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Research article

# PTPN4 NEGATIVELY REGULATES CRKI IN HUMAN CELL LINES

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**Abstract:** PTPN4 is a widely expressed non-receptor protein tyrosine phosphatase. Although its overexpression inhibits cell growth, the proteins with which it interacts to regulate cell growth are unknown. In this study, we identified CrkI as a PTPN4-interacting protein using a yeast two-hybrid, and confirmed this interaction using *in vitro* GST pull-down and co-immunoprecipitation and co-localization assays. We further determined the interactional regions as the SH3 domain of CrkI and the proline-rich region between amino acids 462 and 468 of PTPN4. Notably, overexpression of PTPN4 inhibits CrkI-mediated proliferation and wound healing of HEK293T cells, while knockdown of PTPN4 by siRNA in Hep3B cells enhances CrkI-mediated cell growth and motility. Moreover, our data show that ectopic expression of PTPN4 reduces the phosphorylation level of CrkI in HEK293T cells. These findings suggest that PTPN4 negatively regulates cell proliferation and motility through dephosphorylation of CrkI.

**Key words:** PTPN4, PTPMEG, CrkI, CRK, Cell proliferation, Protein tyrosine phosphatase, Wound-healing assay, Subcellular localization, Protein-protein interactions, Yeast two-hybrid

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Abbreviations used: DAPI – 4',6-diamidino-2-phenylindole; ORF – open-reading frame; PTPs – protein tyrosine phosphatases; RNAi – RNA interference; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

## INTRODUCTION

Protein tyrosine phosphorylation is the net effect of the action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [1, 2]. PTK signaling is the major mechanism for receptor signal transduction, which mediates cell growth, differentiation, host defense, and metabolic regulation [3, 4].

In humans, there are about 80 active protein phosphatases, which can be divided into four distinct classes. The largest classical class, Class I, can be sub-divided into receptor and non-receptor PTPs [5]. PTPN4, a non-receptor PTP that was originally isolated from human umbilical vein endothelial cell cDNA libraries, contains a single PTP domain (amino acids 654 to 913) [6]. The recombinant phosphatase domain possesses PTP activity when expressed in *E. coli*. PTPN4 contains several characteristic motifs outside the catalytic domain, including an erythrocyte cytoskeleton protein 4.1 homology domain and a DHR motif (518 to 599), which has been found in numerous proteins, most of which are localized at membranes, cytoskeleton, or adhesion junctions [7]. There are also two proline-rich sequences at amino acids 389 to 397 and 462 to 468 in the middle of PTPN4, and these could bind to the SH3 domains of some signaling proteins.

Overexpression of PTPN4 in COS-7 cells affects cell growth and colony formation in soft agar, indicating that PTPN4 is involved in the regulation of cell proliferation [8]. Knockdown of PTPN4 abrogated virus-mediated apoptosis [9]. The mouse homolog of PTPN4 is highly expressed in the testes, suggesting the involvement of PTPN4 in meiotic cell division during spermatogenesis [10]. The *Drosophila* homolog PTPN4 is expressed in the neural system and is required for the proper establishment and maintenance of axon projections in the central brain of *Drosophila* [11].

The endogenous PTPN4 only accounts for about $1/3000^{th}$ of the total tyrosine phosphatase activity in COS-7 cells [8]. Overexpression of PTPN4 in cells does not result in detectable changes in the overall state of protein tyrosine phosphorylation, illustrating that the effect of PTPN4 on cell proliferation is likely via specific substrates rather than global dephosphorylation. Two specific substrates of PTPN4 have been reported: the glutamate receptor (GluR) δ2 and the T-cell receptor (TCR) ξ subunits. PTPN4 plays a role in signaling downstream of the GluRs and is involved in cerebellum-dependent motor learning [12]. PTPN4 dephosphorylates the ITAM motif of the TCR ξ subunit and negatively regulates NF-κB and AP-1 activation in Jurkat T cells [13]. How overexpression of PTPN4 slows down cell proliferation remains unclear.

*Crk* is the mammalian homologue of the avian sarcoma virus CT10 gene v-*Crk* [14]. It exists in two alternatively spliced isoforms: CrkI, which contains one SH2 domain and one SH3 domain (SH2-SH3), similar to v-Crk; and CrkII, which contains one SH2 domain and two SH3 domains (SH2-SH3-SH3) [15, 16]. CrkI transmits signals via its SH2 domain, which binds to the phospho-motif Y-X-X-P of many focal adhesion components, such as p130Cas and paxillin [17, 18], and growth factor receptors such as IGF1 receptor [19], EGFR [20] and

PDGFβR [21]. CrkI subsequently associates with guanine nucleotide exchange factors, such as DOCK180 and C3G, via its SH3 domain [22, 23]. CrkI play roles in Src, Abl and Arg cellular tyrosine kinases signaling pathways [24]. In this way, CrkI functions as a signaling adaptor protein to mediate diverse cellular responses, including proliferation, differentiation and migration [25-27]. Overexpression of CrkI has been detected in many types of human cancer cells [16, 28-31]. A possible role has been suggested for CrkI during these tumorigenesis events, specifically in mediating intracellular signaling related to cell motility and proliferation [29].

In this study, we identified CrkI as a novel PTPN4-interacting protein via the yeast two-hybrid approach with a human brain cDNA library. We confirmed this interaction using two approaches: the *in vitro* GST pull-down assay and co-immunoprecipitation and co-localization experiments in human cells. We found that PTPN4 negatively regulates CrkI-mediated cell growth and migration and acts as a protein tyrosine phosphatase to dephosphorylate CrkI. These findings imply that a regulatory role for PTPN4 in cell proliferation is likely through the specific dephosphorylation of the adaptor protein CrkI.

## MATERIALS AND METHODS

### Plasmid construction

The open-reading frame (ORF) of PTPN4 was obtained via PCR from a human brain cDNA library (Clontech). It was subcloned into the pDBLeu vector (Invitrogen) for the yeast two-hybrid screen and into the pCDEF-Myc or pEF-FLAG vector for expression in mammalian cells. All of the PTPN4 deletion mutants were amplified from full-length PTPN4 and introduced into the pDBLeu vector to generate BD-fused proteins. The ORF of full-length CrkI was amplified via PCR from the human brain cDNA library, further subcloned into pPC86 (Invitrogen) for interaction verification in yeast, into bacterial expression vector pGEX-5x-1 (Amersham) to generate GST-fusion protein, and into pEF-FLAG for expression in mammalian cells or the pEGFP-C1 vector (Clontech) for the localization assay. The CrkI deletion mutants SH2 and N-SH3 were amplified from full-length CrkI and cloned in-frame into the pPC86 vector.

