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Tyrosine Phosphoproteomics and Identification of Substrates of Protein Tyrosine Phosphatase dPTP61F in *Drosophila* S2 Cells by Mass Spectrometry-Based Substrate Trapping Strategy

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Recent biochemical and genetic approaches have clearly defined the functional role of critical components in tyrosine phosphorylation-dependent signal transduction. These signaling modulators often exhibit evolutionarily conserved functions across various species. It has been proposed that if protein tyrosine kinases (PTKs), protein tyrosine phosphatases (PTPs), and thousands of their substrates could be identified and characterized, it would significantly advance our understanding of the underlying mechanisms that control animal development and physiological homeostasis. The fruit fly *Drosophila melanogester* has been used extensively as a model organism for investigating the developmental processes, but the state of its tyrosine phosphorylation is poorly characterized. In the current study, we used advanced mass spectrometry (MS)-based shotgun analyses to profile the tyrosine phosphoproteome of *Drosophila* S2 cells. Using immunoaffinity isolation of the phosphotyrosine (pTyr) subproteome from cells treated with pervanadate followed by enrichment of phosphopeptides, we identified 562 nonredundant pTyr sites in 245 proteins. Both this predefined pTyr proteome subset and the total cell lysates were then used as sample sources to identify potential substrates of dPTP61F, the smallest member in terms of amino acid number and molecular weight in the *Drosophila* PTP family and the ortholog of human PTP1B and T Cell-PTP, by substrate trapping. In total, 20 unique proteins were found to be specifically associated with the trapping mutant form of dPTP61F, eluted by vanadate (VO_4^{3-}), and identified by MS analyses. Among them, 16 potential substrates were confirmed as tyrosine phosphorylated proteins, including a receptor PTK PDGF/VEGF receptor, a cytosolic PTK Abl, and several components of SCAR/WAVE complex, which may work in coordination to control actin dynamics. Thus, our data suggest that dPTP61F plays a central role in counteracting PTK-mediated signaling pathways as well as in regulating actin reorganization and remodeling through tyrosine dephosphorylation of critical substrates in *Drosophila* cells.

Keywords: *Drosophila* S2 cells • protein tyrosine phosphatase • Tyrosine phosphoproteomics • substrate trapping • mass spectrometry • SCAR/WAVE complex

Introduction

Reversible tyrosine phosphorylation is one of the most important post-translational modifications because it plays a critical role in regulating a diverse range of biological processes in all eukaryotes. Its dynamic nature is largely controlled by the coordinated actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), the abnormal functioning of which contribute to the disregulation of tyrosine phosphorylation and lead to disease states such as human malignancies, diabetes, and immune disorders.[1,2] The identification and characterization of PTKs, PTPs, and thousands of their substrates, therefore, is of primary importance in delineating this complicated signaling network and in developing therapeutic strategies against various diseases.[1]

A variety of biochemical and genetic approaches have been used extensively over the last two decades to define the functional roles of the critical components involved in tyrosine phosphorylation-dependent signal transduction. More recently, genomic sequence analyses has made possible the identification of these components in a diverse range of multicellular eukaryotes and collectively demonstrated that many signaling modulators exhibit similar functions across various species

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throughout evolution.[3,4] Moreover, the regulatory mechanism that precisely controls the signaling outputs in a number of pathways is also evolutionarily conserved.[5–7] On the basis of these findings, it has been proposed that investigations using model organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, might provide insight into the general mechanism through which eukaryotic signaling networks integrate while avoiding the complicated interpretation of mammalian genetic screens resulting from the redundancy of regulatory components in a given signaling pathway.[8–10] Because it is a simple organism with a relatively small genome readily accessible by genetic manipulation,[8] *Drosophila* can serve as an ideal model system to investigate the concerted actions of PTKs and PTPs in controlling the balance of tyrosine phosphorylation in a complex proteome.[4] Yet, little is known about the overall content of the *Drosophila* phosphotyrosine (pTyr) proteome at the cellular level and biochemical level. The exact functional role of a given *Drosophila* PTK or PTP in regulating signaling cascades also remains mostly undefined. In particular, we need a systematic identification of the physiological substrates of a *Drosophila* PTP to help in the delineation of the mechanism governing the down-regulation of phosphotyrosyl signaling.

Substrate trapping[11–14] is a well-accepted experimental approach to identify the substrates of a given PTP, although there are disadvantages in using this approach. Typically, mutant forms of the PTP are generated. They form a stable complex with their cognate substrates, though their catalytic activity is impaired.[12] In essence, the mutated PTP serves as an affinity trap enabling the capture and isolation of its substrates for later conventional identification by specific antibodies or, in more recent studies, by mass spectrometry (MS).[15,16] Mass spectrometry offers the distinct advantage of identifying potential substrates and their phosphorylation sites independently of the availability of antibodies. However, direct MS-based identification of pTyr sites on the trapped substrates remains daunting as is often limited by the low amount of sample recovered. In contrast, current advances in MS-based proteomic technologies coupled with efficient phosphoprotein and/or phosphopeptide enrichment protocols have facilitated large-scale shotgun phosphoproteomic studies,[17–23] making possible the unambiguous identification of several hundreds of pTyr sites from a single proteome source.[19,21–23] The pTyr sites mapped by such exploratory phosphoproteomic studies allow the construction of a database [19,21] and provide a convenient platform for follow-up genetic or mutagenesis studies aiming to verify the relevant pTyr sites of any pTyr protein of interest.

In this study, we have developed and optimized a generally applicable proteomic protocol for trapping PTP substrate on a specific subset of pTyr proteome derived from a particular biological source that has been 'catalogued' by its constituent identities and pTyr sites. Substrate trapping mutants of *Drosophila* PTP61F (dPTP61F), which has been defined as an ortholog of human PTP1B and T Cell-PTP (TC-PTP),[24] were constructed, expressed, and applied to both total cell lysates and an affinity enriched pTyr proteome subset of *Drosophila* S2 cells. Although there has been a substantial amount of evidence demonstrating the involvement of PTP1B and TC-PTP in the regulation of cell proliferation, migration, survival, and glucose metabolism,[25,26] the potential function of dPTP61F in controlling signal transduction and development in *Drosophila* has not yet been elucidated. This study shows that, among others, components of SCAR/WAVE complex, which work together to reorganize and remodel actin, are the primary sites for dPTP61F to act. Our data, therefore, suggest that dPTP61F plays a central role in regulating actin dynamics involved in *Drosophila* signal transduction and development.

Experimental Procedures

Plasmid Constructs and Purification of Recombinant dPTP61F. cDNA sequences corresponding to amino acids 1–339 of dPTP61F, or the trapping mutants D203A or C237S, were constructed with an HA tag at the N-terminus and cloned into a pET-28a vector, which provided an additional His6 tag to facilitate purification. The constructed plasmids were transformed into *Escherichia coli* (BL21 DE3 strain), and protein expression was induced by adding 1 mM IPTG to bacterial culture incubated at 20 °C for 20 h. The bacterial lysate was prepared in a buffer containing 50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1% Triton X-100, 1 mg/mL lysozyme, and 2 mM DTT. After centrifuging (30 000*g*) the lysate at 4 °C for 10 min, the resulting supernatant was collected and loaded onto a 1 mL Ni-NTA sepharose (Qiagen) column. It was washed with 10 mL each of buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, and 2 mM DTT) containing 20 and 70 mM imidazole. The His6-tagged proteins were then eluted with the same buffer containing 135 mM imidazole. The excess imidazloe was removed by gel filtration using a PD-10 column (GE Healthcare Life Science), and the purified protein was concentrated using a centrifugal Amicon Ultra-4 apparatus (Millipore).

