FUNCTIONAL CHARACTERIZATION OF PROTEIN TYROSINE PHOSPHATASES IN TUMORIGENESIS THROUGH SUBSTRATE IDENTIFICATION

By

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(date) _______________________

*We also certify that written approval has been obtained for any proprietary material contained therein.
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I thank my parents, who have been supportive with unwavering trust and confidence in me during my time in the graduate school. I want to dedicate this work to the nicest person I have ever encountered in my life, my grandfather, who passed away last year.
List of Abbreviations

AAV  Adeno-associated virus
ALL  Acute lymphoblastic leukemia
AML  Acute myeloid leukemia
APC  Adenomatous polyposis coli
AOM  Azoxymethane
ARK  Adhesion-related kinase
Bcl-XL B-cell lymphoma-extra large
Bp   Base pairs
BSA  Bovine serum albumin
CAM  Cell adhesion molecules
Cas  Crk-associated substrate
CRC  Colorectal cancer
CSPG Chondroitin sulfate proteoglycan
DAG  Diacylglycerol
DAPI 4,6-diamidino-2-phenylindole
DNA  Deoxyribonucleic acid
DSP  Dual specificity phosphatase
DTT  Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
<table>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPM2A</td>
<td>Epilepsy, progressive myoclonus type 2A</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FERM</td>
<td>Band 4.1/ezrin/radixin/moesin</td>
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<td>Fig.</td>
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<td>FN</td>
<td>Fibronectin</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>Inositol triphosphate</td>
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<td>JAK</td>
<td>Janus-activated kinase</td>
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<td>Description</td>
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<tr>
<td>kb</td>
<td>Kilo base-pairs</td>
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<td>KI</td>
<td>Knock-in</td>
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<tr>
<td>LAR</td>
<td>Leukocyte common antigen-related</td>
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<tr>
<td>LCK</td>
<td>Lymphocyte-specific kinase</td>
</tr>
<tr>
<td>MAM</td>
<td>Meprin/A5-protein/PTPmu</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK and extracellular signal-regulated kinase</td>
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<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>NHL</td>
<td>Non-Hodgkin lymphoma</td>
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<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
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<tr>
<td>p-</td>
<td>Phospho-</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Polymerase chain reaction</td>
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<tr>
<td>PDB</td>
<td>Protein data bank</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
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<td>Protein kinase A</td>
</tr>
<tr>
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<td>Protein kinase C</td>
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<tr>
<td>PLCγ1</td>
<td>Phospholipase C gamma 1</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>PTK</td>
<td>Protein tyrosine kinase</td>
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<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
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<tr>
<td>PTPN</td>
<td>Protein tyrosine phosphatase nonreceptor-type</td>
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<td>PTPR</td>
<td>Protein tyrosine phosphatase receptor-type</td>
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<tr>
<td>pY</td>
<td>Phospho-tyrosine</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RT</td>
<td>Reverse transcriptase</td>
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<td>SCLC</td>
<td>Small cell lung cancer</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SH</td>
<td>Src-homology</td>
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<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
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<tr>
<td>SHP</td>
<td>Src homology 2 domain-containing phosphatase</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling 3</td>
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<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TRICT</td>
<td>Tetramethylrhodamine isothiocyanate</td>
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<td>Tyr</td>
<td>Tyrosine</td>
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<td>UV</td>
<td>Ultra-violet</td>
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<td>Vaccinia H1</td>
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Functional Characterization of Protein Tyrosine Phosphatases in Tumorigenesis through Substrate Identification

By

PENG ZHANG

Abstract

Tyrosine phosphorylation plays critical roles in literally every cellular process. Highlighting its importance, the dysregulation of tyrosine phosphorylation leads to the pathogenesis of many human diseases including cancers. Tyrosine phosphorylation is regulated by two families of enzymes: PTKs and PTPs. Despite the fact that many PTPs were found mutated in different cancers, the cellular functions of many PTPs are still largely unknown. This work focuses on the elucidation of cellular functions of two PTPs: PTPRT and PTPN14, both of which are mutated in multiple cancer types, including colorectal cancers.

We utilized a proteomic approach to search for substrates of PTPN14 in colorectal cancer cells. We identified and validated that p130Cas is a direct substrate of PTPN14 and PTPN14 dephosphorylates p130Cas at the tyrosine 128 residue. To dissect the role of pY128 p130Cas in colorectal tumorigenesis, we engineered p130Cas Y128F mutant knock-in colorectal cancer cell lines. The p130Cas Y128F mutant cells display reduced transformation ability \textit{in vitro} and xenograft tumor growth \textit{in vivo}. Mechanistically, the p130Cas Y128F mutant cells exhibit impaired Akt signaling through reduced binding to p85.
Our previous studies demonstrated that PTPRT functions as a tumor suppressor and directly dephosphorylates STAT3 at the tyrosine 705 residue. Although it is well-documented that pY705 STAT3 plays an oncogenic role in some cancers, the role of pY705 STAT3 in colorectal tumorigenesis is not well defined. To this end, we engineered STAT3 Y705F mutant KI colorectal cancer cell lines. We demonstrated that STAT3 Y705F mutant cancer cells, display reduced tumorigenicity both in vivo and in vitro. Significantly, we discovered a novel cross-talk between STAT3 and PLCγ1. We also showed that the extracellular fragments of PTPRT mediate homophilic cell-cell interactions and that cancer-derived mutations in the MAM, Ig and FN III domains of PTPRT impair cell-cell adhesion, indicating that these PTPRT mutations are loss-of-function mutations.

Taken together, these studies provide a novel method for substrate identification for PTPs in a systematic, unbiased way. Once candidate substrates are experimentally verified, the specific phosphosite that is subject to dephosphorylation by the PTP of interest can be genetically modified to study its cellular functions. With the improved understanding of the roles of PTPs in cells especially those related to tumorigenesis, we can explore the potential of PTPs as the targets for a new generation of anti-cancer drugs.
Chapter 1

Background and Significance
Overview

Protein phosphorylation is an extremely important post-translational modification. It is especially critical in cellular signaling. Protein phosphorylation can serve as a switch to turn on or turn off signaling events in cells. In this way, protein phosphorylation plays significant roles in literally every cellular process like proliferation, differentiation and migration. Tyrosine phosphorylation is the most well-studied among the three phosphorylations under physiological conditions. The level of tyrosine phosphorylation is tightly regulated by two opposing families of enzymes: Protein Tyrosine Kinases (PTKs) and Protein Tyrosine Phosphatases (PTPs) (as shown in Figure 1-1). In accordance with its central role in cells, dysregulation of tyrosine phosphorylation is implicated in many human diseases including various types of cancers (Andersen et al., 2004).

Tyrosine phosphorylation in cellular signaling

History of protein phosphorylation

The first phosphorylated protein was reported in 1906 by P. A. Levene at the Rockefeller Institute for Medical Research. In this study, a phosphate group was found to be attached to Vitellin, a yolk protein, which caused its unusual acid properties (Levene and Alsberg, 1906). Several other phosphoproteins were identified afterwards. However, it was not until 1954 that protein phosphorylation was understood to be an active enzymatic process. E. P. Kennedy discovered that a phosphate group was transferred from ATP to Casein by an unidentified enzyme in rat liver mitochondria through monitoring radioactivity (Burnett and
Figure 1-1. The process of protein tyrosine phosphorylation/dephosphorylation. PTKs transfer a phosphate group from an ATP molecule to the hydroxyl side chain of the target tyrosine residue; while PTPs remove the phosphate group from the protein. The addition of the negatively charged phosphate group to the protein may cause the conformational change of the protein itself. (Modified from Arena et al., 2005).
Kennedy, 1954). At that time, protein phosphorylation was still underappreciated. In 1979, phosphotyrosine was reported for the first time by Tony Hunter (Eckhart et al., 1979). Together with his subsequent discovery that v-Src could transform chicken cells through tyrosine phosphorylation (Hunter and Sefton, 1980), it opened up an exciting field about a new mechanism of protein regulation in cells. Protein phosphorylation especially tyrosine phosphorylation has been extensively studied and its importance has been recognized as indicated by an increasing amount of publications on the subject (Cohen, 2002). With the advancement of protein techniques and informatics, protein phosphorylation has started to be examined systematically. The so-called phosphatome is believed to lead us to better understand the biological functions of protein phosphorylation particularly those associated with disease development (Julien et al., 2011).

**Effect of phosphorylation on substrates**

Phosphorylation of a protein can alter its properties in many different aspects. Firstly, if a phosphate group is added into a hydrophobic portion of a protein this protein will become polar and extremely hydrophilic. This will induce a conformational change in the protein (Groban et al., 2006). Thus, a new docking site can be created for protein-protein interaction or an existing protein-protein interaction would be disrupted. The conformational change can also increase or decrease an enzyme’s biological activity. There are many reports that phosphorylation can disrupt the auto-inhibitory effect within the structure of an enzyme so that the enzyme can then be fully activated (Adams, 2003). Phosphorylation can also regulate a protein’s stability (Hunter, 2007).
can inhibit ubiquitination and protect the protein from proteasome-mediated degradation (Wang et al., 2006). At the same time, phosphorylation can also mark a protein for degradation by signaling to the ubiquitination machinery (Levkowitz et al., 1999). In addition, it is shown that phosphorylation can control the transport of a protein between subcellular compartments (Khokhlatchev et al., 1998). It may seem conflicting that phosphorylation can induce two opposite effects on the same process. However, the exact effect of phosphorylation on a particular protein all depends on the relative level and inherent nature of the induced conformational change.

**Role of phosphorylation in signaling cascades**

The essential role of phosphorylation in cellular signaling can be exemplified by MAP Kinase signaling cascade. The step-by-step construction of this classical signaling cascade is one of the most important achievements during the late 1980s and early 1990s in cell biology (Seger and Krebs, 1995). MAP Kinase was first identified because of its ability to react to various mitogens and growth factors (Boulton et al., 1990). It was found out that MAP Kinase can only be fully activated by the phosphorylation of a threonine and a tyrosine residue (Payne et al., 1991). The enzyme that catalyzes the phosphorylation of MAP Kinase was named MAP Kinase Kinase also known as MEK, which has dual specificity (Crews et al., 1992). MEK in turn is activated by Raf (MAP3K) through serine/threonine phosphorylation (Kyriakis et al., 1992) while Ras, a small GTPase, recruits Raf to the plasma membrane and activates it (Marais et al., 1995). Ras is itself activated by swapping nucleotides from GDP to GTP, which
is triggered by the event that extracellular ligand binds to its receptors (Boguski and McCormick, 1993). These receptors either have integral kinase activity or associated tyrosine kinases. In such an exquisite way, cells can transmit extracellular signals into cytoplasm and relay the signals into the targets in nucleus. This signaling cascade is further complicated by the fact that on every step there are several isoforms for the effector. This built-in redundancy allows for fine tuning the signal at every stage. In addition to the MAP Kinase cascade, several other signaling cascades have been dissected over the years. (Lodish et al., 2007) They all function in a similar fashion. The complete reversibility of phosphorylation makes it a simple and flexible way for cells to transduce signals so as to adapt to changes in the environment. Compared with other phosphorylations, tyrosine phosphorylation is extensively utilized only in multicellular organisms, which implicates its important roles in cell-cell communication.

Protein tyrosine phosphatases in the human genome

**Protein tyrosine phosphatase identification**

The first protein tyrosine phosphatase PTP1B was purified from human placenta in 1988 (Tonks et al., 1988). Its amino acid sequence was determined by Edman degradation (Charbonneau et al., 1989). Part of its intracellular domain was found to be similar to several other proteins which also showed tyrosine phosphatase activity. This structure was thought to be the core catalytic domain. The first ever PTP gene was cloned from a rat brain library by screening with the amino acid sequence of PTP1B which was derived from the above-
mentioned earlier study. By expressing the coding sequence of the cloned gene in *E. Coli*, the recombinant protein showed strict specificity for phosphotyrosine *in vitro* (Guan et al., 1990). Since then, more and more PTPs were identified. It was determined that there are 107 PTP genes in the human genome based on the presence of the conserved phosphatase domain (Alonso et al., 2004). Many PTPs have additional domains outside the catalytic core. These domains are thought to regulate cellular localization or substrate specificity of these PTPs. The domain structures of PTPs appear to be distinct from those of PTKs. Together with the fact that there are only 90 protein tyrosine kinases in the human genome, it indicates that PTPs do not simply passively reverse the action of PTKs (Alonso et al., 2004). These two groups of enzymes need to be distinctly regulated in cells. These 107 PTPs can be further divided into different families based on the homology of the catalytic domains. Each family has a range of substrate specificities.

**Classical Protein Tyrosine Phosphatases**

38 PTPs are classified as classical PTPs (Alonso et al., 2004). The domain structures of classical PTPs are shown in Figure 1-2. There are two groups within this family: receptor-like PTPs which bear transmembrane domains or nonreceptor PTPs which are cytosolic. All classical PTPs are strictly tyrosine specific. They share a common HC(X)₅R motif in the active site. The cysteine residue is crucial for the phosphatase activity. It is located at the base of the catalytic pocket and forms a thiol phosphate intermediate with the phosphoryl
Figure 1-2. Domain structure of all classical PTPs. Schematic view of the domain composition of all members of the Classical PTP. Abbreviations: BRO, baculovirus BRO homology; CA, carbonic anhydrase-like; Cad, Cadherin like; FERM, band 4.1/ezrin/radixin/moesin homology; FN, fibronectin-like; Ig, Ig, immunoglobulin-like; KIM, KIM, kinase interaction motif; KIND, kinase N lobe-like domain; PDZ, postsynaptic density-95/discs large/ZO1 homology; Pro-rich, proline-rich; Sec14p, Sec14p homology; SH2, src homology 2. (Modified from Alonso et al., 2004).
group in the substrate by acting as a nucleophile (Zhang et al., 1994; Denu and Dixon, 1998). The WPD-loop is another key element in the catalytic domain. The binding of the substrate brings the WPD-loop over the active site so the aspartic acid is close to the leaving group in the intermediate. It then functions as a general acid pronating the oxygen in the tyrosine residue and hydrolyzes the intermediate and converts the cysteine residue back into its resting state (see Figure 1-4). Classical PTPs are very much alike in their structures of the catalytic domain with only minor variations (Andersen et al., 2001; Barr et al., 2009) as represented by the three-dimensional structure of PTP1B shown in Figure 1-3. Interestingly, many RPTPs have two tandem phosphatase domains: D1 (membrane proximal) and D2 (membrane distal). D2 is thought to be catalytically inactive and its function is still largely unknown. The nonreceptor PTPs have more protein-protein interaction domains which indicate that they may be the components of large protein complexes.

**Dual Specificity PTPs**

Dual specificity PTPs are another class of protein tyrosine phosphatases. In addition to tyrosine, they also have activity towards serine/threonine. The prototype DSP VH1 was identified in vaccinia virus (Guan et al., 1991). DSPs share homology in amino acid sequence with classical PTPs for the catalytic domain. Their three-dimensional structures are also similar which indicates that DSPs utilize the same mechanism for dephosphorylation. However, DSPs seem to have a shallower active site pocket than classical PTPs, which allows the selection of serine/threonine as substrates (Camps et al., 2000). Many DSPs are
Figure 1-3. Three-dimensional Structure of PTP1B with a phosphotyrosine. Position of the substrate in the active site is illustrated by the phosphotyrosine ligand (blue). Active-site nucleophile Cys215 (grey) attacks the substrate phosphorus leading to the formation of the cysteinyl-phosphate intermediate. Asp181 within the WPD-loop (cyan), here in the closed conformation, acts as a general acid. (Modified from Tabernero et al., 2008).

Figure 1-4 Common catalytic mechanism of PTPs. The catalytic cysteine is part of the PTP signature motif C(X)5R located in the phosphate-binding loop (P-loop). An invariant aspartic acid, located in the so-called WPD-loop, functions as the general acid/base during hydrolysis (Tautz and Mustelin, 2007).
MAP kinase phosphatases and are important in signal transduction by regulating MAPK activity (Camps et al. 2000). Another important group within DSPs is PTENs, which dephosphorylate inositol phospho-lipids \textit{in vivo}. In addition to DSPs, there are a small number of other PTPs that lie outside the classical PTPs. These PTPs are evolutionarily independent and have a distinct mechanism of action (Alonso et al., 2004).

**Protein tyrosine phosphatases and human diseases**

**Somatic mutations of PTPs in cancers**

Many PTPs have been found to be mutated in various cancer types over the years. The most prominent one is undoubtedly PTEN, a non-classical PTP. PTEN bears somatic mutations in many different cancers with an especially high frequency in prostate cancer (Maehama and Dixon, 1999). With the progress of sequencing techniques recently, more and more PTPs are implicated in tumorigenesis. The tyrosine phosphatome in colorectal cancers was systematically studied for mutational analysis. In this study, somatic mutations were identified in six PTPs (PTPRF, PTPRG, PTPRT, PTPN3, PTPN13, and PTPN14), affecting 26% of the cancer samples (Wang et al., 2004). In addition to this study, somatic mutations of many classical PTPs have also been discovered from other genome wide mutational screenings in several cancer types. The results are summarized in Table 1-1. Since many PTKs have long been established as oncogenes, PTPs were thought to function as tumor suppressors as antagonists of PTKs. Some PTPs were mutated in both alleles in cancer
Table 1-1. Summary of classical PTPs that have been identified to be somatically mutated through genome-wide mutational analyses in different cancers.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal</td>
<td>PTPRF, PTPRG, PTPRT, PTPN3, PTPN13, PTPN14</td>
<td>(Wang et al., 2004)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>PTPRB, PTPRD, PTPRG, PTPRT, PTPRU, PTPN14, PTPRO</td>
<td>(Wei et al., 2011; Berger et al., 2012)</td>
</tr>
<tr>
<td>Breast</td>
<td>PTPRD, PTPRM, PTPRT, PTPN14, PTPB, PTPRD, PTPRF, PRPRZ1, PTPN13</td>
<td>(Nik-Zainal et al., 2012),</td>
</tr>
<tr>
<td>HNSCC</td>
<td>PTPRD, PTPRF, PTPRK, PTPRT, PTPRZ1, PTPN3</td>
<td>(Stransky et al., 2011)</td>
</tr>
<tr>
<td>Lung</td>
<td>PTPRD, PTPRG, PTPN11</td>
<td>(Ding et al., 2008)</td>
</tr>
<tr>
<td>AML</td>
<td>PTPRB, PTPRD, PTPRH, PTPRM, PTPRZ1</td>
<td>(Ding et al., 2012)</td>
</tr>
<tr>
<td>Prostate</td>
<td>PTPRB, PTPRD, PTPRH, PTPRM, PTPRZ1</td>
<td>(Barbieri et al., 2012)</td>
</tr>
<tr>
<td>SCLC</td>
<td>PTPRD, PTPRK, PTPRM, PTPRU, PTPRZ1</td>
<td>(Peifer et al., 2012)</td>
</tr>
<tr>
<td>NHL</td>
<td>PTPRD, PTPRE</td>
<td>(Morin et al., 2011)</td>
</tr>
<tr>
<td>CLL</td>
<td>PTPRB</td>
<td>(Quesada et al., 2012)</td>
</tr>
<tr>
<td>HCC</td>
<td>PTPN14</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>Renal</td>
<td>PTPRE</td>
<td>(Peña-Llopis et al., 2012)</td>
</tr>
</tbody>
</table>
samples as predicted by the “two-hit” model, which indicates they might indeed be tumor suppressors. Through biochemical studies and animal models, the tumor-suppressing roles have been confirmed for several PTPs (Östman et al., 2006).