### Yeast two-hybrid screening

Two-hybrid screening was performed in the ProQuest Two-Hybrid System (Invitrogen). pDBLeu-PTPN4 was transformed into the yeast strain MaV203 as a bait to generate stable BD-PTPN4-transformed yeast cells. These cells were then transformed with a human fetal brain library (Clontech). A total of $2 \times 10^6$ clones were screened on the selection agar plates and positive clones were verified using the β-galactosidase assay. Plasmid DNA was isolated and re-transformed into yeast along with either pDBLeu-PTPN4 or pDBLeu vector control to verify the specific interaction.

**GST pull-down assay**

Both GST and GST-CrkI fusion proteins were expressed in *E. coli* strain BL21 (Stratagene) and purified with glutathione Sepharose 4B beads (Amersham Pharmacia) according to the manufacturer's protocol. Whole cell lysates from HEK293T cells transfected with pEF-PTPN4 were precipitated with anti-FLAG beads (Sigma) and eluted with FLAG peptide (Sigma), then incubated with GST fusion proteins immobilized on the beads for 6 h at 4ºC. The bead-conjugated complexes were washed with cell lysis buffer, boiled in SDS sample buffer and subjected to Western blot analysis.

**Cell culture, transfection and immunoprecipitation**

HEK293T, Hep3B and HeLa cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in 5% $CO_2$ at 37ºC. Plasmids were transfected into cells using Lipofectamine or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations, and were harvested 48 h post-transfection. For immunoprecipitation, harvested cells were washed with ice-cold PBS and lysed in cell lysis buffer consisting of 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 1 mM DTT, 5 mM EDTA (pH 8.0), 1 mM PMSF and protease inhibitor cocktails from Amresco. Cell lysates were pre-cleared with protein A/G-agarose beads (Santa Cruz) at 4ºC for 1 h and then were immunoprecipitated with the indicated antibodies at 4ºC for 6 h. The bead-conjugated complexes were washed with cell lysis buffer, boiled in SDS sample buffer and subjected to Western blot analysis.

**Western blot**

Protein samples were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies at 4ºC overnight, followed by incubation with secondary antibody for 1 h at 25ºC. Immunoreactivity was visualized by enhanced chemiluminescence (Millipore). The specific antibodies included anti-Myc antibody (9E10, Santa Cruz), anti-FLAG antibody (M2, Sigma) and anti-p-Tyr antibody (P-Tyr-100, Cell Signaling).

**Laser-scanning confocal microscopy**

HeLa cells were treated essentially as described previously [32]. HeLa cells grown on glass coverslips were transfected with the construct CrkI-pEGFP and PTPN4-pCDEF-Myc. Transfected HeLa cells were washed with PBS and fixed with 4% paraformaldehyde (pH 7.4) in PBS for 20 min. After permeabilization with 0.1% Triton X-100 in PBS for 10 min, the cells were incubated with an anti-c-Myc antibody at 4ºC overnight, followed by incubation with a Cy3-conjugated anti-mouse IgG antibody (Rockland). Slides were embedded in an anti-fade aqueous mounting medium containing 1 μg/ml DAPI (Sigma), and then evaluated and photographed on a laser-scanning confocal microscope (LSM510

META, Carl Zeiss) equipped with a Zeiss AxioCam HR-cooled CCD camera and a 40× oil-immersion objective.

**RNA isolation and real-time PCR**

RNA was isolated from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. A total RNA sample with a volume of 2 μg was treated with DNase I and reverse-transcribed by the M-MLV reverse transcriptase (Promega). Each primer set amplified a single product as indicated by a single peak during melting curve analyses. Real-time PCR was performed on an Applied Biosystems 7300 Real-Time PCR System using SYBR Green Real-Time PCR Master Mix (TaKaRa). After an initial denaturation at 95ºC for 1 min, amplification was performed with 40 cycles of 95ºC for 30 s and 60ºC for 30 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. The real-time PCR primer sequences of PTPN4 and β-actin are shown in Table 1.

Table 1. Sequences of primers used in this study.

| Primer name | Sequences | Product sizes (bp) |
|---|---|---|
| PTPN4-qRT-FP | TGCTGGCAGAACCTACAATG | 105 |
| PTPN4-qRT-RP | CAACAAGACTTGCCCCTGAT | |
| β-actin-qRT-FP | CCTGGCACCCAGCACAAT | 144 |
| β-actin-qRT-RP | GGGCCGGACTCGTCATACT | |

**RNA interference**

Synthetic siRNA (GenePharma, Shanghai, China) was delivered into Hep3B cells using Lipofectamine 2000 reagent. The nucleotide sequence of the PTPN4 siRNA was 5'ACACCUAGUCAAUACAGAUdTdT3' and that of the luciferase control was 5'UUCUCCGAACGUGUCACGUdTdT3'.

**Cell proliferation analysis**

Cell proliferation was performed using Cell Counting Kit-8 (CCK-8, Dojindo) as described previously [33]. HeLa or Hep3B cells were employed for the transfection and the measurement. After transfection, cells were plated in 96-well plates at 3000 cells per well and cultured in the growth medium. At the indicated time points, 10 μl CCK-8 solution was added to each well and incubated at 37ºC for 1 h, and the cell numbers in triplicate wells were determined by reading the OD at 450 nm.

**Wound-healing assay**

Cell motility was evaluated using the scratched wound-healing assay on plastic plate wells. Briefly, a total of $5 \times 10^5$ HeLa or Hep3B cells were seeded in each well and incubated for 12 h. After transfection, a surface of confluent cells in a monolayer was wounded with a 200-μl regular pipette tip. The cellular

migration was evaluated by measuring the width of the wound at a given point at the indicated time intervals. All of the experiments were repeated at least three times with similar results.