Cell Culture and Lysate Preparation. *Drosophila* S2 cells were grown in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (JRH Biosciences) in the presence of penicillin (100 Units/mL) and streptomycin (100 μg/mL) at 24 °C in a humidified atmosphere for 2 days (to 70% confluence). To increase the level of pTyr, S2 cells were serum-deprived for 16 h, followed by stimulation with 100 μM pervanadate, which has been used extensively as an effective inhibitor specifically to PTPs, at 24 °C for 30 min. The 50 mM pervanadate stock was freshly prepared by adding 5.7 μL of 30% (v/v) H_2O_2 to 500 μL of water, and then mixed with 500 μL of 0.1 M sodium orthovanadate (Na_3VO_4). Cells were harvested in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitor (Complete, Roche) containing either 2 mM Na_3VO_4 or 5 mM iodoacetamide at 4 °C for 30 min to prepare pTyr proteome subset or to prepare the cell extracts used in direct substrate trapping experiments, respectively. Total lysates were centrifuged at 30 000*g* to remove insoluble debris. The supernatant was collected, and the protein concentration was measured using a Protein Assay (Bio-Rad).

Affinity Purification of pTyr Proteome Subset. A sample of total lysates (150 mg of cellular proteins in 50 mL of lysis buffer) prepared from 2×10^8 S2 cells was incubated with 1.5 mL of agarose beads (Sigma) at 4 °C for 1 h to preclear the proteins which are nonspecifically associated with beads. The supernatant was then collected and mixed with 1.5 mL of immobilized anti-pTyr antibody (PT66)-agarose beads (Sigma). After an incubation at 4 °C for 6 h, the agarose beads were washed twice with 45 mL of ice-cold lysis buffer each time, and then twice with 45 mL of ice-cold 1× PBS each time. To elute the pTyr proteins specifically associated with anti-pTyr antibody, agarose beads were reacted with 6 mL of 1× PBS containing 200 mM phenyl phosphate (Calbiochem) at room temperature for 10 min. After centrifugation, the supernatant underwent tyrpsin digestion. Alternatively, to prepare pTyr

proteome subset for use in the indirect substrate trapping experiments, the supernatant was subjected to gel filtration using a PD-10 column that removed excess phenyl phosphate.

Trypsin Digestion of pTyr Proteins and Enrichment of pTyr Peptides. For in-solution trypsin digestion, pTyr proteins were first incubated in a buffer containing 25 mM ammonium bicarbonate, 6 M urea, and 12.5 mM DTT at 37 °C for 1 h. Proteins were then subjected to alkylation with 40 mM iodoacetamide at room temperature for 1 h. Before adding sequencing grade trypsin (Promega), the sample was diluted to a final urea concentration of 1 M. The trypsin digestion was carried out at 37 °C overnight with an enzyme-to-protein ratio of 1:50 (w/w). The reaction was terminated by adding formic acid (0.1% v/v). The digested peptides were loaded onto a C18 SepPak cartridge (Waters) for desalting. Eluted peptides were lyophilized and stored at −20 °C.

To enrich the phosphopeptides by immobilized metal ion affinity chromatography (IMAC), the IMAC column was prepared by incubating the Ni-NTA silica (obtained from a Ni-NTA spin column, Qiagen) sequentially with 0.1 M EDTA, 0.1 M formic acid, followed by 0.1 M Fe(III) chloride in 0.1 M formic acid. The treated silica was further washed with 0.1 M formic acid, and then packed in a 5 cm column (450 μm i.d.). Before loading onto the column, the lyophilized peptide sample was resuspended in 50 μL of 0.1 M formic acid. After sequential washing with 100 μL of 0.1 M formic acid and 250 μL of 0.1 M formic acid/25% acetonitrile, the peptides retained in the column were eluted with 75 μL of 200 mM NaH_2PO_4. Peptides were collected and then subjected to desalting by C18 SepPak cartridges.

Alternatively, trypsin-digested peptides prepared from total lysates of pervenedate-treated S2 cells were reacted with the immobilized anti-pTyr antibody (pY100)-agarose beads (PhosphoScan kit, Cell Signaling Technologies). The pTyr peptides in immunocomplexes were obtained according to manufacturer's instructions.

Direct substrate Trapping Experiments. Excess iodoacetamide in total lysates was first removed by a PD-10 column. For small-scale immunoblotting-based experiments, an aliquot of lysates (2 mg) was reacted with 8 μg of HA-tagged WT, DA, or CS forms of dPTP61F at 4 °C for 30 min, followed by incubation with 10 μL of immobilized anti-HA antibody-agarose beads (Sigma) at 4 °C for 3 h. For larger-scale MS-based experiments, 20 mg of whole cell extracts (isolated from 2 × 10^7 S2 cells) was reacted with 80 μg of a WT or DA form of dPTP61F at 4 °C for 30 min before incubation with 30 μL of immobilized anti-HA antibody-agarose beads at 4 °C for additional 3 h. After centrifugation, agarose beads were washed twice with 1 mLof ice-cold lysis buffer each time, and then twice with 1 mL of ice-cold 1× PBS each time. To elute potential substrates associated with dPTP61F, beads were either boiled in SDS sample buffer or incubated with 0.01∼5 mM sodium orthovanadate (Na_3VO_4) at room temperature for 10 min. Those eluted proteins were resolved in a SDS-PAGE gel, and then subjected to immunoblotting with anti-pTyr antibody (G104, kindly provided by Dr. Nicholas K. Tonks[27]), or subjected to SyproRuby staining for in-gel trypsin digestion[28] followed by LC−MS/MS analysis.

Indirect Substrate Trapping Experiments. The affinity purified pTyr proteins derived from 10^8 S2 cells were reacted with 80 μg of a WT or DA form of dPTP61F at 4 °C for 30 min before incubation with 30 μL of immobilized anti-HA antibody-agarose beads at 4 °C for additional 3 h. After centrifugation, agarose beads were washed twice with 1 mL of ice-cold lysis buffer each time, and then twice with 1 mLof ice-cold 1× PBS each time. To elute potential substrates associated with dPTP61F, beads were incubated with 5 mM Na_3VO_4 at room temperature for 10 min. The eluted proteins were resolved in a SDS-PAGE gel, and then subjected to SyproRuby staining for in-gel trypsin digestion[28] followed by LC−MS/MS analysis.

MS and MS/MS Analyses. For LC−MS/MS analyses on a Micromass Q-Tof Ultima API instrument, samples were dissolved in 6 μL of 0.1% formic acid and loaded onto a 150 μm × 15 mm precolumn packed with 5 μm C18 particles (Nucleosil 120–5 C18, Macherey-Nagel, GmbH & CO. KG). The retained peptides were then eluted and separated on an analytical C18 capillary column (75 μm × 250 mm) packed with the same material. Peptides were eluted using a multistep gradient of 0% B for 4 min, raised to 10% B within 0.5 min, 10–40% B within 105.5 min, 80% B within 0.5 min, and then held at 80% B for additional 9.5 min, at a flow rate of 300 nL/min. Solvent A and B were 0.1% formic acid and 95% acetonitrile in 0.1% fromic acid, respectively. For each cycle, one survey scan (mass range m/z 400–1600) was followed by three data-dependent MS/MS acquisitions on the three most abundant ions. Charge-state recognition was used to exclude singly charged precursor ions. Selected ions were dynamically excluded for 120 s.

