and introducing a stop codon by PCR with primer 3′GTCTGCCCCACGTCCAAGTGAGAATTCCG5′. At 36 h post-transfection, cells were seeded into 96-well plates (10^4 cells/well) with medium containing IL-3 and puromycin (2 μg/ml), resistant cells were screened for expression of mutants by Western blot (WB) after 16 h with Doxy (2 μg/ml). Positive cells were selected for growth in PRL-containing medium without IL-3 and screened for Doxy-inducible expression of mutants by WB. Cells with similar induction were pooled, grown in complete media [RPMI-1640 containing 5% Tet-Free FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 5 ng/ml of PRL] and used for experiments. Prior to stimulation cells were starved overnight in RPMI-1640 containing 5% Tet-Free FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, then cells were stimulated by addition of PRL (100 ng/ml) for the indicated time. Generation of MCF7-TET-ON-SrcDN and MCF7 with shRNA for c-Src and EGFP has been previously described [33]. Mutations of c-Src at W118A or at R175L were generated by site-directed mutagenesis and checked by sequencing. Mutants cloned into pBabe-puromycin were transfected into MCF7 and selected with puromycin (2 μg/ml). Pool of resistant clones were tested for expression of the c-Src mutants by WB and used for experiments. MCF7 derived cell lines were starved for 48 h in DMEM Phenol Red Free for 48 h prior to stimulation with PRL (100 ng/ml) for the indicated time.

2.3. Cell proliferation assay

Proliferation and cellular viability were assayed by cell counting with the Trypan Blue dye exclusion method as previously described [33]. Briefly, cells were collected by centrifugation at 500 ×g for 5 min at room temperature, mixed with a 0.4% Trypan blue solution (1:1). Cell proliferation was determined calculating the number of viable cells by phase contrast microscopy. In all cases, the percentage of dead cells was always lower than 5%, permitting consideration of the number of viable cells as total number of cells. To determine the effect of c-Src mutants on PRL-induced cell proliferation, the proliferation rates between untreated and Doxy-treated cells were compared. Noteworthy is the fact that proliferation was not assayed in absence of PRL because cells became apoptotic within 24 h in this condition [16].

2.4. Cell stimulation and Western blot analysis

W53 Tet-On cell lines were washed with RPMI-1640 to remove PRL and cultured overnight in PRL-depleted medium [RPMI-1640 containing 5% Tet-Free FCS, 4 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml)]. The cultures were incubated overnight with or without Doxy (2 μg/ml). Cells were then stimulated with PRL (100 ng/ml) for different periods of time; control cells were not stimulated with PRL. Cells were collected and washed once in ice-cold PBS and lysed for 30 min at 4 °C with 1 ml per 2×10^7 cells of lysis buffer (LB: 1 PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM

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phenantroline, 1 mM benzamidine hydrochloride, and 1 mM iodoacetamide). The total cell lysates were centrifuged for 30 min at 4 °C and 15000×g, and protein concentration was determined in the supernatants with the BCA protein assay. Protein aliquots were boiled in SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol) and stored at −20 °C for Western blotting. The remaining protein aliquots were incubated for 1 h at 4 °C with the appropriate antibody and collected by incubation for 1 h at 4 °C with 30 µl of protein G-Sepharose beads (Sigma, St. Louis, MO), washed three times with LB and eluted by boiling in 2× SDS sample buffer. For Western blotting analysis, samples were subjected to SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Filters were blocked with 5% fat-free dried milk (Fluka BioChemika, Neu-Ulm, Switzerland) in TTBS (10 mM Tris–HCl pH 7.4, 0.1% Tween-20) or with 5% BSA in TTBS for anti-phospho antibodies. Membranes were incubated with the primary antibody in blocking buffer, washed three times with TTBS, further incubated with the suitable horseradish peroxidase-conjugated secondary antibody, and proteins detected with ECL.

