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BCCIP ASSOCIATES WITH THE RECEPTOR PROTEIN TYROSINE PHOSPHATASE PTPmu

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Abstract

The receptor protein tyrosine phosphatase PTPmu belongs to a family of adhesion molecules that contain cell-cell adhesion motifs in their extracellular segments and catalytic domains within their intracellular segments. The ability of PTPmu both to mediate adhesion and exhibit enzymatic activity makes PTPmu an excellent candidate to transduce signals in response to cell-cell adhesion. In an effort to identify downstream signaling partners of PTPmu, we performed a modified yeast two-hybrid screen using the first tyrosine phosphatase domain of PTPmu as bait. We isolated an interacting clone encoding BRCA2 and CDKN1A (CDKN1A [p21]) interacting protein (BCCIP) from a HeLa cell library. BCCIP is a p21 and BRCA2 interacting protein that has been shown to play roles in both cell cycle arrest and DNA repair. In this manuscript, we confirm the interaction between BCCIP and PTPmu identified in yeast using *in vitro* biochemical studies and characterize BCCIP as a PTPmu binding protein. We demonstrate that BCCIP is phosphorylated by the Src tyrosine kinase and dephosphorylated by the PTPmu tyrosine phosphatase *in vitro*. Furthermore, we show that BCCIP is required for both the permissive and repulsive functions of PTPmu in neurite outgrowth assays, suggesting BCCIP and PTPmu are in a common signal transduction pathway.

Keywords

Receptor protein tyrosine phosphatase; neurite outgrowth; BCCIP; nuclear localization

Protein tyrosine phosphorylation is recognized as an important mode of regulation for many cellular functions. The tyrosine phosphorylation state of cellular proteins is determined by the coordinated activity of protein tyrosine kinases (PTKs) [Machida et al., 2003; Pawson, 2002] and protein tyrosine phosphatases (PTPs) [Tonks, 2006]. PTPs are a diverse group of enzymes that exist as either soluble cytoplasmic forms or as transmembrane, receptor-like proteins [Tonks, 2006]. In this manuscript, we focus on the receptor protein tyrosine phosphatase (RPTP) PTPmu. The extracellular segment of PTPmu contains sequences found in several cell adhesion molecules including a MAM domain, an Ig domain and four FNIII repeats. As suggested by the presence of these domains, PTPmu mediates cell-cell adhesion via homophilic binding [Brady-Kalnay et al., 1993; Gebbink et al., 1993b] meaning the “ligand” for PTPmu is an identical PTPmu molecule on an adjacent cell. The juxtamembrane domain of PTPmu contains a region homologous to the conserved intracellular domain of the cadherins. Cadherins are calcium-dependent cell-cell adhesion molecules that associate with the actin cytoskeleton via catenins [Gumbiner, 2005; Lilien, 2005]. Members of the catenin family bind the intracellular domain of cadherins and include α -catenin, β -catenin, plakoglobin and p120. PTPmu associates with cadherin-catenin complexes [Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995;

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Hiscox and Jiang, 1998; Sui et al., 2005]. Specifically, PTP μ interacts with classical cadherins such as N-cadherin, E-cadherin and R-cadherin [Brady-Kalnay et al., 1998] and VE-cadherin [Sui et al., 2005]. PTP μ has been shown also to interact with p120 catenin [Zondag et al., 2000]. The intracellular domain of PTP μ contains two conserved catalytic domains. Of these two phosphatase domains, only the membrane proximal domain possesses catalytic activity [Gebbinck et al., 1993a].

PTP μ has been implicated previously in the regulation of E-cadherin-mediated cell-cell adhesion [Hellberg et al., 2002] and both N-cadherin and E-cadherin-mediated neurite outgrowth [Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007]. Furthermore, PTP μ has been shown to interact directly with IQGAP1 [Phillips-Mason et al., 2006], a protein involved in the regulation of cadherin-mediated cell-cell adhesion [Kuroda et al., 1998; Li et al., 1999]. In addition to PTP μ 's role in the regulation of cadherin function, PTP μ appears to either directly or indirectly regulate protein kinase C. In support of this, PTP μ was found to interact with the scaffolding protein RACK1 (receptor for activated C-kinase) in a yeast two-hybrid screen [Mourton et al., 2001], and both PTP μ -mediated neurite outgrowth and PTP μ -mediated axon guidance of retinal ganglion cells require PKC δ activity [Ensslen and Brady-Kalnay, 2004; Rosdahl et al., 2002].

In an effort to elucidate PTP μ -mediated signaling events, we set out to identify PTP μ -interacting proteins using a modified yeast two-hybrid screen in which we co-expressed PTP μ with a constitutively active Src tyrosine kinase. The presence of active Src increased the potential to identify proteins that interact in response to tyrosine phosphorylation. In this manuscript, we describe BCCIP as a PTP μ -interacting protein isolated by this modified two-hybrid screen. BCCIP was originally described as TOK-1 (twenty-one and CDK associated protein-1) based on its ability to bind the cyclin-dependent kinase (CDK)-inhibitor, p21^{Waf1/Cip1} (referred to as p21 hereafter) in a cyclin/CDK2/p21 ternary complex and to enhance the inhibitory activity of p21 on CDK2 [Ono et al., 2000]. Independently, a second group identified BCCIP as a protein that interacts with the internal conserved region of BRCA2, and showed that overexpression of BCCIP inhibits the growth of some tumor cell lines [Liu et al., 2001]. TOK-1 and BCCIP are identical proteins existing as two isoforms in humans, BCCIP α and BCCIP β [Liu et al., 2001; Ono et al., 2000]. In addition to interacting with p21, BCCIP has been shown recently to regulate p21 expression in a p53-dependent manner [Meng et al., 2004b]. In lower organisms, including rodents, only one BCCIP isoform exists [Lu et al., 2005]. A yeast homolog of BCCIP, Bcp1 has been identified and demonstrated to function in the nuclear export of a protein involved in the regulation of the yeast PI4P 5-kinase, Mss4 [Audhya and Emr, 2003]. In this manuscript, we demonstrate a direct interaction between PTP μ and BCCIP. Furthermore, we show BCCIP α is tyrosine phosphorylated *in vitro* and a substrate for PTP μ . The best characterized biological function of PTP μ is neurite outgrowth. The catalytic activity of PTP μ is required for it to promote neurite outgrowth of nasal retinal ganglion cells and induce repulsion of temporal retinal ganglion cells [Ensslen-Craig and Brady-Kalnay, 2005]. In this manuscript, we demonstrate that expression of a BCCIP antisense construct blocks both permissive and repulsive activities of PTP μ in neurite outgrowth assays of retinal ganglion cells, suggesting BCCIP and PTP μ are in a common signaling pathway.

MATERIALS AND METHODS

Antibodies

A monoclonal antibody generated against the intracellular domain of PTP μ (SK18) has been described previously [Brady-Kalnay and Tonks, 1993; Brady-Kalnay and Tonks, 1994] and was used to detect PTP μ . An HRP-conjugated antibody to T7 (Novagen, Madison, WI) was used to detect the T7 tag in His-iPTP μ constructs. Antibodies to BCCIP α,β (TOK-1 α,β) and Lamin A/C were purchased from Transduction Laboratories (San Diego, CA). An antibody

that recognizes vinculin was purchased from Sigma Chemical Company (Saint Louis, MO) and a monoclonal antibody to phosphotyrosine (PY99) was obtained from Santa Cruz (Santa Cruz, CA).