## RESULTS

### Identification of the interaction between PTPN4 and CrkI

To identify novel PTPN4-interacting proteins, yeast two-hybrid screening was performed using full-length PTPN4 as the bait to screen a human fetal brain library. Sequence analysis showed that of the 21 positive targets, 3 encode the C-terminal portion of CrkI (amino acids 42 to 204). For confirmation, the full-length CrkI was cloned into pPC86 and co-transformed with pDBleu-PTPN4 into the yeast strain MaV203. As shown in Fig. 1A, the reporter LacZ gene is activated in the CrkI and PTPN4 co-transformed yeast cells, compared to the negative controls (Fig. 1A left panel) and cells with CrkI or PTPN4 alone (Fig. 1A right panel). This interaction between PTPN4 and CrkI was further verified

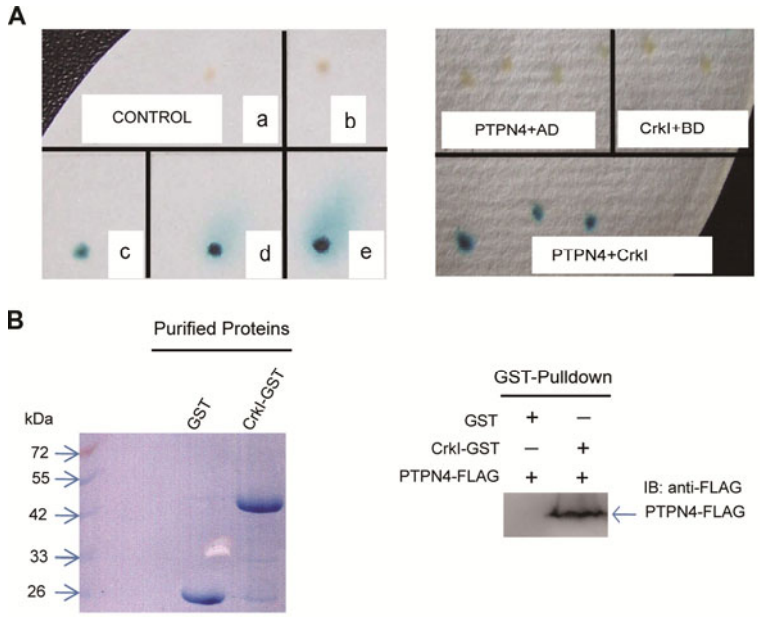


Fig. 1. Identification of the interaction of CrkI and PTPN4. A – Yeast two-hybrid LacZ reporter gene assay for interaction between PTPN4 and CRK. Left panels (a, b, c, d and e): MaV203 yeast controls with different degrees of protein-protein interaction. In the controls from a to e, the degree of interaction is from negative to maximal positive. Right panel: yeast strain MaV203 co-transformed with pDBLeu-PTPN4/pPC86, pPC86-CrkI/pDBLeu or pPC86-CrkI/pDBLeu-PTPN4. The blue color indicates positive interactions and the light brown color indicates no evident interaction. B – GST pull down *in vitro* confirmation of CrkI and PTPN4 interaction. Left panel: recombinant GST and GST-CrkI proteins purified from *E. coli*. Right panel: GST pull-down assay. PTPN4 protein was expressed and purified from HEK293T cells.

using the *in vitro* GST pull-down assay. In the GST pull-down assay, FLAG-tagged PTPN4 protein was expressed in HEK293T and purified with anti-FLAG beads, and GST-CrkI was expressed in *E. coli* and purified with Glutathione Sepharose 4B beads. We found that PTPN4 could efficiently bind to GST-CrkI, but not to GST tag (Fig. 1B), which is consistent with the results of the yeast two-hybrid experiment results.

**Verification of the CrkI-PTPN4 interaction in cells**

To confirm the interaction of CrkI and PTPN4 in cells, FLAG-tagged CrkI and Myc-tagged PTPN4 were co-transfected into HEK293T cells to examine whether CrkI and PTPN4 can be co-immunoprecipitated with specific antibodies. As shown in Fig. 2A, compared with the vector, Myc-tagged PTPN4 was co-precipitated with FLAG-tagged CrkI. These results suggest that the interaction between CrkI and PTPN4 could really exist in cells. To test if the positive result of CrkI and PTPN4 co-IP is caused by a non-specific binding of CrkI, we used PTPN3 as a control, because PTPN3 is a very similar protein to PTPN4 in the PTPN family [34]. As shown in Fig. 2B, PTPN3 cannot be pulled down by CrkI, unlike its near family member PTPN4, which suggests that the interaction of CrkI and PTPN4 is specific.

To provide further evidence of this interaction, immunofluorescence microscopy was used to visualize the subcellular localization of the two proteins in HeLa cells. We found that most CrkI is localized on the cell membrane, as indicated by the GFP-fusion protein. PTPN4 exists in the cell membrane and surrounding cytoplasm, as shown by Cy3-labeled red immunofluorescence (Fig. 2C). Importantly, remarkable co-localization of CrkI and PTPN4 can be easily viewed in the cell membrane area, suggesting that the interaction of CrkI and PTPN4 is physiologically relevant. As shown in Suppl. Fig. 1 in supplementary material at http://dx.doi.org/10.2478/s11658-013-0090-3, we found that there is no significant difference whether CrkI is expressed separately or is expressed with PTPN4 simultaneously. These data indicate that CrkI interacts with PTPN4 both *in vitro* and in cells.

**Identification of domains involved in the CrkI-PTPN4 interaction**

After having shown that CrkI can bind PTPN4 *in vitro* and in cells, we next asked which regions of PTPN4 and CrkI are involved in their interaction. Since CrkI contains two individually separate src-homology domains, SH2 and SH3, and the other isoform of CRK, CrkII, contains an additional C-terminal SH3 domain, we constructed three truncation mutants of CRK: SH2, N-SH3 and C-SH3. Interestingly, we found that the N-SH3 domain, but not the C-SH3 domain, is responsible for this interaction in the yeast two-hybrid assay (Fig. 3A). As the N-SH3 domain is also present in CRKL, which is a homologous gene of CRK, we next tested whether CRKL can also bind PTPN4. Consistent with the idea that the N-SH3 domain mediates the interaction with PTPN4, we found that the full-length CRKL indeed binds PTPN4 protein (Suppl. Fig. 2A in supplementary