For LC−MS/MS analyses on a hybrid linear ion trap 7-T Fourier transform ion cyclotron resonance instrument (LTQ FT; ThermoElectron, Bremen, Germany), samples were dissolved in 6 μL of 0.1% formic acid, and 4 μL of sample was loaded onto a C18 precolumn (150 μm × 20 mm) packed in-house with Magic C18AQ (5 μm, 100 Å, Michrom BioResources). Retained peptides were then separated on an analytical C18 capillary column (75 μm × 300 mm) packed with the same material, using a multistep gradient at a flow rate of 300 nL/min started at 2% B and raised to 8% B in 8 min, after which it was increased to 50% B within 80 min. The gradient was further raised to 85% B in the next 8 min and held at 85% B for additional 8 min. Solvent A and B were 0.1% formic acid and 80% acetonitrile in 0.1% formic acid, respectively. The FT survey scan was recorded in an m/z window between 300 and 1800 Da with the resolution set to 50 000 and the automatic gain control (AGC) target set to 1 000 000 ions. Four data-dependent MS^2 scans were performed in the LTQ in parallel operation, with a total duty cycle of 1.8 s.

Data Analysis and Functional Annotation. The LC−MS/MS data acquired on Q-TOF were processed by Mass Lynx 4.0 (Waters); the raw data of LTQ-FT were converted by BioWorks TurboSEQUEST (Thermo) into a set of peak lists. Both were searched against a composite forward/reverse database[29] of *D. melanogaster* proteins extracted from FlyBase (released version 5.1) using an in-house MASCOT Search engine (version 2.1). Parameters used for queries were trypsin cleavage, up to two missed cleavages, and variable modifications: carbamidomethyl (Cys), oxidation (Met), phosphorylation (Thr/Ser/Tyr). Peptide and MS/MS tolerance for Q-TOF were set at 0.25 Da, and for LTQ-FT, they were set at 10 ppm and 0.6 Da, respectively. The return hits were then manually filtered assuming the only top ranking phosphopeptides would have charge states of 2+ to 4+ and a minimum ion score threshold set to eliminate all reverse database hits. Normalized delta ion scores[30] were also provided for a further assessment of the assigned phosphorylation sites.

For substrate–protein identification, the MS/MS data generated from the analyses of each protein band were searched

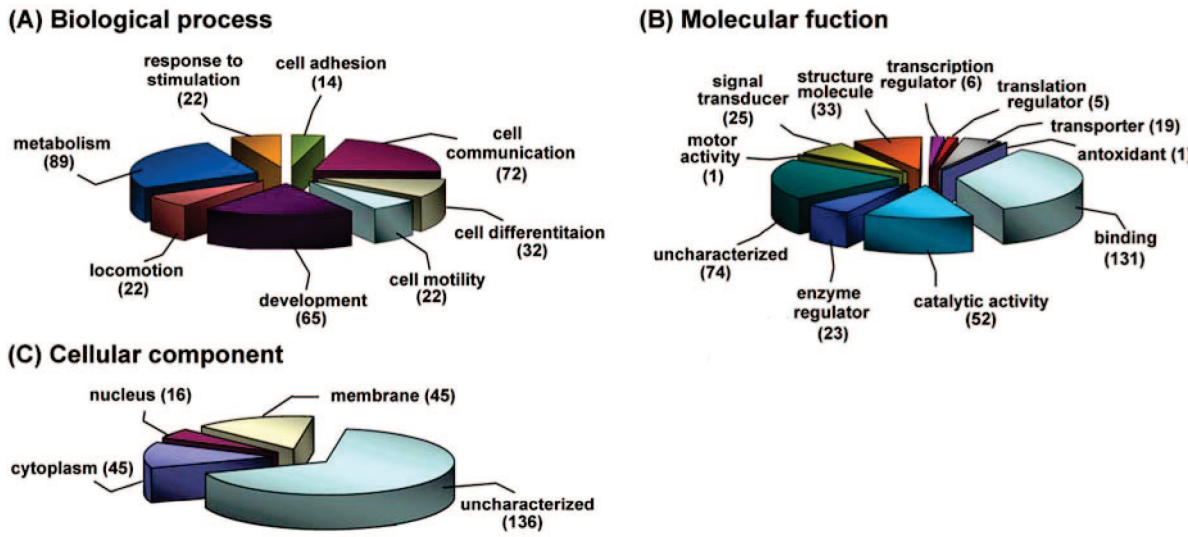

Figure 1. Classification of tyrosine phosphorylated proteins identified in S2 cells treated with pervanadate. The annotations of (A) biological process, (B) molecular function, and (C) cellular component are categorized according to GO description in FlyBase (http://flybase.bio.indiana.edu/). Some proteins are present in more than one category.

against both the *D. melanogaster* database and NCBI nonredundant database, using Mascot and applying the same criteria settings as above. Proteins identified by two or more unique peptides with a combined peptide score set arbitrarily at above 55 or a single unique peptide score above 70 were considered significant. For functional annotation, all identified pTyr proteins were categorized into process, functional, or component classes according to Gene Ontology (GO) terms by GOminer.[31]

Results

Profiling of pTyr Proteome in S2 Cells by Mass Spectrometry. To first catalog the pTyr sites of *Drosophila* S2 cell for subsequent PTP substrate trapping inference, a commonly adopted shotgun LC−MS/MS approach was applied to the tryptic digestion of the anti-pTyr immuno-enriched pTyr proteome subset of S2 cell, as detailed in the Experimental Procedures. The key steps included pervanadate treatment to increase the overall pTyr level before harvesting and the use of phenylphosphate to more specifically elute off the affinity captured pTyr proteins. An additional IMAC enrichment step was then used at the peptide level. This protocol was supplemented by a parallel approach employing the anti-pTyr antibody capture directly at the peptide level. Collated MS/MS data from each of the multiple LC−MS/MS runs on both LTQ-FT and Q-TOF API MS instruments for both experiments were processed and searched individually against a composite forward and reverse database of *D. melanogaster* proteins (FlyBase released version 5.1) using Mascot. After manual filtering by criteria that would collectively eliminate false positive reverse database hits in each data set, the pTyr peptide hits were compiled into a composite database of identified pTyr peptides, as listed in Supplementary Table S1 in Supporting Information. In total, 2459 pTyr peptides (redundant) were identified, deriving from 245 proteins and corresponding to a net total of 562 nonredundant pTyr sites (Supplementary Table S1 in Supporting Information). These identified pTyr proteins were then functionally annotated based on GO terms (Figure 1) and grouped according to their molecular functions, summarized in Table 1. When evaluating the reliability of pTyr site assignment, we found 77.5% of the assigned unique sites (436 out of total 562), or 74% of the confidently identified pTyr peptides (1833 out of total 2459), could be accorded a normalized delta ion score[30] (ΔS) of $\geq$ 0.4, indicating a reasonably high quality database. For those pTyr peptides with a $\Delta S <$ 0.4, 43.5% were assigned by Mascot with alternative pTyr sites as the second best candidate, 36% with alternative pSer/Thr sites, 17% carrying uncertainties at the inferred second phosphorylation site on the same pTyr peptide, and the remaining 3.5% were assigned with different peptide sequences. These identified pTyr proteins were then functionally annotated based on GO terms (Figure 1) and grouped according to their molecular functions, summarized in Table 1.

Construction of a DA Mutant Form of dPTP61F for Substrate Trapping Experiments. dPTP61F is the smallest member in the *Drosophila* PTP family.[24] The full-length protein contains a catalytic domain, five proline rich motifs (PxxP),[32] and a C-terminal tail with alternative spliced regions,[33] which determine the subcellular localization of this phosphatase (Figure 2A). To avoid potential nonspecific interaction between the C-terminal stretch of dPTP61F and other *Drosophila* proteins, constructs encoding truncated forms of recombinant dPTP61F (1–339 aa) containing the catalytic domain and only the first proline-rich motif were generated for substrate trapping and identification (Figure 2A). The wild-type (WT), trapping mutant (D203A, DA), and catalytically dead mutant (C237S, CS) forms of truncated dPTP61F proteins were expressed, purified, and tested for their abilities to trap pTyr proteins from total lysates prepared from S2 cells treated with pervanadate. As revealed by immunoblotting with anti-pTyr antibody (Figure 2B), the DA mutant form of dPTP61F was found to associate with more pTyr proteins in the immunoprecipitated complex than the CS mutant form or wild-type form and was, thus, expected to serve as a better substrate trapping mutant. All subsequent trapping experiments were performed using the DA mutant in parallel with the WT, which served as a negative control.