Cell Culture

Sf9 insect cells (CRL 1711: American Type Tissue Culture, Manassas, VA) were maintained at 27°C in Grace's Insect Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1 µg/ml gentamicin. A549 human non-small cell lung carcinoma cells (CCL 185: American Type Tissue Culture, Manassas, VA) were maintained in F12 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1 µg/ml gentamicin at 37°C, 5% CO₂. LNCaP human prostate cancer cells were maintained in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1 µg/ml gentamicin at 37°C, 5% CO₂. PrEC, normal human prostate epithelial cells (BioWhittaker, Walkersville, MD) were maintained at 37°C, 5% CO₂ in PrEGM growth media (BioWhittaker, Walkersville, MD).

E6 retinal cell cultures were prepared as described previously [Burden-Gulley and Brady-Kalnay, 1999]. Briefly, E6 (stage 28) chick neural retinas were dissociated in 0.25% trypsin, 4Na EDTA (Invitrogen) for 20 minutes, followed by mechanical trituration. Cells were resuspended and cultured in 10% fetal bovine serum (Hyclone), 2% chick serum (Sigma, St. Louis, MO), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.025 µg/ml amphotericin in RPMI (Invitrogen).

Modified Yeast Two-hybrid Screen

We utilized an Interaction Trap/Two-Hybrid system to identify PTPµ interacting proteins originally described by Erica Golemis [Golemis, 1997; Mourtou et al., 2001]. There are four essential components of the system: the bait (PTPµ), the prey (potential interacting proteins from a library) and two reporters (LEU2 and lacZ) that allow detection of the interaction between the bait and prey. Briefly, a construct containing the membrane-proximal catalytic domain of human PTPµ (PTPµD1, amino acids 915-1178) was cloned into pEG202 (*HIS3*) to yield pEG202-D1, and this plasmid was used as bait. The pEG202 plasmid constitutively expresses a chimeric protein containing the DNA-binding domain of LexA fused to the bait. In the absence of an interacting library protein, the LexA-bait fusion is unable to activate either of the two reporters. pEG202-D1 and the β-galactosidase reporter plasmid, pSH18-34 (*URA3*), were co-transformed into the YPH499 yeast strain. The reporter plasmid pSH18-34 carries a LexA operator-lacZ fusion gene. A conditionally expressed HeLa cell cDNA library in the pJG4-5 (*TRP1*) yeast expression vector was introduced into a yeast strain containing a chromosomal copy of the *LEU2* gene (EGY48). The upstream activating sequences for the chromosomal *LEU2* gene in the EGY48 strain were replaced with LexA operators. *LEU2* is required for biosynthesis of leucine. The library proteins are expressed, under an inducible yeast *GAL1* promoter, as fusions to an acid blob that function as a transcriptional activation motif. Library protein expression is induced by plating transformants on medium containing galactose. There is no expression of library proteins in the presence of glucose, which is used as a negative control. The two yeast strains, EGY48 and YPH499, were mated. Potential interactions were detected by growing the mated yeast strain on minimal media containing 2% galactose and 1% raffinose lacking certain amino acids to ensure selective pressure. The two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on media lacking leucine and selected based upon blue color when the yeast are grown in Xgal-containing media. Library proteins that do not interact specifically with the bait fail to grow in the absence of Leu. Yeast that contain PTPµ-interacting proteins will turn blue only when plated in the presence of Xgal and galactose.

In the modified yeast two-hybrid screen, the constitutively active form of the non-receptor protein tyrosine kinase *src* provided by Dr. Jonathan Cooper [Keegan and Cooper, 1996] was ligated into the pSH18-34 plasmid. The pSH18-34/*src* and pEG202-D1 plasmids were used to transform the YPH499 yeast strain. This transformed yeast strain was then mated to the EGY48 strain containing the HeLa cell cDNA library to isolate interacting clones that are dependent upon or enhanced by tyrosine kinase activity.

Expression of Fusion Proteins in *E. coli*

Plasmids containing GST fusion proteins of full length BCCIP α (pGEX-GST-TOK-1 α) and BCCIP β (pGEX-GST-TOK-1 β) were kindly provided by Dr. Hiroyoshi Ariga (Hokkaido University, Japan). GST-BCCIP α , GST-BCCIP β and GST alone were expressed in *E. coli* under the control of the *lac* promoter. The BCCIP fusion proteins and GST were purified from *E. coli* using glutathione Sepharose 4B beads (Amersham Biosciences) and used in the GST pull-down experiments described below. Plasmids containing intracellular GST-PTP μ fusion proteins (GST-iPTP μ :765-1452, GST-iPTP μ :765-1178 and GST-iPTP μ :765-958) were described previously [Phillips-Mason et al., 2006]. These fusion proteins were also expressed in bacteria under the control of the *lac* operon and isolated on glutathione Sepharose as described above.

The pTrcHis plasmid (Invitrogen, Carlsbad, CA) is used to generate T7, 6(His)-tagged fusion proteins in bacteria. Using PCR, we generated a plasmid encoding the juxtamembrane domain and first phosphatase domain of PTP μ (His-iPTP μ :774-1161) that expresses a T7/His-tagged fusion protein containing amino acids 774-1161 of PTP μ . Similarly, a plasmid encoding the second phosphatase domain of PTP μ (His-iPTP μ :1213-1452) that expresses a T7/His-tagged fusion protein containing amino acids 1213-1452 was generated. Finally, a plasmid that expresses a T7/His-tagged fusion protein containing amino acids 915-1161 of PTP μ was generated (His-iPTP μ :915-1161). This construct is similar to the yeast two-hybrid bait (915-1161 vs. 915-1178). The T7/His-tagged fusion proteins were expressed in *E. coli* under the control of the *lac* operon. His-iPTP μ :915-1161 was purified using the PrepEaseTM His-Tagged Protein Purification Kit (USB Corporation, Cleveland, OH).

Baculovirus Expression

A baculovirus encoding BCCIP α was generated by digesting the pGEX-GST-TOK-1 α plasmid with *Bam*HI and *Not*I restriction enzymes and ligating the TOK-1/BCCIP insert into pVL1392 (Pharmlingen) linearized with *Bg*II and *Not*I restriction enzymes. Baculovirus was generated using the BCCIP/pVL1392 plasmid and the BaculoGoldTM system (BD Pharmingen). Sf9 cells were infected with virus stocks, and forty eight hours post-infection, cells were harvested in a lysis buffer containing 20mM Tris, pH 7.5, 1% Triton X-100, 150mM NaCl, 1mM benzamidine, 0.2mM phenyl arsine oxide, 1mM sodium orthovanadate, 0.1mM molybdate and protease inhibitor cocktail (Sigma Chemical Company, Saint Louis, MO), and used in GST pull-down experiments as described below.