2.5. Confocal analysis

W53 Tet-ON-Vo, -SrcK⁻, -SrcDN and -SrcΔK in exponential growth were treated or not overnight with 2 µg/ml Doxy. Cells were prepared as described [16] and incubated with primary antibodies (rabbit anti-Fyn, rabbit anti-Lyn, and mAb c-Src327, diluted 1:50 in PBS-2% BSA) for 1 h at 37 °C and then incubated with a goat anti-rabbit IgG Alexa 488-conjugated antibody or a goat anti-mouse IgG Alexa 647-conjugated antibody (dilution 1:250 in PBS-2% BSA) for 1 h at 37 °C. After mounting in Mowiol medium, coverslips were observed by using a 63×/1.40 oil Plan-Apochromat objective and confocal analyses were performed with a Leica TCS SPII Spectral microscope.

2.6. Preparation of whole mammary gland extracts

The fourth inguinal mammary gland from $Src^{-/-}$ mice (Soriano et al., 1991), or from control $Src^{+/+}$ mice, F_1 (C57Bl6 × 129 Sv) and F2 [C57Bl × F1(C57Bl6 × 129 Sv)] (The Jackson Laboratory, Bar Harbor, ME), was removed at day two of lactation and snap frozen in liquid nitrogen as described [30].

2.7. Statistical analysis

The Student's *t* test was used for analysis of statistical significance of the data.

3. Results

3.1. Effect of c-Src mutants in PRLR-induced cell proliferation

We previously observed that SrcDN inhibited PRLR signaling more efficiently than SrcK⁻ when transiently co-expressed in BaF-3 cells [14]. We then wished to investigate these inhibitions in further details. To this end, we generated BaF-3 TET-ON derived cell lines that constitutively express the PRLR (W53 TET-ON cells) and the c-Src mutants SrcK⁻, SrcDN, or SrcΔK (deletion of the kinase domain and the C-terminal tail) in a conditional manner (Figs. 1A and 3A). Accordingly, treatment of growing cells with Doxycycline (Doxy, 2 µg/ml, 24 h) induced expression of these Src mutants at similar levels (Fig. 1B). Src expression did not affect the level of endogenous members Fyn and Lyn of the Src family. We next determined their incidence on PRL-induced cell proliferation. While Doxy treatment had no effect in control cells (Fig. 1C), it significantly reduced proliferation of cells expressing Src mutants. Interestingly, SrcDN gave the strongest inhibitory effect (83.7% ± 3.2 at 72 h of induction) as compared to SrcΔK (68.6% ± 1.9) and SrcK⁻ (43.9% ± 1.8) (Fig. 1C). These results confirmed our previous data [14] and suggest the involvement of the SH2 and/or SH3 domains of SFK in PRLR-induced cell proliferation.

3.2. Regulation of Jak2/Stat5 pathway by Src kinases

We next investigated the mechanism by which these Src mutants inhibit PRLR-induced mitogenesis in further details. First, we analyzed their impact on PRLR-induced Jak2 signaling. Surprisingly, we found that both SrcDN and SrcΔK inhibited Jak2 activation as well as the phosphorylation of its mitogenic substrate Stat5 (Fig. 2B). In contrast, SrcK⁻ had no effect on these molecular events, which is consistent with the inability of Src pharmacological inhibitors to affect Jak2/Stat5 signaling [14,15]. These findings suggest that the SH2 and SH3 domains of SrcΔK and SrcDN compete with the SH2 and SH3 domains of the endogenous Src members Fyn and Lyn for Jak2 signaling. In accordance with such a competitive mechanism, we observed that Src mutants co-localized with Fyn and Lyn at the plasma membrane (Fig. 3A and B).

3.3. Regulation of PI3K/Akt/Myc and Mek1/2–Erk1/2 pathways by SFK

We next addressed whether Src mutants affect additional pathways that are important for PRLR mitogenic response. Our previous report indicated that SFK kinase activities were required for the induction of both PI3K/Akt/Myc and p70S6K pathways, but they did not affect Mek1/2 and Erk1/2 activities [14,16]. As expected, all Src kinase-dead mutants abrogated the activation of Akt and p70S6K and the up-regulation of Myc (Fig. 4A). This confirms our previous conclusion that SFK regulate PRLR-induced Akt/Myc and p70S6K signaling in catalytic-dependent fashion. Surprisingly, we observed that SrcDN and SrcΔK additionally blocked activation of the Mek1/2–Erk1/2 pathway, while SrcK⁻ had no effect (Fig. 4B), which is in accordance with data obtained with Src-like pharmacological inhibitors [14]. We thus suggest an additional adaptor function of SFK in PRL activation of Mek1/2–Erk1/2.