Identification of Substrates of dPTP61F from S2 Lysates by Mass Spectrometry. As with most other affinity capture methods coupled with MS-based protein identification, a major

Table 1. Identified pTyr[a] Proteins Are Grouped According to Molecular Function

protein	accession (FBpp)	pTyr[b] sites	additional grouping	protein	accession (FBpp)	pTyr[b] sites	additional grouping
1. Signal Transducer Activity							
PDGF-and VEGF-receptor related	0079244	14	9, 10	Signal transducing adaptor molecule	0079732	1	10
daughter of sevenless	0072803	7	10	Pak3-PA	0082763	4	9, 10
Abelson Interacting Protein	0082371	2	8, 10	CG7097-PB	0085691	1	8, 9, 10
roundabout	0071833	2	-	genghis khan	0072194	2	8, 9, 10
auxillin-PA	0078604	4	9, 10	shaggy	0070449	1	9, 10
Down syndrome cell adhesion molecule	0110350	7	10	RacGAP50C-PA	0086767	1	8, 10
Sterile20-like kinase	0072271	3	9, 10	cueball	0072590	1	10
Protein kinase related to protein kinase N	0087682	4	9, 10	CG17528-PC	0110466	1	9, 10
lea-PA	0077606	2	10	corkscrew	0070362	1	9, 10
CG33722-PC	0081295	1	-	Lap1-PA	0086538	1	9, 10
misshapen	0072830	1	8, 9, 10	slipper	0099601	1	9, 10
cdc2-PA	0079641	1	9, 10	downstream of receptor kinase	0086812	1	10
dreadlocks	0077678	1	10				
2. Motor Activity							
Myosin heavy chain-like	0082683	1	6, 9, 10				
3. Antioxidant Activity							
Thioredoxin reductase-1	0071116	1	9, 10				
4. Transporter Activity							
drongo-PB	0077699	1	-	Scamp-PA	0073895	1	10
Na pump alpha subunit	0088502	3	9, 10	CG8062-PA	0074451	2	-
CG5594-PD	0072046	5	-	Malvolio	0088507	1	-
CG8468-PC	0086661	5	-	CG3168-PC	0070944	2	9, 10
yin-PA	0070601	4	-	CG2165-PB	0088198	1	9, 10
CG12773-PA	0070226	1	-	CG7888-PC	0075929	2	-
ubisnap	0072145	3	-	gp210-PA	0085380	1	-
CG11665-PA	0085408	2	-	Syntaxin 7	0078007	1	-
alpha-coatomer protein	0072693	1	10	CG17119-PA	0083750	1	-
5. Structural Molecule Activity							
Lasp-PB	0099495	14	10	Translationally controlled tumor protein	0081820	1	10
Cortactin-PA	0083441	5	10	CG32138-PA	0075517	2	10
Paxillin	0080752	7	10	Cdep-PA	0078479	1	8, 10
enabled	0085768	5	10	Cdep-PE	0110258	2	8, 10
Zyx102EF-PG	0088274	3	6, 10	coracle	0085697	2	10
polychaetoid	0088725	18	9, 10	kst-PC	0089204	1	10
Chd64-PB	0073126	1	10	CG6509-PB	0079831	4	10
rhea-PA	0076353	3	10	arouser	0077695	2	10
hu li tai shao	0085729	2	10	Tenascin major	0078161	2	10
bazooka	0074162	4	10	Ribosomal protein S24	0071846	1	10
Lam-PA	0078733	1	9, 10	Ribosomal protein S29	0081614	1	10
cheerio	0088478	1	10	Arp11-PA	0074529	1	10
Ribosomal protein S3	0083802	2	9, 10	WASp-PA	0084721	1	10
Septin-1	0076897	1	9, 10	CG30084-PF	0099801	1	10
scra-PA	0087985	1	10	unc-115-PC	0081572	1	10
string of pearls	0079500	1	10	Ankyrin	0088238	1	10
6. Transcription Regulator Activity							
cabeza	0073996	1	10	Not1-PB	0087628	1	-
CG11063-PB	0073665	1	10	ypsilon schachtel	0075759	1	10
brain tumor	0080702	1	6, 9, 10				
7. Translation Regulator Activity							
lethal (2) 01424	0100139	7	10	Argonaute 2	0075312	3	10
Eukaryotic-initiation-factor-4G	0088303	3	10	Srp72-PA	0083319	1	10
8. Enzyme Regulator Activity							
RhoGAP15B-PB	0110289	9	-	CG5522-PA	0086177	2	-
GTPase-activating protein 69C	0075713	3	10	Graf-PA	0073979	2	9
CG31048-PA	0084696	1	10	vacuolar peduncle	0073945	1	-
CG6838-PA	0078191	3	-	RhoGAPp190-PB	0074255	3	-
CG30372-PB	0099488	3	-	Ral guanine nucleotide exchange factor 2	0075535	1	-
CdGAPr-PA	0080851	4	10	centaurin gamma 1A	0080123	1	10
CG31158-PA	0083660	1	10	CG10188-PB	0080820	1	-
PP2A-B'-PD	0082974	1	-	Smad anchor for receptor activation	0071564	1	10

Table 1. Continued

protein	accession (FBpp)	pTyr[b] sites	additional grouping	protein	accession (FBpp)	pTyr[b] sites	additional grouping
			9. Catalytic Activity				
TER94-PA	0087479	1	10	CG9311-PA	0075450	2	-
bel-PA	0081374	9	10	Magi-PA	0071559	1	10
par-1-PD	0085648	4	10	Pi3K92E-PA	p0083348	1	-
Abl tyrosine kinase	0075116	3	10	RhoGAP71E-PB	0075320	3	10
CG3523-PA	0077343	1	10	X11L-PA	0074225	2	10
Fak-like tyrosine kinase	0086889	6	4, 10	skittles	0071491	1	-
CG32705-PA	0088747	7	10	purple	0088417	1	-
Phosphotidylinositol 3 kinase 68D	0075818	5	10	Neu3-PB	0099550	1	10
Ack-PA	0073067	3	10	Src oncogene at 42A	0085320	1	10
tre oncogene-related protein	0086731	1	-	CG6701-PA	0086733	2	10
CG10777-PB	0071084	1	10	CG7375-PA	0076445	1	10
CG30069-PA	0086659	5	-	DISCO Interacting Protein 2	0072420	1	-
CG8176-PB	0081543	2	10	CG15817-PB	0084764	1	-
flap wing	0071381	2	-				
			10. Binding				
protein–protein				**nucleic acid**			
Hepatocyte growth factor regulated tyrosine kinase substrate	0079677	4	-	sqd-PA	0082319	4	-
				encore	0073011	2	-
chico-PA	0071008	6	-	CG9705-PB	0075083	1	-
Downstream of kinase	0073119	3	-	translin-PA	0087398	1	-
Ras opposite	0079737	2	-	polyA-binding protein	0085915	1	-
SCAR-PA	0073371	3	-	Ataxin-2	0082555	1	-
bifocal	0074247	2	-	mushroom-body expressed	0078122	1	-
Suppressor of Cytokine Signaling at16D	0082110	1	-	quaking related 54B	0086079	2	-
CG6359-PA	0085235	1	-	Upstream of N-ras	0076384	1	-
Microtubule-associated protein 205	0082541	1	-	quaking related 58E-2	0071739	2	-
specifically Rac1-associated protein 1	0084390	1	-	vasa intronic gene	0080277	1	-
scribbled	0072188	1	-	IGF-II mRNA-binding protein	0073274	1	-
tamo-PA	0086727	1	-	Zn72D-PA	0075248	1	-
Prosap-PA	0082219	2	-	quaking related 58E-1	0071754	3	-
CG8863-PA	0078930	1	-	**others**			
Liprin-alpha-PA	0088340	2	-	Eps-15-PC	0072338	1	-
spri-PG	0088340	1	-	Spinophilin	0072831	2	-
rasputin	0082264	1	-	lethal (1) G0320	0071307	1	-
jdp-PA	0085043	1	-	Centrosomal protein 190 kDa	0082580	1	-
Dynamin associated protein 160	0081031	1	-	expanded	0077719	2	-
sprouty	0073004	1	-				