GST Pull-down Experiments

10 μ g of *E. coli* protein lysate from cells expressing the His-iPTP μ :774-1161 or His-iPTP μ :1213-1452 constructs were incubated with glutathione Sepharose beads which had been bound previously to GST-BCCIP α , GST-BCCIP β or GST alone in lysis buffer containing 20mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1mM benzamidine, 0.2 mM phenyl arsine oxide, 1mM sodium orthovanadate, 0.1 mM molybdate and protease inhibitor cocktail. Samples were rocked overnight at 4°C. The next day, samples were washed 4 times in lysis buffer and incubated at 95°C for five minutes in 2X SDS sample buffer. One fifth of the sample was resolved by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis as described previously [Brady-Kalnay et al., 1995]. A similar experiment was done using 50 μ g of protein

lysate from Sf9 cells expressing BCCIP α incubated with GST-PTP μ fusion proteins immobilized on glutathione Sepharose. Pull-downs were also done using 500 μ g of protein lysate from A549 cells treated with 100 μ M pervanadate for 20 minutes, or untreated. These lysates were incubated with GST, GST-BCCIP α or GST-BCCIP β immobilized on glutathione Sepharose in lysis buffer as described above.

In Vitro Src Kinase Assay

GST-BCCIP α and GST-BCCIP β fusion proteins and GST alone were expressed and purified from bacteria as described above. 30 μ g of each fusion protein was used in an *in vitro* Src kinase reaction. 500 μ l of kinase buffer (50mM Hepes, pH 7.4, 50mM NaCl, 0.1mM NaV, 5mM MgCl₂, 5mM MnCl₂, and 5 mM ATP) was added to 30 μ g of fusion protein along with 5 μ l (15U) of purified Src tyrosine kinase (Catalog number 14-117, Upstate, Lake Placid, NY) or buffer, and the samples were incubated at room temperature for 2 hours. Samples were separated by SDS-PAGE and transferred to nitrocellulose. Tyrosine phosphorylation was detected by immunoblot with an anti-phosphotyrosine antibody. These fusion proteins were subsequently used in pull-down experiments with purified His-iPTP μ :915-1161.

PTP μ Phosphatase Assay

GST-BCCIP α was phosphorylated *in vitro* by Src, as described above. After the Src kinase reaction, the phosphorylated BCCIP α sample was divided into three samples. One sample was kept as a control for the kinase reaction, and the remaining two were washed 3 times in phosphatase buffer (25mM Hepes, pH 7.4, 50mM NaCl and 5 mM DTT). 500 μ l phosphatase buffer was added to the samples, and active GST-iPTP μ :765-1178 isolated as previously described [Phillips-Mason et al., 2006] was added to one sample. Samples were incubated at 37°C for 15 minutes and then stopped by the addition of 2X sample buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. Tyrosine phosphorylation was analyzed by immunoblot with an anti-phosphotyrosine antibody.

Immunocytochemistry

A549, PrEC and LNCaP cells were grown on Lab Tech II chamber slides (Nalge Nunc International Corp., Rochester, NY) for one to two days. Cells were fixed with 4% paraformaldehyde for 10 minutes. Cells were then washed with PBS three times and permeabilized using 0.5% saponin in blocking buffer (20% goat serum, 1% BSA in PBS) for 30 minutes. Primary antibodies were diluted in blocking buffer plus 0.5% saponin and incubated overnight at 4°C. After incubation with primary antibody, cells were rinsed five times with TNT buffer (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween-20). Secondary antibodies (either goat anti-mouse-FITC or goat anti-rabbit-FITC from ICN Biochemicals, Irvine, CA) were diluted in blocking buffer plus saponin and incubated for one hour at room temperature. After incubation with secondary antibody, cells were washed five times with TNT buffer and once with distilled water. Molecular Probes SlowFade® Light Antifade kit was used to minimize quenching. Slides were imaged using a Nikon (Tokyo, Japan) TE200 inverted microscope and images were collected with a Spot RT digital camera and image acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Subcellular Fractionation

A549, PrEc and LNCaP cells were grown in 10 cm dishes to 80–95% confluence. Cells were washed twice with PBS, and 500 μ l of cold lysis buffer (10mM Tris-HCl, pH 7.5, 10mM NaCl, 2mM EDTA and 10% (v/v) glycerol) was added to the cells. Cells were collected by gentle scraping into a microfuge tube, incubated on ice for 10 minutes and then dounced fifty times on ice with a size B pestle. Dounced cells were centrifuged at 3000 $\times$ g for 10 minutes at 4°C to pellet nuclei. The supernatant was collected and centrifuged at 100,000 $\times$ g for 60 minutes

at 4°C; the supernatant of this spin was designated as cytosol. Nuclear pellets were resuspended in 200 µl cold lysis buffer and layered over an equal volume of 50% sucrose (w/v) in lysis buffer and centrifuged at 14,000 × g for 15 minutes at 4°C. Nuclear pellets were resuspended in cold lysis buffer with 0.1 % Triton X-100, briefly sonicated and insoluble material pelleted by centrifuging at 14,000 × g for 4 minutes at 4°C. Protein concentration of the cytosolic and nuclear lysates was determined by the Bradford method [Bradford, 1976], using BSA as standard. Lysates were incubated with equal amounts of 2X sample buffer at 95°C for 5 minutes. Twenty µg of both cytosolic and nuclear proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for analysis by immunoblotting with various antibodies.

Lentivirus Preparation and Cell Infection

A Human full-length PTPµ-GFP construct in pBPSTR1 has been described previously [Burden-Gulley and Brady-Kalnay, 1999]. We constructed a PTPµ-GFP lentiviral construct by inserting the *NotI* fragment from PTPµ-GFP/pBPSTR1 into the lentiviral expression vector pCDH-MCS2 (System Biosciences, Mountain View, California). An intracellular PTPµ-765-1451-GFP construct was generated using PCR. The PCR fragment was inserted into pCDH-MCS2 using the *XbaI* and *NotI* restriction sites in the MCS. VSV-G-pseudotyped lentiviral particles were generated by triple transfection of pCDH-MCS-2 constructs with the packaging plasmids pCMVΔR8.91 and pMD.G into 293T cells using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, California) according to a previously described protocol [Dull et al., 1998]. A549 cells were infected with lentiviral particles concentrated by ultracentrifugation in the presence of 6 µg/ml of polybrene. Immunocytochemistry was performed 24 hours post-infection.

Recombinant HSV Preparation and Cell Infection

The antisense PTPµ-HSV plasmid used to generate recombinant HSV was previously described [Ensslen et al., 2003]. To generate an antisense BCCIP-HSV plasmid, the human BCCIPβ insert was excised from BCCIPβ/pGEX-FLAG via digestion with *BamHI* and *XhoI* restriction enzymes and inserted in the antisense orientation into the pHSVpuc-MCS vector. The virus was produced as described previously [Ensslen et al., 2003] by transfection of the recombinant plasmids into a helper cell line (2–2 Vero cells), followed by infection with the 5d11.1 helper virus. After three amplification cycles, the virus was concentrated by ultracentrifugation and stored at –80°C.

E6 retinal cells and LNCaP cells were infected with 10 µl of HSV in basal media (RPMI and F12, respectively). After a 2 hour incubation, 2X complete media was added to the cells to give a final concentration of 1X. Cells were incubated for a total of 24 hours in the presence of HSV prior to harvesting the cells for protein extraction.