**Other genetic alterations of PTPs in cancers**

PTEN was frequently deleted in prostate, melanoma and other cancers, sometimes even with homozygous deletion (Guldberg et al., 1997; Wang et al., 1998). PTPRG was frequently deleted in renal and lung carcinomas (LaForgia et al., 1991). Additionally, homozygous deletion of PTPN2 was discovered in T-cell ALL (Kleppe et al., 2010). All these deletion events strongly suggest that PTPs are tumor suppressors. Interestingly, PTPN1 was found to be amplified in a subset of gliomas (Kotliarov et al., 2006). However, it is not clear whether the amplification is causative without a functional study.

**Epigenetic change of PTPs in cancers**

Epigenetic change can also influence gene expression. Promoter hypermethylation is frequently associated with gene downregulation or silencing. PTPRM, PTPRT, PTPRR and PTPRZ1 were found to be differentially methylated between healthy and colorectal cancer samples in a recent epigenetic screening (Laczmanska et al., 2012), which suggests that promoter hypermethylation may be a common mechanism to inactivate PTPs during tumorigenesis. In addition to receptor-like PTPs, a nonreceptor PTPN6 (SHP-1) has been well documented to be silenced by hypermethylation especially in several types of blood cancers.
(Oka et al., 2002). The expression of SHP1 could be restored by demethylating agent treatment in cancer-derived cell lines (Chim et al., 2004).

**Oncogenic PTPs**

Although PTPs are generally believed to be tumor suppressors, a few PTPs can function as oncogenes in certain contexts. They usually work by inactivating inhibitory components of signaling pathways. PTPN11 (SHP2) is a prominent example as an oncogenic PTP. Activating mutations of PTPN11 have been discovered in several leukemias (Bentires-Alj et al., 2004). These mutations can disrupt the auto-inhibitory interaction between the catalytic domain and N-terminal SH2 domain in PTPN11. In this way, the normal inactive conformation of PTPN11 becomes active even without substrate binding. It happens that a subset of PTPN11 substrates negatively regulate the Ras-ERK pathway. Thus, the somatic mutations of PTPN11 can actually transduce pro-growth signals and become oncogenic (Qu, 2000). Although there are reports that other PTPs can also display oncogenic behavior, they are still mostly in a preliminary phase of study.

**PTPs and other human diseases**

PTPs are not merely implicated in cancer pathogenesis. Many PTPs have also been associated with human diseases other than cancers. PTPN22 is mainly expressed in lymphoid tissues. A single SNP in PTPN22 was linked to type I diabetes and several other autoimmune diseases (Criswell et al., 2005). This point mutation seems to alter its binding to T cell receptor-signaling Src-family kinases LCK and Fyn (Bottini et al., 2004). PTPN1 is thought to modulate
insulin signaling. PTPN1 knockout mice showed increased Insulin sensitivity and were resistant to obesity on a high-fat diet (Elchebly et al., 1999). PTPN1 was also found to be associated with type II diabetes and obesity in humans (Bento et al., 2004). A mutation in EPM2A which encodes a DSP was shown to cause progressive myoclonus epilepsy, a neurological disorder, which implicates that it plays a critical role in neurons (Minassian et al., 2000). However, the exact mechanism is unclear.

**PTPs as drug targets**

PTKs have long been studied as drug targets. Many inhibitors targeting PTKs are already in clinical use now (Baselga 2006). Compared with PTKs, PTPs are still in an emerging phase as drug targets. The implication of PTPs in multiple human diseases makes them attractive targets for drug development. The discovery that PTPN1 negatively regulates insulin signaling led to great efforts in finding orally available inhibitors for treatment of type II diabetes and obesity. One of the many identified inhibitors, Ertiprotafib, is currently in clinical trials (Zhang and Zhang, 2007). PTPN11 is another PTP that has drawn great attention for inhibitor development (Helmut et al., 2008). Ideally, the identified inhibitors would show greater potency towards activating mutants found in cancer compared to the wild-type proteins. A major obstacle is that the inhibitors need to have selectivity of PTPN11 over closely-related PTPN6 which nevertheless has vastly different cellular functions. At present, none of the PTPN11 inhibitors has reached clinical trial yet. In order to better target PTPs, more studies are needed to look into the molecular mechanisms by which PTPs affect the pathogenesis of
the diseases. With improved understanding, a small molecule library screen coupled with in silico design would result in the identification of successful therapeutics in the future.

**Regulation of Protein tyrosine phosphatases**

**Classical ways of PTP regulation**

PTPs play such important roles in so many cellular signaling pathways and regulate literally every biological process in the cell. It would be expected that PTPs themselves are tightly regulated. Indeed, phosphorylation is a general mechanism for PTP regulation. PTPN12 was demonstrated to be phosphorylated by PKA and PKC on several serine residues (Garton and Tonks, 1994). The phosphorylation of serine decreases its activity by reducing its affinity towards its substrates. Regulation of PTPs by tyrosine phosphorylation has also been described. In addition to phosphorylation, proteolytic cleavage is another mechanism of regulating PTP activity either through degradation or translocation. It was reported that PTPRM is downregulated in GBM due to proteolytic processing and the cleaved intracellular fragment promotes cell migration (Burgoyne et al., 2009b). It was also discovered that UV irradiation can induce calpain-mediated proteolytic degradation of several PTPs including PTPN1 (Galati et al., 2004). In recent years, a few new mechanisms have been revealed for PTP regulation.

**Reversible oxidation**

One emerging mechanism for PTP regulation is reversible oxidation of the
Figure 1-5. Reversible oxidation of PTPs. As shown for PTP1B, and presumably reflective of the classical PTPs in general, oxidation is reversible due to the rapid conversion of the sulphenic acid form of the oxidized cysteine to a novel 5-atom-ring structure, a cyclic sulphenamide, which is promoted by the environment of the catalytic site. (Modified from Tonks, 2006).
conserved cysteine residue in the signature motif of PTPs. Many PTPs have been shown to be oxidized transiently in reaction to various stimuli like the binding of a growth factor to its receptor (Meng et al., 2002). Dependent on the extent of oxidation, it can be reversible as shown in Figure 1-5. Since cysteine is essential for phosphatase activity, PTPs can be inactivated by oxidation and the suppression of PTPs upon growth factor signaling is thus relieved. The production of ROS from normal metabolic activities can also have an impact on the oxidative status of PTPs, which might be an important mechanism for ROS-induced mitogenesis. It was further reported that different PTPs have differential susceptibility to oxidation, which depends on the orientation of the arginine residue in the PTP loop (Groen et al., 2005).

**Ligand binding of RPTP**

The solution of the crystal structure of the D1 domain of PTPRA revealed that D1 domains were organized in symmetrical dimers. The active site of one monomer was occluded by an inhibitory wedge motif in the other monomer (Bilwes et al., 1996). Thus, the activity of PTPRA might be negatively regulated by dimerization. This model shows a stark contrast with that of PTKs, which are activated upon dimerization by auto-phosphorylation. However, the crystal structure of the tandem D1 and D2 domains of PTPRF (LAR) showed that both domains were accessible and only a monomeric configuration was observed (Nam et al., 1999). The D1 domain of PTPRM displayed the same open conformation in the crystal structure as LAR (Hoffmann et al., 1997), which
indicates that the RPTPs may not generally share the feature of dimerization-induced activity modulation.

Several RPTPs including PTPRM display structural similarity to immunoglobulin-like cell adhesion molecules. PTPRM was shown to mediate cell-cell aggregation via homophilic binding: one PTPRM molecule interacts with another PTPRM molecule on a neighboring cell (Brady-Kalnay et al., 1993). The Ig domain of PTPRM was demonstrated to be the homophilic binding site (Brady-Kalnay & Tonks, 1994) while the MAM domain was shown to help determine the specificity of the interaction (Zondag et al., 1995).

Heterophilic ligands for RPTPs have also been reported. PTPRZ1/PTPRB was reported to be the receptor of pleiotrophin, a PDGF-inducible cytokine (Meng et al., 2000). The binding of pleiotrophin inactivates the phosphatase activity and the phosphorylation of its substrate sharply increases. However, it is not clear that dimerization mediates the inactivation. Chondroitin sulfate proteoglycans, important macromolecules in neural development showed high affinity to PTPRS. PTPRS-/- neurons were less responsive to CSPG (Shen et al., 2009). It is worthy further investigation into how the activity of PTPRS was affected by the binding of CSPG.

In order to facilitate the discovery of more ligands of RPTPs, a systematic strategy for large-scale screenings should be developed. It also opens a new route for the design of new drugs that mimic the ligands of RPTPs so that the aberrantly activated signaling pathway in diseases can be specifically suppressed.
Substrate identification of PTPs

Peptide Libraries

As for every enzyme, substrate identification is the first and very crucial step to understand the cellular functions of PTPs. Knowledge about substrates can shed lights on the signaling pathways that PTPs are involved in. Unlike PTKs, whose substrates can be relatively easily traced by radioactive labeling, to identify substrates for PTPs has long been challenging for scientists in the field. Many efforts have been made for the design of successful strategies in the search of physiological substrates of PTPs.

Early efforts for PTP substrate identification were focused on in vitro approaches. Both synthetic chemical and phage display libraries were utilized to screen candidate peptides (Huyer et al., 1998; Garaud and Pei, 2007). Substrates can then be identified by matching the amino acid sequences of the candidate peptides. The major problem with this approach is that many PTPs have broader substrate specificity in vitro than under physiological conditions (Tonks and Neel, 2001).

Substrate trapping

It was first noted that when the nucleophilic cysteine was altered to serine or alanine, some PTPs could be isolated together with their substrates in a complex. However, this interaction between PTPs and their substrates was too weak for other PTPs (Flint et al., 1997). Thus, the application of Cys-Ser/Ala mutant was limited. Efforts were then made to create a new mutant form of PTPs
that were more potent in the application of substrate identification. Ideally, this mutant should keep the affinity for substrates while its phosphatase activity is reduced to an extent so that the enzyme-substrate complex would be stable enough to withstand biochemical experiments. Mutants for several invariant residues at the active site of PTPN1 were generated and their enzymatic activity and affinity towards a substrate peptide were analyzed. It was shown that the Asp-Ala mutant best met the requirements to be utilized as a substrate trap. The mutation from Asp to Ala would suppress the cleavage of the intermediate formed between Cys and substrates and thus stabilize the interaction. Using this DA mutant, EGFR was discovered as the substrate of PTPN1 from the trapping assay. It was validated that the interaction between PTPN1 and EGFR is dependent on the tyrosine phosphorylation on EGFR (Flint et al., 1997).

Ever since this substrate trapping mutant was described, many substrates have been identified for different PTPs. However, the strategy of substrate trapping has several limitations. First, it requires prior knowledge about a PTP. Thus, the search for substrates cannot be done in an unbiased manner. Second, cells need to be treated with pervanadate to elevate the basal level of tyrosine phosphorylation which bears the risk of artificial results. Last, domains outside the catalytic core may regulate the substrate specificity of PTPs. By expressing only the catalytic domain, substrates identified may not be physiologically relevant. To overcome some of the limitations, the combination of gene targeting and substrate trapping has been utilized (Côté et al., 1998).

*Phosphoproteomics*
The progress in the substrate identification of PTPs has been relatively slow-moving because of the limitations of traditional methods. It becomes difficult to identify new targets especially for proteins that have low abundance in cells. The new development of mass spectrometric and bioinformatic techniques open up the possibility of a systematic, unbiased and high-throughput strategy for substrate discovery of PTPs (Macek et al., 2009). Mass spectrometry is well suited for detecting phosphorylation events. MS can identify each phosphorylated peptide and the exact phosphorylated residue within the peptide. New development in MS makes it sensitive enough to be applied in large-scale analysis in a quantitative way. For substrate identification of PTPs, the PTP of interest can be overexpressed in cells. Compared with control cells, differential phosphorylated peptides can be picked up by MS. Experimental verification is then needed to confirm candidates as *bona fide* substrates. Using this strategy, STAT3 was successfully demonstrated to be a substrate of PTPRT (Zhang et al., 2007). To correctly identify candidates from huge amount of data obtained from a large-scale MS analysis has proved to be challenging. Future development is needed to make phosphoproteomics more efficient and accurate with higher throughput.

In this project, we identified substrates for PTPN14 through proteomic analysis to help us understand its roles during tumorigenesis. The same approach can also be applied to other PTPs that are implicated in cancer development so that our improved knowledge of PTPs in tumorigenesis would lead to better targets for cancer treatment.
Chapter 2

Identification and functional characterization of p130Cas as a substrate of protein tyrosine phosphatase nonreceptor 14

A modified version of this chapter was previously published as:

Abstract

Protein tyrosine phosphatase nonreceptor type 14 (PTPN14) is frequently mutated in a variety of human cancers. However, the cell signaling pathways regulated by PTPN14 largely remain to be elucidated. Here, we identify a list of potential substrates of PTPN14 using a phospho-proteomic approach. We show that p130 Crk-associated substrate (p130Cas) is a direct substrate of PTPN14 and that PTPN14 specifically regulates p130Cas phosphorylation at tyrosine residue 128 (Y128) in colorectal cancer (CRC) cells. We engineered CRC cells homozygous for a p130Cas Y128F knock-in mutant and found that these cells exhibit significantly reduced migration and colony formation, impaired anchorage-independent growth, slower xenograft tumor growth in nude mice and have decreased phosphorylation of AKT. Furthermore, we demonstrate that SRC phosphorylates p130Cas Y128 and that CRC cell lines harboring high levels of pY128Cas are more sensitive to SRC family kinase inhibitor Dasatinib. These findings suggest that p130Cas Y128 phosphorylation may be exploited as a predictive marker for Dasatinib response in cancer patients. In aggregate, our studies reveal a novel signaling pathway that has an important role in colorectal tumorigenesis.
Introduction

Reversible tyrosine phosphorylation governs numerous signaling pathways that regulate cell proliferation, apoptosis, adhesion and migration (Blume-Jensen and Hunter, 2001). Protein tyrosine phosphorylation is coordinately controlled by protein tyrosine kinases and protein tyrosine phosphatases (PTPs) (Blume-Jensen and Hunter, 2001). Many protein tyrosine kinases have been identified to be oncogenes that are crucial to tumorigenic processes (Brognard and Hunter, 2011). Increasing evidence indicates that PTPs also have important roles in tumorigenesis (Julien et al., 2011). In a systematic mutational analysis of the PTP gene family in colorectal cancers (CRCs), protein tyrosine phosphatase nonreceptor type 14 (PTPN14) was identified as one of the six PTPs that are mutated in CRCs (Wang et al., 2004). Recent studies revealed that PTPN14 is also mutated in breast, head and neck, kidney, liver, ovarian and skin cancers (Sjöblom et al., 2006; van Haaften et al., 2009; TCGA 2011; Li et al., 2011; Stransky et al., 2011), suggesting that PTPN14 may play an important role in tumorigenesis of multiple types of cancers.

PTPN14, also known as Pez, PTP36 and PTPD2, consists of an N-terminal FERM (four-point-one, ezrin, radixin, moesin) domain and a C-terminal phosphatase domain (Wyatt and Khew-Goodall, 2008). It has been shown that PTPN14 may regulate cell–cell adhesion, cell–matrix adhesion, cell migration and cell growth (Ogata et al., 1999; Wadham et al., 2000, 2003). Interestingly, a recent study indicates that PTPN14 also regulates TGF-β gene expression,
thereby modulating epithelial–mesenchymal transition (Wyatt et al., 2007). Knockdown of the PTPN14 homolog in zebrafish results in developmental defects (Wyatt et al., 2007). Although PTPN14 was shown to regulate tyrosine phosphorylation of β-catenin (Wadham et al., 2003), it remains to be determined how this regulation impacts tumorigenesis. Given that PTPN14 is mutated in various cancers including CRCs, it is important to identify more substrates of PTPN14 in CRC cells. In this study, we identified a list of putative substrates of PTPN14 using a phospho-proteomic approach. We further validated p130 Crk-associated substrate (p130Cas) as a critical PTPN14 substrate.

p130Cas was originally identified as a Crk-associated protein (Reynolds et al., 1989; Matsuda et al., 1990). It is also known as breast cancer antiestrogen-resistant protein, because p130Cas overexpression renders breast cancer cells resistant to Tamoxifen treatment (Dorssers et al., 1993). Moreover, p130Cas is overexpressed in a subset of breast cancers (Cabodi et al., 2006). Transgenic mice overexpressing p130Cas shorten the latency of Her2/neu-induced breast cancer development. Furthermore, p130Cas is required for SRC-mediated transformation (Burnham et al., 1996; Honda et al., 1998). Together, these studies suggest that p130Cas is a key player in tumorigenesis. p130Cas is an adaptor protein that mediates integrin and growth factor signaling (Defilippi et al., 2006). It contains several conserved domains for protein–protein interactions. At the N-terminus, there is a substrate domain with 15 YXXP repeats, which can be heavily phosphorylated (Defilippi et al., 2006). Here, we report that PTPN14 dephosphorylates the tyrosine 128 (Y128) residue, one of the YXXP repeats, on
p130Cas. We further demonstrated that regulation of Y128 p130Cas phosphorylation has a critical role in CRC tumorigenesis.

Results

Identification of p130Cas as a substrate of PTPN14 using a phospho-proteomic approach

To identify potential substrates of PTPN14, we constructed two stable SW480 CRC cell lines expressing either a wild-type (WT) PTPN14 PTP domain or a PTPN14 D1079A-trapping mutant PTP domain. It is well-documented that PTP-trapping mutants can trap phospho-substrates in their catalytic pockets and protect the substrates from dephosphorylation, thereby potentially enriching the phospho-substrates (Flint et al., 1997; Blanchetot et al., 2005). Proteins were extracted from these cell lines as well as the parental SW480 cells and digested with trypsin. Phospho-tyrosine (Py)-containing peptides were enriched using anti-pY antibody columns. Peptides from each cell line were profiled by mass spectrometry analyses (Figure 2-1a). pY peptides were quantified as described by Rush et al (Rush et al., 2005). Those pY containing peptides that were either downregulated in the cell lines overexpressing the WT phosphatase or upregulated in the cell line overexpressing the trapping mutant compared with the parental cells are potential candidate substrates (Figure 2-1b and Table 2-1). Of these candidate substrates, we chose to further characterize p130Cas, because the pY128 containing peptides of p130Cas were enriched over 150-fold.
Figure 2-1. Identification of potential substrates of PTPN14 using a phospho-proteomic approach. (a) Schematic diagram of a phospho-proteomic approach to identify potential substrates of PTPN14. Cell lysates were made from the parental SW480 CRC cells, stable clones expressing either a WT PTP domain or a PTPN14 D1079A trapping mutant PTP domain. Proteins were digested with trypsin and pY containing peptides were enriched by anti-pY antibody (pY100)-conjugated beads. Peptide mixtures were profiled by mass-spectrometry. (b) List of the top candidate substrates of PTPN14. ND not detected.
Table 2-1. Candidate substrates of PTPN14.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession#</th>
<th>Site</th>
<th>Sequence</th>
<th>SW480</th>
<th>WT-1</th>
<th>WT-2</th>
<th>DA-1</th>
<th>DA3-2</th>
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<td>NF_005722</td>
<td>857</td>
<td>VSYTHYLYPERPSYLER</td>
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<td>8.2</td>
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in the cell line overexpressing the PTPN14-trapping mutant phosphatase (Figure 2-1b).

