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A Human Tyrosine Phosphatase Interactome Mapped by Proteomic Profiling

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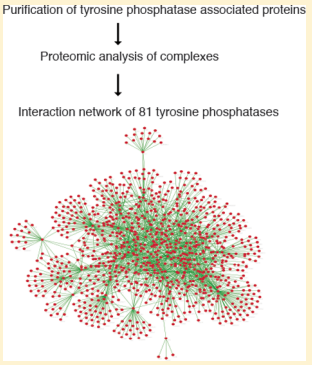
Supporting Information

ABSTRACT: Tyrosine phosphatases play a critical role in many cellular processes and pathogenesis, yet comprehensive analysis of their functional interacting proteins in the cell is limited. By utilizing a proteomic approach, here we present an interaction network of 81 human tyrosine phosphatases built on 1884 high-confidence interactions of which 85% are unreported. Our analysis has linked several phosphatases with new cellular processes and unveiled protein interactions genetically linked to various human diseases including cancer. We validated the functional importance of an identified interaction network by characterizing a distinct novel interaction between PTPN5 and Mob1a. PTPN5 dephosphorylates Mob1a at Y26 residue. Further, we identify that PTPN5 is required for proper midbody abscission during cytokinesis through regulation of Mob1a dephosphorylation. In conclusion, our study provides a valuable resource of tyrosine phosphatase interactions, which can be further utilized to dissect novel cellular functions of these enzymes.



KEYWORDS: *phosphatase, interactome, multiprotein complexes, PTPN5, Mob1a, cytokinesis*

INTRODUCTION

Protein phosphorylation is a fundamental post-translational modification employed by an organism to regulate a plethora of biological processes such as cell proliferation, apoptosis, migration, differentiation, metabolism, and many more.[1] Protein kinases add one or more phosphate groups to target proteins, while phosphatases remove the phosphate group from their target proteins.[2] Substrate phosphorylation/dephosphorylation often acts as a switch to regulate activity of key cellular proteins during the signal transduction process.[3] Thus, any alteration in the equilibrium between kinases and phosphatases results in development and progression of various diseases, such as cancer, neurodegenerative, or inflammatory disorders.[2,4]

Depending on the substrate residue they act upon, protein phosphatases are broadly classified into two classes such as (A) tyrosine phosphatases and (B) serine/threonine phosphatases.[5] In humans, protein tyrosine phosphatases (PTPs) constitute 107 members,[6] nearly analogous in range to 90 protein tyrosine kinases, indicating a similar degree of intricacy between the two enzyme classes. Protein tyrosine phosphatases are further divided into four categories: the class I, II, and III Cys-based PTPs and the Asp-based PTPs containing EYA phosphatases. Class I is further distributed into two subfamilies (i) classical PTPs (receptor and nonreceptor type) and (ii) dual specificity protein phosphatases (DSPs/VH1-like) that consist of Map kinase phosphatases, atypical DSPs, slingshots, PTEN group, CDC14 phosphatases, the phosphatases of regenerating liver (PRLs) and the myotubularins (MTMRs) all of which dephosphorylate both Ser/Thr and Tyr residues. Class II PTP consists of a unique tyrosine-specific low molecular weight phosphatase. Class III PTPs contain CDC25 phosphatases, which play a critical role in cell cycle progression.

Historically kinases have been widely studied and their importance is well established; however, recent studies made it eminently clear that phosphatases play an equally important role in different cellular processes.[7] Despite their essential role in many cellular processes and pathogenesis, a comprehensive analysis of tyrosine phosphatases and their functional interacting proteins in a cell is limited. Hence, we performed a systematic proteomic study to identify protein−protein interactions of 81 available protein tyrosine phosphatases in human cells. Our protein−protein interaction map has linked phosphatases to a multitude of biological processes and revealed a novel association of protein phosphatases to protein complexes. Also, the phosphatase interaction network connects to proteins associated with various human diseases, in particular enriched with cancer-associated proteins. Finally, this study provides an important resource for future studies to assign new functions for phosphatases and to devise approaches to perturb phosphatase−protein interactome for intervening various human diseases.

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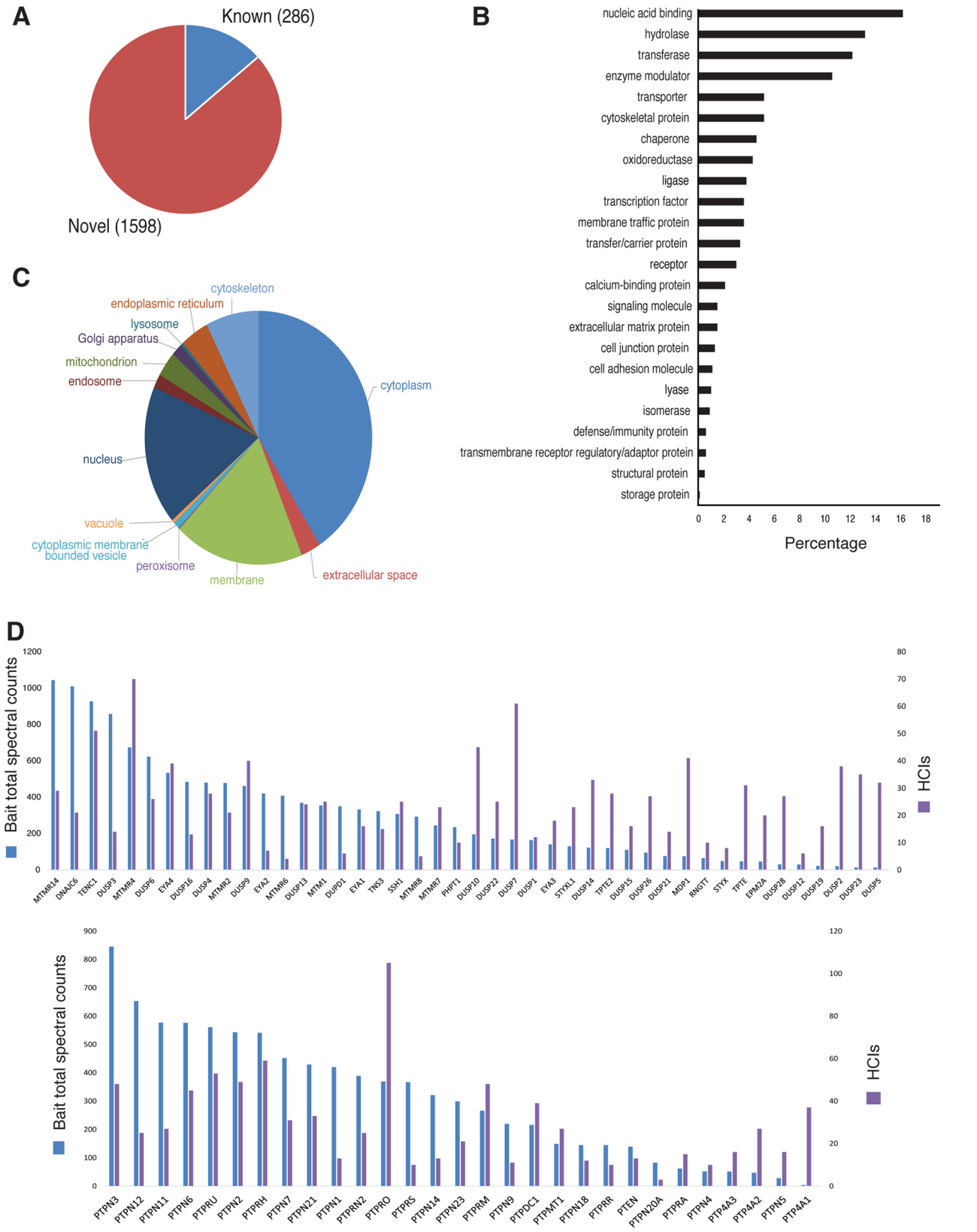