Bonhoeffer Stripe Assay

The stripe assay used was a modified version of the Bonhoeffer method [Vielmetter et al., 1990] previously described [Burden-Gulley et al., 2002]. Briefly, tissue culture dishes were coated with nitrocellulose [Lagenaur, 1987] and allowed to dry before applying a silicon lane matrix to the culture dish. Alternating stripes of laminin and PTPµ-Fc chimera containing Texas-Red conjugated BSA (for visualization) were generated as follows for single stripe assays. 80 ng of PTPµ-Fc chimera described previously [Rosdahl et al., 2003], was injected into the channels of the silicon lane matrix, incubated for 10 minutes, aspirated and then replaced with a new aliquot of PTPµ-Fc substrate for two additional 10 minute incubations. Any remaining binding sites within the lanes were blocked with bovine serum albumin (BSA; fraction V; Sigma) and rinsed with calcium-magnesium free phosphate buffer (CMF). The matrix was removed and 1.75 µg of laminin (BTI, Stoughton, MA) was spread across the lane

area and incubated for 20 minutes. Explants from E8 chick nasal retina were grown on the alternating stripes of laminin and PTP μ -Fc for 48 hours in 10% fetal bovine serum (Hyclone), 2% chick serum (Sigma, St. Louis, MO), 2 mM L-glutamine, 2 U/ml penicillin, 2 μ g/ml streptomycin and 5 ng/ml amphotericin in RPMI (Invitrogen). Quantitation of the stripe assays was performed using a rating scale previously described [Ensslen and Brady-Kalnay, 2004; Walter, 1987]. Neurites that show no preference for either substrate are assessed at 0. Neurites that grow exclusively on one substrate are assessed at 3. Neurites that grow mainly on the laminin lanes with an occasional neurite crossing over PTP μ lanes, are assessed at 2, while an assessment of 1 is given when there is a significant amount of neurite crossing but a tendency to fasciculate on laminin. For mixed substrate stripe experiments, 160ng PTP μ -Fc mixed with 0.9 μ g laminin containing Texas Red BSA was used as the first substrate as described above. For the second substrate, 1.75 μ g of laminin was spread over the lane area. The entire dish was blocked with BSA and rinsed with RPMI. When viral perturbations were used, HSV was added at the time of explant. Statistical significance between the different treatments was determined using an unpaired *t* test.

RESULTS

Modified yeast two-hybrid analysis

A modified yeast two-hybrid screen was performed to identify PTP μ interacting proteins. In this modified scheme, a constitutively active Src kinase was co-expressed as a strategy for identifying protein-protein interactions that are dependent upon, or strengthened by, tyrosine phosphorylation. This modified yeast two-hybrid scheme has been used previously to identify protein interactions that are enhanced by phosphorylation [Keegan and Cooper, 1996; Lioubin et al., 1996]. Yeast containing either the first phosphatase domain of PTP μ as bait or the first phosphatase domain of PTP μ and constitutively active Src were mated with yeast containing a HeLa cell cDNA library. Only yeast containing both bait and prey plasmids will grow on galactose, and the β -galactosidase reporter will be activated only when interaction of the bait and a prey protein occurs. There is no expression of library proteins when the yeast are plated on glucose. Using this system, we isolated a prey construct encoding full length BCCIP α from the HeLa cell library. PTP μ and BCCIP α interacted in yeast on galactose only in the presence of constitutively active Src as indicated by β -galactosidase staining of the yeast colony (Fig. 1A). These data suggest the interaction between BCCIP and PTP μ is directly or indirectly stabilized by Src kinase activity in the yeast system. BCCIP is a p21 [Meng et al., 2004b; Ono et al., 2000] and BRCA2 [Liu et al., 2001; Lu et al., 2005] interacting protein. In human cells, two BCCIP isoforms, BCCIP α and BCCIP β , are expressed as the result of alternative splicing [Liu et al., 2001; Ono et al., 2000]. The isoforms are identical through the first 258 amino acids but have unique C-termini. BCCIP α has a 64 amino acid C-terminal domain, while BCCIP β has a 58 amino acid C-terminus. In lower organisms, including rodents, only one BCCIP isoform exists [Lu et al., 2005]. To date, no isoform-specific functions have been demonstrated for BCCIP.

BCCIP and PTP μ interact in vitro

The first phosphatase domain of PTP μ (amino acids 915-1178) was used as the bait in our modified yeast two-hybrid screen. To verify that BCCIP was interacting with this domain, lysates from bacteria expressing PTP μ constructs corresponding to the juxtamembrane domain (JMD) and first phosphatase domain (D1) of PTP μ (His-iPTP μ :774-1161) and the second phosphatase domain (D2) of PTP μ (His-iPTP μ :1213-1452), shown in figure 1C, were incubated with BCCIP-GST fusion proteins isolated on glutathione Sepharose (Fig. 1B) in a pull-down assay (Fig. 1D). Figure 1D shows BCCIP interacts specifically with the His-iPTP μ :774-1161 protein. Furthermore, both BCCIP α and BCCIP β are equally efficient at interacting with PTP μ in this system. These results suggest that the PTP μ binding site on BCCIP

lies within the N-terminal 258 amino acids that both BCCIP isoforms share. GST was used as a negative control and did not interact with either phosphatase domain of PTP μ (Fig. 1D). Figure 1C shows that approximately equal amounts of the His-iPTP μ :774-1161 and His-iPTP μ :1213-1452 protein were expressed. It should be noted that there is a small amount of His-iPTP μ :1213-1452 protein binding to BCCIP α and β (Fig. 1D), this is most likely due to the fact that there is 33% identity and 53% similarity between the D1 and D2 phosphatase domains of PTP μ .

BCCIP α is a substrate for the Src tyrosine kinase and the PTP μ tyrosine phosphatase in vitro

In order to investigate the role of Src phosphorylation on the PTP μ /BCCIP interaction, we first determined whether BCCIP was a substrate for Src *in vitro*. BCCIP α and BCCIP β GST fusion proteins isolated on glutathione Sepharose were incubated with Src tyrosine kinase in an *in vitro* kinase assay. Tyrosine phosphorylation of the BCCIP fusion proteins was determined by immunoblotting with an anti-phosphotyrosine antibody. Figure 2A shows that BCCIP α , but not BCCIP β is a substrate for Src *in vitro*. There are no tyrosine residues in the BCCIP α unique region. There are six tyrosine residues in the region common to both BCCIP α and BCCIP β ; three of the six residues (Y64, Y71 and Y257) are predicted to be candidates for phosphorylation by NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos). Because both BCCIP isoforms contain all three tyrosine residues that have the potential to be phosphorylated, it is possible that there is some aspect of the unique region of BCCIP β that prevents phosphorylation, or some aspect of the unique region of BCCIP α that promotes phosphorylation of these residues. Of the three residues which have phosphorylation potential, perhaps the most interesting is Y257 which resides just one amino acid away from the end of the 258 amino acid sequence that the two BCCIP isoforms share. The fact that only BCCIP α is phosphorylated by Src may explain why this isoform was isolated in the modified yeast two-hybrid screen.