3.4. A role for Jak2 in PRLR-induced Erk1/2 signaling cascade

Since SrcDN and SrcΔK inhibited both Jak2/Stat5 and Mek1/2–Erk1/2 pathways, we searched for a link between these biochemical events. To this end, we generated a W53 cell line with inducible expression of kinase-dead Jak2ΔK mutant (Fig. 5A). As expected, induction of Jak2ΔK by Doxy abrogated both Jak2 signaling (Fig. 5B) and cellular proliferation induced by PRL (58.7 ± 1.7% inhibition) (Fig. 5C). Interestingly, Jak2ΔK also altered PRL-induced Mek1/2 and Erk1/2 activation (Fig. 5D), in agreement with a role for Jak2 mediating SFK-induced Erk1/2 activities. In contrast, Jak2ΔK neither affected SFK activation nor Akt/Myc and p70S6K signaling (Fig. 5E). These results suggest that Jak2 specifically mediates SFK–Erk1/2 pathway without impacting on the SFK/Akt/Myc and p70S6K cascades.

3.5. Role of c-Src in PRL stimulation of Jak2 and Stat5 in MCF7 human breast cancer cells

Since PRL plays an important role in breast cancer [5–7], we next investigated whether a similar mechanism operates in these cancer cells. To this end, we used MCF7 breast cancer cells that express SrcDN upon Doxy addition (MCF7-TET-ON-SrcDN cells) [33]. Doxy addition for 1 day allowed the expression of SrcDN by 5 fold (Fig. 6A) without affecting the level of Fyn. Cells were then serum-starved for 48 h in the absence or the presence of Doxy, then stimulated with PRL for 15 min for signaling induction. As expected, PRL induced both Jak2 activation and Stat5 phosphorylation. Interestingly, SrcDN significantly inhibited these biochemical events (Fig. 6A), indicating that c-Src also mediates PRL-induced Jak2 signaling in MCF7 cells. In accordance with an adaptor function, expression of kinase active Src