[a] A full listing of details from MS/MS data can be found in Supplementary Table S1 in Supporting Information. [b] Nonredundant pTyr sites.

shortcoming is false-positive identification resulting from nonspecific association and the usual contamination with the abundant immunoglobulin chains from the antibodies employed in immuno-affinity capture. To optimize identification of true substrates trapped by the DA mutant, the commonly adopted method of boiling the immunocomplex in SDS-PAGE loading buffer for direct gel running and Western blot analysis should be avoided. Not only would it result in an abundance of dissociated IgG heavy and light chains from the supposedly immobilized antibodies, many other proteins would be equally recovered from the immunocomplexes of wild-type and DA mutant alike (Figure 3A), which argues against a desirable specificity.

For a more specific elution, vanadate (VO_4^{3-}), which normally functions as a reversible inhibitor of PTPs[34] and is generally believed to interact with PTPs as a transition state analogue,[34,35] was examined for its ability to compete with substrates already trapped in the catalytic pocket of dPTP61F DA mutant. As demonstrated by immunoblotting, it was found that more pTyr proteins could be eluted from the immunocomplex with increasing concentrations of sodium orthovanadate (Na_3VO_4, dissolved in water for releasing of VO_4^{3-} as a competitive substrate) in the incubation buffer (Figure 3B). We found that 5 mM Na_3VO_4 was found to be optimum, since higher concentrations of Na_3VO_4 (up to 10 mM) did not further improve the efficiency of eluting potential substrates (data not shown). Under these optimized experimental conditions, only a limited number of nonsubstrate proteins were dissociated from the WT form of dPTP61F (Figure 3C). Furthermore, the abundant proteins such as IgG were not found in the eluted fractions (Figure 3C). The clean background and the significant intensity of protein bands afforded by the eluates from the immunocomplex of the DA mutant but not the WT suggests that they may act as true substrates of dPTP61F. These protein bands (A1−A7) were individually excised from the gel, tryptic-digested, and subjected to LC−MS/MS analyses, leading to identification of 9 potential substrates (Table 2 and Supplementary Table S2 in Supporting Information). With the exception of A4 and A6 bands (Hem and Hsc-70-4), all other identified proteins were related to known tyrosine phosphorylation signaling pathways and carried at least one single pTyr peptide detected using the pTyr proteomic approach (Table 2).

Identification of Substrates of dPTP61F from Enriched pTyr Proteome Subset of S2 Cells by Mass Spectrometry. To further increase the opportunity for trapping and identifying

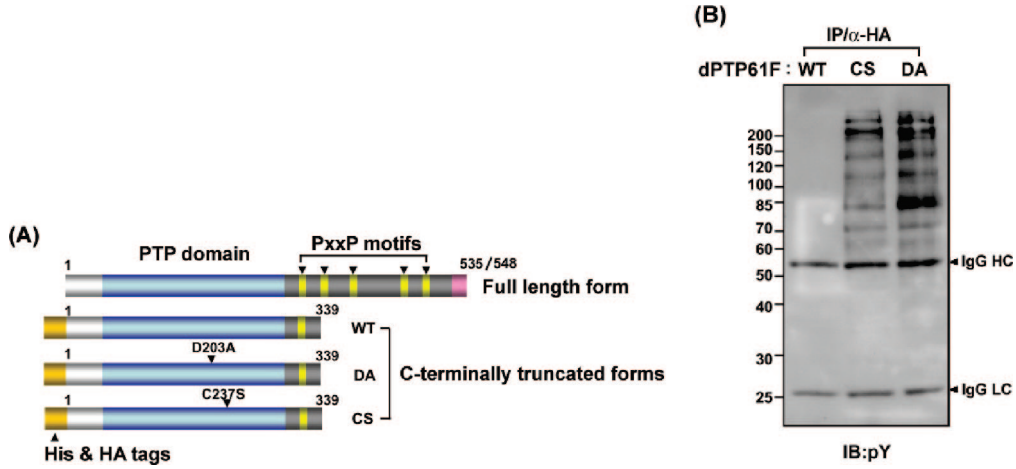

Figure 2. (A) Schematic view of the full-length forms (amino acid 1–535 for the nucleus-localized form (M_r 61 kDa), and 1–548 for the membrane-bound form (M_r 62.1 kDa)), and three C-terminally truncated variants (amino acid 1–339) of dPTP61F. The full-length forms of dPTP61F are characterized by a PTP domain (blue), five proline rich motifs (PxxP, yellow), and an alternative splicing region (pink) at the C-terminal tail. Truncated constructs, which were used in this study, contain His and HA tags (orange) at the N-terminus and one proline rich motif near the C-terminal end. The wild-type (WT) form retains an active PTP domain, whereas a point mutation in the catalytic domain at aspartate 203 to alaine and cysteine 237 to serine were introduced in the DA and CS mutant forms, respectively. (B) An aliquot of total lysates (2 mg) prepared from S2 cells treated with pervanadate was incubated with HA-tagged WT, DA, or CS mutant form of dPTP61F (8 μg). After immunoprecipitation of dPTP61F with anti-HA-tag antibody, the associated proteins were eluted by SDS-PAGE sample buffer, resolved by SDS-PAGE, and then visualized by immunoblotting with anti-pTyr antibody.