To determine whether tyrosine phosphorylation of BCCIP α altered the ability of BCCIP to interact with PTP μ , pull-downs were performed using the phosphorylated BCCIP fusion proteins and purified His-iPTP μ :915-1161 which encompasses the first phosphatase domain similar to the yeast two hybrid bait (amino acids 915-1161 vs. 915-1178). The bottom panel of Figure 2A (WB: α -PTP μ) shows Src phosphorylation did not have a significant effect on the ability of BCCIP α to bind to PTP μ . Furthermore, BCCIP β , which is not a substrate for Src tyrosine kinase *in vitro*, binds PTP μ . Therefore, the Src-dependence of the PTP μ /BCCIP interaction observed in the modified yeast two-hybrid screen, suggests Src is likely to indirectly regulate their interaction in complex systems. These data also suggest that PTP μ and BCCIP interact directly, since both the BCCIP and PTP μ used in these experiments were purified proteins.

Since BCCIP α is a substrate for Src *in vitro*, we were curious to determine whether BCCIP α was a substrate for PTP μ as well. Figure 2B shows that BCCIP α is indeed a substrate for PTP μ *in vitro*. These data strengthen the hypothesis that the interaction of these proteins is involved in cellular signaling in response to tyrosine phosphorylation.

The PTP μ /BCCIP interaction domain

To characterize further the region of PTP μ that interacts with BCCIP, we performed pull-downs using a series of PTP μ deletion constructs fused to GST (Fig. 3A). Lysates from Sf9 cells expressing BCCIP α were incubated with PTP μ -GST fusion proteins and assayed for their ability to interact with BCCIP α in a pull-down assay. Figure 3B shows that the smallest GST-PTP μ construct tested (GST-iPTP μ :765-958) was able to bind BCCIP. The yeast two-hybrid bait comprises amino acids 915-1178 of PTP μ . Together, these data suggest the minimal overlapping sequence in the PTP μ constructs that interact with BCCIP is amino acids 915-958.

Localization of endogenous BCCIP

PTP μ is a transmembrane protein stabilized at cell-cell contacts [Brady-Kalnay et al., 1995; Gebbink et al., 1995]. BCCIP is a cytosolic protein that contains a potential nuclear localization sequence. Previous studies have shown BCCIP to be predominantly nuclear [Liu et al., 2001; Ono et al., 2000]. Currently, there are only commercial antibodies that recognize the human BCCIP protein effectively. Therefore, we examined the localization of BCCIP in several human cell lines that endogenously express BCCIP (Fig. 4A). Immunocytochemistry was performed using a monoclonal antibody that recognizes both BCCIP α and BCCIP β . In all three cell lines examined, BCCIP was present in both the cytoplasm and the nucleus, suggesting BCCIP shuttles between the cytoplasm and nucleus. Both A549 human lung cancer cells and PrEC human prostate cells have endogenous PTP μ , but LNCaP human prostate cancer cells have down-regulated PTP μ protein expression by an unknown mechanism [Hellberg et al., 2002]. Figure 4A shows LNCaP and PrEC cells have a similar staining pattern of BCCIP, suggesting the presence of PTP μ protein does not change BCCIP localization in these cells. Adding PTP μ back to LNCaP cells via a lentiviral expression system did not change the localization of BCCIP (data not shown).

To verify the localization of BCCIP demonstrated by immunocytochemistry, we did subcellular fractionation of A549, LNCaP and PrEC cells. Somewhat surprisingly, BCCIP α appeared to be predominately, if not completely, in the cytoplasm and BCCIP β was both nuclear and cytosolic (Fig. 4B). These data are consistent with our immunocytochemistry data showing both a cytoplasmic and nuclear pool of BCCIP. The predominant cytoplasmic pool of BCCIP α was unexpected and revealed only by doing cell fractionation and immunoblot analysis, since the BCCIP isoforms cannot be distinguished by immunocytochemistry with the commercially available BCCIP α,β antibody. Our data are not in full agreement with the results of Ono, et al. [Ono et al., 2000], which demonstrate exogenous BCCIP is a predominately nuclear protein. The source for this discrepancy could be due to the fact that Ono, et al. [Ono et al., 2000] performed immunocytochemistry on Cos-7 cells over-expressing BCCIP. Consistent with our data, images from Liu, et al. [Liu et al., 2001] of MCF-7 cells stained for BCCIP, clearly demonstrate endogenous BCCIP is both nuclear and cytoplasmic. We would like to note that we performed immunocytochemistry in A549 cells with a polyclonal antibody specific to BCCIP α [a gift from Dr. Hiroyoshi Ariga, (Hokkaido University, Japan), data not shown]. With the BCCIP α -specific antibody, BCCIP α appeared to be mainly cytoplasmic, in agreement with our cell fractionation data. From these data in combination with the cell fractionation data, we concluded that the commercially available antibody directed against both BCCIP α and BCCIP β , has a higher affinity for native BCCIP β than native BCCIP α . This may explain why immunocytochemistry performed with the commercial antibody shows a greater nuclear pool than cell-fractionation. Overall, these combined data indicate BCCIP α and BCCIP β have both cytoplasmic and nuclear localization.

Endogenous PTP μ associates with BCCIP in pull-down assays

To gain additional evidence that PTP μ and BCCIP interact in vivo, we attempted to co-immunoprecipitate endogenous BCCIP and PTP μ from mammalian cells. Our attempts were unsuccessful, in part, due to the lack of antibodies capable of immunoprecipitating BCCIP. However, we were able to pull-down endogenous PTP μ from A549 cells using BCCIP-GST fusion proteins (Fig. 5A). To evaluate the role of tyrosine phosphorylation in this interaction, A549 cells were treated with or without the tyrosine phosphatase inhibitor pervanadate prior to making cell lysates. Pervanadate treatment elevates phosphotyrosine levels by inhibiting tyrosine phosphatases. Figure 5A shows that both BCCIP α and BCCIP β can pull down endogenous PTP μ , however, BCCIP α was much more efficient. The presence of pervanadate had no effect on the interaction. Full length PTP μ , which migrates ~200 kDa on SDS-PAGE, is proteolytically processed by a furin-like protease, generating two fragments of ~100 kDa

each, which remain tightly associated by a non-covalent mechanism [Brady-Kalnay and Tonks, 1994]. Therefore, PTP μ immunoreactive bands are recognized at both 200 kDa and 100 kDa on an immunoblot. BCCIP appears to predominately interact with the cleaved, intracellular form of PTP μ (Fig. 5A). These data support the yeast two-hybrid data in that BCCIP α appears to have a greater affinity for PTP μ than BCCIP β in human cell lines and that BCCIP may preferentially interact with the cleaved, intracellular form of PTP μ .