Figure 1. Analysis of human protein tyrosine phosphatase interactome. (A) High confidence interactions mediated by phosphatases and interacting proteins was compared with known interactions, and the distribution of known and novel interactions was plotted. (B) Interactors were analyzed by PANTHER and their GO distribution into various functional protein classes, and subcellular localization (C) was shown. (D) Expression of each phosphatase (represented by bait total spectral counts, blue) and HCIs (purple) found in respective phosphatase purification was plotted in a clustered graph.

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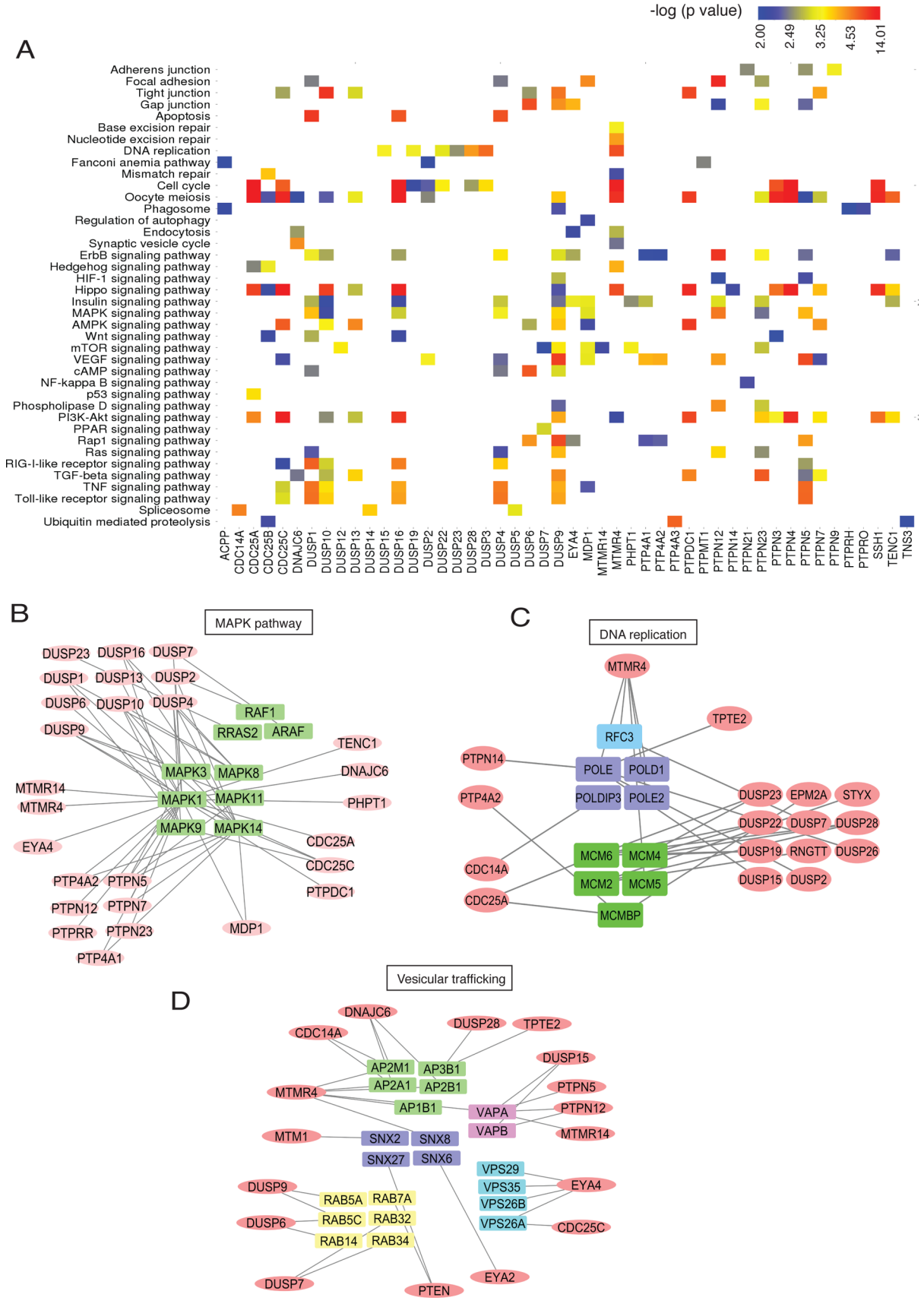

Figure 2. Association of phosphatases with pathways and cellular processes. (A) KEGG pathway mapping was performed using the interaction data set and the pathway enrichment was plotted for each phosphatase. Enrichment *p*-values were calculated using the Fisher test and further corrected by the FDR method. (B) Interaction network of phosphatases and the proteins associated with MAPK pathway, (C) DNA replication, and (D) vesicular trafficking.