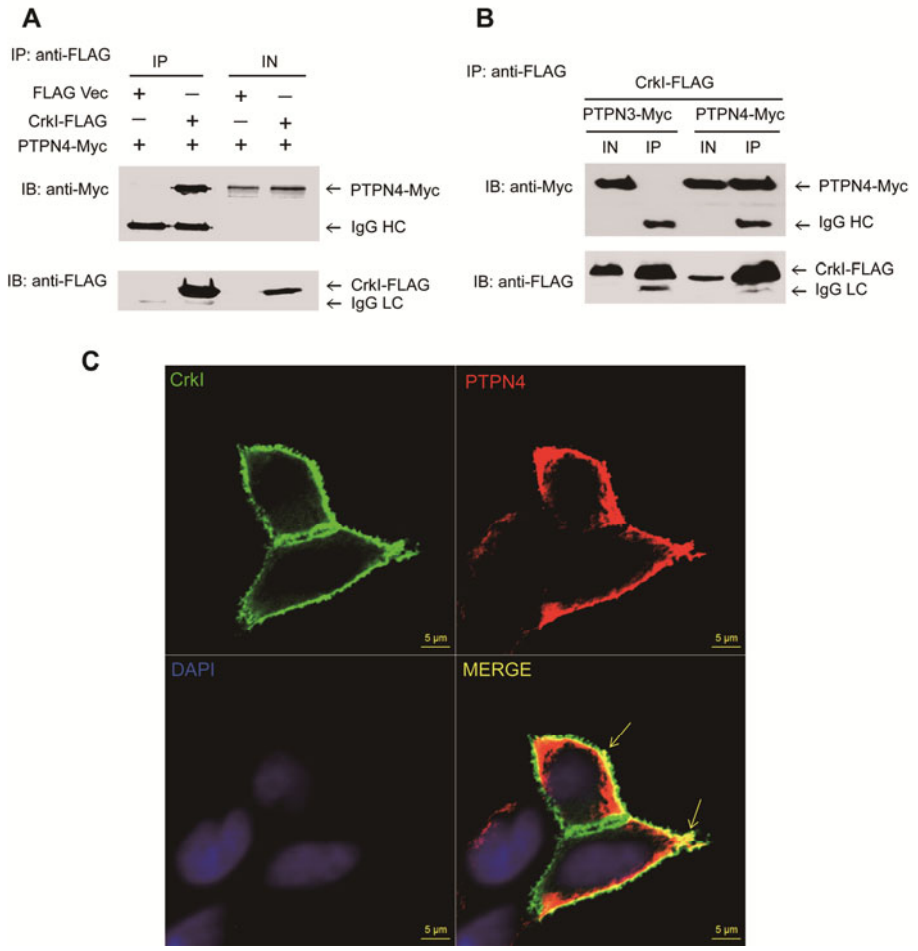


Fig. 2. Confirmation of the interaction of CrkI and PTPN4 in cells. A – Co-immunoprecipitation confirmation of CrkI and PTPN4 interaction in HEK293T cells. CrkI-FLAG and PTPN4-Myc were co-transfected into HEK293T cells. FLAG-tagged vector and PTPN4-Myc were co-transfected as a control. Lysate was immunoprecipitated with anti-FLAG antibody, and then analyzed by immunoblotting with anti-FLAG antibody or anti-c-Myc antibody. IN: input, 5% of cell lysate; IP: immunoprecipitation. B – Different family members of PTPN present different CrkI binding activities. CrkI expressed with PTPN3 or PTPN4 in HEK293T cells, respectively. Lysate was immunoprecipitated with anti-FLAG antibody, and then analyzed by immunoblotting with anti-FLAG antibody or anti-c-Myc antibody. IN: input, 10% of cell lysate; IP: immunoprecipitation. C – Co-localization assay of CrkI and PTPN4. HeLa cells were co-transfected with PTPN4-Myc and CrkI-GFP plasmids. Immunofluorescence staining was performed using an anti-c-Myc antibody. The merged images showed the co-localized positions (yellow). These images were visualized using a confocal laser-scanning microscope.

material at http://dx.doi.org/10.2478/s11658-013-0090-3). Furthermore, this interaction can also be confirmed by GST pull-down assay (Suppl. Fig. 2B), suggesting that the interaction of the SH3 domain and PTPN4 can be employed by other CRK-homologous protein.

Based on the domain analysis of PTPN4 and a previous report that the SH3 domain can bind canonical proline-rich motifs [35], we generated three truncation mutants of PTPN4: PT01, which is PTPN4 without C-terminal PTP;

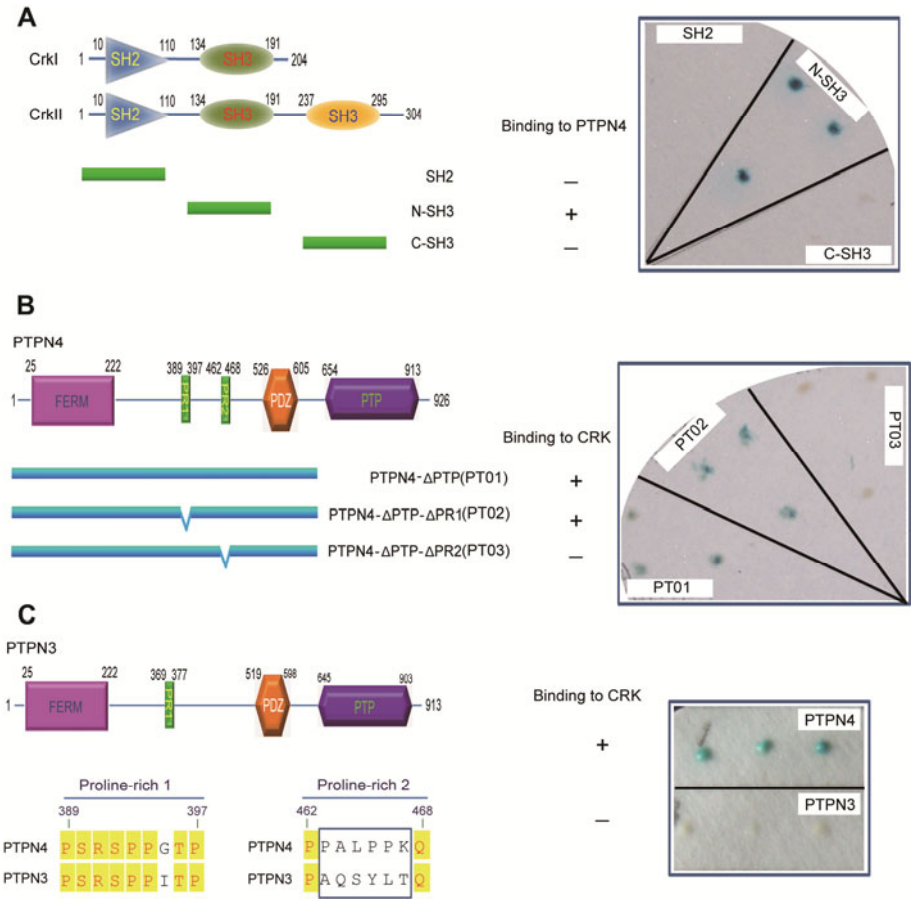


Fig. 3. Interactional domain mapping of CrkI and PTPN4. A – Mapping of the CrkI-binding region inside PTPN4. Left panel: schematic representation of different PTPN4 mutants. Three PTPN4 mutants were separately co-transformed with CrkI into yeast strain MaV203. Left panel: β-galactosidase assay was utilized to test the interactional region. B – Mapping of the PTPN4-binding region inside CrkI. A schematic representation of different CrkI truncated mutants is shown in the left panel. C – Confirmation of CrkI-specific binding activity using Y2H. The most differences in PTPN4 and PTPN3 existed in their proline-rich 2 (PR2) regions. PTPN3 does not have a complete PR2 region. The same amino acids are marked in yellow and the significantly different amino acids in the PR2 region are boxed.