**PTPN14 regulates phosphorylation of p130 at the tyrosine 128 residue in CRC cells**

We set out to determine if PTPN14 indeed regulates p130Cas Y128 phosphorylation. To this end, we first generated a pY128 p130Cas-specific antibody. As shown in Figure 2-2, the antibody recognized a phospho-signal on the WT p130Cas. In contrast, it failed to detect any signal on p130Cas Y128F mutant protein (Figure 2-2), indicating that this antibody specifically recognizes phosphorylated Y128 of p130Cas. We then performed Western blot analyses on the aforementioned SW480 CRC cell lines using this antibody. Consistent with the proteomic data, compared with parental cells, overexpression of WT PTPN14 phosphatase resulted in reduced levels of pY128 p130Cas (Figure 2-4a), whereas overexpression of the trapping mutant led to increased levels of this phosphorylation (Figure 2-4a). Conversely, knockdown of PTPN14 by two independent short hairpins (shRNAs) resulted in increased levels of pY128 p130Cas in a genetically engineered DLD1 CRC cell line (Figure 2-4b), in which the endogenous PTPN14 proteins are epitope-tagged with 3xFLAG (Zhang et al., 2008). Taken together, these data demonstrated that PTPN14 regulates phosphorylation of p130Cas Y128 in CRC cells.
Figure 2-2. Anti-pY128 Cas antibody is specific. HEK293 cells were transfected with Flag-tagged WT or Y128F mutant p130Cas plasmid. Cell lysates were immunoprecipitated with anti-Flag antibodies and the immunocomplexes were resolved on SDS-PAGE gel and blotted with the indicated antibodies.

Figure 2-3. PTPN14 trapping mutant traps p130Cas protein in SW480 CRC cell lysates. GST, GST-WT and GST-D1079A trapping mutant PPTN14 PTP domain were expressed in E. Coli, purified and resolved on a SDS-PAGE gel (bottom panel). Arrow indicates the GST fusion proteins. Equal amounts of the purified proteins were mixed with SW480 cell lysates in the phosphatase trapping assay. The trapped p130Cas proteins were detected by Western blot (top panel).
**PTPN14 directly dephosphorylates p130Cas**

To determine whether PTPN14 directly regulates p130Cas Y128 phosphorylation, we first employed a substrate-trapping assay developed by Flint et al. (Flint et al., 1997). WT or substrate-trapping mutant (D1079A) of PTPN14 phosphatase domain was fused to GST and expressed in E. coli. GST fusion proteins were purified and attached on Glutathione beads. Equal amounts of WT and mutant GST-fusion proteins were used for a trapping assay (Figure 2-4c). As shown in Figure 2-4c and Figure 2-3, the trapping mutant fusion proteins pulled down p130Cas abundantly from cell lysates made from two different CRC cell lines DLD1 and SW480. In contrast, the WT GST-fusion proteins only pulled down minimal amount of p130Cas, whereas GST alone did not bind to p130Cas. Second, when mixed with phosphorylated p130Cas substrates in vitro, the WT PTPN14 GST fusion proteins dephosphorylated p130Cas at the Y128 site in comparison with controls with equal amounts of GST or the enzymatically inactive mutant proteins (trapping mutant) (Figure 2-4d). In aggregate, our results showed unequivocally that p130Cas is a direct substrate of PTPN14. However, we failed to detect protein–protein interaction between PTPN14 and p130Cas in DLD1 cell lysates by a co-immunoprecipitation assay (data not shown), suggesting that the two proteins only interact transiently when the enzymatic reaction occurs.

**SRC phosphorylates p130Cas at Tyr128**

Having demonstrated that PTPN14 dephosphorylates Y128 of p130Cas,
Figure 2-4. Validation of p130Cas as a substrate of PTPN14. (a) Overexpression of WT PTPN14 PTP domain decreases p130Cas Y128 phosphorylation, whereas the trapping mutant enriches pY128 Cas in CRC cells. Cell lysates made from the cell lines used in Figure 1 were blotted with the indicated antibodies. (b) Knock-down of PTPN14 leads to increased levels of pY128 Cas in CRC cells. Cell lysates from stable clones of DLD1 CRC cells expressing scramble shRNAs or shRNAs against PTPN14 were blotted with the indicated antibodies. N14-1 and N14-2 are two independent shRNAs against PTPN14. (c) A PTPN14 trapping mutant traps p130Cas protein. GST, GST-WT and GST-D1079A trapping mutant PTPN14 PTP domains were expressed in *E. coli*, purified and resolved on a SDS-polyacrylamide gel electrophoresis (bottom panel). Arrow indicates the GST fusion proteins. Equal amounts of the purified protein were mixed with DLD1 cell lysates in the phosphatase trapping assay. The trapped p130Cas proteins were detected by Western blot (top panel). (d) PTPN14 dephosphorylates pY128 Cas *in vitro*. Phosphorylated p130Cas proteins were incubated with the indicated GST fusion proteins. The pY128 Cas proteins were quantified by Western blot analysis.
we set out to identify the kinase that phosphorylates this site. Because SRC kinase has been shown to phosphorylate p130Cas at several tyrosine residues other than Y128, we elected to test whether SRC could also phosphorylate Y128 on p130Cas. Three lines of evidence indicated that SRC indeed phosphorylates this site (1): Treatment of DLD1 cells with a SRC inhibitor AZD0530 led to dephosphorylation of pY128 p130Cas (Figure 2-5a); (2) Knockdown of SRC by two independent shRNAs also resulted in reduced levels of pY128 p130Cas in DLD1 cells (Figure 2-5b); (3) Overexpression of SRC led to increased levels of pY128 on p130Cas (Figure 2-5c).

Colon cancer cell lines harboring higher levels of pY128 p130Cas are more sensitive to the killing by SRC family kinase inhibitor Dasatinib

SRC family kinase inhibitor Dasatinib is used in clinic to treat chronic myelogenous leukemia and there are several ongoing clinical trials using Dasatinib in combination with other drugs to treat colon cancers (Montero et al., 2011). To test if p130Cas Y128 phosphorylation associates with Dasatinib inhibition, we treated six colon cancer cell lines with various doses of Dasatinib and the IC50s of these cell lines were shown in up panel of Figure 2-5d. Remarkably, colon cancer cell lines harboring higher levels of pY128 p130Cas were more sensitive to growth inhibition by Dasatinib (Figure 2-5d).

Phosphorylation of p130Cas Y128 can be stimulated by epidermal growth factor (EGF)
Figure 2-5. SRC phosphorylates the Y128 residue of p130Cas. (a) SRC kinase inhibitor treatment downregulates pY128 p130Cas. DLD1 cells were treated with the indicated concentrations of SRC inhibitor AZD0530. Cell lysates were blotted with the indicated antibodies. (b) Overexpression of SRC results in increased levels of pY128 p130Cas. HEK 293 cells were transfected with either a Myc-tagged SRC plasmid or an empty vector. Cell lysates were blotted with the indicated antibodies. (c) Knock-down of SRC downregulates pY128 Cas in CRC cells. Cell lysates from stable clones of DLD1 cells expressing scramble shRNAs or shRNAs against SRC were blotted with the indicated antibodies. SRC-1 and SRC-2 are two independent shRNAs against SRC. (d) CRC cells harboring high levels of pY128 Cas are more sensitive to SRC inhibitor Dasatinib-induced growth inhibition. The indicated CRC cell lines are treated with a serial concentration of Dasatinib. The IC50s of each cell line from three independent experiments are plotted. The levels of pY128 Cas in each cell line are quantified by Western blot analysis.
Given that we have identified the kinase and phosphatase that regulate p130Cas Y128 phosphorylation, it is of interest to identify stimuli that may activate this phosphorylation in CRC cells. To this end, we treated DLD1 cells with various growth factors and cytokines including EGF, fibroblast growth factor, platelet-derived growth factor, interleukin-6 and vascular endothelial growth factor. Among them, EGF could robustly stimulate p130Cas Y128 phosphorylation after 30 min treatment (Figure 2-6A). In contrast, other growth factors and cytokines, including platelet-derived growth factor, failed to activate p130Cas Y128 phosphorylation, although platelet-derived growth factor was able to activate ERK1/2 (Figure 2-6B). Furthermore, EGF also induced phosphorylation of p130Cas Y128 in multiple CRC cells including RKO cells (Supplementary Figure 2-6A). Given that EGF signaling has a critical role in CRC tumorigenesis and that anti-EGFR antibodies are approved by the Food and Drug Administration to treat CRC patients (Yarom and Jonker, 2011), we chose to study p130Cas Y128 phosphorylation in the context of EGF stimulation.

**Engineering p130Cas Y128F mutant knock-in (KI) CRC cells**

To rigorously test whether regulation of p130Cas Y128 phosphorylation is critical to colorectal tumorigenesis, we set out to engineer p130Cas Y128F knock-in (KI CRC) cell lines. The adeno-associated virus (AAV) targeting system was used to engineer the KI cell lines because of its high homologous recombination frequency in somatic cells (Hirata et al., 2002; Kohli et al., 2004; Zhang et al., 2008). We first chose to knock-in the p130Cas Y128F mutant allele
Figure 2-6. EGF stimulates Y128 Cas phosphorylation in CRC cells. The indicated cells were serum-starved and stimulated with either EGF or PDGF for the indicated time. Cell lysates were blotted with the indicated antibodies.
into the human colon cancer cell line RKO, because we had shown that p130Cas Y128 phosphorylation can be activated by EGF in RKO cells as described above and that RKO cells are widely used for gene targeting (Du et al., 2010). The targeting strategy is outlined in the schematic diagram in Figure 2-7a. After the first round of gene targeting, seven targeted clones were identified out of 96 geneticin-resistant clones screened. To ensure the presence of the mutant allele, genomic polymerase chain reaction (PCR) products of exon 3, which encodes the Y128 residue, from the targeted clones were sequenced. Five of the seven targeted clones harbored p130Cas Y128F mutant allele. Two clones were infected with adenovirus expressing Cre-recombinase to excise the neomycin-resistant gene (Figure 2-7a) and targeted for the second allele to generate homozygous KI clones. To confirm that there was no WT p130Cas allele expression in the homozygous p130Cas Y128F mutant clones, the genomic PCR products of p130Cas were sequenced. As expected, both WT p130Cas alleles were replaced by the p130Cas Y128F mutants in the homozygous KI clones (Figure 2-7b). Furthermore, Western blot analyses showed that p130Cas proteins were expressed in the homozygous KI cells but they remained unphosphorylated at residue 128 after EGF stimulation, whereas p130Cas proteins in the parental cells were heavily phosphorylated at Y128 post-EGF stimulation (Figure 2-7c). These data indicated that we had successfully engineered p130Cas Y128F mutant KI cells. We chose two independently derived heterozygous and homozygous KI clones for in-depth analyses, and both clones behaved similarly in all the studies described below. To ensure that what
we observed with RKO cells is not cell-line specific, we used the same method to
generate p130Cas Y128F mutant DLD1 cells (Figure 2-7).

**In vitro, p130Cas Y128F mutant CRC cells are reduced in properties
predicative of in vivo tumorigenicity**

When grown under normal tissue culture conditions (McCoy’s 5A supplemented with 10% FBS), the average doubling times of the DLD1 p130Cas Y128F mutant clones increased by 1.5 h in comparison with the parental cells (Figure 2-8), whereas no doubling time difference was observed between RKO parental and the mutant clones (Figure 2-8). Cell cycle profiling showed slightly increased G1 populations in the p130Cas Y128F homozygous KI clones derived from both DLD1 and RKO cells (Figure 2-10). To test whether p130Cas Y128F mutant affects tumorigenicity correlated responses in vitro, we performed colony formation and soft agar assays with the p130Cas mutant KI cells. Compared with the parental cells, homozygous p130Cas Y128F KI RKO and DLD1 cells exhibited 3–6-fold (P<0.001) reduced abilities to form colonies in colony-formation assays (Figure 2-9a). Similarly, homozygous p130Cas mutant CRC cell clones formed ~25-fold (P<0.001) less foci in soft agar assay than their WT counterparts (Figure 2-9b). Interestingly, the RKO heterozygous KI clones, but not these of DLD1 heterozygous KI clones, displayed significant (P<0.001) reduction in colony numbers and soft-agar foci with respect to WT cells (Figures 2-9a and b).
Figure 2-7. Generation of p130Cas Y128F mutant knockin CRC cells. (a) Diagram of the KI construct. (b) Genomic sequences of the parental (WT), heterozygous (Het) and homozygous (Hom) KI cells. The rectangular boxes indicate codons for the Cas amino acid 128 position. (c) The parental (WT) and p130Cas Y128F homozygous KI CRC cells were serum-starved and stimulated with EGF. Cell lysates were blotted with the indicated antibodies.
The p130Cas mutant RKO cells are less tumorigenic in vivo

Tumorigenicity of the KI cells was also tested in a more stringent in vivo model. For these studies, p130Cas Y128F homozygous, heterozygous clones or the parental RKO and DLD1 cells were injected subcutaneously into nude mice. After 35 days of growth, WT cells formed tumors in all mice injected, whereas the p130Cas Y128F homozygous RKO KI clones failed to form tumors in two of the five mice injected (Figure 2-11a). The average tumor volumes of p130Cas Y128F homozygous RKO KI clones were 30-fold smaller than those produced by the parental cells (P<0.001) (Figure 2-11b). However, no significant difference in xenograft tumor growth was observed between the DLD1 homozygous KI clones and the parental (Figure 2-12). Both RKO and DLD1 heterozygous KI clones formed similar sizes of tumors to those of parental cells (Figures 4-11a and b, and Figure 2-12).

The p130Cas Y128F mutant CRC cells display defects in cell spreading and migration

Given that both PTPN14 and p130Cas are involved in cell adhesion and migration, we set out to determine how p130Cas Y128 phosphorylation impacts cancer cell adhesion and migration (Wadham et al., 2003; Wyatt and Khew-Goodall, 2008). Boyden chamber cell migration assay showed that the p130Cas Y128F mutant cells exhibited significantly reduced ability in cell migration (Figure 2-13). When grown on coverslips coated with fibronectin, the majority of parental
Figure 2-8. Doubling time of p130Cas Y128F mutant cells. Cells of the indicated genotypes were analyzed in log phase. Cell numbers of each clone were counted for two consecutive days using a hemocytometer and doubling times were calculated. Average doubling times of the three independent experiments are plotted. *p<0.05, t test.
RKO and DLD1 cells spread fully and displayed a fibroblast-like morphology (Figures 4-14A and B). In contrast, most of the p130Cas Y128F DLD1 mutant cells were not fully spreading (Figures 4-14A and B). The percentages of fully-spreading mutant RKO cells were also significantly reduced, although not as dramatic as the mutant DLD1 cells. However, no apparent focal adhesion defect was observed with the mutant cells (Figure 2-14A).

**AKT signaling is impaired in the p130Cas Y128F mutant Ki cells**

We demonstrated that phosphorylation of the p130Cas Y128 residue has an important role in colorectal tumorigenesis. To gain insights into the effects of this phosphorylation on downstream signaling, we examined how the p130Cas Y128F Ki affects phosphorylation of signaling molecules in CRC cells after EGF stimulation. It is well-documented that EGF receptors, once they are engaged by their ligands, activate multiple well-characterized signaling pathways including Ras-MAPK, PI3K-AKT, PLC-γ and STATs (Avraham and Yarden, 2011). We tested the phosphorylation status of 27 sites on 16 proteins that could be potentially modulated by EGF signaling (Table 2-2). In both RKO and DLD1 CRC cells, phosphorylation of AKT Thr308 was significantly reduced in p130Cas Y128F Ki cells in comparison with the parental cells (Figures 4-15a and b). However, the kinetics of AKT activation appeared to be faster in RKO cells than in DLD1 cells (Figures 4-15a and b). Although no difference in ERK1/2 phosphorylation was observed between the RKO parental and Y128F Ki cells, ERK1/2 phosphorylation levels were elevated in DLD1 p130Cas Y128F mutant...
Figure 2-9. p130Cas Y128F mutant CRC cells are less tumorigenic in vitro. (a) p130Cas Y128F mutant CRC cells form fewer colonies. Cells from indicated clones were plated in six-well plates in triplicates. Cells were grown for 14 days and stained with crystal violet. Colony numbers were counted and plotted for each of the clones. (b) p130Cas Y128F mutant CRC cells impair anchorage-independent growth. CRC cells of the indicated clones were mixed in 0.4% soft agar and plated in six-well plates in triplicates. Cells were grown for 30 days. Colony foci were counted and plotted for each of the clones. *P<0.0001, t test. Het and Hom indicate the heterozygous and homozygous KI clones respectively.
Figure 2-10. Cell-cycle profiles of p130Cas Y128F mutant cells. Cells of the indicated genotypes were harvested during the log phase and fixed with methanol. Cells were then incubated at 37°C for 30 min in 5% normal goat serum diluted in PBS. Propidium iodide solution was used to stain cells at 4°C for 1 hour. Cells were analyzed on an Epics XL flow cytometer. WinMDI2.9 was used for data analysis. Cell debris and aggregates were excluded on PI gating. Percentages of G1, S and G2/M populations were determined by histograms generated by WinDi2.9. Representative histograms of the indicated clones are shown. Averages of percentage of cells in G1, S and G2/M phases from three independent experiments are shown as mean ± SD.
Figure 2-11. The RKO p130Cas Y128F mutant cells are less tumorigenic in vivo. Athymic nude mice were injected subcutaneously with cells from the indicated clones. Tumor sizes were measured weekly for 5 weeks. Mice were then sacrificed and tumors were harvested. (a) Tumors grown from the RKO clones. Each black rectangle indicates tumors harvested from a mouse. (b) Average sizes of the tumors formed by the indicated clones were plotted.
Figure 2-12. Xenograft tumor formation by DLD1 parental and p130Cas Y128F KI cells. Athymic nude mice were injected subcutaneously with cells from the indicated clones. Tumors sizes were measured weekly for 4 weeks. Mice were then sacrificed and tumors were harvested.

Figure 2-13. p130Cas Y128F mutant CRC cells exhibit migration defects. Cells of the indicated genotypes were assayed for cell migration in Boyden chambers coated with fibronectin. Numbers of migrated cells per field were quantified.
cells, suggesting that a compensatory effect occurred in the DLD1 mutant cells.