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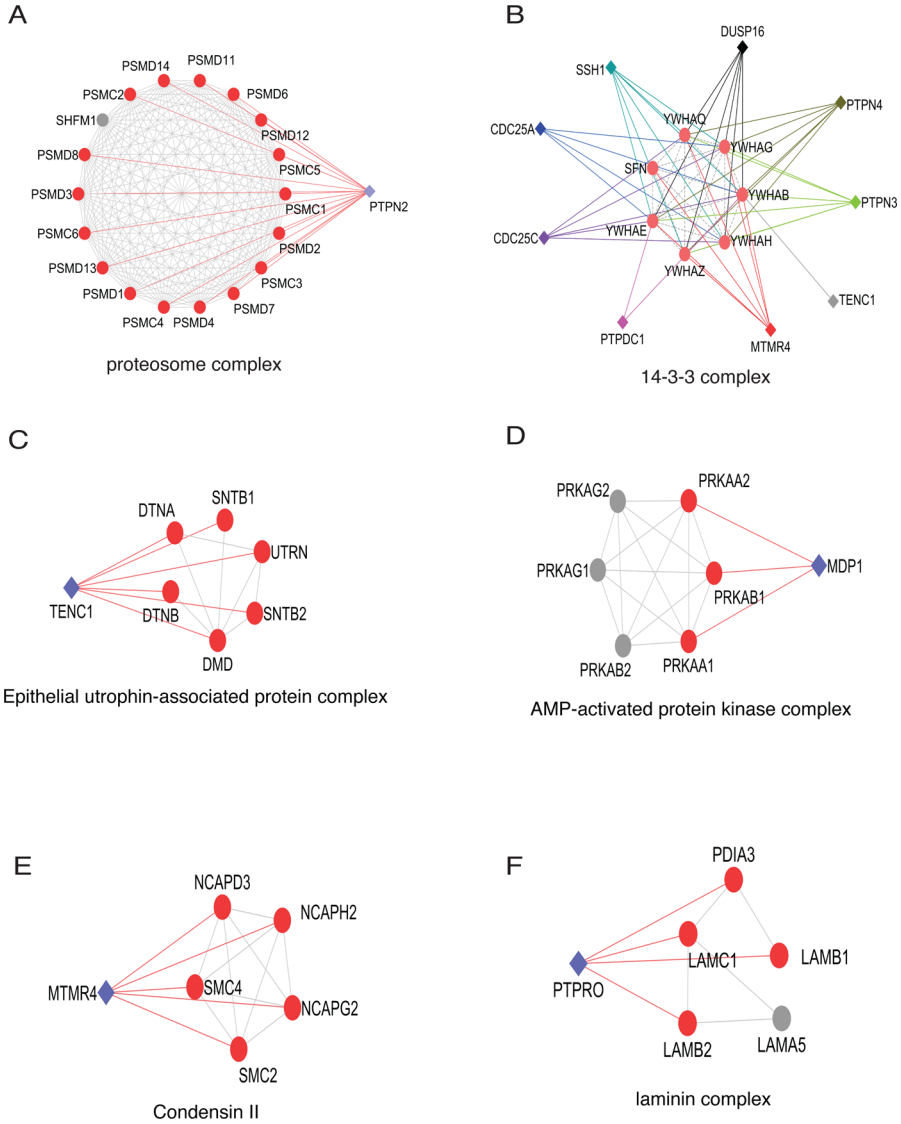

Figure 3. Association of phosphatases to multiprotein complexes. (A–F) The interactors from each phosphatase was searched against the COMPLEAT database and the representative phosphatase–multiprotein complexes were shown.

RESULTS

Systematic Analysis of Human Protein Tyrosine Phosphatase Interactome

To establish a human phosphatase protein interaction network, we performed a systematic proteomic study using tandem affinity purification followed by mass spectrometry analysis (TAP-MS). We cloned 81 human phosphatases (Table S1) in a gateway compatible triple tagged (SBP-Flag-S protein) vector and each of them was individually expressed in HEK293T cells. Protein complexes were isolated after two rounds of affinity purification by using streptavidin binding protein and S protein tag, and the interacting proteins were identified by using LC–MS/MS analysis (Figure S1A). A total of 41 872 interactions were obtained from 81 phosphatase purifications (Table S2). To filter out the nonspecific interactors we used CRAPOME[8] tools to compare out data set against control GFP, eight other nonphosphatase purifications and CRAPOME control purifications. CRAPOME analysis generated four different scores Empirical Fold Change Score (FCA and FCB), Saint Score (SS), Interaction Specificity Score (IS) and a CompPASS WD Score. By using a SAINT score cut off of 0.8, FCA> 3, FCB > 2.5, IS > 1, and WD score >1, we identified 1884 high confident interactions (HCIs) mediated by 916 proteins (HCIPs) for 80 purified phosphatases (Table S3). A comparison of our data with known interactions revealed 286 (~15%) known interactions and 1598 (~85%) novel interactions in the list (Figure 1A). Two recent studies[9,10] have profiled the interaction network of human phosphatases by using the proximity based interaction approach and affinity purification approach. Interestingly, we found only a very small overlap in the interactors in our data compared to these two published data sets (Figure S1B,C). The limited percentage of overlap between our data from the earlier published interactome data set might be due to the differences in the cell lines used, purification approaches, varied affinity tags, and the position of tag (N-terminus vs C-terminus) in the protein.

Functional classification of HCIPs using PANTHER[11] indicated wide distribution of interactors in diverse protein classes (Figure 1B), and varied cellular localization (Figure 1C) clearly suggesting that human protein tyrosine phosphatases play a role in wide variety of biological processes. Although

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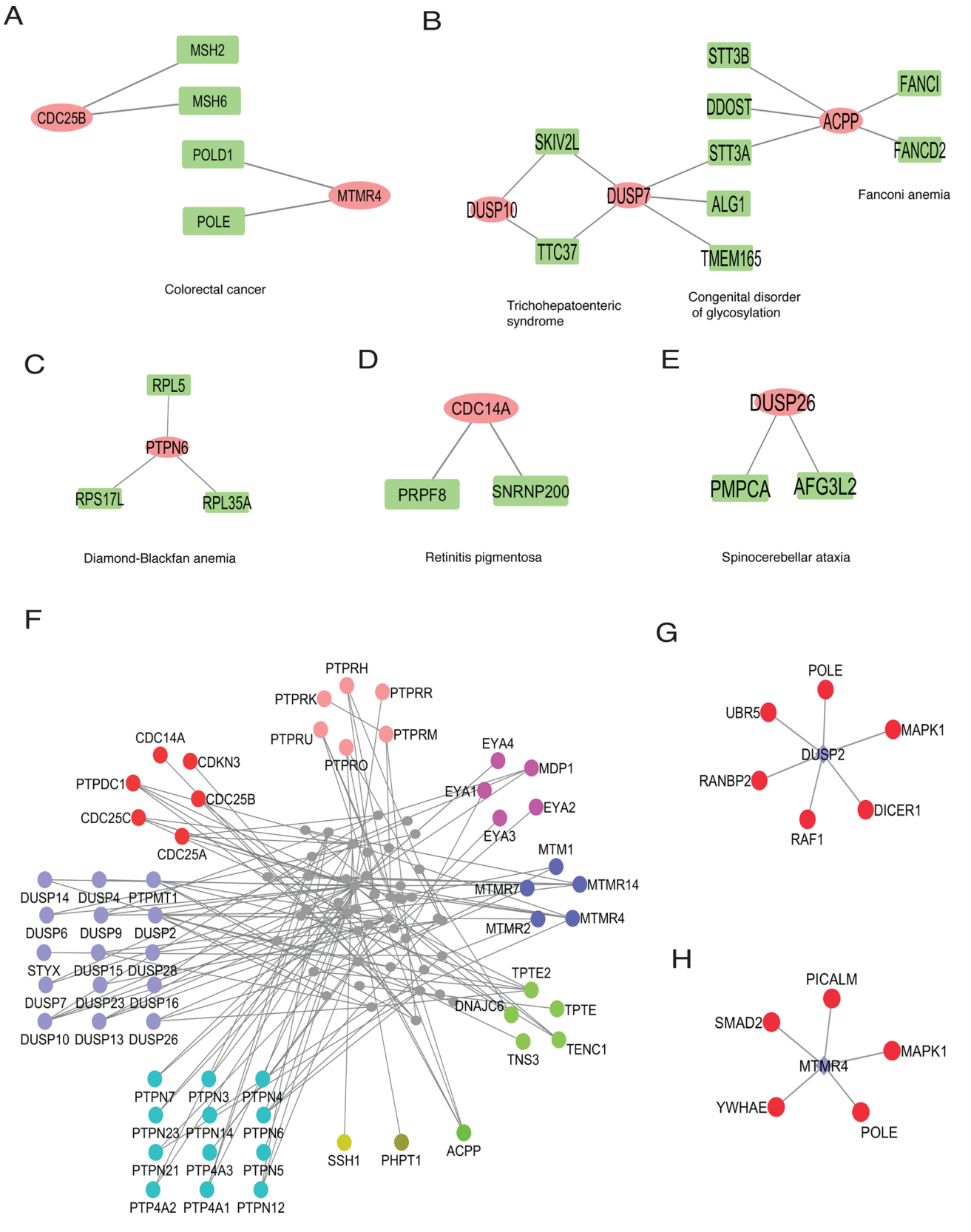