PT02, which is PT01 without the N-proline-rich motif; and PT03, which is PT01 without the C-proline-rich motif. Notably, we found that PT02 can bind CrkI, just like the wide-type full-length PTPN4 or PT01. By contrast, PT03 has lost the ability to bind CrkI (Fig. 3B). These results demonstrate that the C-proline-rich motif from amino acids 462 to 468 in PTPN4 is essential for the interaction with CrkI. Considering that there is no PR2 region in PTPN3, we used the yeast two-hybrid assay to assess whether this region is enough for CrkI to differentiate PTPN4 from PTPN3. As shown in Fig 3C, PTPN3 does not bind to CrkI, which further confirms that CrkI binds to PTPN4 in a specific manner.

**PTPN4 inhibits CrkI-mediated cell growth and mobility**

Since cells overexpressing PTPN4 grow more slowly and reach confluence at a lower density [8], and PTPN4's interacting partner CrkI functions in intracellular signaling related to cell motility and proliferation [29], we reasoned that PTPN4 may inhibit cell growth and mobility through CrkI. Consistent with this idea, the cell proliferation assay revealed that co-expression of PTPN4 and CrkI significantly inhibited HeLa cell growth compared with that of controls transfected with CrkI alone (Fig. 4A). To test the hypothesis that PTPN4 affects CrkI-mediated cell mobility, we evaluated the wound-healing ability in HeLa cells transfected with CrkI. This analysis demonstrated that co-expressed PTPN4 negatively affected CrkI-mediated cell mobility from the first day post-PTPN4 transfection. The vector-transfected control cells reached confluence on the second day after PTPN4 transfection. By contrast, the PTPN4-transfected cells showed a significantly slower healing rate, as shown in Fig. 4B. These findings support our hypothesis that PTPN4 negatively regulates cell growth and mobility through its interaction with CrkI. To further confirm our hypothesis, we transfected a deletion version of PTPN4 (PTPN4-ΔPR2) with CrkI into HeLa cells. As shown in Fig. 4B (lower panel), PTPN4-ΔPR2 did not significantly negatively regulate the wound-healing process, just like the empty vector, which suggests that PR2-deletion of PTPN4 abolished the activity of PTPN4 in cells.

**RNAi of PTPN4 enhances CrkI-mediated cell growth and mobility**

To further assess whether endogenous PTPN4 plays a negative role in CrkI-mediated cell proliferation and mobility, we employed synthetic small interference RNA (siRNA) to knock down the endogenous PTPN4, and then measured the proliferation of these cells. Real-time RT-PCR was utilized to screen for a suitable cell line that has an appreciable *PTPN4* expression and can be used for the RNAi experiment. As shown in Fig. 5A, we found that *PTPN4* is expressed in several cell lines, with the highest expression in Hep3B hepatocellular carcinoma cells. Therefore, the knockdown of PTPN4 was performed in Hep3B cells, as shown in Fig. 5B. Our data showed that siRNA against *PTPN4* significantly enhanced cell proliferation in CrkI-transfected Hep3B cells compared with the control siRNA-treated cells (Fig. 5C). We also tested whether knockdown of PTPN4 can promote the mobility of Hep3B. Consistent with the above observations that the RNAi of PTPN4 helps CrkI-

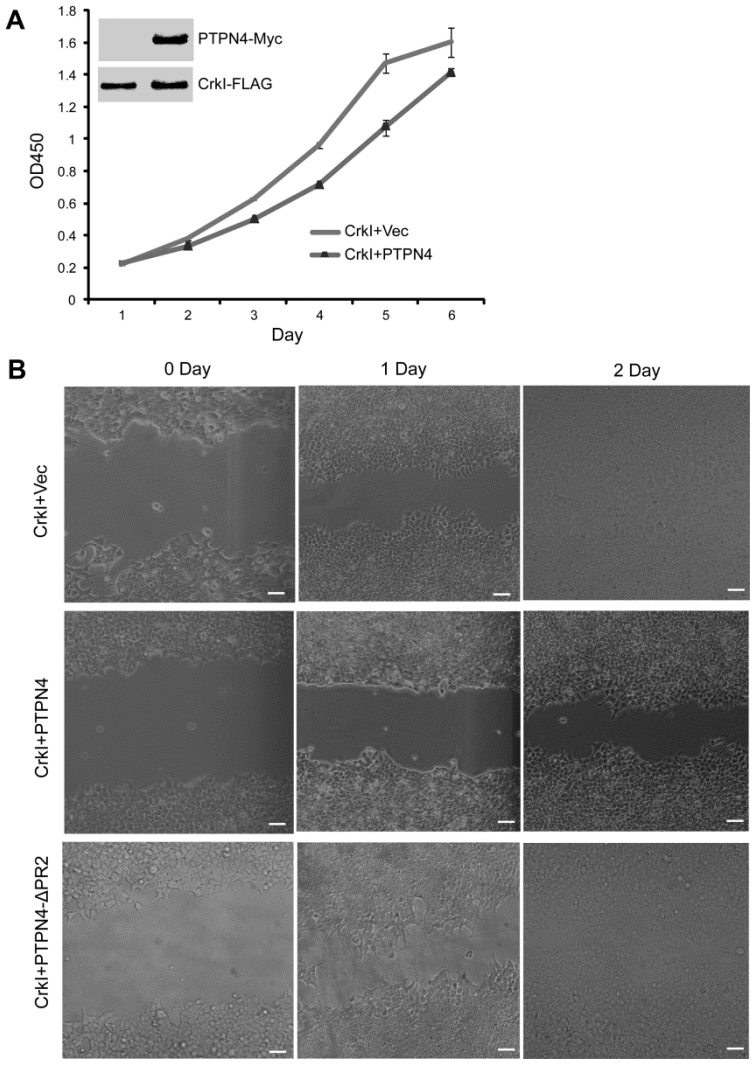


Fig. 4. Co-expressed PTPN4 inhibits CrkI-promoted proliferation of HeLa cells and delays the wound-healing process. A – Growth curves of PTPN4 and CrkI co-transfected HeLa cells. Cellular proliferation was measured using Cell-Counting Kit-8. The empty vector was also co-transfected in HeLa cells and used a control. Error bars show the standard deviation. B – Wound-healing assay of PTPN4 and CrkI co-transfected HeLa cells. PTPN4-ΔPR2 plasmid was used as a control. The width of the wound is measured every 12 h; the pictures taken at 0, 24 and 48 h are shown here. The experiments were repeated three times with similar results. Bars = 50 μm.