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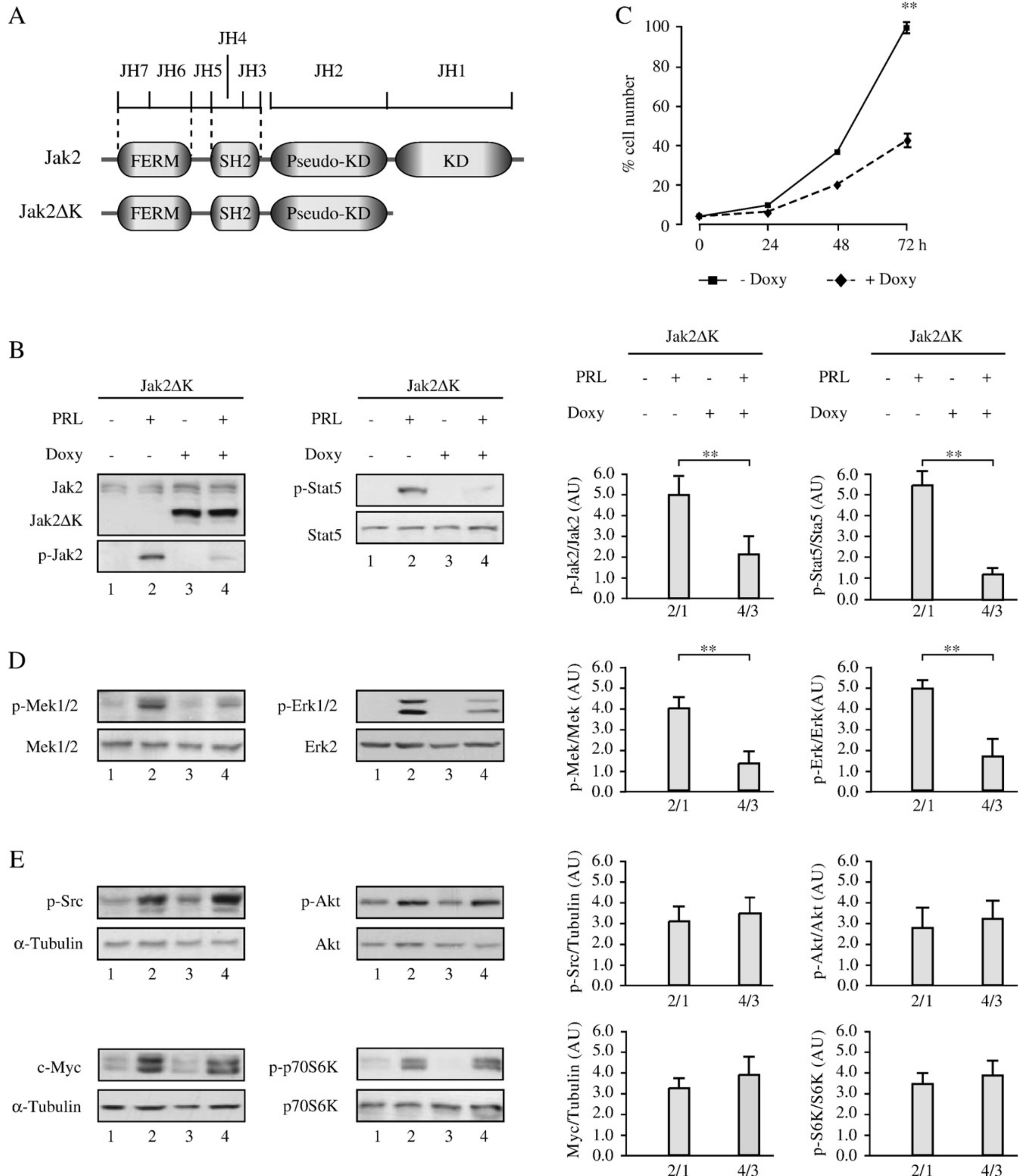

Fig. 5. Role of Jak2 on cell proliferation and signaling. (A) Schematic structure of Jak2 wild type and Jak2ΔK mutant. (B) Exponentially growing cultures (5×10^5 cells/ml) were treated as in Fig. 2A. Protein extracts (20 µg) were used to determine by WB the expression of Jak2ΔK, p-Jak2 and p-Stat5. Jak2 endogenous expression was used as loading control. For Stat5 loading control, the membrane was reblotted with anti-Stat5 antibody. (C) Cell proliferation assay was carried out as in Fig. 1C. D, WB detection of p-Mek1/2 and p-Erk1/2 was carried out as in Fig. 4B. E, Detection of p-Y416-c-Src, p-Akt, Myc and p-p70S6K was determined by WB as described in Materials and methods. The values were normalized and quantified as in Fig. 2B. The results represent one of three independent experiments. Columns, means; bars, standard error; $*p\leq0.05$ ($n=3$); $**p\leq0.01$ ($n=3$); $***p\leq0.001$ ($n=3$).

with inactive SH2 (R175L) or SH3 (W118A) domains, both inhibited Jak2 signaling (Fig. 6B). Furthermore, similar results were obtained in cells where Src has been depleted by a specific RNA interference approach (Fig. 6C). Interestingly, the level of Fyn was not affected by our shRNA, suggesting a specific function for Src in this signaling process. We thus concluded that c-Src also mediates, in a kinase activity-independent manner, PRLR-induced Jak2 signaling in breast cancer cells.