Figure 4. Linking protein phosphatase interactome to diseases phenotypes. (A) Interaction network of phosphatases and the proteins associated with colorectal cancer, (B) trichohepatoenteric syndrome, congenital disorder of glycosylation, Fanconi anemia; (C) Diamond-Blackfan anemia; (D) retinitis pigmentosa; and (E) spinocerebellar ataxia (from OMIM database) built by cytoscape is shown. (F) Interaction network of phosphatases and cancer-linked proteins (from COSMIC database) built by cytoscape. (G) Cancer-linked proteins in complex with DUSP2 and (H) MTMR4 is indicated.

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expression of SFB tagged phosphatases varied over a wide range, the number of HCIs did not corelate with the bait expression (Figure 1D), thus highlighting the unbiased nature of the list of interactors identified in our study. A correlation would be expected if overexpression leads to nonphysiological interactions, which is clearly not the case in this study.

Phosphatase Association with Signaling Pathways and Cellular Processes

To further understand the functional role of these interactions, we annotated them to KEGG pathways (13). Importantly, several key cellular signaling pathways such as PI3-K, Hippo-YAP, Wnt, Hedgehog, HIF-1, mTOR, Ras-MAPK, AMPK, RAP1, and VEGF were highly enriched for HCIPs of different phosphatases (Figure 2A). We found several known as well as novel phosphatase associations enriched among these cellular signaling pathways. For example, we found that DUSP phosphatases associated with components of MAPK pathway, consistent with earlier studies. But, at the same time other phosphatases such as CDC25A and CDC25C, various PTPNs, EYA4, MDP1, MTMR4, and MTMR14 were found to be associated with proteins of the MAPK pathway, implicating new functions for these phosphatases in this pathway (Figure 2B). Also, as expected several already known cellular functions associated with phosphatases were enriched in our analysis. For example, CDC25 phosphatases were found to interact with cell cycle proteins, and dual specific phosphatases (DUSPs) interact with proteins in the MAPK signaling pathway. In addition to known associations, several novel cellular functions have been enriched in the analysis. For instance, phosphatases belonging to the DUSP family were associated with proteins in DNA replication (Figure 2C). Although phospho-dependent regulation of DNA replication is very well-known, the enzymatic machineries that control these processes are relatively unknown. Thus, these novel phosphatase associations will provide a better understanding of regulation of DNA replication. Interestingly, we also found several phosphatases associated with the RAB/VPS/SNX family of proteins (Figure 2D), possibly indicating new functions for these phosphatases in vesicular trafficking. Given that roles of phosphatases in regulation of vesicular trafficking were limited so far, this data may be used to further explore roles of individual phosphatases in this critical cellular process. In fact, in support of this hypothesis we recently assigned a previously unknown role of PTEN in endosome maturation based on its interaction with Rab7.[12] In conclusion, our enrichment analysis in addition to providing known functions also linked poorly studied protein phosphatases to specific biological processes and cellular signaling pathways.

Association of Tyrosine Phosphatases to Multiprotein Complexes

One of main advantages of affinity purification is ability to capture protein complexes associated with baits. Association of a phosphatase with a specific protein complex may suggest its role in a specific pathway. We next used COMPLEAT, a protein complex enrichment analysis tool,[13] to find out protein complexes associated with individual phosphatase baits (Table S5). We found many novel interactions of phosphatases with multiprotein complexes. This analysis highlighted many broad features of phosphatase interactome. For instance, interaction of PTPN2 with proteasome complex (Figure 3A), 14-3-3 complex with multiple phosphatases (Figure 3B), TENC1 with utrophin associated complex (Figure 3C), MDP1 with AMPK kinase protein complex (Figure 3D), MTMR4 with condensin complex (Figure 3E), and PTPRO with laminin complex (Figure 3F) were identified in our study. Several of these phosphatase−multiprotein complex interactions were previously unknown. In the above said examples, PTPN2 might have a role in regulation of proteasome activity. TENC1 may have a role in connecting the cytoskeleton to the extracellular matrix by interacting with the utrophin associated complex. MDP1 may play a key role in regulating cellular energy metabolism by interacting with AMPK. MTMR4 may have a role in chromosome assembly and segregation through its interaction with the condensin complex. PTPRO phosphatase may influence cell differentiation, migration, and adhesion by interacting with the laminin complex. Thus, association of multiprotein complexes may provide indications of previously unexplored functions of the phosphatases.

Linking Protein Phosphatase Interactome to Diseases Phenotypes

Numerous studies have reported multiple alterations in genomic loci such as mutations or deletions in many human diseases. But in many cases how these genomic alterations lead to development of diseases is poorly understood. To understand how phosphatases are involved in the disease pathways and to find components of biochemically related proteins linked to particular disease phenotype we integrated the information on these altered genomic loci into phosphatase interaction network. We used OMIM annotated diseases linked genes and analyzed for interaction of phosphatases with these diseases linked genes. We identified 244 disease-linked proteins that interact with 76 phosphatases (Table S6). We found several diseases such as 3 M syndrome, Charcot-Marie-Tooth disease, Parkinson disease, cardimyopathies, Cowden syndrome, Fanconi anemia, and X-linked mental retardation linked to phosphatases (Table S6). We found clusters of similar disease-associated proteins interacting with multiple phosphatases. For example, proteins involved in colorectal cancer (MSH2, MSH6, POLD1, and POLE) were present in complexes of CDC25B and MTMR4 as well (Figure 4A). On the other hand, we also found single phosphatase connected with proteins from diverse diseases. For example, ACCP associate with proteins linked to congenital disorder of glycosylation (DDOST, STT3A, and SST3B) as well as proteins linked to Fanconi anemia (FANCD2 and FANCI). Similarly, DUSP7 phosphatase interacts with ALG1, TMEM165, and STT3A (all linked to congenital disorder of glycosylation) and SKIV2L and TTC37 (proteins associated with trichoheptoentric syndrome) (Figure 4B). Furthermore, we found phosphatases interacting with distinct proteins involved in the same disease. For example, PTPN6 interacts with proteins involved in Diamond-Blackfan anemia (RPS17L, RPL35A, and RPL5) (Figure 4C), CDC14A interacts with proteins associated with Retinitis pigmentosa (PRPF8, and SNRNP200) (Figure 4D), and DUSP26 interacts with components linked with spinocerebellar ataxia (PMPCA and AFG3L2) (Figure 4E). This data may suggest the presence of multiple levels of phosphatase-dependent regulation in these diseases.