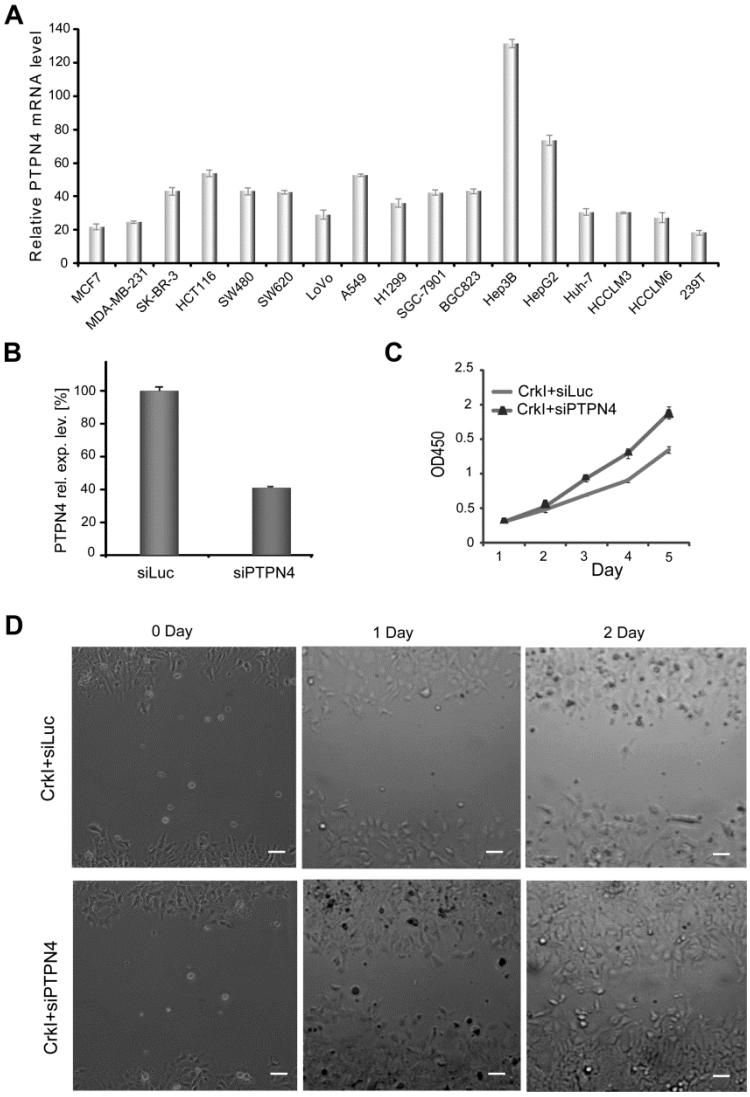


Fig. 5. Knockdown of PTPN4 enhances CrkI-mediated cell growth and cell motility in Hep3B cells. A – The expression pattern of PTPN4 in cell lines. Quantitative real-time RT-PCR analysis on 17 different cell lines from a variety of tissues showed that PTPN4 was strongly expressed in Hep3B and HepG2. B – Knockdown of PTPN4 using siRNA. The siRNA target to luciferase was used as a control. C – Growth curves of knockdown of PTPN4 in CrkI-transfected Hep3B cells. siLuc was used as a control. Error bars are shown. D – Wound-healing assay of silence of PTPN4 in CrkI-transfected Hep3B cells. The width of the wound is measured every 12 hours; the pictures taken at 0, 24 and 48 h are shown here. The experiments were repeated three times. Bars = 50 μm.

transfected Hep3B cell growth, we found that siPTPN4 accelerates the healing rate in Hep3B cells transfected with CrkI in comparison with the control siRNA-transfected cells (Fig. 5D). These data further supported the notion that PTPN4 negatively modulates CrkI-medicated cell growth and mobility.

**PTPN4 dephosphorylates CrkI in cells**

Inasmuch as PTPN4 is a protein tyrosine phosphatase that interacts with CrkI and presumably participates in CrkI-mediated cellular functions, we hypothesized that PTPN4 negatively regulates CrkI activity via dephosphorylation. To examine this hypothesis, we co-transfected PTPN4 and CrkI in HEK293T cells and measured the phosphorylation status of CrkI in cells. CrkI-FLAG protein was purified by immunoprecipitation with an antibody against FLAG 48 h post-transfection, and then incubated with an anti-phosphor-Tyr antibody to determine the phosphorylation level. Notably, we found that the phosphorylation level of CrkI in PTPN4-transfected cells was significantly reduced compared to the control cells (Fig. 6A). These results demonstrate that PTPN4 is a protein tyrosine phosphatase for CrkI in cells. To further confirm that PTPN4 regulates the phosphorylation level of CrkI in cells, we transfected CrkI with PTPN4-ΔPR2 to detect its phosphorylation status. As shown in Fig. 6B, the phosphorylation level of CrkI with PTPN4-ΔPR2 is much higher than the level of CrkI with PTPN4-WT, which suggests that abolishing the CrkI binding of PTPN4 is associated with damage to the dephosphorylation function.

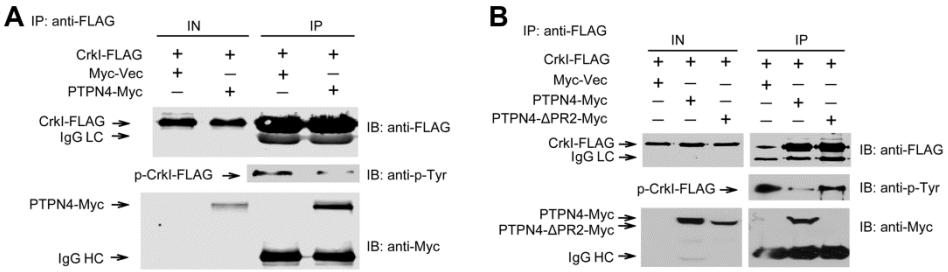


Fig. 6. Ectopic PTPN4 reduces the phosphorylation level of CrkI in HEK293T cells. A – PTPN4-Myc and CrkI-FLAG were co-transfected into HEK293T cells; Myc-vector and CrkI-FLAG were co-transfected as the control. Lysate was immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting with anti-p-Tyr, anti-FLAG and anti-Myc. The phosphorylation level of CrkI was detected by anti-p-Tyr antibody. IN: input, 5% of cell lysate; IP: immunoprecipitation. B – Confirmation of PTPN4 dephosphorylation of CrkI by using PTPN4-ΔPR2 as a control. IgG LC: IgG light chain; IgG HC: IgG heavy chain. CrkI protein was detected by anti-FLAG antibody. WT or ΔPR2-PTPN4 protein was detected by anti-Myc antibody. Phosphorylation level of CrkI was detected by anti-p-Tyr antibody. IN: input, 10% of cell lysate; IP: immunoprecipitation.