**The p130Cas Y128F mutant proteins bind less p85 than the WT p130Cas**

The above results indicate that AKT is a critical downstream mediator of the p130Cas pY128 signaling. It is well-documented that AKTs are activated by phosphatidylinositol 3-kinase (PI3K) (Bader et al., 2005). PI3K, which consists of a p85 regulatory subunit and a p110 catalytic subunit (Bohnacker et al., 2009), converts phosphatidylinositol 4, 5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (Bader et al., 2005; Bohnacker et al., 2009). Phosphatidylinositol 3, 4, 5-trisphosphate then recruits AKT to the plasma membrane, which, in turn, activates AKT (Bohnacker et al., 2009). PI3K becomes activated when it is recruited to the membrane by interaction between p85 and pY residues on membrane-bound receptors or adaptor proteins (Bohnacker et al., 2009). Interestingly, several studies demonstrate that membrane-associated p130Cas activates PI3K through interaction with p85 (Li et al., 2000; Riggins et al., 2003), and that this interaction is dependent on tyrosine phosphorylation of the substrate-binding domain on p130Cas (Riggins et al., 2003). Given that the Y128 belongs to one of the YXXP repeats (Defilippi et al., 2006), we postulated that the reduced AKT activity in the p130Cas Y128F mutant cells might be due to weaker binding affinity of the mutant p130Cas protein to p85. Indeed, as shown in Figure 2-15c, less p85 proteins associated with the p130Cas Y128F mutant compared
Figure 2-14. Focal adhesions and spreading of parental (WT) and p130Cas Y128F mutant cells. (a) The WT and mutant cells were grown on coverslips coated with fibronectin, fixed and stained with TRITC-conjugated Phalloidin and anti-vinculin antibodies. (b) Fully-spreading fibroblast-like cells were quantified. * P < 0.005; ** P < 0.001.
with the WT p130Cas. In contrast, the p130Cas Y128F mutation did not affect its binding with CRK, a known p130Cas interacting protein (Figure 2-15c) (Reynolds et al., 1989; Matsuda et al., 1990). Under these conditions, we failed to detect protein interactions between p130Cas and focal adhesion kinase (FAK).

**Discussion**

Using a phospho-proteomic approach, we identified and validated p130Cas as a substrate of PTPN14. PTPN14 dephosphorylates an unappreciated Y128 residue on p130Cas protein. PTPN14-regulated p130Cas Y128 phosphorylation has an important role in colorectal tumorigenesis, because the genetically engineered p130Cas Y128F mutant CRC cells exhibit reduced abilities in colony formation, anchorage-independent growth, cell migration and xenograft tumor growth. PTPN14 is identified to be mutated in various human cancers including breast, colon, head and neck, kidney, liver, ovarian and skin cancers (Wang et al., 2004; Solomon et al., 2008; van Haaften et al., 2009, 2009; 2011; Li et al., TCGA 2011; Stransky et al., 2011; Wei et al., 2011), thereby providing genetic evidence suggesting that this PTP has a role in tumorigenesis. In a fraction of tumors, one allele of PTPN14 is mutated whereas the other allele is lost (Wang et al., 2004), consistent with a ‘two-hit’ tumor suppressor model. Therefore we postulate that PTPN14 may function as a tumor suppressor. Consistent with this notion, PTPN14 negatively regulates an oncogenic target pY128 p130Cas, because the nonphosphorylatable p130Cas Y128F KI DLD1
Figure 2-15. Reduced AKT phosphorylation in the p130Cas Y128F mutant cells. (a, b) Parental and p130Cas Y128F mutant (KI) cells were serum-starved and stimulated with EGF for the indicated time. Cell lysates were blotted with the indicated antibodies. (c) The p130Cas Y128F mutant proteins show weaker binding affinity to p85. Cell lysates from either the parental or p130Cas Y128F mutant (KI) cells were immunoprecipitated with an anti-p130Cas antibody and blotted with the indicated antibodies.
Table 2-2. List of phospho-antibodies.

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and RKO cells exhibit reduced colony-formation ability and soft-agar growth (Figure 2-9). Furthermore, the RKO p130Cas Y128F mutant cells grow much slower as xenograft tumors, although the p130Cas Y128F mutation in DLD1 cell does not affect xenograft tumor growth. We hypothesize that this discrepancy is due to a compensatory effect in the DLD1 p130Cas mutant cells. In support, we observed that ERK activities are upregulated in the DLD1 p130Cas mutant KI cells, but not in the RKO mutant KI cells (Figures 2-15a and b).

We also demonstrated that SRC is the kinase that phosphorylates p130Cas Y128 residue (Figure 2-5). Interestingly, CRC cell lines harboring high levels of pY128 p130Cas are more sensitive to SRC inhibitor Dasatinib-induced growth inhibition than those cell lines with low levels of pY128 p130Cas (Figure 2-5d). Dasatinib is used in the clinic to treat chronic myelogenous leukemia, and there are several ongoing clinical trials using Dasatinib in combination with other drugs to treat solid tumors including colon cancers (Montero et al., 2011). Our data suggest that pY128 p130Cas may be exploited as a prediction marker for Dasatinib response in cancer patients. It is worth noting that SRC also phosphorylates other sites of the YXXP repeats on p130Cas including the Y253 that regulate transformed cell growth and migration (Goldberg et al., 2003; Patwardhan et al., 2006). As the future directions, it is of interest to determine if PTPN14 dephosphorylates the other sites and if there is a cross talk between the Y128 phosphorylation and the other Y residues of the YXXP repeats.

The oncogenic signaling of p130Cas Y128 phosphorylation seems to be mediated by the PI3K/AKT pathway, as we showed that AKT T308
phosphorylation, a key to AKT activation, is reduced in both DLD1 and RKO p130Cas Y128 mutant KI cells (Figures 2-15a and b). It has been reported previously that p130cas can activate PI3K through binding to the regulatory subunit p85 (Li et al., 2000; Riggins et al., 2003). Here, we demonstrate that p130Cas pY128 phosphorylation is crucial for its binding to p85. Although the p130Cas interacting protein CRK is shown to activate PI3K through the focal adhesion kinase-p85 interaction (Akagi et al., 2002), our results indicate that the p130Cas Y128F mutant does not affect its binding to CRK.

Lastly, the phospho-proteomic approach used in this study represents a novel method to globally identify potential substrates of PTPs. Identification of substrates of PTPs has been a challenge. Although phosphatase-trapping pull-down has been widely used to validate PTP substrates, it has limited success in identification of new substrates (Liang et al., 2007). We show here that, coupling with global phospho-proteomic profiling, overexpression of the trapping mutant PTPN14 PTP domain can enrich the target pY containing peptides and provides a systematic approach to identify candidate substrates. We also successfully employed a similar method to identify substrates of protein tyrosine phosphatase receptor T (Zhang et al., 2007). We further validated that STAT3 and paxillin are the substrates of protein tyrosine phosphatase receptor T (Zhang et al., 2007; Zhao et al., 2010). Therefore, our studies suggest that the phospho-proteomic approach is a generally applicable method for identification of PTP substrates.
Materials and methods

Cell lines

DLD1, HCT116, HT29, RKO, SW480, LOVO and HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All the CRC cells were maintained in McCoy’s 5A media plus 10% FBS. HEK 293T cells were maintained in DMEM media plus 10% FBS.

Establishment of cell line stably expressing PTPN14 catalytic domain

The catalytic domain of PTPN14 was PCR amplified using primers 5’-CGGGATCCCCGGGAAGAGAATCGAGTTGA-3’ and 5’-CCGCTCGAGGAGCTGGATTGGGGTGATTA-3’. The PCR products were cloned into the pcDNA4-Myc-HisB (Invitrogen, Carlsbad, CA, USA) by BamHI and XhoI, which were incorporated into the primers. The PTPN14 D1079A mutant catalytic domain was made by site-directed mutagenesis with primers 5’-AATATACTGACTGGCCAGCTCACGGCTGTCCAGAAGA-3’ and 5’-TCTTCTGGACAGCCGTGAGCTGGCCAGTCAGTATATT-3. Stable clones of SW480 cells were obtained according to the manufacturer’s instructions and were maintained in medium containing Zeocin (100 μg/ml) plus blasticidin (10 μg/ml). Protein expression was induced by addition of 1 μg/ml of doxycycline.

Profiling pY peptides

Cell lysates were prepared under denaturing conditions in the presence of
vanadate. Following tryptic digestion, peptides were concentrated and partitioned into three fractions by reversed-phase solid-phase extraction. The phosphopeptides from each fraction were bound to agarose beads conjugated with the phosphotyrosine-specific antibody pTyr-100. After thorough washing, peptides were eluted from the immobilized antibody with dilute acid and analyzed by nanoflow liquid chromatography/mass-spectrometry (LC-MS/MS) using an ion trap mass spectrometer. Lists of credible phosphopeptide sequence assignments were assembled.

**Generation of anti-p130Cas pY128 antibody**

Rabbits were immunized with a pY128 p130Cas peptide (KAQQGL(pY)QVPgp) conjugated to bovine serum albumin (BSA). The antiserum was first absorbed to a BSA-Sepharose 4B column and then passed twice through BSA-conjugated unphosphorylated peptide KAQQGL Y Q VPgp columns.

**Stimulation of CRC cells with growth factors**

DLD1 and RKO cells were serum-starved for 24 h and stimulated with EGF (200 ng/ml), platelet-derived growth factor (10 ng/ml), fibroblast growth factor (10 ng/ml), vascular endothelial growth factor (10 ng/ml) and interleukin-6 (10 ng/ml) for various times.
**Western blot analysis**

Cells were lysed in lysis buffer (10 mM Tris–HCl, pH 8.0; 100 mM NaH₂PO₄; 8M urea; 1 mM Na₃VO₄; 20 mM NaF; 80 μM β-glycerophosphate; 20 mM sodium pyrophosphate). Western blots were performed as previously described. Antibodies used include anti-FLAG (M2; Sigma, St. Louis, MO, USA), anti-ERK1/2, anti-SRC, anti-pERK1/2, anti-AKT, anti-pAKT (Cell Signaling, Danvers, MA, USA) and anti-p130Cas (BD Biosciences, San Jose, CA, USA).

**Phosphatase substrate-trapping assay**

Substrate-trapping was performed as described (Zhang et al., 2007) and modified as follows. Ten million cells were treated with 100 μM pervanadate for 30 min and collected by centrifugation. The cell pellet was lysed with 1 ml of lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 × complete protease inhibitor cocktail, 1 mM EDTA, 1 mM Benzamidine), treated with 5 mM iodoacetic acid on ice for 5 min, neutralized by addition of 10 mM DTT for 15 min, and subjected to centrifugation at 16,000 g for 30 min to remove debris. GST–PTPN14 bound beads were incubated with this lysate at 4 °C for 1 h. The beads were pelleted and washed three times for 5 min with lysis buffer supplemented with 1 mM DTT. The beads were then boiled and aliquots analyzed by SDS–Polyacrylamide gel electrophoresis and Western blotting.
**In vitro phosphatase assay**

HEK293T cells overexpressing FLAG-tagged p130Cas proteins were treated with 50 μM pervanadate for 30 min and lysed in RIPA buffer. Cas proteins were immunoprecipitated with anti-FLAG antibody-conjugated agarose beads. The immune complexes were washed twice in wash buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) with the phosphatase inhibitors (10 mM NaF and 2 mM Na$_3$VO$_4$), twice in the same buffer without the phosphatase inhibitors, once in ST buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl) and once in phosphatase assay buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM DTT). The p130Cas immunocomplexes were then incubated with equal amounts of either GST alone, GST-PTPN14, or the trapping mutant at 37 °C for 30 min. Western blots were performed to quantitate p130Cas pY128.

**shRNA knockdown**

shRNA lentiviral vectors were purchased from Sigma. Lentiviruses were packaged in HEK-293T cells with the transfection of lentiviral vectors together with pMD2.G VSVG and pCMV delta R8.74. Twenty-four hours after transfection, lentiviruses were harvested and purified and then were used to transduce DLD1 cells. Stable clones were then selected in medium containing 2.5 μg/ml of puromycin.

**Targeted KI of p130Cas Y128F mutant allele**

Somatic cell gene targeting was performed as described (Zhang et al.,
Briefly, a 1.3-kb fragment from intron 2 to intron 3 of the p130Cas locus containing the exon 3 sequences was amplified 20 cycles from genomic DNA using primers 5’-GGGAA AG/ideoxyU/GGGTTTGCTGAGGGCGACGGG-3’ and 5’-GGAGACA/ideoxyU/GGTGATTGAGGGTGGCTGGGCCCTT-3’. The coding sequences for Y128 were then mutated from TAC (Tyr) to TTC (Phe) by site-directed mutagenesis using primers 5’-GGCTCAGCAAGGCCTCTTCCAAGT CCCGGGTCCCAAG-3’ and 5’-AAGAGGCCTTGCTGAGCC-3’. This mutated fragment was used as the left homologous arm. Another 1.0-kb fragment from intron 3 of the p130Cas gene was also amplified 20 cycles from genomic DNA as the right arm using primers 5’-GGTCCCA/ideoxyU/GGCATTCCAGGAGTGCTGTG-3’ and 5’-GGCATAG/ideoxyU/GTTCATCTGTGTGGGGTGTGG-3’. The left and right homologous arms were cloned into pAAV-USER-Neo-LoxP vector using USER system as previously described. The targeting adeno-associated viruses were packaged in 293T cells (a T75 flask at 70% confluence) by transfecting equal amounts of the targeting vector, pHelper and pRC plasmids (3 μg each). Viruses were harvested 72 h post-transfection. DLD1 and RKO cells were infected with the p130Cas knock-in targeting viruses and selected with geneticin for 20 days. The geneticin-resistant clones were then screened for homologous recombination by 35 cycles of genomic PCR with primers derived from the neomycin-resistant gene 5’-GTTGTGCCCCAGTCATAGCCG-3’ and the upstream region of the left homologous arm 5’-GGGCCACATGGAGCAGCCTAC C-3’. Confirmatory genomic PCR was also performed with positive clones identified using primers derived from the neomycin-resistance gene (5’-TCTGGA
TTCATCGACTGTGG-3’) and the downstream region of the right homologous arm (5’-GCATGGGTGTTCTGATCTGTGG-3’). The DNA fragments from the screening PCRs were then sequenced to ensure the presence of the mutant Y128F alleles. In order to target the second allele with the same targeting virus, correctly targeted clones were infected with adenoviruses expressing the Cre-recombinase to delete the drug selection marker. To select clones with successful deletion of the drug selection marker, 30 cycles of genomic PCR were performed to amplify a ~200 bp genomic fragment in which the Lox P site was inserted (using primers 5’-CACGC AGCTGGGAGGGCAGAAG-3’ and 5’-CTCTGGGTCCATTCACATCCATC-3’). The heterozygous KI clones were infected with the same targeting virus to target the second allele and the neomycin-resistant gene was excised as described above.

**Colony formation assay**

DLD1 and RKO cells were trypsinized, counted twice using a hemocytometer, and placed into six-well plates at 200 cells per well. Cells were grown for 14 days before staining with Crystal Violet (Sigma). The experiment was repeated three times with two replicates each. Average numbers of colonies from each experiment were plotted.

**Focus formation assay in soft agar**

Soft agar assays were performed as described (Chapter 3). Briefly, DLD1 and RKO clones were trypsinized, counted twice using a hemocytometer, and
plated at 5000 cells/ml in top plugs consisting of 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, ME, USA) and McCoy’s 5A medium. After 30 days, the colonies were photographed and counted. The experiment was repeated three times with two replicates each. Average numbers of colonies from each experiment were plotted.

**Xenografts**

Five million cells were injected subcutaneously and bilaterally into 4- to 6-week-old female nude mice (five nude mice in each group). Tumor formation and size were assessed by weekly caliper measurements of the length and width of the tumors. Tumor volumes were calculated using the formula: Volume= (width)² × length/2. After 35 days, the mice were sacrificed and tumors were harvested.

**Focal adhesion assay**

Focal adhesion assay was performed using the Actin Cytoskeleton and Focal Adhesion Staining Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. Briefly, cells were seeded on coverslips treated with Fibronectin. The next day, cells were fixed with 4% paraformaldehyde in 1 × PBS for 15 min and washed once. Cells were then permeabilized with 0.1% Triton X-100 in 1XPBS for 5 min and washed twice. Cells were incubated in anti-Vinculin antibody (1:200 dilution) for 1 h and followed by three washes. Cells were then incubated in Alexa 488-conjugated secondary antibody (1:400) and TRITC-conjugated Phalloidin (1:250) for 1 h and were washed three times. Coverslips
were mounted on a slide and were visualized under Zeiss LSM 510 Confocal Microscope (Zeiss, Maple Grove, MN, USA).

**Boyden chamber cell migration assay**

Transwell membranes (pore size 8.0 μm; Corning Incorporated., Corning, NY, USA) were coated with 50 μg/ml fibronectin. Cells were detached with 2 mM EDTA and resuspended in serum-free medium with 0.1% of BSA. Five hundred thousand cells were added to the upper compartment of the transwell chamber in the wells of a 24-well plate and allowed to migrate to the underside of the inserts for 24 h. Nonmigrating cells on the upper membrane were removed with a cotton swab, and cells that had migrated and become attached to the bottom surface of the membrane were fixed and stained with crystal violet. Migrated cells were counted microscopically. The experiments were repeated three times with two replicates for each cell line. Average numbers of migrated cells from each experiment were plotted.

**Statistical analysis**

We applied the $t$ test to compare the mean between two groups assuming unequal variances. For xenograft growth, we performed MANOVA analysis.
Chapter 3

Cross-talk between Phospho-STAT3 and PLCγ1 Plays a Critical Role in Colorectal Tumorigenesis

A modified version of this chapter was previously published as:

Abstract

Hyperphosphorylation at the Y705 residue of signal transducer and activator of transcription 3 (STAT3) is implicated in tumorigenesis of leukemia and some solid tumors. However, its role in the development of colorectal cancer is not well defined. To rigorously test the impact of this phosphorylation on colorectal tumorigenesis, we engineered a STAT3 Y705F knock-in to interrupt STAT3 activity in HCT116 and RKO colorectal cancer cells. These STAT3 Y705F mutant cells fail to respond to cytokine stimulation and grow slower than parental cells. These mutant cells are also greatly diminished in their abilities to form colonies in culture, to exhibit anchorage-independent growth in soft agar, and to grow as xenografts in nude mice. These observations strongly support the premise that STAT3 Y705 phosphorylation is crucial in colorectal tumorigenesis. Although it is generally believed that STAT3 functions as a transcription factor, recent studies indicate that transcription-independent functions of STAT3 also play an important role in tumorigenesis. We show here that wild-type STAT3, but not STAT3 Y705F mutant protein, associates with phospholipase Cγ1 (PLCγ1). PLCγ1 is a central signal transducer of growth factor and cytokine signaling pathways that are involved in tumorigenesis. In STAT3 Y705F mutant colorectal cancer cells, PLCγ1 activity is reduced. Moreover, overexpression of a constitutively active form of PLCγ1 rescues the transformation defect of STAT3 Y705F mutant cells. In aggregate, our study identifies previously unknown cross-talk between STAT3 and the PLCγ1 signaling pathways that may play a critical role in colorectal tumorigenesis.
Introduction

Signal transducer and activator of transcription 3 (STAT3) is thought to be an oncogene (Levy and Inghirami, 2006). Several lines of evidence support such a premise. First, persistent STAT3 activation has been detected in leukemia and in a variety of solid tumors including breast, brain, pancreas, ovarian, and squamous cell carcinomas of head and neck (SCCHN) cancers, and melanomas (Bowman et al., 2000). Second, constitutively active STAT3 transforms rat and mouse cells and dominant negative STAT3 blocks Src-induced transformation in vitro (Bromberg et al., 1998, 1999). Interestingly, recent studies show that the mitochondrial functions of STAT3 may be important factors in tumorigenesis, because its mitochondrial activity appears to be required for Ras-mediated tumor transformation (Gough et al., 2009; Wegrzyn et al., 2009). Third, transgenic mice with keratinocytes expressing constitutively active STAT3 develop hyperproliferative dermatologic disorders in vivo (Sano et al., 2005). Fourth, targeted deletion of STAT3 in skin cells prevents epithelial cancers in mice (Chan et al., 2004), and targeting STAT3 specifically in B and T cells prevents development of lymphomas and myelomas (Chiarle et al., 2005).