Further, we also matched phosphatase interactome to the COSMIC (cancer gene census) data set that contains genes mutated in human cancers. Out of 81 phosphatases analyzed, 56 phosphatases were associated with cancer-linked proteins (Table S7). Overall, we identified 47 interactors in phosphatase

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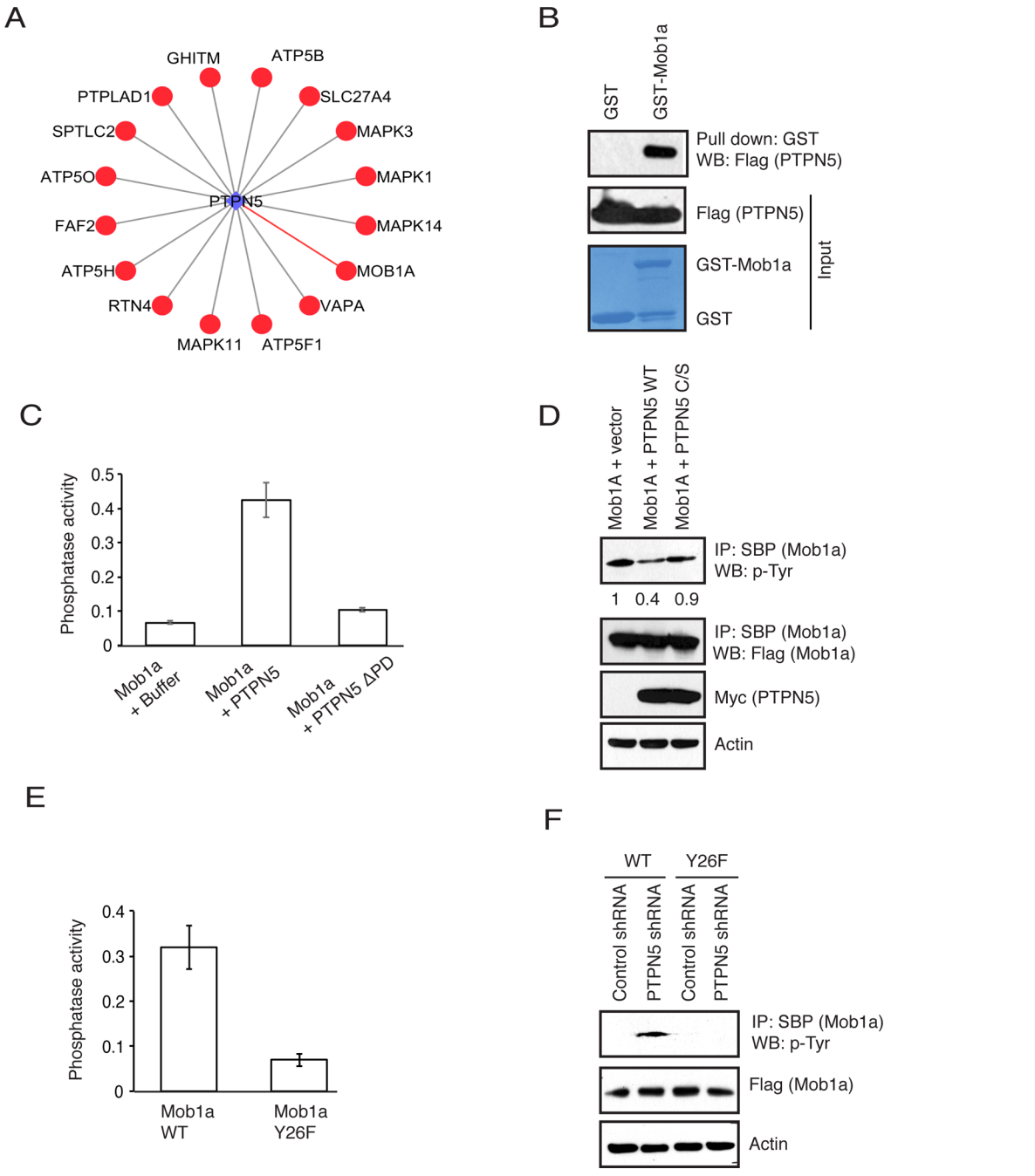

Figure 5. Mob1a is a novel PTPN5 interacting protein. (A) Network representation of PTPN5 and its associated proteins constructed by using Cytoscape. (B) GST or GST-Mob1a fusion protein was immobilized on agarose beads and a pull down assay was performed by incubating the extracts from 293T cells expressing SFB-tagged PTPN5. The *in vitro* interaction of PTPN5 with Mob1a was assessed by immunoblotting with an anti-Flag antibody. (C) Mob1a phosphorylated *in vitro* by using 293T lysate was incubated with bacterially expressed wild type PTPN5 and phosphatase domain deletion (ΔPD) mutant. The amount of released phosphate was assayed by using the malachite green reagent (A_{620} nm). Data represent mean absorbance from three independent experiments, $P < 0.05$. (D) Cells were transfected with SFB-Mob1a along with either vector control, wild type PTPN5 (WT), or catalytically inactive PTPN5 (C/S) mutant. Mob1a was pulled down using SBP beads, and levels of phosphorylation were detected by blotting with phospho-tyrosine antibody. (E) *In vitro* phosphorylated wild type Mob1a or Y26F mutant was used as substrate in a phosphatase release assay to assess PTPN5 phosphatase activity. The amount of released phosphate was assayed, and the data from three independent experiments were plotted, $P < 0.05$. (F) HeLa cells expressing either control shRNA or PTPN5 shRNA were transfected with SFB-tagged wild type (WT) Mob1a or Y26F mutant. Levels of Mob1a tyrosine phosphorylation were detected by blotting with phospho-tyrosine antibody after the Mob1a was pulled down with the use of streptavidin (SBP) beads.

interactome that are genetically linked to various types of tumors forming a total of 131 interactions (Figure 4F and Table S6). We found many known as well as novel phosphatase interactions with proteins linked to different cancers. For example, DUSP2 complex has POLE, MAPK1, DICER1, RAF1, RANBP2 and UBR5 all linked to different cancers (Figure 4G). MTMR4 interacts with SMAD2, MAPK1, PICALM, POLE and YWHAE (Figure 4H), all of which are well-designated cancer associated proteins either as oncogenes or tumor suppressors. Since majority of these interactions between phosphatases and