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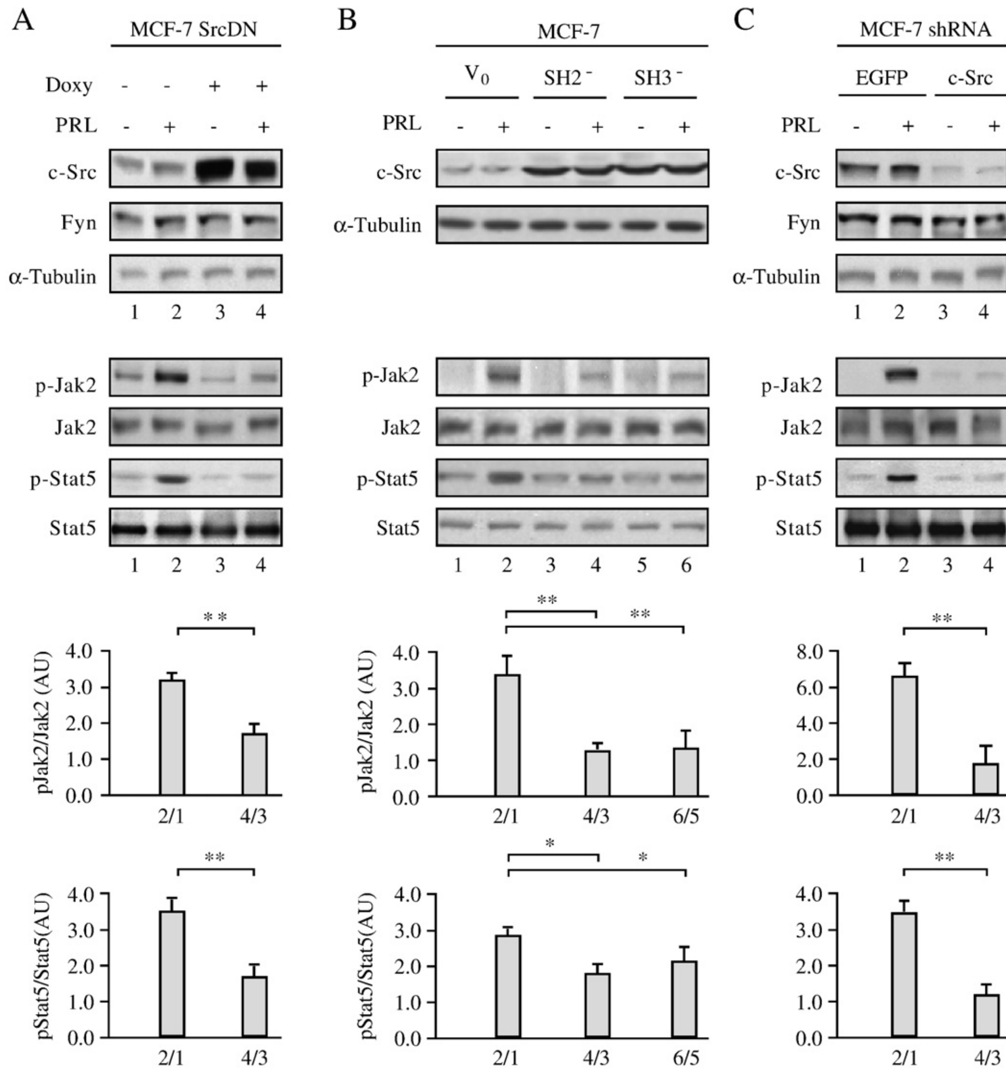

Fig. 6. Role of c-Src in PRL stimulation of Jak2 and Stat5 activation in MCF7. Exponentially growing cell cultures of MCF7-TET-ON-SrcDN (A), MCF7 cells expressing empty pBabe-Puro (V0) or containing c-Src-W118A (SH3⁻) or c-Src-R175L (SH2⁻) (B) and of c-Src-shRNA-MCF7 and EGFP-shRNA-MCF7 (C) were transferred for 48 h to serum and phenol red free DMEM for starvation. The MCF7-TET-ON-SrcDN cultures were maintained during this time both 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 by WB the levels of c-Src, Fyn, and Jak2 and Stat5 phosphorylation. Membranes were reblotted with α-tubulin, anti-Jak2 and anti-Stat5 for loading controls. The quantified values were normalized to their loading controls and the degree of activation of Jak2 and Stat5 was quantified by the determining the ratio of +/− PRL stimulation for each cell line. Columns, means; bars, standard error; $*p \leq 0.05$ ($n=3$); $**p \leq 0.01$ ($n=3$).