## DISCUSSION

In this study, we identified CrkI as a novel PTPN4-interacting protein using the yeast two-hybrid technique, and verified this interaction using the *in vitro* GST pull-down assay and cell co-immunoprecipitation and co-localization assays. All of the CrkI and most of the PTPN4 localized surrounding the cell membrane, where CrkI mediated a variety of cell signals including cell growth from the outer environment of the cell. Previous studies suggested that neither of the two known PTPN4-interacting proteins (TCR ξ and GluR δ2) is associated with the PTPN4-mediated pathway that inhibits cell proliferation [12, 13]. Our data revealed CrkI as the first PTPN4-interacting partner involved in cell proliferation and motility.

Overexpressed PTPN4 suppresses CrkI-mediated cell growth and motility in HEK293T cells. Importantly, knockdown of PTPN4 in Hep3B cells facilitates the overexpressed CrkI-mediated cell proliferation and migration, which is in line with observations on overexpressed PTPN4 in HEK293T cells. Notably, CrkI is a previously recognized oncoprotein that can enhance cell growth and motility [30, 36].

Our data support the model that CrkI acts as at least one of the PTPN4 downstream signaling proteins that are regulated by PTPN4 dephosphorylation. CrkI and CrkII, the two alternatively spliced isoforms of CRK, play important cellular roles, probably through different molecular mechanisms [37]. Y221 in the C-SH3 of CrkII is a defined regulatory site [38], whereas CrkI does not contain this tyrosine residue, but holds much stronger transformative activity as compared with CrkII [30, 31]. In contrast to previous studies that suggest that CrkI may function in a constitutively active form [39], our findings indicate that the phosphorylation status of CrkI can be altered by phosphatases like PTPN4 and some other unknown kinase(s).

However, two questions remain unanswered. First, which specific site of CrkI can be regulated by PTPN4, as there are eight tyrosine residues in CrkI? Second, which kinase or kinases can alter the phosphorylation status of CrkI?

The interaction mapping data have shown that only the second proline-rich motif of PTPN4 can bind CrkI, suggesting that the binding of CrkI to the proline-rich region is highly specific. The mechanism may also apply to other CrkI-interacting proteins. On the other hand, the N-SH3 domain of CrkI, which is responsible for the interaction with PTPN4, has helped us to identify two other N-SH3-containing members of the CRK family, CrkII and CRKL, as PTPN4-interacting partners. Further investigation is needed to determine whether CrkII and/or CRKL really interact with PTPN4 in cells, and whether CrkII and/or CRKL can also be regulated by PTPN4.

In summary, our study provides the first description of a molecular mechanism of how PTPN4 mediates the inhibition of cell proliferation. We propose that PTPN4 directly dephosphorylates CrkI and thus PTPN4 negatively regulates CrkI-mediated cell growth and mobility.

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## REFERENCES

1. Hubbard, S.R. and Till, J.H. Protein tyrosine kinase structure and function. **Annu. Rev. Biochem.** 69 (2000) 373-398.
2. Zhang, Z.Y. Protein tyrosine phosphatases: structure and function, substrate specificity, and inhibitor development. **Annu. Rev. Pharmacol. Toxicol.** 42 (2002) 209-234.
3. Rudolph, J. Inhibiting transient protein-protein interactions: lessons from the Cdc25 protein tyrosine phosphatases. **Nat. Rev. Cancer** 7 (2007) 202-211.
4. Vang, T., Miletic, A.V., Arimura, Y., Tautz, L., Rickert, R.C. and Mustelin, T. Protein tyrosine phosphatases in autoimmunity. **Annu. Rev. Immunol.** 26 (2008) 29-55.
5. Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J. and Mustelin, T. Protein tyrosine phosphatases in the human genome. **Cell** 117 (2004) 699-711.
6. Gu, M.X., York, J.D., Warshawsky, I. and Majerus, P.W. Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. **Proc. Natl. Acad. Sci. USA** 88 (1991) 5867-5871.
7. Gu, M. and Majerus, P.W. The properties of the protein tyrosine phosphatase PTPMEG. **J. Biol. Chem.** 271 (1996) 27751-27759.
8. Gu, M., Meng, K. and Majerus, P.W. The effect of overexpression of the protein tyrosine phosphatase PTPMEG on cell growth and on colony formation in soft agar in COS-7 cells. **Proc. Natl. Acad. Sci. USA** 93 (1996) 12980-12985.
9. Prehaud, C., Wolff, N., Terrien, E., Lafage, M., Megret, F., Babault, N., Cordier, F., Tan, G.S., Maitrepierre, E., Menager, P., Chopy, D., Hoos, S., England, P., Delepierre, M., Schnell, M.J., Buc, H. and Lafon, M. Attenuation of rabies virulence: takeover by the cytoplasmic domain of its envelope protein. **Sci. Signal.** 3 (2010) ra5.
10. Park, K.W., Lee, E.J., Lee, S., Lee, J.E., Choi, E., Kim, B.J., Hwang, R., Park, K.A. and Baik, J. Molecular cloning and characterization of a protein tyrosine phosphatase enriched in testis, a putative murine homologue of human PTPMEG. **Gene** 257 (2000) 45-55.