Latent cytoplasmic STAT3 becomes activated through phosphorylation of Y705 by cytoplasmic nonreceptor tyrosine kinases including Janus-activated kinase (JAK) and Src (Darnell, 2005). Phosphorylated STAT3 dimerizes through reciprocal Src Homology 2 (SH2)-phosphotyrosine interaction and accumulates in the nucleus (Bowman et al., 2000). Therefore, STAT3 activates the
transcription of a wide array of genes including B-cell lymphoma-extra-large (Bcl-XL) and suppressor of cytokine signaling 3 (SOCS3) (Bowman et al., 2000). Although the kinases that phosphorylate the Y705 residue of STAT3 are well defined in epithelial and hematopoietic cells, the phosphatases that specifically dephosphorylate pY705 have received little attention.

We previously identified STAT3 as a direct substrate of protein tyrosine phosphatase receptor T (PTPRT) (Zhang et al., 2007). PTPRT is mutated in colon, lung, stomach, and skin (melanoma) cancers (Wang et al., 2004). Moreover, PTPRT knockout mice are highly susceptible to azoxymethane-induced colon tumors (Zhao et al., 2010), indicating that PTPRT normally functions as a tumor suppressor. Our finding that PTPRT specifically dephosphorylates STAT3 at the Y705 residue supports a critical role for regulation of STAT3 Y705 phosphorylation in colorectal tumorigenesis. Although STAT3 is implicated in oncogenesis of leukemia, skin, and head and neck cancers (Levy and Inghirami, 2006), the impact of STAT3 Y705 phosphorylation in colorectal tumorigenesis has not heretofore been well defined. Here we show that successful knock-in (KI) of the STAT3 Y705F mutant allele into 2 different colorectal cancer cell lines results in mutant colorectal cancer cells that are less tumorigenic both in vitro and in vivo. The results of this study further suggest that modulation of tumorigenicity is at least partially dependent on STAT3 cross-talk with phospholipase Cγ1 (PLCγ1) through effects on S1248 phosphorylation. PLCγ1 is a key signaling molecule that hydrolyzes phosphatidylinositol-4,5-biophosphate to generate inositol-1,4,5-triophosphate (IP3) and 1,2-
diacylglycerol, which, in turn, activate intracellular Ca\(^{2+}\) and protein kinase C (PKC) signaling pathways that are implicated in tumorigenesis (Wells and Grandis, 2003). In support, we show that colorectal cancer cells carrying STAT3 mutated in Y705 also exhibit reduced PKC activities.

**Results**

**Engineering STAT3 Y705F knock-in colorectal cancer cell lines**

To rigorously test whether regulation of STAT3 Y705 phosphorylation is critical to colorectal tumorigenesis, we set out to engineer STAT3 Y705F KI colorectal cancer cell lines. The AAV targeting system was used to engineer the KI cell lines because of its high homologous recombination frequency in somatic cells (Hirata et al., 2002; Kohli et al., 2004). We first chose to knock in the Y705F STAT3 mutant allele into the human colon cancer cell line HCT116, because we had shown that STAT3 can be activated by IL-6 in HCT116 cells (Zhang et al., 2007) and because HCT116 had been widely used for successful gene targeting by homologous recombination (Kohli et al., 2004; Zhang et al., 2008). The targeting strategy is outlined in the schematic diagram in Figure 3-1A. After the first round of gene targeting, 4 targeted clones were identified out of 192 geneticin resistant clones screened. To ensure the presence of the mutant allele, STAT3 exon 22 genomic PCR products of the targeted clones were DNA sequenced. Three of the 4 targeted clones harbored STAT3 Y705F mutant allele
Figure 3-1. (a) Diagram of the KI construct. (b, c) PCR products of the KI clones after excision of the neomycin resistance gene by Cre recombinase. P indicates parental cells; WT/KI indicates Y705F STAT3 heterozygous KI cells; KI/KI indicates Y705F STAT3 homozygous KI cells; arrow indicates the KI allele. (d, e) Parental, Y705F heterozygous and Y705F homozygous cells were starved for 18 hours and treated with or without IL-6 for 30 minutes. Western blots were performed with pSTAT3 or STAT3 antibodies.
Figure 3-2. Genomic sequences of STAT3 Y705 KI cells. Genomic fragments surrounding the mutation site were PCR amplified from the indicated clones and sequenced. Rectangle boxes indicate either WT (TAC) or mutant (TTC) codons.
Two clones were infected with adenovirus expressing Cre-recombinase to excise the neomycin resistance gene (Fig. 3-1B) and targeted for the second allele to generate homozygous KI clones. To confirm that there was no wild-type (WT) STAT3 allele expression in the homozygous Y705F STAT3 clones, the RT-PCR products of STAT3 were sequenced. As expected, both WT STAT3 alleles were replaced by the STAT3 Y705F mutants in the homozygous KI clones (Fig. 3-2). Furthermore, Western blot analyses showed that STAT3 proteins were expressed in the homozygous KI cells but they remained unphosphorylated at residue 705 after IL-6 stimulation, whereas STAT3 proteins in the parental and heterozygous cells were heavily phosphorylated post–IL-6 stimulation (Fig. 3-1D). These data indicated that we had successfully engineered STAT3 Y705F KI cells. We chose 2 independently derived heterozygous and homozygous KI clones for in-depth analyses and both clones behaved similarly in all the studies described in the following text. To ensure that what we observe with HCT116 cells is not cell line specific, we used the same method to generate STAT3 Y705F mutant RKO cells (Fig. 3-1C and E).

**STAT3 Y705F mutant fails to activate its target genes**

Our previous studies showed that IL-6 induces STAT3 translocation from the cytoplasm to the nucleus and activates transcription of its target genes, Bcl-XL and SOCS3 in colorectal cancer cells (Zhang et al., 2007). To delineate the role of STAT3 phosphorylation in the translocation process, current experiments examined whether IL-6 induces STAT3 Y705F mutant protein translocation. This
translocation process was directly monitored in parental and homozygous STAT3 Y705F mutant HCT116 and RKO cells using immunofluorescence staining. As shown in Figure 3-3A and Supplementary Figure 3-4A, STAT3 Y705F mutant proteins remain diffused on IL-6 stimulation, whereas WT STAT3 proteins accumulate in the nucleus in parental cells. Then, we utilized gene expression analyses by RT-PCR to show that IL-6 fails to activate gene transcription of Bcl-XL and SOCS3, 2 of the STAT3 target genes, in STAT3 Y705F homozygous KI cells (Fig. 3-3B; Fig. 3-4B). Taken together, these data showed that STAT3 Y705F mutant is unresponsive to IL-6 stimulation, and strongly suggest that STAT3 Y705 phosphorylation is critical for its activation in CRC cells.

**STAT3 Y705F mutant CRC cells grow slower than parental cells in tissue culture**

When grown under normal tissue culture conditions (McCoy's 5A supplemented with 10% FBS) over a 4-day period, both HCT116 and RKO STAT3 Y705F homozygous KI cells grew slower than their parental cells (Fig. 3-5A). Although the doubling times of the parental HCT116 and STAT3 Y705F mutant cells were not significantly different (Fig. 3-6A), the average doubling times of the RKO STAT3 Y705F mutant clones increased by 4 hours in comparison with the parental cells (Fig. 3-6A). Consistently, compared with WT cells, the STAT3 Y705 mutant RKO cells had an elevated G1 population, whereas no cell cycle profile difference was observed among the HCT116 parental and mutant clones (Fig. 3-6B). Given that STAT3 is also involved in
Figure 3-3. STAT3 Y705F mutant proteins fail to activate its target genes in HCT116 cells. (a) Wild-type and STAT3 Y705F homozygous KI HCT116 CRC cells were starved for 18 hours and stimulated with IL-6 for 30 minutes. Cells were fixed and stained with anti-STAT3 antibodies. Scale bar = 10 μm. (b) Wild-type and STAT3 Y705F homozygous KI HCT116 CRC cells were starved for 18 hours and stimulated with IL-6 for the indicated times. Gene expression of STAT3 target genes, Bcl-XL and SOCS3, were assayed by RT-PCR. GAPDH served as the control.
Figure 3-4. STAT3 Y705F mutant proteins fail to activate its target genes in RKO cells.  
(a) Wild-type and STAT3 Y705F homozygous KI RKO CRC cells were starved for 18 hours and stimulated with IL-6 for 30 minutes. Cells were fixed and stained with an anti-STAT3 antibody. Scale bar: 10 µm.  
(b) Wild-type and STAT3 Y705F homozygous KI RKO CRC cells were starved for 18 hours and stimulated with IL-6 for the indicated times. Gene expression of STAT3 target genes, Bcl-XL and SOCS3, were assayed by RT-PCR. GAPDH served as the control.
tumor invasion and metastasis (Devarajan and Huang, 2009), we set out to determine whether the STAT3 Y705 mutation affects colorectal cancer cell migration and invasion. As shown in Figure 7, no significant difference was found between the parental and the homozygous KI cells on fibronectin, collagen IV, and Matrigel matrices.

**In vitro, STAT3 Y705F mutant colorectal cancer cells are reduced in properties predicative of in vivo tumorigenicity**

To test whether STAT3 Y705F mutant affects tumorigenicity-correlated responses in vitro, we carried out colony formation and soft agar assays with the STAT3 mutant KI cells. Compared with the parental cells, homozygous STAT3 Y705F KI HCT116 and RKO cells exhibited 3- to 5-fold ($P < 0.001$) reduced abilities to form colonies in colony-formation assay (Fig. 3-5B). Similarly, homozygous STAT3 mutant colorectal cancer cell clones formed approximately 3-fold ($P < 0.001$) less foci in soft agar assay than their WT counterparts (Fig. 3-5C). Interestingly, all of the heterozygous KI clones also displayed significant ($P < 0.05$) reduction in colony numbers and soft agar foci with respect to WT cells (Fig. 3-5B and C).

**STAT3 Y705F mutant colorectal cancer cells are less tumorigenic in vivo**

Tumorigenicity of the KI cells was also tested in a more stringent in vivo model. For these studies, STAT3 Y705F homozygous, heterozygous clones or the parental HCT116 and RKO cells were injected subcutaneously into nude
Figure 3-5. STAT3 Y705F mutant CRC cells are less tumorigenic in vitro
(a) $1 \times 10^5$ cells of indicated clones were plated in 6-well plates. Duplicate wells of each clone were counted daily for 4 consecutive days using a hemocytometer. Each well was counted three times. Average cell numbers of each time point from three independent experiments are plotted. (b) Cells from indicated clones were plated in 6-well plates in triplicates. Cells were grown for 14 days and stained with crystal violet. Colony numbers were counted and plotted for each of the clones. (c) CRC cells of the indicated clones were mixed in 0.4% soft agar and plated in 6-well plates in triplicates. Cells were grown for 30 days. Colony foci were counted and plotted for each of the clones. * $P < 0.05$, ** $P < 0.001$, t test.
Figure 3-6. Doubling times and cell-cycle profiles of STAT3 Y705F mutant cells. (a) Cells of the indicated genotypes were analyzed in log phase. Cell numbers of each clone were counted for two consecutive days using a hemocytometer and doubling times were calculated. Average doubling times of three independent experiments are plotted. * p=0.28; ** p=0.21; *** p=0.0072; **** p=0.0087; t test of means between WT and KI/KI cells. (b) Cells of the indicated genotypes were harvested during the log phase and fixed with methanol. Cells were then incubated at 37°C for 30 min in 5% normal goat serum diluted in PBS. Propidium iodide solution was used to stain cells at 4°C for 1 hour. Cells were analyzed on an Epics XL flow cytometer. WinMDI2.9 was used for data analysis. Cell debris and aggregates were excluded on PI gating. Percentages of G1, S and G2/M populations were determined by histograms generated by WinDI 2.9. Representative histograms of the indicated clones are shown. Averages of percentage of cells in G1, S and G2/M phases from three independent experiments are shown as mean ± SD.
Figure 3-7. STAT3 Y705F mutant does not affect cell migration and invasion. Cells of the indicated genotypes were grown in Boyden chambers coated with either Collagen IV or Fibronectin and assessed for their ability to migrate through porous 8 μm membranes. Average numbers of migrated cells from three independent experiments are plotted. (a) Collagen IV. (b) Fibronectin. (c) Matrigel.
mice. Tumor formation and size were assessed by weekly caliper measurements. After 21 days of growth, WT cells formed tumors in all mice injected, whereas each of the STAT3 Y705F homozygous KI clones failed to form tumors in at least 1 of the 5 mice injected (Fig. 3-8A). The average tumor volumes of STAT3 Y705F homozygous KI clones were 10-fold smaller than those produced by the parental cells for both the HCT116 ($P < 0.001$) and RKO ($P < 0.0001$) colorectal cancer cell lines (Fig. 3-8B). Furthermore, the development of tumor sizes over time for STAT3 Y705F homozygous KI clones was significantly slower than that of the parental cells for both the HCT116 ($P < 0.001$) and RKO ($P < 0.0001$). Notably, the average tumor sizes of STAT3 Y705F heterozygous KI clones were also significantly ($P < 0.05$) less than those of parental cells (Fig. 3-8B).

**STAT3 modulates PLC$\gamma$1 activity**

The studies above showed that phosphorylation of the STAT3 Y705 residue plays a critical role in colorectal tumorigenesis. It was also desirable to gain insights into the effects of this phosphorylation on downstream signaling. In these studies, we examined how the STAT3 Y705F KI affects phosphorylation of other signaling molecules in colorectal cancer cells after IL-6 stimulation. It is well documented that IL-6 activates multiple well-characterized signaling pathways including Ras-MAPK, PI3K-AKT, and PLC$\gamma$. Therefore, we tested the phosphorylation status of 26 sites on 17 proteins on the basis of their ability to respond to IL-6 stimulation (Table S1). Among these candidates, PLC$\gamma$1 S1248 phosphorylation was consistently elevated in STAT3 Y705F mutant cells in
Figure 3-8. STAT3 Y705F mutant CRC cells are less tumorigenic in vivo. Athymic nude mice were injected subcutaneously with cells from the indicated clones. Tumors sizes were measured weekly for 3 weeks. Mice were then sacrificed and tumors were harvested. (a) Tumors grown from HCT116 clones. (b) Average sizes of the indicated clones were plotted.
comparison with the parental cells (Fig. 3-9A; Fig. 3-10). As S1248 phosphorylation negatively regulates PLCγ1 activity, this result suggests that PLCγ1 is less active in STAT3 Y705F mutant cells. In support, pPKC levels, an immediately downstream target of PLCγ1, were also reduced in the STAT3 Y705F mutant cells (Fig. 3-9A; Fig. 3-10).

We speculated that STAT3 might physically interact with PLCγ1 leading to modulation of its activity. In support, STAT3 was shown to form complexes with PLCγ1 under both overexpression and physiologic conditions, as shown by reciprocal immunoprecipitations (Fig. 3-9B and C). Interestingly, the WT, but not the STAT3, Y705F mutant proteins interact with PLCγ1. Furthermore, the STAT3 proteins immunoprecipitated by PLCγ1 were phosphorylated (Fig. 3-9C). These results suggest that STAT3 phosphorylation may play a role in its physical interaction with PLCγ1. In support, the STAT3-PLCγ1 interaction was readily detected when cells were stimulated with IL-6 (Fig. 3-9D), but was barely detectable under starvation conditions. Moreover, this interaction is mediated by the 2 SH2 domains of PLCγ1, as recombinant PLCγ1 SH2 domains pulled STAT3 down from HCT116 lysates (Fig. 3-9E).

**Constitutively active PLCγ1 rescues the colony formation defect of STAT3 Y705F mutant cells**

The functional significance of the cross-talk between STAT3 and PLCγ1 was then tested by determining whether constitutively active PLCγ1 mutant could phenotypically rescue STAT3 Y705 mutant cells. A PLCγ1 triple mutant construct
(D1019L, Y509A, and F510A), which is well documented to be constitutively active (Everett et al., 2009), was introduced into STAT3 Y705F mutant HCT116 cells. As shown in Figure 5F, the mutant PLCγ1 partially rescued the colony formation defect of the STAT3 Y705F mutant cells, suggesting that PLCγ1 is a critical downstream mediator of STAT3 oncogenic signaling.

**Discussion**

Using genetically engineered STAT3 Y705 KI colorectal cancer cells, we show that STAT3 phosphorylation can regulate the efficiency of colorectal tumorigenesis. Unexpectedly, extension of studies of the STAT3 Y705F mutant also shows that crosstalk between STAT3 and PLCγ1 pathways may provide further mediating mechanisms that modulate colorectal tumorigenesis.

PLCγ1 is a major signal transducer of growth factor and cytokine signaling (Patterson et al., 2005). PLC hydrolyzes phosphatidylinositol-4, 5-biophosphate to generate IP3 and DAG. Although IP3 modulates intracellular Ca$^{2+}$ signaling, DAG activates PKCs (Patterson et al., 2005). Results of our studies show, for the first time, that STAT3 modulates PLCγ1 activity. In this regard, decreased PKC activities in STAT3 Y705F mutant colorectal cancer cells suggest that STAT3 activates PLCγ1 (Fig. 3-9; Fig. 3-10). However, it remains to be determined whether STAT3 can regulate intracellular Ca$^{2+}$ signaling. Furthermore, our data suggest that activation of PLCγ1 by STAT3 requires physical interaction between the 2 proteins and that this interaction seems to be mediated by STAT3 pY705...
Figure 3-9. STAT3 cross-talks with PLCγ1. (a) Parental and STAT3 Y705F KI cells were stimulated with IL-6 for the indicated time. Cell lysates were blotted with the indicated antibodies. See Fig. S5 for quantification. (b) HEK 293 cells were transfected with the indicated plasmids. Cell lysates were immunoprecipitated with either anti-Flag or anti-Myc antibodies and blotted with the indicated antibodies. Arrow indicates co-immunoprecipitated Flag-STAT3. (c) Cell lysates from parental and STAT3 Y705F KI
cells were immunoprecipitated with antibodies against either STAT3 or PLCγ1 and blotted with the indicated antibodies. (d) HCT116 cells were serum-starved for 16 hours and then stimulated with or without 10 ng/ml of IL-6 for 30 minutes. Cells were then lysed and immunoprecipitated with either anti-PLCγ1 antibodies or control IgG. Immunocomplexes were resolved on 8% SDS-PAGE and Western blots were performed with the indicated antibodies. (e) HCT116 cell lysates were incubated with 1 μg of either GST-PLCγ1-SH2 domains or GST alone for 1 hour at 4°C. The pull-down mixtures were resolved on SDS-PAGE and blotted with an anti-STAT3 antibody. (f) STAT3 Y705F KI cells were transfected with either an empty vector or a vector expressing PLCγ1 triple mutant (D1019L, Y509A and F510A). Colony formation assay was performed. * P < 0.05, ** P < 0.001, t test. Western blot was performed to show expression of myc-tagged PLCγ1 mutant protein.
Figure 3-10. Quantification of PLCγ1, PKC and PTEN phosphorylation. (a) Parental and STAT3 Y705F KI cells were stimulated with IL-6 for the indicated time. Cell lysates were blotted with the indicated antibodies. Intensity of each band was quantified using Image J software. The ratios of phospho-protein vs total protein at time point 0 of WT cells were set as 1 (reference points). The ratios of other time points were normalized against the reference points. Average ratios of three independent experiments are plotted. (b) PLCγ1; (c) PKC and (d) PTEN.
Table 3-1. List of phospho-antibodies.