3.6. Role of c-Src in the control of Jak2/Stat5 pathway in mammary gland

Finally, we wished to confirm the important role for this Src/Jak2 signaling in a physiological setting. Since Jak2/Stat5 is essential for PRL-induced mammary gland differentiation, we analyzed the role of c-Src in this biological process. We found a reduction of Jak2/Stat5 activities in extract of mammary gland that were isolated from *Src*$^{-/-}$ mice at day 2 of lactation (Fig. 7A). Accordingly, Src was not detected in *Src*$^{-/-}$ glands (Fig. 7B). However, a low amount of phosphorylated SFK was observed in these *Src*$^{-/-}$ tissues, which was attributed to the presence of another activated member of the Src family, including Fyn (Fig. 7B). Altogether, these results also support a role for Src in the propagation of Jak2/Stat5 pathway in normal mammary tissue.

4. Discussion

Our report indicates that SFK control Jak2 activation in response to PRL through a tyrosine kinase-independent mechanism. Since Jak2 is considered as the canonical tyrosine kinase of PRLR signaling [1,20], this suggests that SFK are key regulators of PRLR biological responses. This conclusion is based on regulatory effects of SrcDN and SrcΔK mutants on Jak2 signaling in W53-derived cells. Interestingly, this uncovered Src function has not been observed with the use of SrcK⁻. This may be explained, at least in part, by a scaffolding function for SFK in this signaling process. The c-Src interfering mutants used here co-localized with Fyn and Lyn [endogenous SFK members stimulated by PRL in W53 cells [14]]. They are catalytically inactive, but their folding presumably differs. In SrcK⁻, the carboxy-terminal Y527, being phosphorylated by CSK, could bind intra-molecularly to SH2 domain, causing an inactive conformation, which restrains accessibility of SH2 and SH3 and induces an inhibition of Y416 autophosphorylation. The Y527 is absent in SrcDN and SrcΔK and their SH3 and SH2 domains are presumably capable of forming signaling complexes. Accordingly with these folding differences, immunoprecipitation of these Src mutants with MAb 327, which specifically recognizes c-Src, from Doxy-induced W53 cells shows increased Y416 phosphorylation of SrcDN upon PRL stimulation that is not detected in SrcK⁻ expressing cells (Supplementary Fig. 1). In addition, assuming that Y416-SrcDN is exposed, it could serve as a competitive substrate for both the kinase activity of Fyn and Lyn and their binding to

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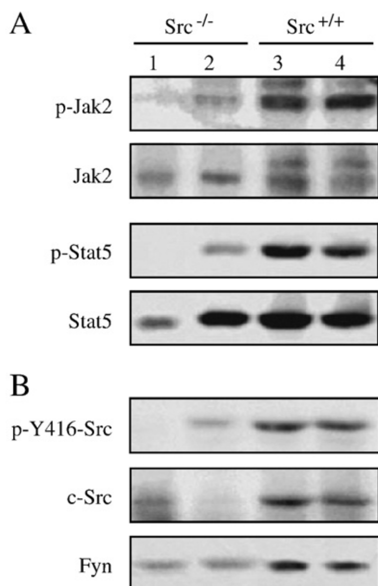

Fig. 7. Role of c-Src in the control of Jak2/Stat5 pathway in mammary gland. Inguinal mammary glands isolated at day two of lactation from *Src*$^{-/-}$ (lanes 1 and 2) and *Src*$^{+/+}$ (lanes 3 and 4) mice were homogenized in lysis buffer and 100 µg of total protein from supernatants were used for WB analyses of: (A) SFK activation with anti-p-Y416-c-Src, c-Src and Fyn expression; (B) Phosphorylation and expression of Jak2 and Stat5.

downstream targets. Consistently, immunoblotting detection of pY416 in extracts from W53 cells upon Doxy-induction of SrcDN shows that PRL stimulation highly increases Y416-phosphorylation, which is inhibited by preincubation of cells with the SFK tyrosine kinase inhibitor PP2 (Supplementary Fig. 2). This finding suggests a mechanism of trans-phosphorylation among SFK members.