11. Whited, J.L., Robichaux, M.B., Yang, J.C. and Garrity, P.A. PTPMEG is required for the proper establishment and maintenance of axon projections in the central brain of *Drosophila*. **Development** 134 (2007) 43-53.
12. Hironaka, K., Umemori, H., Tezuka, T., Mishina, M. and Yamamoto, T. The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor delta 2 and epsilon subunits. **J. Biol. Chem.** 275 (2000) 16167-16173.
13. Young, J.A., Becker, A.M., Medeiros, J.J., Shapiro, V.S., Wang, A., Farrar, J.D., Quill, T.A., Hooft van Huijsduijnen, R. and van Oers, N.S. The protein tyrosine phosphatase PTPN4/PTP-MEG1, an enzyme capable of dephosphorylating the TCR ITAMs and regulating NF-kappaB, is dispensable for T cell development and/or T cell effector functions. **Mol. Immunol.** 45 (2008) 3756-3766.
14. van der Geer, P., Hunter, T. and Lindberg, R.A. Receptor protein-tyrosine kinases and their signal transduction pathways. **Annu. Rev. Cell Biol.** 10 (1994) 251-337.
15. Watanabe, T., Tsuda, M., Makino, Y., Konstantinou, T., Nishihara, H., Majima, T., Minami, A., Feller, S.M. and Tanaka, S. Crk adaptor protein-induced phosphorylation of Gab1 on tyrosine 307 via Src is important for organization of focal adhesions and enhanced cell migration. **Cell Res.** 19 (2009) 638-650.
16. Rodrigues, S.P., Fathers, K.E., Chan, G., Zuo, D., Halwani, F., Meterissian, S. and Park, M. CrkI and CrkII function as key signaling integrators for migration and invasion of cancer cells. **Mol. Cancer Res.** 3 (2005) 183-194.
17. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y. and Hirai, H. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. **EMBO J.** 13 (1994) 3748-3756.
18. Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., Tanaka, S., Nakamura, S. and Hattori, S. CRK protein binds to two guanine nucleotide-releasing proteins for the Ras family and modulates nerve growth factor-induced activation of Ras in PC12 cells. **Mol. Cell. Biol.** 14 (1994) 5495-5500.
19. Beitner-Johnson, D. and LeRoith, D. Insulin-like growth factor-I stimulates tyrosine phosphorylation of endogenous c-Crk. **J. Biol. Chem.** 270 (1995) 5187-5190.
20. Hashimoto, Y., Katayama, H., Kiyokawa, E., Ota, S., Kurata, T., Gotoh, N., Otsuka, N., Shibata, M. and Matsuda, M. Phosphorylation of CrkII adaptor protein at tyrosine 221 by epidermal growth factor receptor. **J. Biol. Chem.** 273 (1998) 17186-17191.
21. Antoku, S. and Mayer, B.J. Distinct roles for Crk adaptor isoforms in actin reorganization induced by extracellular signals. **J. Cell Sci.** 122 (2009) 4228-4238.
22. Akakura, S., Kar, B., Singh, S., Cho, L., Tibrewal, N., Sanokawa-Akakura, R., Reichman, C., Ravichandran, K.S. and Birge, R.B. C-terminal SH3

domain of CrkII regulates the assembly and function of the DOCK180/ELMO Rac-GEF. **J. Cell Physiol.** 204 (2005) 344-351.
23. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T. and Matsuda, M. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. **Genes Dev.** 12 (1998) 3331-3336.
24. Bell, E.S. and Park, M. Models of crk adaptor proteins in cancer. **Genes Cancer** 3 (2012) 341-352.
25. Park, T.J. and Curran, T. Crk and Crk-like play essential overlapping roles downstream of disabled-1 in the Reelin pathway. **J. Neurosci.** 28 (2008) 13551-13562.
26. Matsuki, T., Pramatarova, A. and Howell, B.W. Reduction of Crk and CrkL expression blocks reelin-induced dendritogenesis. **J. Cell Sci.** 121 (2008) 1869-1875.
27. Feller, S.M. Crk family adaptors-signalling complex formation and biological roles. **Oncogene** 20 (2001) 6348-6371.
28. Linghu, H., Tsuda, M., Makino, Y., Sakai, M., Watanabe, T., Ichihara, S., Sawa, H., Nagashima, K., Mochizuki, N. and Tanaka, S. Involvement of adaptor protein Crk in malignant feature of human ovarian cancer cell line MCAS. **Oncogene** 25 (2006) 3547-3556.
29. Wang, H., Linghu, H., Wang, J., Che, Y.L., Xiang, T.X., Tang, W.X. and Yao, Z. W. The role of Crk/Dock180/Rac1 pathway in the malignant behavior of human ovarian cancer cell SKOV3. **Tumour Biol.** 31 (2010) 59-67.
30. Takino, T., Nakada, M., Miyamori, H., Yamashita, J., Yamada, K.M. and Sato, H. CrkI adapter protein modulates cell migration and invasion in glioblastoma. **Cancer Res.** 63 (2003) 2335-2337.
31. Miller, C.T., Chen, G., Gharib, T.G., Wang, H., Thomas, D.G., Misek, D.E., Giordano, T.J., Yee, J., Orringer, M.B., Hanash, S.M. and Beer, D.G. Increased C-CRK proto-oncogene expression is associated with an aggressive phenotype in lung adenocarcinomas. **Oncogene** 22 (2003) 7950-7957.
32. Wan, B., Wang, X.R., Zhou, Y.B., Zhang, X., Huo, K. and Han, Z.G. C12ORF39, a novel secreted protein with a typical amidation processing signal. **Biosci. Rep.** 30 (2010) 1-10.
33. Wan, B., Zhou, Y.B., Zhang, X., Zhu, H., Huo, K. and Han, Z.G. hOLFML1, a novel secreted glycoprotein, enhances the proliferation of human cancer cell lines in vitro. **FEBS Lett.** 582 (2008) 3185-3192.
34. Bauler, T.J., Hendriks, W.J. and King, P.D. The FERM and PDZ domain-containing protein tyrosine phosphatases, PTPN4 and PTPN3, are both dispensable for T cell receptor signal transduction. **PLoS ONE** 3 (2008) e4014.
35. Schumacher, C., Knudsen, B.S., Ohuchi, T., Di Fiore, P.P., Glassman, R.H. and Hanafusa, H. The SH3 domain of Crk binds specifically to a conserved proline-rich motif in Eps15 and Eps15R. **J. Biol. Chem.** 270 (1995) 15341-15347.
36. Feller, S.M., Knudsen, B. and Hanafusa, H. c-Abl kinase regulates the protein binding activity of c-Crk. **EMBO J.** 13 (1994) 2341-2351.

37. Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T. and Shibuya, M. Two species of human CRK cDNA encode proteins with distinct biological activities. **Mol. Cell. Biol.** 12 (1992) 3482-3489.
38. Ren, R., Ye, Z.S. and Baltimore, D. Abl protein-tyrosine kinase selects the Crk adapter as a substrate using SH3-binding sites. **Genes Dev.** 8 (1994) 783-795.
39. Watanabe, T., Tsuda, M., Tanaka, S., Ohba, Y., Kawaguchi, H., Majima, T., Sawa, H. and Minami, A. Adaptor protein Crk induces Src-dependent activation of p38 MAPK in regulation of synovial sarcoma cell proliferation. **Mol. Cancer Res.** 7 (2009) 1582-1592.