Intracellular PTP μ is found in the nucleus

Recently, the proteolytic cleavage of some RPTPs has been described [Aicher et al., 1997; Anders et al., 2006; Haapasalo et al., 2007]. A common theme among the RPTPs investigated is sequential cleavage resulting in fragments that can localize to distinct areas of the cell, including the nucleus. Proteolytic cleavage of PTP μ has not been studied thoroughly, however, cleavage of a closely related phosphatase, RPTP κ , has been reported [Anders et al., 2006]. Anders et al., show RPTP κ is sequentially cleaved by furin, then ADAM 10 to release an extracellular fragment, followed by a γ -secretase-dependent cleavage within the interior of the lipid bi-layer to release an intracellular fragment that translocates to the nucleus. To test whether related phosphatases were cleaved in a similar manner, Anders et al. used PTP μ in a series of experiments. The results of Anders et al. suggest PTP μ is proteolytically processed similar to RPTP κ , and that an intracellular PTP μ fragment is generated [Anders et al., 2006]. This intracellular fragment of PTP μ could potentially translocate to the nucleus but that was not tested by Anders et al. Interestingly, we can see a pool of endogenous PTP μ in the nucleus of A549 lung cancer cells by immunocytochemistry using an antibody directed against the intracellular domain of PTP μ (Fig. 5B). The nuclear staining of PTP μ is most evident in low cell density cultures. Whereas, in high cell-density cultures, PTP μ is localized to cell-cell contacts as previously described [Brady-Kalnay et al., 1995; Gebbink et al., 1995]. To support the notion that the nuclear PTP μ seen by immunocytochemistry is a cleaved intracellular fragment, we made an intracellular fragment of PTP μ (amino acids 765-1451) fused to a GFP tag. When we expressed this construct in A549 cells, it went predominantly to the nucleus (Fig. 6A). We performed immunocytochemistry on A549 cells expressing iPTP μ :765-1451-GFP using an anti-BCCIP antibody to show both PTP μ and BCCIP reside in the nucleus at the same time, demonstrating the potential for PTP μ and BCCIP to interact in the nucleus. Full-length PTP μ fused to GFP localized at cell-cell contacts (Fig. 6B), indicating that addition of the GFP tag did not alter subcellular location. PTP μ could modulate BCCIP activity in the nucleus or perhaps BCCIP could shuttle PTP μ in or out of the nucleus.

Antisense BCCIP Perturbs PTP μ Function

PTP μ function is well characterized in the chick retina where PTP μ has been shown to be a permissive substrate for E8 nasal retinal ganglion cell (RGC) neurite outgrowth and axon guidance [Burden-Gulley et al., 2002; Ensslen and Brady-Kalnay, 2004; Ensslen-Craig and Brady-Kalnay, 2005]. To determine whether BCCIP regulates PTP μ -mediated neurite outgrowth, we performed Bonhoeffer stripe assays in the presence of BCCIP antisense expression. Stripe assays are used to measure the preference of neurites to grow on different substrates.

To ensure that the HSV encoding antisense PTP μ and antisense BCCIP reduce PTP μ and BCCIP protein expression, respectively, we measured protein expression of HSV-infected cells by immunoblot. For PTP μ expression, E6 retinal cells were infected with antisense PTP μ -HSV, antisense BCCIP-HSV or uninfected for 24 hours. Infection of retinal cells with antisense PTP μ -HSV reduced the overall PTP μ protein levels by 60% (Fig. 7A). This reduction in protein is similar to what we have previously published with the antisense PTP μ -HSV construct [Ensslen et al., 2003; Ensslen-Craig and Brady-Kalnay, 2005; Oblander et al., 2007]. Antisense BCCIP-HSV had little to no effect on PTP μ protein expression. Since there are no available

antibodies to BCCIP that recognize chick protein, we determined the efficiency of the antisense BCCIP-HSV, in LNCaP cells because we can detect BCCIP in these human cells with an available antibody and they can be infected with HSV. LNCaP cells were infected with antisense BCCIP-HSV, the HSV vector alone, or uninfected for 24 hours. Infection with antisense BCCIP-HSV reduced both BCCIP isoforms by an average of 55% based on three separate experiments (Fig. 7B).

Both PTP μ and laminin are permissive substrates for E8 chick nasal RGCs and neurites grow freely on these substrates (Fig. 8, control). We have previously shown that both downregulation of PTP μ protein via antisense and over-expression of a catalytically inactive PTP μ block neurite outgrowth on a PTP μ substrate [Ensslen-Craig and Brady-Kalnay, 2005]. When antisense BCCIP is added to the cultures, PTP μ is no longer a permissive substrate for the neurites (neurites do not grow on PTP μ lanes), suggesting BCCIP is required for PTP μ 's permissive function (Fig. 8). Note that the neurites retain their ability to grow on laminin, demonstrating the specificity of the BCCIP antisense effect for the PTP μ substrate lane. These results suggest BCCIP protein expression is required for PTP μ to promote neurite outgrowth. Green fluorescence protein (GFP) was used as a negative control for viral infection. PTP μ antisense was used as a positive control.

PTP μ is expressed as a gradient in the chick retina with the nasal region having low PTP μ expression and the temporal region having high PTP μ expression [Burden-Gulley et al., 2002]. The differences in PTP μ expression yield different outcomes in neurite outgrowth assays. As mentioned above, PTP μ is a permissive substrate for E8 chick nasal RGCs. In contrast, PTP μ is a repulsive substrate for E8 chick temporal RGCs where PTP μ expression is high [Ensslen and Brady-Kalnay, 2004; Ensslen-Craig and Brady-Kalnay, 2005]. To further investigate the role of BCCIP in PTP μ function, we used a mixed stripe assay to determine whether BCCIP was also involved in PTP μ 's repulsive activity. Mixed stripe assays combine a permissive substrate (laminin) with a repulsive substrate (PTP μ) [Ensslen-Craig and Brady-Kalnay, 2005]. When the repulsive signal is blocked, the neurites grow on the permissive component (laminin) of that lane. In E8 chick temporal RGCs, PTP μ 's repulsive activity is dominant over laminin [Ensslen-Craig and Brady-Kalnay, 2005], therefore, under control conditions, E8 chick temporal RGCs only grow on laminin lanes and avoid lanes that contain both laminin and PTP μ (mixed stripes, Fig. 9). This confirms PTP μ is repulsive to temporal RGCs and that PTP μ signaling is dominant over laminin. As expected, the presence of PTP μ antisense blocked the repulsive activity of PTP μ in the mixed stripes, allowing the neurites to grow on the permissive laminin substrate also present in the lane as previously demonstrated [Ensslen-Craig and Brady-Kalnay, 2005]. When cultures are grown in the presence of BCCIP antisense, the lanes containing both PTP μ and laminin are no longer repulsive (Fig. 9, BCCIP-AS), indicating PTP μ function and signaling has been impaired. GFP and PTP μ antisense were used as negative and positive controls, respectively.

DISCUSSION

While many investigators have studied RPTP functions, it has been more problematic to identify signals that are directly transduced by RPTPs in response to cell adhesion or "ligand" binding. The identification of RPTP signaling pathways represents a major area of investigation in this field. To identify additional PTP μ interacting proteins, we used a modified yeast two-hybrid screen [Keegan and Cooper, 1996; Lioubin et al., 1996] to isolate proteins that bind PTP μ in the presence of the Src tyrosine kinase. In this system, proteins expressed in yeast are phosphorylated by an exogenously expressed constitutively active Src tyrosine kinase. We isolated BCCIP α in this screen as a protein that binds to the first phosphatase domain of PTP μ . This interaction was confirmed *in vitro*. Furthermore, we have demonstrated that

BCCIP α is a substrate for both the Src tyrosine kinase and the PTP μ tyrosine phosphatase *in vitro*.