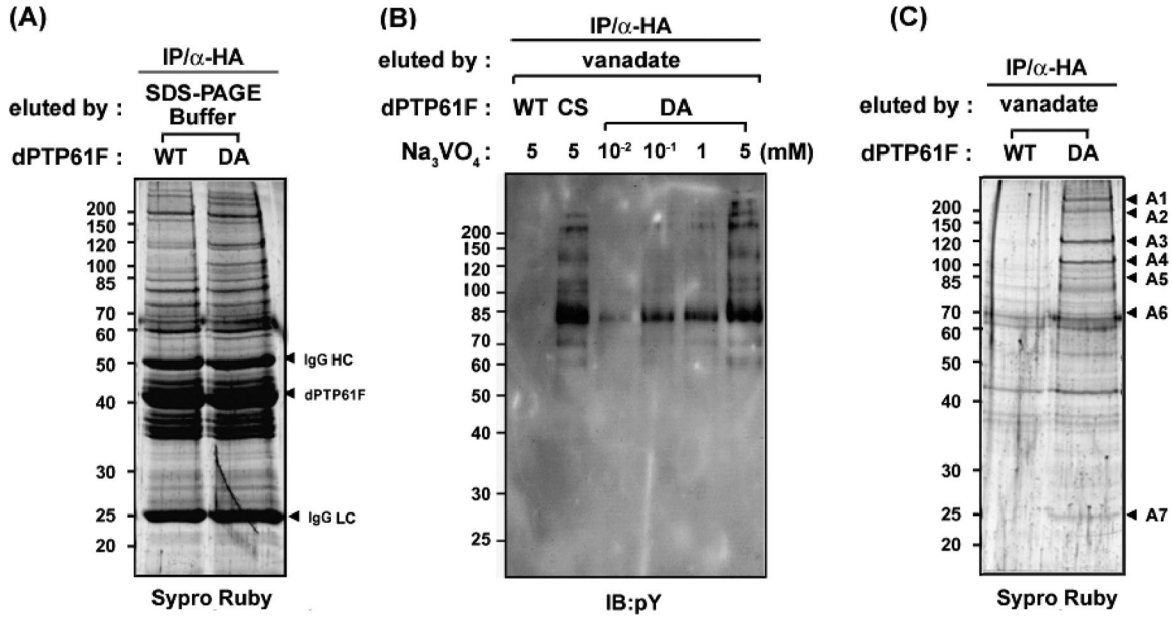

Figure 3. (A) An aliquot of total lysates (20 mg, corresponding to 2 $\times$ 10^7 cells) prepared from S2 cells treated with pervanadate was incubated with HA-tagged WT form or DA mutant form of dPTP61F (80 μg). After immunoprecipitation of dPTP61F with anti-HA-tag antibody, the associated proteins were eluted by SDS-PAGE sample buffer, resolved in a SDS-PAGE gel, and then visualized by SyproRuby staining. Arrowheads indicate the position of IgG heavy chain (IgG HC), truncated forms of dPTP61F, and IgG light chain (IgG LC) in an SDS-PAGE gel. (B) An aliquot of total lysates (2 mg) prepared as described in (A) was incubated with an HA-tagged WT, CS, or DA form of dPTP61F (8 μg). After immunoprecipitation, potential substrates of dPTP61F were eluted from the immunocomplexes by vanadate (10^{-2} − 5 mM, as indicated in the figure), and then visualized by immunoblotting with anti-pTyr antibody. (C) An aliquot of total lysates (20 mg) prepared as described in (A) was incubated with an HA-tagged WT form or DA mutant form of dPTP61F (80 μg). dPTP61F was immunoprecipitated, and potential substrates were eluted by vanadate (5 mM), resolved in a SDS-PAGE gel, and then visualized by SyproRuby staining. Those proteins (bands A1−A7) eluted specifically from the complex of DA mutant form of dPTP61F were excised from the gel, in-gel digested with trypsin, and then subjected to MS-based analyses.

more of the pTyr substrates, the same experimental approach was used with the immuno-affinity enriched pTyr proteome subset (Figure 4A). Our rationale was that some of the potential substrates may be preferentially enriched by the anti-pTyr antibody PT66 we used and would, thus, present a greater opportunity of capture by the DA mutant, especially in the absence of most other non-pTyr cellular proteins. Potential substrates eluted by 5 mM Na_3VO_4 from the immunocomplex of the DA mutant but not the WT were similarly resolved by SDS-PAGE (Figure 4B), and identified by LC−MS/MS analyses of the tryptic digests from the excised protein bands (B1−B8). In total, 16 proteins were identified (Table 2 and Supplementary

Table 2. Summary of Potential Substrates of dPTP61F

accession (FBpp)	protein description	sample preparation[a]	unique peptide	protein score	MW	phosphotyrosine site[b]
			Signal Transducer Activity			
0110350	Down syndrome cell adhesion molecule	A1	12	371	224 kDa	^{1656}GGQKDVYpYpDVVYpNQTMGPGATLDKR^{1680} ^{1686}DELGYpIAPPNRK^{1697} ^{1698}LPPVPGSNYpNTCDR^{1711} ^{1730}RNPNLYpEELK^{1739} ^{1978}GNYpGAVKR^{1985}
0079244	PDGF- and VEGF-receptor related	A2	4	105	170 kDa	^{990}MSDNYpELHR^{998}
		B3	19	810		^{999}DTNGGGLKYpANVGFPIHSYpINEPHNNNTQPPTHR^{1032} ^{1048}TGSGTATYpSYpDR^{1059} ^{1153}SMYRGDNYpKK^{1162} ^{1153}SMYpRGDNYpKK^{1162c} ^{1261}FANMLGEDVASHYpLDLNNPYpMQSNIEYpMKK^{1290} ^{1291}QSTDYpLALMGSPDELAPAAPR^{1311c} ^{1312}YpVNGHIVPDIR^{1322} ^{1435}QQYpVTPTPSPR^{1445} ^{1452}LNGEPSENYpVNMKPPRK^{1468} ^{1474}TTTGGGGAAAGASTEAFSNPSYpQPLSTVNEK^{1504}
0082371	Abelson Interacting Protein	B8	6	263	51 kDa	^{235}TPPVVNPPQVPSHYpAPNYpPIGHPK^{258}
0086812	Downstream of receptor kinase	A7	3	119	24 kDa	^{199}KGIFPATYpVTPYHS^{211}
			Strcture Molecular Activity			
0088725	Polychaetoid	B1 and B2	12	415	230 kDa	^{380}GSSGGAAQEDFYpSSRR^{395} ^{395}RQLYpEER^{401} ^{887}AVVQDDVQAEYpITR^{900} ^{983}SVPNANGRPLPPTGQSHEYpGR^{1003} ^{1065}TDYpGKYSR^{1072} ^{1073}NNSVTQADYpTKLPK^{1086} ^{1271}GSAFELYpR^{1278} ^{1302}YpDDYYNMPPPAAHPSQGHHMQR^{1323c} ^{1330}YPHERPPHAQDPNYpYGHYpGTSR^{1351c} ^{1330}YPHERPPHAQDPNYYpGHYpGTSR^{1351c} ^{1330}YpPHERPPHAQDPNYYGHYpGTSR^{1351c} ^{1435}GVQRPYYPDEYPTYpPAGFEER^{1455} ^{1509}MPHHQDNGYGYDQYDLYpANR^{1528c} ^{1664}EFSPQSSSSQSEASNSEAIYpQSRR^{1687} ^{1636}NHLYpQSKR^{1648} ^{1613}FYPGAANEQTEEEPLYpQSRR^{1633} ^{1703}DQIYpQTRR^{1709}
0099495	Lasp	B6	7	252	74 kDa	^{80}IQSNVKYpHADFEK^{92} ^{115}HISNVAYpHGDLEK^{127} ^{250}AQQQQLHDPYpAHYpQQPQALR^{269} ^{411}RSAASVVAYpDGNSK^{424} ^{425}QQVAAGPGAAQNHLQQLYpASPNYpAAVTPSENSINVK^{460} ^{485}IADYpDPLTDGPR^{496} ^{504}SSTTLVYpSSEPR^{515} ^{516}GNQGGNSVYpPK^{526} ^{527}RIGSVSDIDPANGIYpGSLTAAEQAHQQQK^{555} ^{556}HQQYYpQQVQMMQQQEHPPQQQQMR^{579c} ^{556}HQQYpYQQVQMMQQQEHPPQQQQMR^{579c} ^{580}QQPSYpSSLQEK^{590}
			Enzyme Regulator Activity			
0110289	RhoGAP15B	A2	3	74	169 kDa	^{12}RLYPELRPANYpENIEIR^{28}
		B3	5	146		^{174}YpASNASLDCSESSHSGK^{190} ^{434}KNYpEHIELR^{442} ^{69}LILQENNSDSQQPIYpGPDANENVQEPVYpATPRPAPR^{104c} ^{668}AKPYpLSRMPESANKASGPVDLASASR^{693} ^{1214}KSDELIYpSQK^{1223} ^{1259}YpYAAAETLKDAVK^{1271c} ^{1259}YYpAAAETLKDAVK^{1271c}
			Catalytic Activity			
0073067	Ack	B4	2	72	118 kDa	^{973}LQQQQQLDGAHQLYpAPVPSDYGR^{996} ^{630}LQPPPYpQMPPTYpSNTMEFVQK^{650}
0075116	Abl tyrosine kinase	B3	2	57	171 kDa	^{516}LMRDDTYpTAHAGAK^{529} ^{928}YpGTLPK^{933} ^{1495}LTIYpATPIAK^{1504}
			Translation Regulator Activity			
0088303	Eukaryotic-initiation-factor-4G	B3	1	71	149 kDa	^{919}STSHSKYpTQQAPPTR^{933} ^{738}SSSAYpGGSHSQRGDNGNLR^{756} ^{699}NEQLSAQYpFGTLSSTTPGGSQGGSGK^{724}