<table>
<thead>
<tr>
<th>Antibodies</th>
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<tbody>
<tr>
<td>P-Akt (Ser-473)</td>
</tr>
<tr>
<td>P-Akt (Thr-308)</td>
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<tr>
<td>P-CrkL (Tyr-207)</td>
</tr>
<tr>
<td>P-FAK (Tyr-397)</td>
</tr>
<tr>
<td>P-FAK (Tyr-576/577)</td>
</tr>
<tr>
<td>P-FAK (Tyr-925)</td>
</tr>
<tr>
<td>P-GSK 3β(Ser-9)</td>
</tr>
<tr>
<td>P-p130CAS (Tyr-165)</td>
</tr>
<tr>
<td>P-p130CAS (Tyr-249)</td>
</tr>
<tr>
<td>P-p130CAS (Tyr-410)</td>
</tr>
<tr>
<td>P-p44/42 Map Kinase (Thr-202/204)</td>
</tr>
<tr>
<td>P-Paxillin (Tyr-118)</td>
</tr>
<tr>
<td>P-Paxillin (Tyr-31)</td>
</tr>
<tr>
<td>P-PDK1 (Ser-241)</td>
</tr>
<tr>
<td>P-PI3K p85 (Tyr-458)/ p55 (Tyr-199)</td>
</tr>
<tr>
<td>P-PLCγ1(Ser-1248)</td>
</tr>
<tr>
<td>P-PLCγ1(Tyr-783)</td>
</tr>
<tr>
<td>P-PTEN (Ser-380)</td>
</tr>
<tr>
<td>P-Raf (Ser-259)</td>
</tr>
<tr>
<td>P-SHP-2 (Tyr-542)</td>
</tr>
<tr>
<td>P-SHP-2 (Tyr-580)</td>
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<tr>
<td>P-Src (Tyr-416)</td>
</tr>
<tr>
<td>P-Src (Tyr-527)</td>
</tr>
<tr>
<td>P-Stat1 (Tyr-701)</td>
</tr>
<tr>
<td>P-Stat3 (Ser-727)</td>
</tr>
<tr>
<td>P-Stat5 (Tyr-694)</td>
</tr>
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and the SH2 domains on PLCγ1. Given that numerous studies suggest that PLCγ1 is involved in tumor progression (Wells and Grandis, 2003), it is not surprising that overexpression of constitutively active PLCγ1 can partially rescue the colony formation defect of STAT3 Y705F KI colorectal cancer cells. Data presented here strongly suggest that a transcription-independent function of STAT3 is at least partially involved in critical control of tumor transformation and that the general belief that STAT3 mainly functions through its transcriptional activity is an oversimplification. In this regard, recent studies show that the mitochondrial functions of STAT3 may be important factors in tumorigenesis, because its mitochondrial activity appears to be required for Ras-mediated tumor transformation (Gough et al., 2009; Wegrzyn et al., 2009). Notably, however, our studies show that PLCγ1 only partially rescues the defect in the STAT3 Y705F mutant cells suggesting that STAT3 transcriptional activity plays an important role in regulating STAT3’s oncogenic functions.

Our data indicate that STAT3 Y705F mutant proteins fail to accumulate in the nucleus on IL-6 stimulation (Fig. 3-3; Fig. 3-4). However, the unphosphorylated STAT3 proteins can still be imported into the nucleus, as shown by the observation that a significant portion of STAT3 Y705F mutant protein is localized in the nucleus in both HCT116 and RKO colorectal cancer cells (Fig. 3-3). This observation is consistent with a previous study showing that STAT3 nuclear importation is independent of tyrosine phosphorylation and that the N-terminal coiled-coil domain of STAT3 is important for its nuclear importation (Corvinus et al., 2005). In fact, numerous studies show that STAT3
proteins translocate and accumulate in the nucleus after cytokine and growth factor stimulation (Levy and Inghirami, 2006). In this regard, the data from our laboratory show unequivocally that the nuclear accumulation of STAT3 is dependent on STAT3 Y705 phosphorylation. Although we showed that STAT3 Y705F mutant proteins failed to activate IL-6–induced target genes, Bcl-XL and SOCS3, our data do not exclude the possibility that the mutant STAT3 can activate transcription of other non-canonic STAT3 target genes. Interestingly, recent studies showed that overexpression of the STAT3 Y705F mutant induces expression of genes that are distinct from those induced by pSTAT3 (Yang et al., 2005).

STAT3 activity is upregulated in various cancers including colorectal cancers (Bowman et al., 2000). Although STAT3 has not yet been found to be mutated in human cancers, kinases and phosphatases that regulate STAT3 Y705 phosphorylation are mutated in a variety of cancers that result in STAT3 activation. Activating mutations of JAK2 are found in the majority of myeloproliferative neoplasms (Quintás-Cardama et al., 2011), whereas inactivation mutations of phosphatases PTPRT and PTPRD that dephosphorylate STAT3 are found in various solid tumors (Wang et al., 2004; Solomon et al., 2008; Veeriah et al., 2009). Although our previous study showed that STAT3 is a direct substrate of PTPRT that is mutated in colorectal cancers (Zhang et al., 2007), this study clearly shows that regulation of STAT3 Y705 phosphorylation plays a critical role in colorectal tumorigenesis, because STAT3 Y705F homozygous KI colorectal cancer cells grow slower than the parental cells.
both in tissue culture and in tumor xenograft models. Interestingly, STAT3 Y705F heterozygous KI cells also exhibit reduced tumorigenicity, suggesting that STAT3 Y705F protein may act as either a dominant negative or haploid-insufficiency mutant. Although our data cannot distinguish these 2 possibilities, several previous studies suggest that STAT3 Y705F mutant has a dominant negative effect (Kaptein et al., 1996; Bromberg et al., 1998; Rivat et al., 2004).

Finally, our data indicate that inactivation of STAT3 in colorectal cancer cells slows down tumor growth both in tissue culture and in tumor xenograft models (Figs. 3-5 and 3-8). Interestingly, RKO STAT3 Y705F KI cells progress slower through cell cycle, but no cell cycle defect was observed in HCT116 STAT3 Y705F mutant cells. It is possible that HCT116 STAT3 mutant cells grow slower through other mechanisms (e.g., the cells may be less responsive to growth factor stimulation). Our study suggests that the STAT3 signaling pathway may be a good target for colorectal cancer therapy. It is conceivable that interference with STAT3 phosphorylation, dimerization, and DNA binding processes or inhibition of its upstream kinase(s) could be exploited to inhibit the tumorigenic growth of colorectal cancers. Indeed, peptides, peptidomimetics and small chemical compounds that target STAT3 dimerization have shown promising inhibition of \textit{in vitro} growth of breast cancer cells as well as cancer cells from other tissue types (Turkson et al., 2001, 2004a; Yu and Jove, 2004). Recently, a combination of \textit{in silico} approaches and chemical screens led to identification of several small molecules that potently inhibit STAT3 activities and cause breast cancer cell death in both \textit{in vitro} and \textit{in vivo} models (Song et al.,
2005; Siddiquee et al., 2007). Further, platinum compounds that block the DNA binding of phosphorylated STAT3 were also found to inhibit cancer cell growth (Turkson et al., 2004b). Along the same line, Dr. Jennifer Grandis' group has successfully used a decoy oligonucleotide that blocks the transcription activity of STAT3 to inhibit tumor growth of SCCHN xenografts which harbor persistently active STAT3 (Leong et al., 2003; Xi et al., 2005). Moreover, targeting STAT3 activation by inhibiting upstream kinases with chemical compounds also caused potent antitumor effects (Nam et al., 2005). Our data provide a strong rationale for exploring existing and emerging STAT3 inhibitors alone and in combination as targeted therapy for colorectal cancers. Furthermore, our STAT3 Y705F mutant clones and their parental cells should provide ideal reagents for testing the specificity of STAT3 inhibitory compounds that target STAT3 Y705 phosphorylation.
Materials and Methods

Cell culture

HCT116, RKO, and HEK 293T cells were obtained from the American Type Culture Collection. HCT116 and RKO colorectal cancer cells were maintained in McCoy 5A media plus 10% FBS. HEK 293T cells were maintained in Dulbecco's modified Eagle's medium media plus 10% FBS.

Somatic cell gene targeting

Somatic cell gene targeting was conducted as described (Zhang et al., 2008; Du et al., 2010). Briefly, a 1.3-kb fragment from intron 21 to intron 22 of the STAT3 locus containing the exon 22 sequences was amplified 25 cycles from genomic DNA, using primers 5’-TGACCAACTAGTCTGCTTACTGAATGCGACTCACAG-3’ and 5’-TGACCACGCGGAGGGTCCTTTCTCATTCCACCTTA-3’. The coding sequences for Y705 were then mutated from TAC (Tyr) to TTC (Phe) by site-directed mutagenesis, using primers 5’-TCCCAGGCCTGCCCATTCC TGAAGACCAAGTTATC-3’ and 5’-AATGggcagcgcCGCTGGGA-3’. This mutated fragment was used as the right homologous arm and cloned in to an pAAV-Neo-Lox P vector with restriction enzymes SpeI (left cloning site) and SacII (right cloning site). Another 1.2-kb fragment from intron 21 of the STAT3 gene was also amplified 25 cycles from genomic DNA as the left arm, using primers 5’-TGACCACTGAGTCCCGTCAACGCATTTCTAACTGTA-3’ and 5’-TGACCAG AATTCATCTGCCTCGGAGGACTGATTTGA-3’. The targeting adeno
associated viruses (AAV) were packaged in 293T cells (a T75 flask at 70% confluence) by transfecting equal amounts of the targeting vector, pHelper and pRC plasmids (3 μg each). Viruses were harvested 72 hours post-transfection. HCT116 and RKO cells were infected with the STAT3 KI targeting viruses and selected with geneticin for 20 days. The geneticin resistant clones were then screened for homologous recombination by 35 cycles of genomic PCR with primers derived from the neomycin resistance gene 5’-GTTGTGCCAAGTCATA GCCG-3’ and the upstream region of the left homologous arm 5’-TCAGTTTCTTT GGCCCCAAAGT-3’. Confirmatory genomic PCR was also carried out with positive clones identified by primers derived from the neomycin resistant gene (5’-TCTGG ATTCATCGACTGTGG-3’) and the downstream region of the right homologous arm (5’-TAGGCGCCTCAGTCGTATCT-3’). The DNA fragments from the confirmatory PCRs were then sequenced to ensure the presence of the mutant Y705F alleles. To target the second allele with the same targeting virus, correctly targeted clones were infected with adenoviruses expressing the Cre-recombinase to delete the drug selection marker. To select clones with successful deletion of the drug selection marker, 30 cycles of genomic PCR were carried out to amplify an approximately 200-bp genomic fragment in which the Lox P site was inserted (using primers 5’-GCAGATGGAGCTTCCAGAC-3’ and 5’-CGCCTGGGAAGAAGAAAAC-3’). The heterozygous KI clones were infected with the same targeting virus to target the second allele and the neomycin resistance gene was excised as described earlier.
**Plasmid transfection**

Cells were plated 1 day prior to transfection to achieve a 70% confluence at the time of transfection. Plasmids were transfected with the Lipofectamine Transfection Reagent (Invitrogen; catalogue no. 18324-020) according to the manufacturer’s instructions. To transfect a T75 flask of HEK 293 cells, 9 μg of plasmids were mixed with 54 μl of Lipofectamine Transfection Reagent and 1.5 mL of OptiMEM (Invitrogen; catalogue no. 31985070). The transfection mixture was then incubated with cells at 37°C for 4 hours. Cells were subsequently cultured in normal medium after washing once with Hank’s balanced buffer.

**Western blot**

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with complete protease inhibitor mixture and phosphatase inhibitors [50 mmol/L Tris-HCl (pH 8.0), 0.5% triton X-100, 0.25% sodium deoxycholate, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 80 μmol/L β-glycerophosphate, and 20 mmol/L sodium pyrophosphate]. Western blots were carried out essentially as described (Yu et al., 2008). Antibodies used included anti-pY705 STAT3 (catalogue no. 9139) antibody, anti-STAT3 antibody (catalogue no. 9145; Cell Signaling Technology), and the antibodies listed in the Table 3-1.

**Immunofluorescence staining**

Cells were seeded on glass cover slips and grown to 50% confluence and
serum starved for 18 hours. A subset of cells was treated with 10 ng/mL of interleukin-6 (IL-6) for 30 minutes following by fixation with 4% paraformaldehyde for 30 minutes at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 at room temperature for 5 minutes and then blocked with Image-iT FX signal enhancer (Invitrogen) at room temperature for 30 minutes. Immunofluorescent staining was done with anti-STAT3 antibody (Santa Cruz Biotechnology) and the Alexa 488–conjugated anti-rabbit secondary antibody (Invitrogen). Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI; 1 μg/mL) at room temperature for 20 minutes. Images were captured with a LSM 510 META confocal microscope (Carl Zeiss MicroImaging).

**Flow cytometry**

Cell were harvested during the log phase of growth and fixed with methanol. Cells were then incubated at 37°C for 30 minutes in 5% normal goat serum diluted in PBS. Propidium iodide (PI) solution (100 μg/mL; 0.1% NP40; 0.1% sodium azide) was used to stain cells at 4°C for 1 hour. Cells were analyzed on an Epics XL flow cytometer (Beckman Coulter). WinMDI2.9 was used for data analysis. Cell debris and aggregates were excluded on PI gating. Percentages of G1, S, and G2–M populations were determined by histograms generated by WinMDI2.9.

**Reverse transcriptase-PCR**

Total RNAs were isolated from 1 million cells by using the Qiagen RNeasy
Mini Kit according to the manufacturer's instructions and cDNAs were synthesized by the SuperScript III First-Strand Synthesis System (Invitrogen). The 5'-TCCCAGAAAGGATACAGCTGG-3' and 5'-ACTGAAGAGTGAGCCCAGCAG-3' primers were used to PCR amplify Bcl-XL. The 5'-AGCTGGTACTCGTCCTTTGA-3' and 5'-AGGCTCCTTTGTGGACTTCAG-3' primers were used to amplify SOCS3. The PCR products were resolved on 1% agarose gel. PCR conditions: Platinum Taq polymerase (Invitrogen; catalogue no. 10966083) was used and the PCR mixtures were prepared according to the manufacturer's instructions; the mixtures were run on a GeneAmp PCR system 9700 (Applied Biosystems) at 94°C for 30 seconds, 55°C for 30 seconds, 70°C for 30 seconds for 25 cycles.

**Colony formation assay**

HCT116 and RKO cells were trypsini zed, counted twice by using a hemocytometer, and placed into 6-well plates at 400 cells per well. Cells were grown for 14 days before staining with Crystal Violet (Sigma). The experiment was repeated 3 times with 2 replicates each. Average numbers of colonies from each experiment were plotted.

**Focus formation assay in soft agar**

HCT116 and RKO clones were trypsini zed, counted twice by a hemocyto meter, and plated at 5,000 cells/mL in top plugs consisting of 0.4% Sea Plaque agarose (FMC Bioproducts) and McCoy's 5A medium. After 30 days, the
colonies were photographed and counted. The experiment was repeated 3 times with 2 replicates each. Average numbers of colonies from each experiment were plotted.

**Boyden chamber cell migration and invasion assay**

Cell migration and invasion assay was carried out as previously described (Miao et al., 2009). Transwell membranes (pore size 8.0 μm; Corning Inc.) were coated with 50 μg/mL fibronectin, 12.5 μg/mL of collagen IV or 1 mg/mL of Matrigel. The matrices were coated on membranes according to the manufacturer's instructions. Specifically, Matrigel (BD Biosciences) was thawed overnight at 4°C and then diluted in serum-free medium to 1 mg/mL, and 100 μl of the diluted Matrigel was added to upper chamber of 24-well and incubated at 37°C for 2 hours. Cells were detached with 2 mmol/L EDTA and resuspended in serum-free medium with 0.1% of bovine serum albumin. Five hundred thousand cells were added to the upper compartment of the transwell chamber in the wells of a 24-well plate and allowed to migrate to the underside of the inserts for 24 hours. Complete medium containing 10% FBS was added in the lower compartment as a chemoattractant. Nonmigrating cells on the upper membrane were removed with a cotton swab, and cells that had migrated and become attached to the bottom surface of the membrane were fixed and stained with crystal violet. Migrated cells were counted microscopically at 200× magnification. Five randomly chosen fields were counted for each transwell membrane. The
experiments were repeated 3 times with 2 replicates for each cell line. Average numbers of migrated cells per field from each experiment were plotted.

Xenograft

Five million cells were injected subcutaneously and bilaterally into 4- to 6-week-old female nude mice (5 nude mice in each group). Tumor formation and size were assessed by weekly caliper measurements of the length and width of the tumors. Tumor volumes were calculated by the formula: Volume = (width)² × length/2. After 21 days, the mice were sacrificed and tumors were harvested.

Construction of PLCγ1-myc tag and PLCγ1 mutant plasmid

To facilitate molecular cloning, we constructed a pCMV-USER-3xMyc vector by inserting a USER cassette into the pCMV-3Tag-2A plasmid (Agilent Technologies). Briefly, primers 5'-AATTCGATATCGCTGAGGTCCCATCTAGAGGATCCTCTAGACTATGCCTCAGC-3' and 5'-TCGAGCTGAGGCATAGTCTAGAGGATCCTCTAGATGGGACCTCAGCGATATCG-3' were annealed together and cloned into the pCMV-3Tag-2A plasmid, using EcoRI and XhoI restriction enzymes. The open reading frame of PLCγ1 was PCR amplified from a human PLCγ1 cDNA purchased from Open Biosystems, using primers 5'-GGTCCCA/ideoxyU/TGGCGGGCGTCGCGACCCCCT-3' and 5'-GGCATAG/ideoxyU/TTAAAGAGAGCACTTCCACA-3' and then cloned into the pCMV-USER-3xMyc vector by using the USER cloning system (New England Biolabs) according to the
manufacturer's instructions. The construct was sequenced to ensure no mutation was introduced by PCR.

Constitutively active PLCγ1 Y509A, F510A, and D1019L mutations were then introduced into the Myc-tagged PLCγ1 plasmid by 2-step site-directed mutagenesis. The first step created the PLCγ1 Y509A and F510A double mutant, using primers 5′-TGACCACCATGGGCACACTCTCACA-3′ and 5′-TGACCAA GATCTTGCTGCTAGTCAGAACGGCGCGTGGGATACCAC-3′. The second step generated the D1019L mutation in the above double-mutant plasmid, using primers 5′-GGGTCAATACTGGAGAGTAGCTCTGCCCCCTAGGG-3′ and 5′-CTCTCCTCCAATTATGACCCT. The details of site-directed mutagenesis methods are described in Chapter 2.