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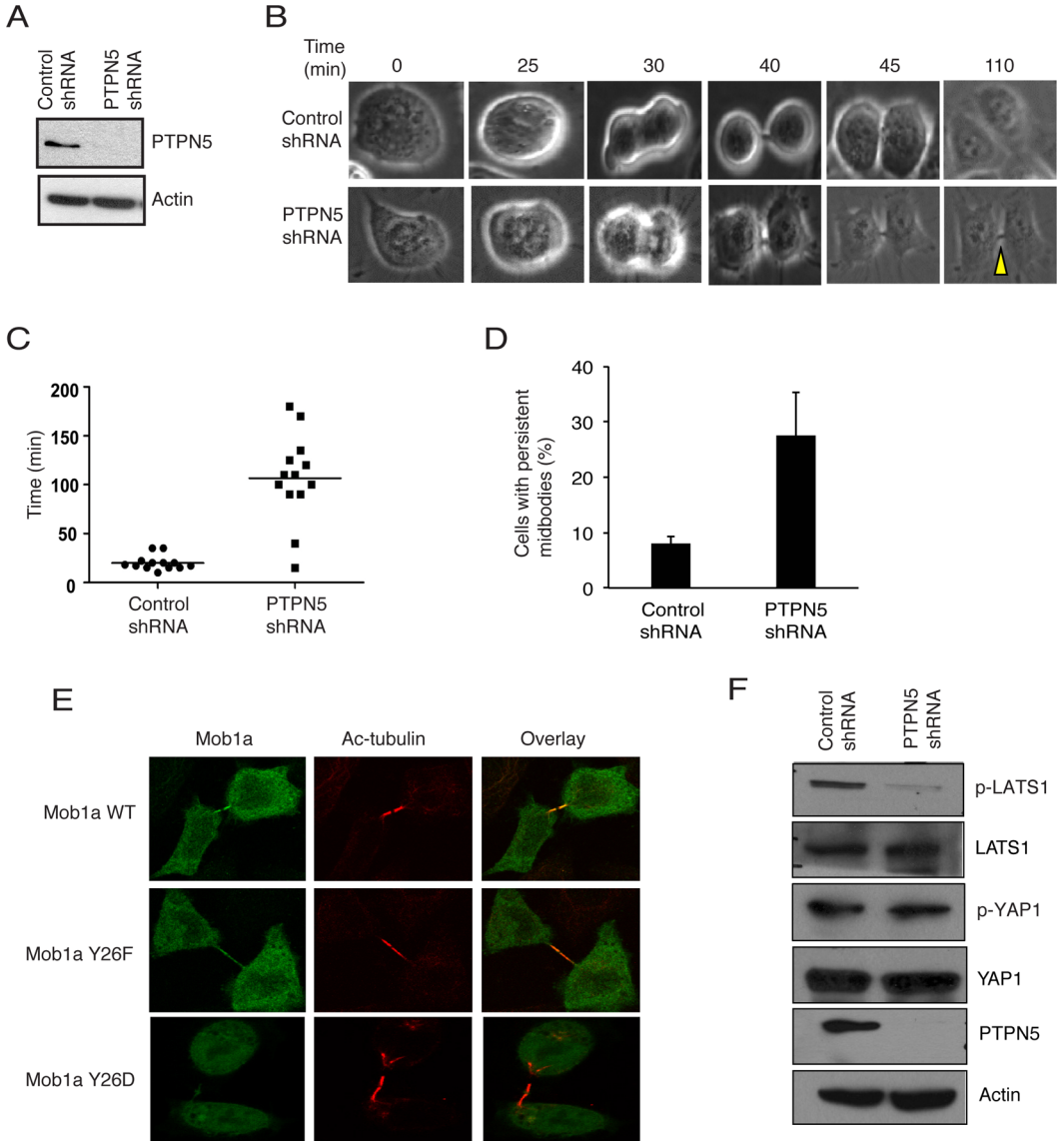

Figure 6. PTPN5−Mob1a interaction is functional during cytokinesis. (A) PTPN5 was depleted in Hela cells by using shRNA and (B) the transition of cells through mitosis and cytokinesis was analyzed by live cell time-lapse microscopy. Rounding of cells was marked as time = 0. Arrow indicates persistent midbody with the divided cells. (C) Time taken by each cell from mitotic entry to separation of midbodies after cytokinesis was calculated using live cell imaging, and the data was plotted for control and PTPN5 depleted cells (n = 13), $P < 0.05$. Cells taking longer than 200 min for separation were not included in the analysis. (D) The number of persistent midbodies in control and PTPN5 shRNA cells were counted after staining with acetylated tubulin antibody followed by immnoflourescence imaging. Data is presented from three independent experiments, $P < 0.05$. (E) SFB tagged Mob1a WT, Mob1a Y26F, and Mob1a Y26D were expressed in Hela cells and the localization of Mob1a is detected by immunofluorescence after staining with Flag antibody. Acetylated-tubulin was used to stain midbody. (F) Hela cells were transfected with control or PTPN5 shRNA and the levels of indicated phospho-proteins or total proteins was detected by Western blotting with their specific antibodies.

the disease linked proteins were unexplored so far, our phosphatase-disease associated interactome presents a rich source for developing new directions in investigating disease pathways controlled by phosphatases.

Functional Importance of PTPN5-Mob1a Interaction

To validate the functional importance of the identified interaction network, we chose to characterize one of the novel interactions identified in our study. We tested the functional significance of PTPN5 interaction with Mob1a. PTPN5 also known as STEP (striatal-enriched protein tyrosine phosphatase) is a nonreceptor tyrosine phosphatase that is mainly expressed in the brain regions such as striatum, cortex, and hippocampus.[14] Several substrates such as p38, Pyk2, ERK1/2, Fyn, and NMDA receptors were identified for PTPN5 phosphatase activity.[15−18] Although PTPN5 is shown to play a major role in synaptic function and is involved in pathophysiology of several neurological disorders including Huntington's disease, Alzheimer's disease, fragile X syndrome, Parkinson's disease, and schizophrenia,[19] its cellular functions are poorly understood. In our interaction analysis, in addition to its known interactors such as MAP kinases, we uncovered several novel PTPN5 associated proteins among which an uncharacterized interaction with Mob1a was repeatedly found (Figure 5A). Mob1 expressed in two isoforms (Mob1a & Mob1b) is a conserved coactivator of NDR and the LATS family of kinases in the Hippo signaling pathway and acts as a tumor suppressor by restricting proliferation and promoting apoptosis.[20,21] In

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addition, Mob1 is shown to be functionally important for cytokinesis during mitotic exit.[22,23] Thus, we tested if PTPN5 participates in any of these cellular roles by interacting with Mob1a.