Table 2. Continued

accession (FBpp)	protein description	sample preparation[a]	unique peptide	protein score	MW	phosphotyrosine site[b]
			Binding (major in protein–protein)			
0076142	SH3PX1	B7	7	293	63 kDa	ND
0082541	Specifically Rac1-associated protein 1	A3	3	107	149 kDa	^{1263}CFPPPQHPSVISSSSHYpQDPQK^{1287}
		B4	28	1348		
0078162	HEM	A4	24	905	129 kDa	ND
		B5	26	1075		
0082264	Rasputin	A5	2	74	74 kDa	^{676}GAGNGQSGGGNYpGRR^{690}
0082514	Hsc-70-4	A6	3	145	71 kDa	ND
		B7	2	79		
0079737	SCAR	B6	10	527	67 kDa	^{220}ALVHGETLMPNNVIYpR^{235} ^{236}TPNSMVNEEAGYpGNMGVYpDTRPPRPNSpIELNR^{267c}
0088691	Hepatocyte growth factor regulated tyrosine kinase substrate	A4	3	66	85 kDa	^{311}EYpHQQPEEATNPELAK^{326} ^{311}EYHQQPEEATNPELAKYpLNR^{330} ^{229}SGPRPADSELPAEYpLNSTLAQQVQTPAR^{256} ^{332}SYpWEQR^{336}
			Binding (all others)			
0081675	Fragile X related	B6	2	60	76 kDa	ND
0085915	poly(A)-binding protein	B7	5	172	69 kDa	^{385}MQQLGQIYpQPNAASGFFVPTLPSNQR^{410}
0073274	IGF-II mRNA-binding protein	B7	2	67	62 kDa	^{10}LNNNISNNYpYQQK^{22c}

[a] The sample prepared for substrate trapping experiments were from (A) whole cell extractsand, and (B) immuno-affinity enriched pTyr proteome subset from pervanadate treated S2 cells. The numbers refer to corresponding protein bands as annotated in Figures 3B and 4C. [b] Phosphorylaiton sites were detected in pTyr proteomic apporches (details as in Supplementary Table S1 in Supporting Information). [c] Delta ions scores of pTyr peptides were <0.4. ND, not detected.

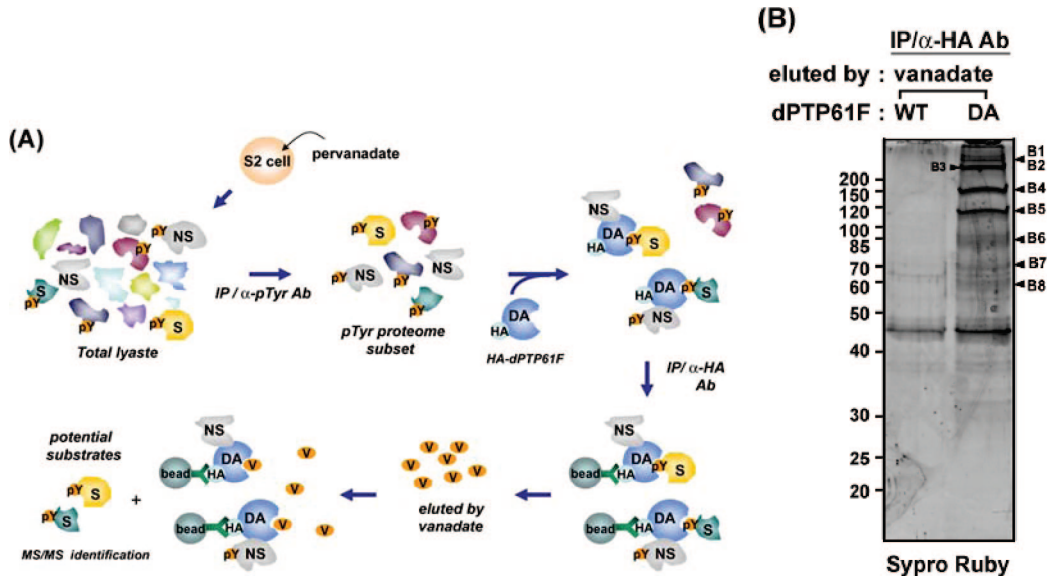

Figure 4. (A) The workflow of large-scale substrate trapping experiment using a pTyr subproteome as a source for identifying potential substrates of a PTP. Details are described in the text. NS, nonspecifically associated protein; S, potential substrate; V, vanadate; DA, DA mutant form of dPTP61F; HA, HA tag. (B) A pool of tyrosine phosphorylated proteins were immunoprecipitated from total lysates prepared from 10^8 S2 cells stimulated with pervanadate. These proteins were eluted from the immunocomplexes by phenylphsophate and then incubated with an HA-tagged WT form or DA mutant form of dPTP61F. After immunoprecipitation with anti-HA antibody, potential substrates of dPTP61F were eluted by vanadate (5 mM), resolved in a SDS-PAGE gel, and then visualized by SyproRuby staining. Those proteins (bands B1−B8), eluted specifically from the complex of DA mutant form of dPTP61F, were excised from the gel, in gel-digested with trypsin, and then subjected to MS-based analyses.

Table S2 in Supporting Information). Among them, 11 were not found by trapping experiment on total lysates (Table 2). Conversely, four out of the nine potential substrates identified from the total lysates were not reproducibly captured from the enriched pTyr proteome subset. Taken together, 20 unique proteins were identified from the two data sets, with a majority (16) having one or more pTyr peptides identified by the pTyr proteomic approach (Table 2). Using the emPAI or Mascot protein score as an indicator of abundance,[36] the three most intensely stained bands (B3, B4, and B5) correspond to PDGF/VEGF-related protein (FBpp0079244), specifically Rac1-associated protein 1 (FBpp0082541), and HEM (FBpp0078162), respectively. These were also captured by the DA mutant from total lysates and ran as bands A2, A3, and A4, respectively.

Discussion

Despite its well-recognized importance, few studies have attempted to characterize tyrosine phosphorylation-dependent signaling cascades in *Drosophila* cells and embryos. In several widely accessed phospho-databases such as PhosphoELM (http://phospho.elm.eu.org) and Phosphosite (http://www.

phosphosite.org), data set for *Drosophila* pTyr protein is not included. Likewise, only a few records of fly pTyr proteins can be found in the Swiss-Prot database. Very recently, a phosphoproteome resource, PhosphoPep (http://www.phosphopep.org), has been established. This database is devoted entirely to *Drosophila* phosphoproteins based on the phosphoproteomic data sets contributed by a single research team using *Drosophila* Kc167 cells as the sole biological source.[19] At a PeptideProphet probability score of >0.9 and a dCn threshold of 0.1, a total of just over 10 000 distinct phosphorylation sites were reported, of which phospho-Ser, -Thr, and -Tyr represent 78%, 19%, and 3%, respectively. In other words, approximately over 300 pTyr sites were identified with reasonable confidence through data sets collated from multiple analyses of enriched phosphopeptides from total lysates of Kc 167 cells grown under several media conditions including nutrient rich, nutrient depleted, and nutrients containing inhibitors of protein phosphatase 1 and 2A, but not pervanadate, as used in this work.