These conformational differences among c-Src mutants are also reflected in their efficacy to interfere with Fyn and Lyn signaling. Consequently, SrcDN is more efficient than SrcΔK, while SrcK^{-} is the least active mutant to impede PRL-activated cell proliferation. We believe that SrcDN and SrcΔK mutants may interfere with the capacity of endogenous Fyn and Lyn to activate Jak2 signaling.

Similarly, in MCF7 human breast cancer cells with c-Src depletion or by expression of SrcDN [33], PRL is unable to stimulate Jak2. Moreover, Src-SH2^{-} and Src-SH3^{-} defective mutants further support the role of the scaffold functions of SFK in the control of PRL activation of Jak2. Due to the inactivation of the SH2 or the SH3 domains, the kinase activity of these mutants is constitutively activated; nevertheless, both of them significantly reduce PRL stimulation of Jak2. These results are consistent with inability of SrcK^{-} expression and the SFK-selective inhibitors to alter PRL activation of Jak2 in W53 and MCF7 cell lines [14,15]. In HC11 cells, a kinase inactive Src reduces PRL stimulation of b-casein expression [34], while Src-like inhibitors have no effect on lactogenic activation of Stat5 [35]. Our data, concerning SH2 and SH3 defective Src mutants in MCF7 cells, suggest that these adaptor domains of SFK are involved in the control of Jak2 activation by PRL. While these data argue that targeting this signaling pathway may be of therapeutic value in this type of cancer, this would predict that Src kinase inhibitors might be inefficient. Therefore, small inhibitors of Src-SH2/3 functions could be more effective in this case.

Finally, our results seem to indicate that this regulatory process also operates under physiological conditions as exemplified in lactating mammary glands from *Src*$^{-/-}$ mice. It should be noticed that in these animals a residual level of Jak2 activity is detected but this may be attributed to the activation of additional upstream tyrosine kinases to be identified, probably another SFK member. There is evidence for redundant functions between Src kinases [36]; although mammary gland development and lactation is not altered in the *Fyn*$^{-/-}$ mice (M. Richert, H. Watkin, and S.M. Anderson, unpublished data), it is significantly altered in *Src*$^{-/-}$ mice [30].

The results presented here also confirm an essential role of SFK kinase activity in the control of Akt/Myc and p70S6K pathways, which are consistent with data previously published [14–16]. Interestingly, it has been recently shown in MCF7 that inhibition of SFK by PP1 reduces PRLR internalization and activation of Akt, but not Jak2 [17]. This may not be restricted to PRL signaling as an SFK/Akt pathway has been reported upon stimulation by serum, estradiol and EGF in mammary epithelial mammary cells [29,33,37]. It should be noticed that, while expression of Jak2ΔK does not affect PRL-induced Akt activation in our study, this kinase is not activated in conditional *Jak2*$^{-/-}$ mammary epithelial cells stimulated with “growth factors enriched medium” [21]. Therefore, we cannot exclude a possible scaffolding function for Jak2 in Src-mediated Akt activation in our model.

Finally, our observations suggest the existence of an SFK/Jak2/Erk1/2 pathway, which is also regulated by a scaffolding function of SFK. This data agrees with a role of SFK in the regulation of Erk1/2 previously reported in mammary epithelial cells [15,29,33]. However, the role of Jak2 in the control of Erk1/2 activation has not been clearly established. For instance, a link between Jak2 and Erk1/2 has been reported upon growth hormone, angiotensin or PDGF stimulation [38–40], while PRL stimulates Erk1/2 in conditional *jak2*$^{-/-}$ mammary epithelial cells [21]. Furthermore, in NIH-3 T3 fibroblasts expressing PRLR short form, which is unable to activate Jak2, PRL promotes Erk1/2 activation and proliferation [41]. Therefore the importance of a Jak2/Erk1/2 pathway may be dependent upon the biological model system and/or the extracellular stimulus.

In summary, our study uncovers the existence of a scaffolding function for SFK in the induction of SFK/Jak2/Stat5 and SFK/Jak2/Erk1/2 pathways by PRL. Moreover, these data reinforce the pivotal role for SFK in PRL signaling both in normal and transformed cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2009.10.013.

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