**Glutathione S-transferase–fusion protein pull-down**

Sequences encoding the 2 tandem SH2 domains of PLCγ1 were amplified by PCR from PLCγ1 cDNA, using primers 5′-TGACCAGAATTCGAGAAGTGGTACGGGAAG-3′ and 5′-TGGTCACTCAGGTACAGGGGGTGCTTCTCA-3′. The PCR product was then cloned into pGEM-6p-1 (GE Healthcare), using EcoRI and XhoI restriction enzymes. Recombinant proteins were expressed and purified from *Escherichia coli*. Twenty million HCT116 cells were lysed in RIPA buffer with complete protease inhibitor mixture and phosphatase inhibitors (see Western blot section for the recipe) for STAT3 pull-down. GST–PLCγ1–SH2 domain fusion protein (1 μg) bound beads were incubated with the cell lysate at 4°C for 1 hour. The beads were washed and then boiled and the
 aliquots were analyzed by SDS-PAGE and Western blotting. Equal amount of glutathione S-transferase (GST) beads were treated identically as a control.

**Statistical analyses**

All statistical analyses were carried out by the SAS software (SAS Institute). We applied the t test to compare the means between 2 groups assuming unequal variances. For xenograft growth, we carried out MANOVA for repeated measurements to test whether there is an overall difference in the tumor sizes by testing group difference as well as whether there was a difference in development of tumor sizes over time between the 2 groups by testing the interaction between time and group.
Chapter 4

Cancer-derived mutations in the fibronectin III repeats of PTPRT inhibit cell-cell aggregation

A modified version of this chapter was previously published as:

Abstract

The receptor protein tyrosine phosphatase T (PTPRT) is the most frequently mutated tyrosine phosphatase in human cancer. PTPRT mediates homophilic cell-cell aggregation. In its extracellular region, PTPRT has cell adhesion molecule-like motifs, including a MAM domain, an immunoglobulin domain and four fibronectin type III (FNIII) repeats. Tumor-derived mutations have been identified in all of these extracellular domains. Previously, we determined that tumor-derived mutations in the MAM and immunoglobulin domains of PTPRT reduce homophilic cell-cell aggregation. In this manuscript, we describe experiments in which we evaluated the contribution of the FNIII repeats to PTPRT-mediated cell-cell adhesion. Our results demonstrate that deletion of the FNIII repeats of PTPRT result in defective cell-cell aggregation. Furthermore, all of the tumor-derived mutations in the FNIII repeats of PTPRT also disrupt cell-cell aggregation. These results further support the hypothesis that mutational inactivation of PTPRT may lead to cancer progression by disrupting cell-cell adhesion.
Introduction

Cancer progression and metastasis is in part influenced by changes in cell adhesion and tyrosine phosphorylation mediated by cell adhesion molecules (CAMs) (Wheelock et al., 2008; Paschos et al., 2009), as well as tyrosine kinases and tyrosine phosphatases, respectively (Freiss and Vignon, 2004; Östman et al., 2006). Receptor protein tyrosine phosphatases (RPTPs) comprise a family of proteins that have extracellular CAM domains coupled to cytoplasmic tyrosine phosphatase domains, and thus can impact cancer progression by regulating both cell adhesion and tyrosine phosphorylation (Brady-Kalnay, 2001; Östman et al., 2006).

The type IIB PTPRM-like subfamily of RPTPs includes PTPRM, PTPRT, PTPRK, and PTPRU (Brady-Kalnay, 2001; Aricescu et al., 2006). The extracellular segment of the PTPRM-like RPTPs contain motifs found in CAMs, including a MAM domain, an immunoglobulin (Ig) domain and four fibronectin type III (FNIII) repeats (Brady-Kalnay, 2001; Östman et al., 2006; Aricescu et al., 2008). PTPRM subfamily members bind homophilically (i.e. the “ligand” for PTPRM is an identical PTPRM molecule on an adjacent cell) (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994; Zondag et al., 1995; Cheng et al., 1997; Yu et al., 2008). The Ig domain of PTPRM is responsible for promoting homophilic interactions (Brady-Kalnay and Tonks, 1994), and proper cell surface localization (Del Vecchio and Tonks, 2005). However, both the MAM and Ig domains as well as the first two FNIII repeats are required for efficient cell-cell
adhesion of PTPRM (Brady-Kalnay and Tonks, 1994; Zondag et al., 1995; Cismasiu et al., 2004; Aricescu et al., 2006, 2007, 2008). PTPRT and PTPRK also mediate homophilic cell-cell adhesion (Sap et al., 1994; Yu et al., 2008). However, PTPRU2 has not yet been demonstrated to mediate cell-cell adhesion or aggregation although in vitro binding studies indicate it can bind homophilically (Cheng et al., 1997).

All type IIB RPTPs are potential tumor suppressor genes (McArdle et al., 2001; Yan et al., 2006; Flavell et al., 2008; Burgoyne et al., 2009a). Notably, PTPRT is the most frequently mutated phosphatase gene in human cancers including colon, lung, skin and stomach cancers (Wang et al., 2004; Forbes et al., 2008). Defective adhesion mediated by PTPRT may be at least one mechanism of cancer progression. In fact, cancer derived mutations in the MAM and Ig domains of PTPRT are defective in mediating cell-cell aggregation (Yu et al., 2008).

In addition to the tumor-derived mutations in the MAM and Ig domains of PTPRT previously described (Wang et al., 2004; Yu et al., 2008), the majority of extracellular domain mutations are located in the FNIII repeats of PTPRT (Wang et al., 2004). Given the importance of FNIII repeats in PTPRM-mediated adhesion and aggregation (Aricescu et al., 2007), we tested the contribution of the FNIII repeats of PTPRT to PTPRT-mediated adhesion. We determined that deletion constructs where each of the extracellular domains of PTPRT is removed, including the FNIII repeats, are defective in mediating cell-cell aggregation of Sf9 cells. Furthermore, when we engineered the cancer-derived
mutations in the FNIII repeats of these PTPRT mutant proteins, they also result in defective cell-cell aggregation. These results demonstrate the importance of the FNIII repeats in contributing to proper cell-cell adhesion and suggest that adhesion mediated by PTPRT is likely important for the tumor suppressor activity of PTPRT.

Results

Deletions in the MAM, Ig, and FNIII repeat domains of PTPRT result in defective cell-cell aggregation

To test whether a protein mediates cell-cell aggregation or adhesion, one has to express the protein of interest in the non-adhesive cells. Otherwise, one cannot measure the aggregation or adhesion of a single molecule in the sea of other adhesion molecules naturally expressed in adhesive cells. This is the main reason that the non-adhesive Sf9 or Drosophila S2 cells are widely used models for such studies. To our knowledge, nearly all cell lines of epithelial origin are adhesive cells and thus are not suitable for these studies. Introduction of putative adhesion molecules into non-adhesive Drosophila S2 insect cells has been used to demonstrate adhesive functions for fasciclin II, connectin, Dtrk, ARK, Neuroglian, and Capricious molecules directly (Snow et al., 1989; Nose et al., 1992; Pulido et al., 1992; Bellosta et al., 1995; Hortsch et al., 1995; Shinzakameda et al., 2006). In a similar approach, we and others demonstrated that the full-length form of PTPRM induced aggregation, via a homophilic binding
mechanism, when expressed in nonadhesive Sf9 insect cells, which are derived from the Fall army-worm Spodoptera frugiperda (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Zondag et al., 1995). We have also used this system for analyzing PTPRT (Yu et al., 2008). Examples of other cell adhesion molecules that have been studied in Sf9 cells include L1 (Gouveia et al., 2008), Galectin-3 (Inohara and Raz, 1995) and C-CAM1 (Phan et al., 2001). Insect S2 and Sf9 cells are extremely useful systems to analyze adhesive mechanisms of cell adhesion molecules.

The MAM domain, Ig domain, and first two-FNIII repeats are essential for PTPRM cell-cell aggregation (Brady-Kalnay and Tonks, 1994; Zondag et al., 1995). To test whether these domains are the minimal essential regions required for PTPRT cell-cell aggregation, baculoviral PTPRT deletion constructs of the MAM domain, Ig domain, and each of the four individual FNIII repeats were made and expressed in non-adhesive Sf9 insect cells (Figure 4-1). All the PTPRT deletion constructs were expressed and appropriately trafficked to the cell surface (Figure 4-1). To test whether these domains contribute to PTPRT-dependent adhesion, Sf9 cells were evaluated for their ability to mediate cell-cell aggregation when expressing the deletion constructs. Aggregation assays with Sf9 cells expressing the deletion constructs were conducted alongside aggregation assays using Sf9 cells expressing wild-type PTPRT, which mediates cell-cell aggregation (Figure 4-2) and serves as a positive control (Yu et al., 2008). Deletion of any of the extracellular domains of PTPRT resulted in defective cell-cell aggregation compared to aggregation of wild-type PTPRT.
Figure 4-1. PTPRT deletion constructs are all expressed at the cell surface. Constructs containing deletions of one extracellular domain of PTPRT each were generated and expressed in Sf9 cells. Total cellular protein and cell surface protein were isolated and immunoblotted with the SK18 antibody.
expressing Sf9 cells (Figure 4-2). Deletion of the fourth FNIII repeat was the least severe where ~23% aggregation was observed. Deletion of the MAM domain, Ig domain and FNIII repeats 1, 2 or 3 resulted in 5–16% aggregation. When the level of aggregation was quantified, we determined that all of the deletion constructs resulted in a large and statistically significant reduction in cell-cell aggregation (Figure 4-2, p-value < 0.005). Together with our previous study, these results demonstrate that all of the extracellular domains of PTPRT are necessary for PTPRT-mediated cell-cell aggregation.

**Tumor-Derived PTPRT Mutations in the FNIII repeats result in defective cell-cell aggregation**

We next tested the effects of human tumor-derived mutations in the FNIII repeats of PTPRT on PTPRT-mediated cell-cell aggregation. We recently demonstrated that tumor-derived mutations in the MAM and Ig domains of PTPRT are defective in cell-cell aggregation using the Sf9 aggregation assay (Yu et al., 2008), and given the above results, we hypothesized that mutations in the FNIII domains would likewise result in defective aggregation. Ten human tumor-derived mutations are located in the second, third and fourth FNIII repeats of PTPRT (Wang et al., 2004). To test whether these mutations also affect cell-cell aggregation, we engineered the ten human mutations by site-directed mutagenesis using the full-length PTPRT baculoviral construct as a template (see Figure 4-3). The ten mutations are expressed in Sf9 cells at the expected molecular weight and at similar levels to full-length human PTPRT (Figure 4-3).
Figure 4-2. Deletion of any of the extracellular domains of PTPRT disrupts cell-cell aggregation. Sf9 cells expressing deletion constructs of the individual extracellular domains of PTPRT were allowed to aggregate for 30 minutes prior to imaging. Scale bar equals 100µm. The percentage of Sf9 cell-cell aggregation for each deletion protein was calculated from a minimum of three experiments. An asterisk indicates a statistically significant reduction in aggregation (p<0.005).
Figure 4-3. PTPRT protein containing tumor-derived mutations in their FNIII domains are expressed at the cell surface. Ten mutations in the FNIII domains of PTPRT were generated by site-directed mutagenesis and expressed in Sf9 cells. Total cellular surface proteins were isolated and analyzed by immunoblotting with the SK18 antibody.
All of these mutants are expressed at the cell surface (Figure 4-3). When these mutant PTPRT proteins were expressed in Sf9 cells and tested in aggregation assays, we observed that all of the mutant PTPRT proteins resulted in fewer and smaller aggregates than wild type PTPRT (Figure 4-4). When the level of aggregation was quantified, we determined that all of the FNIII mutations significantly reduced cell-cell aggregation (16–43%) compared to cells expressing wild type PTPRT (68% aggregation, p-value<0.008, Figure 4-4).

Discussion

PTPRT is the most highly mutated tyrosine phosphatase in human colon carcinomas. We and others found that PTPRT is also mutated in lung, gastric and skin (melanoma) cancers (Wang et al., 2004; Forbes et al., 2008). The spectrum of mutations, which included nonsense mutations and frameshifts, suggested that these mutations were inactivating (Wang et al., 2004). Biochemical analyses demonstrated that missense mutations in the catalytic domains of PTPRT diminished its phosphatase activity and overexpression of PTPRT inhibited colon cancer cell growth. Moreover, we showed previously that the tumor-derived mutations in the MAM and Ig domains of PTPRT are defective in cell-cell adhesion (Yu et al., 2008). All these data suggest that PTPRT normally functions as a tumor suppressor gene. The experiments presented in this manuscript evaluate the contribution of the individual extracellular domains of PTPRT to PTPRT-mediated adhesion. We demonstrate that in addition to the
MAM and Ig domains, the FNIII repeats are required for PTPRT-mediated adhesion. Importantly, we also show that PTPRT-mediated adhesion is impaired by human tumor-derived mutations in those FNIII repeats. These studies indicate that all the tumor-derived mutations located in the extracellular domain of PTPRT are loss-of-function mutations, thereby providing further evidence that PTPRT is a tumor suppressor. This notion is further supported by our recent study showing that PTPRT knockout mice are highly susceptible to colon specific carcinogen azoxymethane (AOM)-induced colon cancer (Zhao et al., 2010).

PTPRM-mediated cell-cell aggregation requires the MAM domain, Ig domain, and the first two FNIII repeats (Brady-Kalnay and Tonks, 1994; Zondag et al., 1995; Cismasiu et al., 2004; Aricescu et al., 2006, 2007). The entire extracellular domain of PTPRM is predicted to make a rigid conformation with extensive interfaces between each of the domains (Aricescu et al., 2006). The minimal adhesive unit for functional cell-cell aggregation is the MAM domain, Ig domain and the first two FNIII repeats (Brady-Kalnay and Tonks, 1994; Zondag et al., 1995; Aricescu et al., 2006). However, the FNIII repeats appear to play a role in spacing the key homophilic interaction domain(s) a certain distance from the plasma membrane. Deletion of the FNIII repeats changed the intercellular spacing of two opposing membranes (Aricescu et al., 2007). Cell-cell aggregation assays of the FNIII deletion constructs were not performed in that study. The amino acid residues of the interfaces between the extracellular domains are highly conserved between members of the type IIB RPTP subfamily (Aricescu et al., 2006, 2007). Therefore it is likely that the PTPRT adhesive interface
resembles that of PTPRM. Together our data support the hypothesis that the essential regions for PTPRT-mediated adhesion resemble those of PTPRM.

To gain insight into how the point mutations may alter the adhesive function of PTPRT, we modeled the human tumor derived PTPRT mutations onto the crystal structure of PTPRM (Figure 4-5). The crystal structure of PTPRM includes only the first three FNIII repeats (Aricescu et al., 2007). Four of these mutations fall within the second FNIII (FNIII-2) repeat (I395V, Y412F, R453C, and Q479E). It is important to note that FNIII-2 is required for efficient cell-cell adhesion (Aricescu et al., 2006). Two of the mutations fall in the third FNIII (FNIII-3) repeat (S492F, N510K). The mutation within the linker region between FNIII-1 and FNII-2 (Y412F) could alter the flexibility or positioning between the two domains. The other mutations (I395V R453C, Q479E, S492F, N510K) appear to be clustered in surface exposed regions and/or near the linker regions between FNIII-2 and FNIII-3 that could alter flexibility, position of individual FNIII repeats and/or protein-protein interactions. While these mutations do not correspond to the adhesive interface hypothesized for PTPRM based upon a single static crystal structure (Aricescu et al., 2007), some of the mutations fall within the FNIII-2 repeat which is required for adhesion, and all of these surface modifications would likely alter either the three-dimensional topology and/or cis/trans interactions of PTPRT.

Other type IIB RPTPs also function as tumor suppressor genes, by regulating adhesion and/or tyrosine phosphorylation of effector proteins. Expression of PTPRM is reduced in human glioblastomas (GBM) and cell lines
Figure 4-4. Tumor derived mutations in FNIII domains of PTPRT are defective in cell-cell aggregation. Sf9 cells expressing the mutated PTPRT proteins were allowed to aggregate for 30 minutes prior to imaging. Scale bar equals 100µm. The percentage of Sf9 cell-cell aggregation for each mutant protein was calculated from a minimum of three experiments. An asterisk indicates a statistically significant reduction in aggregation (p<0.008).
Rubey et al., 2009a). Reduction in full-length PTPRM expression with a concomitant increase in a proteolytically processed cytoplasmic fragment of PTPRM is observed in GBM cells and both are correlated with decreased adhesion and increased migration observed in the disease (Rubey et al., 2009a, 2009b). Over-expression of the full-length form of PTPRM suppressed migration of GBM cells and reduces their survival (Rubey et al., 2009b). Of note, at the same time that stable cell adhesion is reduced due to the loss of full-length PTPRM expression in GBM cells, a catalytically active cytoplasmic fragment of PTPRM is released and translocates to the nucleus (Rubey et al., 2009b). Catalytic activity of this fragment is also necessary for cell migration and viability of GBM cells (Rubey et al., 2009b). Therefore, both the tyrosine phosphatase activity of the cytoplasmic fragment of PTPRM and the loss of PTPRM-mediated adhesion may contribute to the invasiveness of GBM cells.

PTPRK is also implicated in tumor progression. PTPRK expression is reduced in melanoma cell lines and human tissue biopsies (McArdle et al., 2001), and in Hodgkin’s lymphoma cells (Flavell et al., 2008). Over-expression of PTPRK in Hodgkin lymphoma cells reduces cellular proliferation and survival (Flavell et al., 2008). Like PTPRM, PTPRK is proteolytically processed (Anders et al., 2006), potentially as a result of aberrant glycosylation (Kim et al., 2006). It is not clear whether adhesion or catalytic activity of PTPRK is required for its tumor suppressor activity (Kim et al., 2006; Flavell et al., 2008). Of note, the cleaved cytoplasmic domain fragment of PTPRK is catalytically active, translocates to the nucleus and promotes β-catenin-mediated transcription (Anders et al., 2006).
Signaling via the PTPRK cytoplasmic fragment might augment the loss of stable cell-cell adhesion and may promote tumor progression.

Although speculative, it is interesting to postulate that tyrosine phosphatase activity and or cleavage of PTPRT will also be an important element of its tumor suppressor activity, as has been demonstrated for PTPRM and PTPRK. Interestingly, we identified and validated STAT3 as a direct substrate of PTPRT (Zhang et al., 2007). STAT3 is latent transcription factor that translocates from the cytoplasm to the nucleus after being phosphorylated at the Y705 residue. It is possible that the cleaved intracellular fragment of PTPRT, which contains the phosphatase domains, translocates to nucleus and dephosphorylates STAT3. Besco and colleagues found that PTPRT associates with the adhesion molecule E-cadherin and its binding partner p120 catenin, which are PTPRT substrates (Besco et al., 2006). p120 also translocates to the nucleus and associates with the Kaiso transcription factor (Daniel and Reynolds, 1999). Given the frequency and distribution of mutations in PTPRT in human colon and other cancers, a more thorough understanding of its biological function is warranted.
Figure 4-5. Structural modeling of the point mutations of PTPRT. The point mutations (indicated in red) evaluated in this manuscript were modeled onto the equivalent sites in the crystal structure of PTPRM. The PTPRT mutations likely alter cell-cell aggregation either by altering the three-dimensional topology and/or cis/trans interaction of PTPRT.
**Materials and methods**

**Baculovirus generation**

Baculoviruses were generated by transfecting cells with the BaculoGold™ Linearized Baculovirus DNA (BD Biosciences, San Diego, CA) according to the manufacturer’s protocol. The baculovirus for wild-type PTPRT was previously described (Yu et al., 2008).