BCCIP is a relatively novel protein. It was identified independently by two groups as a p21 [Ono et al., 2000] and BRCA2 [Liu et al., 2001] interacting protein. BCCIP has been shown to inhibit cell growth [Liu et al., 2001; Meng et al., 2004a] and to regulate p21 expression in a p53-dependent manner [Meng et al., 2004b]. In addition, BCCIP plays a role in homologous DNA repair [Lu et al., 2005; Lu et al., 2007], chromosome stability [Meng et al., 2007a], and p53 transactivation [Meng et al., 2007b]. A BCCIP ortholog has been identified in *Ustilago maydis* and is implicated in DNA repair and homologous recombination like its mammalian counterpart [Mao et al., 2007]. A yeast homolog of BCCIP was cloned and named Bcp1 [Audhya and Emr, 2003]. Bcp1 interacts with PI4, 5P2 phosphoinositide kinase (PI4P 5-kinase) in yeast. The PI4P 5-kinase shuttles from the plasma membrane to the nucleus, and this process is controlled by phosphorylation [Audhya and Emr, 2003]. Bcp1 binds PI4 5-kinase and controls its nuclear export [Audhya and Emr, 2003]. These authors hypothesize that the physiological function of Bcp1 is to control nuclear export of signaling proteins that regulate cell growth, including PI4P 5-kinase, p21/Cdk2 and BRCA-2. Our data suggest that there are sufficient cytoplasmic pools of BCCIP α and BCCIP β isoforms. We hypothesize that BCCIP may shuttle between the cytosolic and nuclear compartment in response to signal transduction. Based on recent data demonstrating some RPTPs including PTP μ undergo multiple cleavage events generating fragments that may enter the nucleus [Anders et al., 2006; Haapasalo et al., 2007] it is exciting to postulate that BCCIP and an intracellular cleavage product of PTP μ may translocate to the nucleus in response to a cell signaling event.

Loss of contact inhibition of growth and adhesion-based signals are necessary for cancer progression. It is interesting to note that both BCCIP and PTP μ expression levels change in cancer cells [Hellberg et al., 2002; Liu et al., 2001]. BCCIP expression is altered in some breast and brain tumor cells [Liu et al., 2001], and BCCIP is down regulated in both cancers of the kidney [Meng et al., 2003] and astrocytic brain tumors [Meng et al., 2007a]. Normal prostate cells express PTP μ whereas LNCaP prostate carcinoma cells do not [Hellberg et al., 2002]. We used this cancer cell model system to analyze the role of PTP μ in the regulation of cell adhesion. Normal epithelial architecture is maintained by E-cadherin, a homophilic cell-cell adhesion molecule. Disrupting E-cadherin or its associated proteins, catenins, correlates with invasion and metastasis [Christofori and Semb, 1999; Van Aken et al., 2001]. Changes in E-cadherin, N-cadherin, α -catenin, β -catenin, plakoglobin and p120 catenin expression have been observed in many types of carcinomas [Kallakury et al., 2001a; Kallakury et al., 2001b; Van Aken et al., 2001]. PTP μ has previously been shown to interact with the E-cadherin complex [Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995]. LNCaP cells express normal levels of E-cadherin and catenins, but do not mediate either PTP μ - or E-cadherin-dependent adhesion [Hellberg et al., 2002]. Re-expression of PTP μ restored cell adhesion to both PTP μ and to E-cadherin [Hellberg et al., 2002], demonstrating a role for PTP μ in the regulation of E-cadherin function. LNCaP cells were used in this manuscript and express both BCCIP α and β . In future studies, we will investigate how PTP μ -dependent cell adhesion regulates signaling and perhaps contact inhibition of growth via BCCIP.

Recently, BCCIP has been shown to promote p53 transcription activity [Meng et al., 2007b]. Down regulation of BCCIP protein abrogates p53-mediated upregulation of p21 in response to radiation. The mechanism by which BCCIP appears to regulate p53 activity is by facilitating the formation of active p53 tetramers [Meng et al., 2007b]. This is of interest to us because p53 can regulate neurite outgrowth [Bacsi, 2005; Di Giovanni, 2006]. PTP μ is well established as both a permissive and repulsive cue for RGC neurite outgrowth [Burden-Gulley et al., 2002; Ensslen-Craig and Brady-Kalnay, 2005]. In addition, PTP μ is required for both N-cadherin [Burden-Gulley and Brady-Kalnay, 1999] and E-cadherin [Oblander et al., 2007]-

mediated neurite outgrowth. The data presented in this manuscript clearly demonstrate BCCIP is required for PTP μ -mediated neurite outgrowth. Future studies will investigate the molecular mechanism by which BCCIP regulates PTP μ -dependent neurite outgrowth and cell-cell adhesion.

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Abbreviations

PTP	protein tyrosine phosphatase
RPTP	receptor protein tyrosine phosphatase
MAM	Meprin-A5-Mu
FNIII	fibronectin III
GST	glutathione S-transferase
CDK	cyclin-dependent kinase
TOK-1	twenty-one and CDK associated protein 1
BCCIP	BRCA2 and CDKN1A interacting protein
RGC	retinal ganglion cell

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Figure 1. PTP μ and BCCIP α interact in a modified yeast two-hybrid screen and *in vitro*

A. The final results of the modified yeast two-hybrid screen are shown. Yeast containing PTP μ and BCCIP α were grown on medium containing either galactose or glucose. Galactose induces the expression of the prey protein (BCCIP α) in the yeast. When grown on Xgal-containing media, the PTP μ /BCCIP α strain turns blue on galactose only in the presence of the Src tyrosine kinase. **B.** GST, GST-BCCIP α and GST-BCCIP β fusion proteins were isolated on glutathione beads as described in "Materials and Methods". These GST fusion proteins were used in the pull-down assay described below. **C.** Bacterial lysates from cells expressing T7-tagged His-iPTP μ :774-1161 and His-iPTP μ :1213-1452 before (–) and after (+) induction with IPTG were separated by SDS-PAGE and protein expression confirmed by immunoblotting with a T7 antibody. **D.** Post-induction bacterial lysates were used as the input for GST pull-downs. Bacterial lysates of cells expressing either the first (His-iPTP μ :774-1161) or second (His-iPTP μ :1213-1452) phosphatase domain of PTP μ were incubated with GST-tagged BCCIP α , BCCIP β or GST alone preloaded on glutathione beads. The ability of His-iPTP μ :774-1161 and His-iPTP μ :1213-1452 to associate with BCCIP was determined by separating the associated proteins by SDS-PAGE followed by immunoblotting with a monoclonal antibody to the T7 tag present in the His-iPTP μ :774-1161 and His-iPTP μ :1213-1452 fusion proteins. To ensure equal amounts of the fusion proteins were used in the pull-down assay, the anti-T7 immunoblot

from the GST pull-downs was stripped and immunoblotted with an anti-GST antibody and shown in **B**.