Our current study, therefore, distinguishes itself from others by virtue of being the most comprehensive cataloging of *Drosophila* protein pTyr sites undertaken to date. At a stringency comparable to the aforementioned studies,[19] we reported a total of over 560 unique pTyr sites. This achievement was not due to superior MS instrumentation but due to the fact that we focused specifically on pTyr, using anti-pTyr antibody enriched pTyr proteome subset as our starting material for proteomic analyses. At present, it is unclear how the pTyr state and sites of S2 cells vary from those of Kc167 cells. Although they are both embryo cells, they have different derivation histories. Furthermore, the culture medium used may also affect the overall phosphorylation level. In addition, we used pervanadate in the culture to specifically inhibit PTPs. Another level of complexity is associated with uncertainty in site specific determination and the different statistical means in assessing the confidence level in assignment. Given these considerations, it is apparent that there are pTyr sites in S2 cells reported in this study that were also identified in Kc167 cells and elsewhere, as well as a significantly larger proportion that was reported for the first time. A systematic site-by-site comparison seems irrelevant at this stage. Suffice to point out here that, of the 136 pTyr sites that can be identified from the PhosphoPep database at a PeptideProphet probability score cutoff of 0.99, corresponding to 0 false positive (FP) in decoy database (DD), and a dCn greater than 0.1, only 29 pTyr sites are also found in our own data set of 436 pTyr sites generated using a comparable stringency criteria of 0% FP in DD and a normalized delta ion score (ΔS) $\geq$ 0.4 as threshold. Given the genetic and epigenetic variations and the somewhat surprisingly low overlapping, any publicly available database of pTyr may best serve as references for hypothesis driven or targeted investigations applied to each specific system, as suggested by the authors assembling the PhosphoPep database and informatic tools.[19]

In our case, our pTyr proteomic studies aimed specifically at cataloging as many of the pTyr sites carried on an enriched pTyr proteomic sample from S2 cells used to trap dPTP61F substrate. Although the substrate trapping technique has been used extensively since it was first introduced more than a decade ago,[12] the high rate of false positives in mis-regarding associated proteins as true substrates has always been a primary concern. An important contribution of this study is its advanced technique using vanadate as eluting reagent. Because of vanadate's unique chemical characteristics which allow it to act as a competitive inhibitor in the catalytic pocket of PTP, it does not dissociate those proteins interacting nonspecifically with other regions of a PTP. In the conventional method, the SDS-PAGE loading buffer is used to release as many interacting proteins including those associated with both DA (or other mutant) and WT forms of PTP, which are not true PTP substrates captured through specific interactions with the catalytic domain of a PTP DA mutant. Our results indicate that the use of vanadate significantly increased the reliability and specificity of the substrate trapping technique, allowing us to identify as many as 20 potential substrates of dPTP61F (Table 2).

Two parallel experimental approaches were adopted in which either the total cell lysates or the enriched pTyr proteome subset was used as the sample source for substrate trapping. *A priori*, it was expected that the latter would constitute a better source material as it was largely devoid of most non-pTyr proteins otherwise present in high abundance. More potential substrates specifically eluted by vanadate were indeed recovered. However, we found that the 16 unique proteins thus identified did not include all the 9 identified from total cell lysates. One contributing factor for this miss may be that the anti-pTyr antibody (PT66) we used failed to retain all pTyr proteins equally well in the initial step. During the MS-based identification steps, a gel-based approach has the added advantage of limiting visualization and analysis to only protein bands that are specifically present in the eluates from the immunocomplex of DA mutant and not the WT, without resorting to quantitative labeling which would require that true positives be distinguished from the background in a shotgun analysis. In either case, the recovery of substrates from the immunocomplex proves to be too low for successful identification of pTyr sites by MS analysis, which is a common reported shortcoming for all MS-based substrate trapping.[15,16] pTyr sites on the identified potential substrates could instead be inferred from the catalogue of pTyr sites mapped independently for the pTyr subproteome under investigation, as was done in this study. It is, however, clear that the identified pTyr sites for any potential substrate may be incomplete and that the experimental approach does not allow distinguishing the actual pTyr site that was acted on by the PTP in question.

In a system such as *Drosophila* in which the extent of pTyr was mostly under explored, the mere identification of pTyr sites alone would provide significant insight into which mammalian orthologs were similarly tyrosine-phosphorylated. In general, the nature of MS-based phosphoproteomic data is such that positive identification is more informative than inference based on negative results, that is, failure of detection, which may be partially caused by a myriad of factors, not necessarily due to its actual absence. In the context of substrate trapping, all potential substrates identified naturally required independent validation using a different means and the pTyr proteins that were direct substrates versus those that were pulled down due to tight association with these primary targets needed to be distinguished. We are, nevertheless, confident that most, if not all, of the proteins identified by our substrate trapping method directly interact with the catalytic pocket of dPTP61F, or tightly associate with those substrates that do.

On the basis of these considerations, it is of interest to note that a receptor tyrosine kinase (PDGF/ VEGF receptor related) and a cytosolic tyrosine kinase (Abl tyrosine kinase) were among those identified potential substrates (Table 2), indicating that dPTP61F may act to down-regulate tyrosine phosphory-

lation-dependent signaling by directly dephosphorylating tyrosine kinases. PTP1B, the human ortholog of dPTP61F, has been shown to act on PDGF receptor[37] and $p210^{Bcr\text{-}Abl}$,[38] which is an oncogenic form of Abl. Thus, dPTP61F and PTP1B may play similar roles in conserved signaling pathways throughout the evolution. Our data further indicated that several important components constituting the SCAR/WAVE complex, including Abelson interacting protein (Abi), specifically Rac1 associated proteins (Sra-1), SCAR/WAVE itself (Table 1), and Kette/Nap1/HEM (Gu, H. Y. and Meng, T. C., unpublished data) were tyrosine-phosphorylated and could be isolated as potential substrates of dPTP61F (Table 2).

It has been demonstrated that the assembly of Abi/Sra-1/Kette complex promotes the stability of SCAR/WAVE, thus, leading to the activation of Arp2/3 for the generation of actin-based protrusions in mammalian and fly[39–42] cells. Recent studies have further indicated that tyrosine phosphorylation of mammalian SCAR/WAVE by Abl is required for actin polymerization to occur at the cell periphery.[43,44] In *Drosophila* cells, the coordinated action of Abl and dPTP61F has been recently shown to regulate the pTyr level of Abi,[45] which in turn regulates the activation of SCAR/WAVE complex. Our data now suggest that dPTP61F may suppress the kinase activity of Abl through tyrosine dephosphorylation, as well as possibly through acting directly on Abi, Sra-1, and/or SCAR/WAVE. We propose that the dPTP61F-dependent tyrosine dephosphorylation of those components is needed to counteract Abl-mediated activation of SCAR/WAVE complex, thus, according dPTP61F a biological role in controlling actin polymerization and remodeling in *Drosophila* cells. Further investigations are required to examine whether all the aforementioned components are direct substrates of dPTP61F *in vivo*.

Abbreviations: MS, mass spectrometry; PTK, protein tyrosine kinases; PTP, protein tyrosine phosphatases; pTyr, phosphotyrosine; TC-PTP, T cell PTP; dPTP61F, *Drosophila* PTP61F; IMAC, immobilized metal ion affinity chromatography; DA, D203A mutant form of dPTP61F; CS, C237S mutant form of dPTP61F; GO, gene ontology; Abi, Abelson interacting protein; Abl, Abl tyrosine kinase; Sra-1, Specifically Rac-1 associated protein 1.

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Supporting Information Available: All details about identified pTyr peptides from MS/MS data can be found in Supplementary Table S1. Supplementary Table S2 lists the matched peptides of identified potential substrates of dPTP61F. This material is available free of charge via the Internet at http://pubs.acs.org.

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