**Sf9 cell culture**

Insect Sf9 cells from (BD Biosciences) were maintained at 27°C in Graces Insect Medium (BD Biosciences). The medium was supplemented with 10% FBS from (Hyclone, Logan, UT) and Gentamicin (MP Biomedicals, Solon, OH). Viruses were added to Sf9 cell medium to induce the expression of proteins of interest. Cells were harvested 30 hours post-infection for further analysis.

**Generation of PTPRT deletion constructs**

The PTPRT-ΔMAM, PTPRT-Δlg, PTPRT-ΔFNIII-1, PTPRT-ΔFNIII-2, PTPRT-ΔFNIII-3 and PTPRT-ΔFNIII-4 constructs were generated by fusion PCR using the full-length PTPRT pVL1393-V5-His vector as the template. The primers 5’-GAAGATCTATGGCGAGCCTCGCCGCG-3’ and 5’-CCGGAATTCGGATCCTGGGGCGCTCTGAGCCCGGG-3’ were used to generate the PTPRT-ΔMAM construct; the primers 5’-GCCTGGAGGGCTCTCTCTGCATGGATGAGCAAGGAC-3’ and 5’CAAT GGCTCTTGCTCATCCATGCAGAGCGCCCGCAGACCGCATCGGATCCT-3’ were used to generate the PTPRT-ΔMAM construct.
3’ were used to generate the PTPRT-ΔIg construct; the primers 5’-CTGCACA CTTGGTCTGGTTTTCCAGATCAGCTCCCGGTA-3’ and 5’-TACGCAGGAGC GTCGTGAAAAACCAGGACCAAGTGTCAGAG-3’ were used to generate the PTPRT-ΔFNIII-1 construct; the primers 5’-CCGCCAGGGCCTCCCCTCACCAGG GAAAGACGTCCAGGAG-3’ and 5’-CTCCTGGAACGTCTTCCTCGGTGAGGGG AGGCCCTGGCCGG-3’ were used to generate the PTPRT-ΔFNIII-2 construct; the primers 5’-GAGGAGCTGG TTGGTGCAAGACTAAAATTTCAGCTCCATCCA-3’ and 5’-TGGATGGAGCTGGAA TTTTAGTCTGACCACCAGGTCACCTC-3’ were used to generate the PTPRT-ΔFNIII-3 construct and the 5’-GTCACCACTCGGATT GCCACCGGTGCCTCCACCAGAATT-3’ and 5’-AATTCTGGGTGGAGGCACC GGTGGCAATCCCGAGTGGTGAC-3’ were used to generate the PTPRT-ΔFNIII-4 construct.

**Immunoblotting**

Sf9 cells were lysed in a standard lysis buffer (20 mM Tris·HCl, pH 7.5, 1% Triton X-100, 150 mM sodium chloride, 2 mM Benzamidine, 2.5μg/ml Aprotinin, 2.5μg/ml Leupeptin, and 0.5μg/ml Pepstatin). Samples were solubilized in 2X SDS loading buffer and separated by SDS-PAGE. Samples were transferred to a nitrocellulose membrane (Whatman, Piscataway, NJ) and immunoblotted with SK18 (Brady-Kalnay et al., 1993), which cross-reacts with PTPRT.

**Cell surface expression of PTPRT**

Cell surface expression of PTPRT protein in baculovirus infected Sf9 cells
was determined using a modified biotin-avidin reaction with the Cell Surface Protein Isolation Kit (catalog# 89991, Pierce, Rockford, IL), as previously described (Yu et al., 2008). Briefly, Sf9 cell media was removed and the flasks were incubated with rocking for 30 minutes at 4°C in 5ml of 0.25mg/ml Sulfo-NHS-SS-Biotin in 1X PBS. The cells were mechanically removed from the flask, centrifuged, washed, and lysed according to the manufacturer’s protocols. To isolate cell surface expressed PTPRT the cell lysate was added to a NeutrAvidin Gel column and incubated for 60 min at room temperature (RT) with rocking prior to washing and eluting proteins with 200μl of sample buffer containing a final concentration of 55mM DTT for 60min at RT. The flow through was run on 6% SDS-PAGE gels and transferred to nitrocellulose. Total cell surface protein levels were normalized by immunoblotting cell lysates with an anti-actin antibody (JLA20). The normalized cell surface protein was run on 6% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with antibodies to PTPRM that also recognized PTPRT (SK18) followed by secondary antibody conjugated to HRP. The HRP signal was detected using a Fluor-S MAX MultImager (Bio-Rad Laboratories Inc., Hercules, CA).

**Aggregation Assays**

Sf9 cells were harvested by trituration. The cell suspensions were added to glass scintillation vials and incubated at 27°C at 90 rpm in a gyratory shaker for 30 minutes. For a given condition, the cell suspension was transferred to a 100mm petri dish either prior to (0 time point) or 30 minutes after aggregation.
Images were captured at 10X magnification with a Nikon TE-200 inverted fluorescent microscope (Tokyo, Japan). To quantify cell aggregates, pictures of four randomly selected fields were taken per dish. The area of each object (single cells and aggregates) was determined by the Metamorph software (Molecular Devices) using an auto-threshold setting for light objects and appropriate size filters that allowed the counting of cells and aggregates but not debris. The four replicates were combined to yield average readings per condition. The percent aggregation was then calculated as the average aggregate area at 30 minutes minus the average aggregate area at zero minutes divided by the average aggregate area at 30 minutes [(N30-N0)/N30]. A minimum of three independent experiments was performed per condition. Statistical significance was determined in Microsoft Excel using Student’s T-test. Error bars indicate standard error.

Site-directed mutagenesis

The I395V, Y412F, R453C, Q479E, S492F, N510K, T605M, V648G, A707T, and L708P tumor-derived mutations (Wang et al., 2004) were generated by fusion PCR using the full-length PTPRT pVL1393-V5-His vector as the template. The primers 5'-TGCCCACAGAACGTGGAAGTCGTAGACATCAGAGCC-3' and 5'-CTTCCACGTTCTGTGGGCCA-3' were used as the mutagenic primers for the I395V mutant; the primers 5'CAGTGGGAGCCCTTCGGCTTCGAGGGTGACCCGCTGCCAT-3' and 5'-AAG CCGAAGGGCTCCCACTG-3' were used as the mutagenic primers for the Y412F mutant; the primers 5'-CTACACC
CTGCGAGGCCCTGTGCCCTCATGACCACCTCCG-3’ and 5’-ACAGGCCTCGCA
GGGTGTAG-3’ were used as the mutagenic primers for the R453C mutant; the
primers 5’-GAGCGAGGAGCTGGTGGGAGACTGAGGAAGACGTCC-3’ and
5’-CCACCACCAGCTCTCGC TC-3’ were used as the mutagenic primers for
the Q479E mutant; the primers 5’-TGTTTCTCTAGAATTCTCAAGGGG
CCT TT-3’ and 5’-ATGAATTCTAGAGGAACA-3’ were used as the mutagenic
primers for the S492F mutant; the primers 5’-TCCAGTGAAAACCTCTCCAAGGA
GACCAATGGGGGTACATCA-3’ and 5’-CTTGGGGAGGTTTTCACCAGGA-3’ were
used as the mutagenic primers for the N510K mutant; the primers 5’-CCATTGA
ATGAGACAGACATGACCACACAG TGATGCTG-3’ and 5’-ATGTCTGTCTCAT
TCAATGG-3’ were used as the mutagenic primers for the T605M mutant; the
primers 5’-ATTATTGAGTGCTTTTCGAGGCCTATCGGAAT-3’ and
5’-CCCCAAAA GCACTCAATAT-3’ were used as the mutagenic primers for the
V648G mutant; the primers 5’-CTACAGCATCTACTCCAGACACTCAGCAAG
CCAATGG-3’ and 5’-TCTGAAGTAG ATGCTGTAG-3’ were used as the
mutagenic primers for the A707T mutant; the primers 5’-AGCATCTACTCCAGG
CACCAGAAAAAGCAATGGAG AG-3’ and 5’-GGTGCTTGGAAGTAGATGCT-
3’ were used as the mutagenic primers for the L708P mutant.

Mapping of PTPRT mutations onto the PTPRM crystal structure

The sequence of PTPRM and PTPRT were aligned using CLUSTALW.
Mutations probed in this study were mapped using this sequence alignment onto
the PTPRM structure (PDB ID: 2V5Y). The figure was prepared utilizing PyMOL 1.2r1 (http://pymol.org).
Chapter 5
Discussion and Future Directions
Summary

Many PTPs, including PTPN14 and PTPRT, have been reported to be mutated in a variety of human cancers. We set out to understand why, in particular, these two PTPs are mutated in different tumor types and what possible roles they play during tumorigenesis. We utilize a proteomic approach to identify their physiological substrates to gain insight into the underlying signaling pathways the PTPs regulate. We first identify p130Cas as a direct substrate of PTPN14. PTPN14 dephosphorylates p130Cas at Tyr 128. This phosphorylation is important for tumorigenesis as demonstrated by the reduced tumorigenicity of p130Cas Y128F mutant cells. The effect is probably mediated through impaired Akt signaling as p130Cas Y128F mutant binds less with p85, an important regulator of PI3K-AKT signaling pathway. STAT3 has been previously reported to be a substrate of PTPRT by our laboratory (Zhang et al., 2007). However, how the regulation of STAT3 by PTPRT plays out in tumorigenesis is not fully elucidated. We discovered that STAT3 can interact with PLCγ1 and this interaction is abolished if the Tyr705 of STAT3 is mutated to Phe. The cross-talk between STAT3 and PLCγ1 may be a downstream effector of PTPRT-mediated tumorigenesis. We also demonstrate that somatic mutations in the extracellular domain of PTPRT can affect cell-cell adhesion. The tumorigenic effect of PTPRT inactivation may be partly mediated through the impairment of cell-cell adhesion.
Discussion and future directions

Is PTPN14 a tumor suppressor?

Our data in Chapter 2 suggest that PTPN14 may have tumor-suppressing roles. In addition, there are two recent reports which indicate that PTPN14 is a potential tumor suppressor by inhibiting the activity of YAP, a transcriptional co-activator (Liu et al., 2012; Wang et al., 2012). However, no unequivocal evidence is available to support it so far. The most convincing proof would be that PTPN14 knockout mice develop cancers. In a study that reveals PTPN14 as a regulator of lymphatic development, a mouse model with a gene trap in the locus of PTPN14 was used (Au et al., 2010). In this mouse model, the expression of PTPN14 was interrupted after the FERM domain. However, the leakiness of the gene trap was reported in this mouse model. Therefore, it is not suitable to study the phenotypes of PTPN14 knockout. By searching the database in the international knockout mouse consortium, there are targeted PTPN14 knockout mouse ES cells available (http://www.knockoutmouse.org/search_results?criteria=ptpn14). We can use the ES cells to generate PTPN14 knockout mice and examine whether the mice display any phenotype. If the knockout mice do not develop colorectal cancer or cancer in other organs spontaneously, we can use specific carcinogens like AOM to try to induce certain cancers as described in (Zhao et al., 2010). If no tumor development is observed in either case, PTPN14 may just be a modifier of tumorigenesis and exert its effect through interaction with other tumor suppressors. By using the mouse model, we can better understand the in vivo cellular functions of PTPN14 and facilitate further studies.
**Are cancer-derived mutations of PTPN14 tumorigenic?**

Although many somatic mutations of PTPN14 have been identified in a variety of cancers, the functional consequences of these mutations are largely unknown. It is unlikely that all of these mutations are causative of tumorigenesis, which makes it necessary to distinguish driver mutations from passenger ones. Therefore, we need to characterize these mutations through functional studies to determine which of them are indeed tumorigenic. Since we propose that PTPN14 is a tumor suppressor, any tumorigenic mutation of PTPN14 should be loss-of-function or dominant negative as in some cases only one of the two copies of PTPN14 is mutated. However, PTPN14 has not been reported to dimerize or oligomerize in cells. It is more likely that there may be a haploinsufficient effect of PTPN14. Interestingly, the majority of the mutations are actually localized outside the conserved phosphatase domain although we would expect that the majority of the mutations to be inside the catalytic domain if the phosphatase activity is essential for the tumor-suppressing function of PTPN14. To understand how the extra-catalytic mutations affect the activity of PTPN14 will shed new light on the roles of PTPN14 in tumorigenesis. There are at least two possibilities. First, although some mutations lie outside the catalytic domain they may affect phosphatase activity indirectly, likely through changing the intramolecular conformation of PTPN14. This can be tested by looking at the phosphorylation of the substrates when the corresponding mutants are introduced. Second, some mutations may induce tumorigenesis in a phosphatase-independent way. The
mutation can abolish an existing protein-protein interaction and alter downstream signaling. One possible way to study this is to compare the interacting proteins between wild-type and mutant PTPN14 either endogenously or in an overexpression system. By identifying oncogenic PTPN14 mutations, it can be utilized in the future to classify cancer patients based on their mutational status and develop individualized therapy that targets the signaling pathways activated by the inactivation of PTPN14.

**Are there any additional substrates of PTPN14?**

Our laboratory has so far experimentally verified two substrates for PTPRT: STAT3 and Paxillin (Zhang et al., 2007; Zhao et al., 2010), one substrate for PTPN14: p130Cas. It is unlikely that both PTPs only have this limited number of substrates in cells. By identifying more substrates, we will get a more complete picture of the signaling networks regulated by the PTP. For PTPN14, there are several additional candidates from the proteomic analysis that might be physiological substrates. Lyn is a member of the Src kinase family. Lyn has been reported to be a mediator of epithelial-mesenchymal transition and frequently linked to leukemias (Goldenberg-Furmanov et al., 2004; Choi et al., 2010). Moreover, the phosphosite on Lyn that can be potentially regulated by PTPN14, Tyr507, is a known functional phosphosite and there are commercially available phosphorylation specific antibodies, which makes it relatively easy to test whether Lyn is a *bona fide* substrate for PTPN14. Another candidate substrate is p120-Catenin which is encoded by the gene CTNND1. p120-Catenin
can affect cell-cell adhesion through interaction with E-cadherin (Lu, 2010). Its expression is frequently altered in cancer (Castillo et al., 2010). The binding between E-cadherin and p120-catenin was shown to be dependent on the tyrosine phosphorylation of p120-Catenin (Roura et al., 1999). If PTPN14 can dephosphorylate p120-catenin, it is possible that a large complex of diverse components including p120-Catenin, PTPN14 and E-cadherin regulate cell-cell adhesion in an intricate manner. To demonstrate that these proteins are real substrates of PTPN14, the substrates should be pulled down by the trapping mutant of PTPN14, their phosphorylation needs to be shown to be regulated by PTPN14 and PTPN14 should be able to dephosphorylate these proteins *in vitro*. Once they are confirmed as substrates, we can look into signaling pathways modulated by the substrates.

**Is phosphorylation of p130Cas Y128 a predictive marker for sensitivity to Src inhibitors?**

Src is the first known proto-oncogene (Stehelin et al., 1976). It has since been extensively studied for its roles in tumorigenesis. Src itself is a very important kinase in the cellular signaling and is involved in many biological processes that are required in tumor growth, progression, invasion and metastasis (Yeatman, 2004). Src is thus an attractive target for new cancer therapy. Several Src inhibitors are currently in clinical trials for the treatment of solid tumors and leukemias (Kim et al., 2009). As for every targeted therapy, the results for unselected population are limited as patients may have different
genetic makeups, which makes them respond differently to specific inhibitors. It is tempting to develop biomarkers that are predictive of patients’ prognosis of a targeted therapy. Our preliminary data demonstrate that the levels of p130cas Y128 phosphorylation in various colorectal cancer cell lines correlate with their sensitivity to the Src inhibitor, Dasatinib. It suggests that we can investigate the possibility of using p130Cas Y128 phosphorylation level as a prognostic marker for the response to Src-inhibitor based cancer therapy. We can first expand our study in cell lines from other cancer types, especially those that are currently under investigation for the efficacy of Src inhibitors, to see if the correlation persists. In addition to the data we obtained from cell lines, we can also test the level of p130Cas Y128 phosphorylation in cancer patient samples. If the phosphorylation level correlates with the patients’ response to Src inhibitor, in the future we can pre-screen patients based on the phosphorylation level of p130Cas Y128 before we place them on the Src-inhibitor based treatment. In this way, the targeted therapy will be more effective and efficient.

Is the interplay between STAT3 and PLCγ1 important for PTPRT-associated tumorigenesis?

In Chapter 3, we unexpectedly discover the crosstalk between STAT3 and PLCγ1 in CRC cells. Notably, only wild-type STAT3 can interact with PLCγ1 but not the STAT3 Y705F mutant. In STAT3 Y705F mutant cells, the phosphorylation of PLCγ1 at S1248 is elevated compared to wild-type cells. This phosphorylation actually inhibits the activity of PLCγ1 (Park et al., 1992). It is very likely that
STAT3 brings a serine/threonine phosphatase into the complex with PLCγ1 so that the S1248 phosphorylation of PLCγ1 is kept at a low level in the wild-type cells. The assembly of the complex is obviously dependent on the Y705 phosphorylation of STAT3. In cancer cells that have PTPRT inactivated, STAT3 would be hyper-phosphorylated at Y705. We expect that more STAT3 would form a complex with PLCγ1 and thus the phosphorylation of PLCγ1 at S1248 be reduced from the normal levels. As a result, PLCγ1 would be hyperactive in PTPRT-inactivated cells. To demonstrate that the crosstalk between STAT3 and PLCγ1 is important for PTPRT-associated tumorigenesis, we need to first mapping the region of interaction in STAT3 and PLCγ1 through the construction of a series of deletion mutants of both proteins. After the region is identified, we can design small peptides to block the interaction. If the crosstalk is important for PTPRT-associated tumorigenesis, its disruption would reduce the tumorigenicity of PTPRT-inactivated cancer cells. Once the importance is validated, we can screen a chemical library to discover small molecule inhibitors that specifically disrupt the interaction between STAT3 and PLCγ1, which would also have the therapeutic potential for treating cancer patients who carry PTPRT mutations.

Does PTPRT-mediated adhesion contribute to its tumor-suppressing role?

Although we show that cancer-derived mutations in the extracellular domain of PTPRT impair cell-cell adhesion in Chapter 4 and Yu et al., 2008, there is no direct evidence that links PTPRT-mediated adhesion to its role as a tumor suppressor. It is very likely that PTPRT exerts its tumor-suppressing effect
through its function in cell adhesion in addition to its regulation on tyrosine phosphorylation. We can generate a mouse strain with one of the cancer-derived mutations that have been shown to cause cell adhesion defects (Chapter 4 and Yu et al., 2008). We can first examine if there is any spontaneous tumor formation in the PTPRT mutant knock-in mice. It is possible that the mutation would not have any effect on tumor formation but on later stages of tumor development. Since cell adhesion is closely related to tumor invasion and metastasis, it is likely that the PTPRT mutations can affect metastasis. We can cross the PTPRT mutant knock-in mice with mice of an existing tumor model like Apc (Min/+ ) and compare the rate of metastasis in the two groups of mice. Ideally, we expect to see a higher rate of metastasis in double mutant mice. By establishing the link between PTPRT-mediated adhesion and tumorigenesis, we can achieve a better understanding of the mechanisms how PTPRT inactivation leads to tumor development.