First by using a GST-pull down assay, we confirmed that PTPN5 interacts with Mob1a (Figure 5B). Full length PTPN5 contains several domains including a C-terminal phosphatase domain, N-terminal transmembrane domain, a central KIM domain, and a KIS domain. Immunoprecipitation analysis using various deletion mutants (Figure S2A) suggested that Mob1a interacts with the KIS domain of PTPN5 (Figure S2B). On the other hand, Mob1a has two distinct interaction surfaces on N-terminus and C-terminus of the protein to interact with PTPN5 (Figure S2C,D). As the KIS domain of PTPN5 provides substrate specificity, we next tested if Mob1a acts as a substrate for PTPN5 phosphatase activity. Indeed full length PTPN5, but not phosphatase domain deleted PTPN5 (ΔPD), readily dephosphorylated Mob1a (Figure 5C). Further, expression of wild type PTPN5, but not a catalytically dead mutant of PTPN5 (C496S), significantly reduced tyrosine phosphorylated Mob1a in cells (Figure 5D). Earlier phosphoproteomic studies have identified Y26 residue as one of the potential phosphorylation sites on Mob1a.[24] In fact, treatment of cells with sodium orthovanadate enhances the phosphorylation of wild type Mob1a, but mutation of Y26 residue abolished its phosphorylation (Figure S3A), thus confirming that Y26 is the major tyrosine phosphorylation site of Mob1a in cells. Therefore, we next tested if PTNP5 dephosphorylates this residue. Our *in vitro* phosphate release assays suggested that mutation of Y26 residue has significantly reduced the release of phosphate by PTPN5 (Figure 5E). In addition, depletion of PTPN5 in cells enhanced the phosphorylation of wild type Mob1a but mutation of Y26 residue abolished its phosphorylation (Figure 5F), thus confirming Y26 as a site of dephosphorylation on Mob1a by PTPN5.

As Mob1a is an important component in the regulation of microtubule stability during mitotic exit,[23] we hypothesized that PTPN5 via interacting with Mob1a might participate in the control of cytokinesis during mitotic exit. To test this hypothesis, PTPN5 was depleted by using shRNA (Figure 6A) and the progression of mitotic cells was observed by using live cell imaging. PTPN5 depleted cells progressed through mitosis similar to control cells but took longer time to accomplish abscission (Figure 6B). While control cells disassembled their midbodies and completed cytokinetic abscission by 45 min of entry in to mitosis, PTPN5 depleted cells showed defective cytokinesis with unseparated midbodies for longer hours (Figure 6C). No significant changes were observed in the other cell cycle stages upon PTPN5 depletion (Figure S3B). In addition, by immunostaining with acetylated tubulin we found that PTPN5 depleted cells remained connected by thin cytoplasmic bridges containing persistent midbodies (Figure S3C and Figure 6D). As Mob1A localizes to midbodies during cytokinetic abscission, we then tested whether PTPN5 controls midbody abscission through regulating Mob1A localization via its dephosphorylation. Mob1A readily localizes to midbodies, whereas its phosphomimetic mutant Y26D fails to do so (Figure 6E), suggesting that Mob1A dephosphorylation at this site by PTPN5 is critical for its midbody localization. Defective Mob1A localization in turn leads to inactivation of LATS1 kinase as depletion of PTPN5 resulted in severe reduction of LATS1 phosphorylation (Figure 6F). Interestingly, down stream activation of Hippo pathway components such as Yap1 phosphorylation were unaffected by PTPN5, suggesting that the PTPN5 regulated Mob1a function is specific to cytokinetic abcision. Taken together, our data revealed a new function for PTPN5 in regulating cytokinetic abscission based on its interaction with Mob1a.

DISCUSSION

In this study, we have assembled a comprehensive human tyrosine protein phosphatase interaction network by using a systematic affinity purification coupled with mass spectrometry. Our network has yielded up to 85% novel interactions along with 286 previously known phosphatase-protein interactions. Upon mapping interactors through functional enrichment analysis, we revealed several previously unassigned cellular functions to phosphatases.

Tyrosine phosphatases are critically involved in various biological processes such as cell proliferation, cell cycle, development and apoptosis and thereby also contribute to pathogenesis of different human diseases.[2,4] But unlike their counterpart kinases, protein phosphatases have long been regarded as secondary players in phosphoprotein homeostasis and received less attention. Although, several studies over the past decade have assigned important cellular roles for these enzymes, most studies were focused on individual phosphatases and their function in a specific biological phenotype. Large scale studies to decipher the involvement of human phosphatases in biological processes and diseases along with their complexity of protein networks is limited. Interaction proteomics with advances in purification methods, mass spectrometer instrumentation, and powerful computational tools resulted in the identification of high-confidence interaction proteomes of different classes of human proteins[25−27] and thus can be applied to dissect phosphatase biology as well. Many studies have employed a wide variety of techniques to determine protein−protein interactions and map protein complexes. However, affinity purification is probably the best currently available method to isolate protein complexes from cells under near-physiological conditions. In comparison with other interaction techniques such as yeast two-hybrid analysis, which measures only binary interactions and largely direct interactions, affinity purification coupled with mass spectrometry (AP-MS) identifies large multiprotein complex interactions. But, on the other hand limitations such as the presence of nonspecific interacting proteins has been attributed to the AP-MS method. Thus, to overcome this challenge of abundant nonspecific interactors, we employed a tandem affinity purification approach to map specific phosphatase interactions. This methodology has been extensively used in our earlier studies to successfully identify several functional protein complexes in cells for individual proteins.[28−31] Identification of a large number of novel interactions of protein phosphatases in this study further testifies the success of this approach.

It may be noted that our study was solely performed using HEK 293T cells and hence the interacting network, cellular processes, and signaling pathways found here may have certain limitations due to cell line or cell-type specificity. However, HEK 293T is the most preferred cell system and used routinely in large-scale protein interactome studies to identify new protein−protein interactions.[25,32] Several important biological insights have been provided in these studies using 293T cells, which were later extended in other cell types as well. In addition, although some phosphatases are majorly expressed in specific tissues, their functions may not be restricted to

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particular cell type. The level of expression might be varied but their functional interactions might be intact or they could have additional functions. For example, PTPN6 (SHP-1), although expressed primarily in hematopoietic cells, and functions as an important regulator of signaling pathways in hematopoietic cells, has been shown in many studies to function in other cell types such as hepatic carcinoma cells, endometrial cells, and oligodendrocytes, etc. Thus, our study may be used as a valuable resource to assign previously unknown cellular functions for many phosphatases based on their interactors.

Although several protein complexes have been identified in our study, it may still be possible that this interaction network may have missed many weak, transient, and low abundant interactions. Given that enzyme−substrate interactions tend to be transient in nature, future studies may consider including additional criteria during purification approaches. For example, catalytically inactive trap mutants of phosphatases may be utilized to isolate the protein complexes to capture the associated substrates. In fact, we used this approach in our earlier study to successfully identify a functional substrate for PTEN phosphatase,[12] and thus it may be applied to other phosphatases too. In addition, including a protein cross-linking step before purification may enhance the chances of stabilizing the phosphatase−substrate interactions. Further, it is also possible that phosphatases may interact with their substrates in a context-dependent manner. For instance cell cycle phosphatases such as CDC25 may interact with their relevant substrates in a particular phase of the cell cycle. Thus, for such kind of context specific phosphatases, one may need to consider purifying the protein complexes from cells arrested in relevant cellular environments.

Although getting reliable phosphatase−substrate interactions is challenging, we could obtain at least a significant fraction of phosphatase-associated proteins that act as substrates in our study. For example, the DUSP family of phosphatases is well-known to regulate magnitude and duration of MAPK signaling by dephosphorylating MAP kinases. In our phosphatase interactions, we could identify 41 different MAPK interactions with DUSPs out of which 31 are known interaction. In addition, we validated functional interaction of PTPN5-Mob1a in our study, which incidentally again turned out to be phosphatase−substrate interactions. Thus, in parallel with other approaches, this study may further be effectively used to map the unknown phosphatase−substrate interactions. In conclusion, this interactome will be helpful in assigning functions to a large number of poorly characterized protein tyrosine phosphatases and facilitates expanding the role of well-studied phosphatases.