Figure 2. BCCIP α is an *in vitro* substrate for both the Src tyrosine kinase and the PTP μ tyrosine phosphatase

A. GST, GST-BCCIP α and GST-BCCIP β fusion proteins were isolated on glutathione Sepharose and used in an *in vitro* Src kinase assay. Following phosphorylation by Src, the GST fusion proteins were used in a pull-down assay with purified His-iPTP μ :915-1161. The pull-down samples were separated by SDS-PAGE, transferred to nitrocellulose and analyzed as follows. The top panel is an immunoblot using an anti-phosphotyrosine antibody and shows BCCIP α is a substrate for Src *in vitro*. The bottom panel is the same membrane immunoblotted for PTP μ (SK18). The center panel is a Ponceau stain of the membrane showing equal loading of the GST fusion proteins. **B.** GST-BCCIP α was isolated on glutathione Sepharose and

phosphorylated in vitro by Src. After phosphorylation the sample was divided into three samples and either untreated (control), or washed with a phosphatase buffer and incubated with (+) or without (–) GST-iPTP μ :765-1178. The phosphorylation status of BCCIP α was determined by immunoblotting with an anti-phosphotyrosine antibody. The bottom panel is a Ponceau stain of the membrane to show equal loading of GST-BCCIP α .

Figure 3. The PTP μ /BCCIP interaction domain

A. Schematic of intracellular GST-PTP μ constructs used in pull-down experiments. All constructs contain a GST sequence at the N-terminus. A gray oval represents the juxtamembrane domain (JMD) and black box represents the first (PTP D1) and second (PTP D2) phosphatase domains. The yeast two-hybrid bait construct is included for reference. **B.** GST-iPTP μ constructs and GST alone were immobilized on glutathione Sepharose and incubated with equal amounts of lysate from Sf9 cells expressing BCCIP α . Interaction of BCCIP with the GST-PTP μ fusion proteins was determined by separating associated proteins by SDS-PAGE followed by immunoblotting with a monoclonal antibody to BCCIP. We observe two bands with the monoclonal antibody to BCCIP when we immunoblot lysates from Sf9 cells expressing BCCIP α . This is most likely due to post-translational modification or cleavage. The BCCIP immunoblot was stripped and reprobed with an anti-GST antibody to verify GST fusion protein loading (WB: α -GST).

Figure 4. Localization of endogenous BCCIP

A. Immunocytochemistry was performed on A549, PrEC and LNCaP cells using a monoclonal antibody that recognizes both BCCIP α and BCCIP β . In all three cell lines, BCCIP localizes to both the cytoplasm and the nucleus. DAPI staining was included to define the nuclei. **B.** A549, PrEC and LNCaP cells were fractionated into nuclear and cytosolic fractions. Isolated fractions were separated by SDS-PAGE and proteins detected by immunoblot using a monoclonal antibody to BCCIP. Purity of the cell fractions was verified using vinculin as a cytosolic (C) marker and lamin A/C as a nuclear (N) marker. There are prominent bands corresponding to BCCIP α and BCCIP β in the cytosolic fraction (BCCIP α and BCCIP β are 50 and 45 kDa, respectively). Upon longer exposure, a low level of BCCIP α can be detected in the nuclear fraction.

Figure 5. BCCIP binds endogenous PTP μ

A. A549 cells were treated with control medium (-) or pervanadate (+) for 20 minutes. Cells were lysed and equal amounts of protein lysate were incubated with GST, GST-BCCIP α and GST-BCCIP β immobilized on glutathione Sepharose. Associated PTP μ was detected by resolving associated proteins by SDS-PAGE, transferring to nitrocellulose membrane and immunoblotting with an intracellular antibody to PTP μ (SK18). PTP μ is present in the cell as a full length, 200 kDa form and a proteolytically processed form which yields a fragment of ~100 kDa. BCCIP α predominately interacts with the cleaved intracellular form of PTP μ in human cells. **B.** Immunocytochemistry was performed on A459 cells using an intracellular antibody to PTP μ (SK18). In low density cell cultures PTP μ localizes to nascent cell-cell

contacts and a fraction of PTP μ is seen in the nucleus. At high density cell cultures PTP μ is stabilized at cell-cell contacts.

Figure 6. Exogenously expressed intracellular PTPμ localizes to the nucleus

A. A549 cells were infected with lentivirus encoding an intracellular PTPμ construct, iPTPμ:765-1451-GFP. Immunocytochemistry was performed 24 hours post-infection using a primary monoclonal antibody that recognizes both BCCIPα and BCCIPβ and a secondary antibody conjugated to Texas Red. iPTPμ:765-1451-GFP and BCCIP show predominantly nuclear localization, defined by DAPI staining, with some diffuse cytoplasmic staining. **B.** A549 cells were infected with lentivirus encoding full-length PTPμ-GFP. Images of PTPμ-GFP localization were taken 24 hours post-infection and show full-length (FL) PTPμ-GFP is stabilized at cell-cell contacts.

A Retinal cells **B LNCaP cells** **Figure 7. Antisense downregulation of PTPμ and BCCIP proteins**

A. Lysates from E6 retinal cells infected with HSV-antisense PTPμ were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an antibody to PTPμ (SK18). Densitometric measurement of the PTPμ bands showed a 60% reduction in PTPμ protein. Antisense BCCIP had little to no effect PTPμ protein levels. **B.** Lysates from LNCaP cells infected with HSV-antisense BCCIP were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an antibody to BCCIP. Densitometric measurement of the BCCIP showed an average 55% reduction of BCCIP protein expression based on three separate experiments. For both A and B, immunoblots were stripped and reprobed with an antibody to vinculin as a protein loading control.

Figure 8. BCCIP regulates PTPμ-mediated nasal neurite outgrowth
BCCIP antisense expression perturbs PTPμ-mediated neurite outgrowth. Explants from E8 nasal retina were grown on alternating stripes of PTPμ or laminin only lanes. Explants were incubated with GFP-HSV, antisense PTPμ-HSV or antisense BCCIP-HSV virus or without any virus (control). In both the control and GFP-HSV virus control explants, nasal neurites cross freely onto a PTPμ substrate. In the explants incubated with antisense BCCIP, nasal neurites no longer cross onto the PTPμ substrate lanes, or avoid these lanes, suggesting BCCIP is required for a permissive response to the PTPμ substrate in those lanes. Antisense PTPμ-HSV was included as a positive control for nasal neurite avoidance. The average degree of avoidance (mean ± STD) was plotted for each condition as described under “Materials and Methods”. An asterisk denotes a statistically significant change in nasal neurite outgrowth ($p < 0.0001$).

Figure 9. BCCIP is required for PTPμ-dependent temporal repulsion
Explants from E8 temporal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes. Explants were incubated with or without virus as described above and neurites were evaluated for crossing from the laminin lanes to the PTPμ mixed substrate lanes. In both the control and GFP-HSV virus control explants, neurites avoid the PTPμ mixed substrate lanes. In the explants incubated with antisense BCCIP, neurites crossed the PTPμ-containing lanes, suggesting BCCIP is involved in the regulation of PTPμ-dependent repulsion of temporal neurites. Antisense PTPμ-HSV was included as a positive control for blocking temporal repulsion. The average degree of avoidance (mean $\pm$ STD) was plotted for each condition as described under “Materials and Methods”. An asterisk denotes a statistically significant change in temporal neurite repulsion ($p < 0.0001$).