MATERIALS AND METHODS

Plasmids and siRNAs

cDNA library of human protein phosphatases was purchased from Open biosystems. All cDNAs were PCR amplified and cloned into donor vector pDONOR201 (invitrogen) using gateway cloning and then moved to the triple tagged (S-Protein/Flag/Streptavidin binding protein) SFB tagged destination vector for expression. List of primers used in the study were shown in Table S8. All clones were verified by sequencing and checked for expression in 293T cells. Myc-PTPN5, HA-Mob1a, GST-Mob1a, and MBP-PTPN5 constructs were all generated by gateway cloning. Point mutants for Mob1a Y26F, Mob1a Y26D, ΔPD PTPN5, PTPN5 deletion mutants, and Mob1a deletion mutants were generated by PCR-based site-directed mutagenesis and cloned into SFB, GST, MBP- and Myc-tagged destination vectors. Lentiviral based shRNAs for PTPN5 were purchased from Open biosystems.

Antibodies

The following antibodies have been used in this study: HA (Bethyl Laboratories), PTPN5, phospho-tyrosine, phospho-LATS1, phospho-YAP1, LATS1, YAP1, Mob1a (all from Cell Signaling technologies), Flag, Myc, actin, YAP1 (Santa Cruz Biotechnologies), Ac-α-tubulin (Abcam).

Cell Culture and Transfection

HEK293T and HeLa cells were maintained in RPMI containing 10% DBS and 1% penicillin and streptomycin. All cell lines were obtained from American Type Culture Collection, which were tested and authenticated by the cell bank using their standard short tandem repeats (STR)−based techniques. Cells were also continuously monitored by microscopy to maintain their original morphology and also tested for mycoplasma contamination by using DAPI staining. Cells were transfected with various plasmids using PEI (Polysciences) according to the manufacturer's protocol. Briefly, the plasmid diluted in serum-free RPMI medium was mixed with PEI (1 μg μL^{-1}) in 1:3 ratio. After incubating the DNA−PEI mixture at room temperature (RT) for 15 min, the complexes were added to cells to allow the transfection of plasmid.

Tandem Affinity Purification and Mass Spectrometry Analysis

HEK293T cells expressing SFB-tagged phosphatases were lysed with NETN buffer (20 mM TrisHCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 1 μg/mL of each pepstatin A and aprotinin on ice for 30 min. Cell debris were removed by centrifugation and cell lysates were incubated with streptavidin sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The bound protein complexes were washed three times with NETN and then eluted with 2 mg/mL of biotin (Sigma) for 90 min at 4 °C. The eluates were incubated with S-protein agarose beads (Novagen) for 1 h at 4 °C and then washed three times with NETN. The proteins bound to S-protein agarose beads were boiled in 2X SDS dye for 5 min and then resolved by SDS-PAGE and visualized by Coomassie Blue staining. Protein samples were allowed to run into the resolving gel up to 1 cm and the gel was sliced into one piece (containing all proteins) and sent for mass spec analysis. Mass spectrometry analysis was done by the Taplin Biological Mass Spectrometry Facility at Harvard University.

Protein Sequence Analysis by LC−MS/MS

Excised gel bands were cut into approximately 1 mm^3 pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure. Gel pieces were washed and dehydrated with acetonitrile for 10 min followed by removal of acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces was carried out with 50 mM ammonium bicarbonate solution containing 12.5 ng/μL modified sequencing-grade trypsin (Promega, Madison, WI) at 4 °C. After 45 min, the excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces. Samples were then placed in a 37 °C room overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac (~1 h). The samples were then

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stored at 4 °C until analysis. On the day of analysis the samples were reconstituted in 5−10 μL of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nanoscale reverse-phase HPLC capillary column was created by packing 2.6 μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter $x \approx 30$ cm length) with a flame-drawn tip. After equilibrating the column, each sample was loaded via a Famos auto sampler (LC Packings, San Francisco CA) onto the column. A gradient was formed, and peptides were eluted with increasing concentrations (gradient of 2% to 30%) of solvent B (97.5% acetonitrile, 0.1% formic acid) for 1 h. As peptides eluted they were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases from UniProt (http://www.uniprot.org/taxonomy/9606) with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher Scientific, Waltham, MA). All databases include a reversed version of all the sequences, and the data were filtered to one percent peptide false discovery rate.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[33] partner repository with the data set identifier PXD006698.

Submission details: Project Name, Tyrosine phosphatase interaction network; Project Accession, PXD006698, Reviewer account details: Username, reviewer12074@ebi.ac.uk; Password: YoXG6ID4.

Data Filtering and Data Analysis

The interactome data was filtered using CRAPOME tools. Saint score more than 0.8 and FCA more than 2, FCB > 2.5, IS > 1, and WD score >1 was used to filter data. Bait and prey associations retained after SAINT score cutoff were further subjected to id mapping using Uniprot id mapping and HGNC gene symbols in order to represent them using most recent protein identifiers. Uniform protein identifier representation was used across different sets of PPIs identified in this study as well as those retrieved from iRefIndex.

PPI data was downloaded from iRefIndex (Razick et al., 2008) (April 2015 release downloaded from http://irefindex.org/), and BIOGRID a meta-database which compiles known human PPIs from major public resources. From this only human PPIs were considered (both interactors are human proteins). KEGG pathway enrichment was done using ConsensusPathDB, and pathway with p value < 0.01 was selected. GO analysis was done using PANTHER, and protein complexes were identified by using COMPLEAT. Network was visualized using Cytoscape.

Immunoprecipitation and Western Blotting

Cells were transfected with various plasmids using polyethylenimine transfection reagent. For immunoprecipitation assays, cells were lysed with NETN buffer. The whole cell lysates obtained by centrifugation were incubated with 2 μg of specified antibody bound to either protein A or protein G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The immunocomplexes were then washed with NETN buffer four times and applied to SDS-PAGE. Immunoblotting was performed following standard protocols.

Immunofluorescence

Cells grown on coverslips were fixed with 3% paraformaldehyde solution in PBS containing 50 mmol/L sucrose at room temperature for 15 min. After permeabilization with 0.5% Triton X-100 buffer containing 20 mmol/L HEPES pH 7.4, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 300 mmol/L sucrose at room temperature for 5 min, cells were incubated with indicated antibodies at 37 °C for 60 min. After washing with PBS, cells were incubated with rhodamine-conjugated secondary antibody at 37 °C for 20 min. Nuclei were counterstained with DAPI. After a final wash with PBS, coverslips were mounted with glycerin containing paraphenylenediamine.

Recombinant Protein Purification

GST, GST Mob1a (wild type and Y26F mutant) or MBP-tagged PTPN5 full length and D4 deletion mutant were transformed into BL21 DE3 competent cells and the transformants were screened on antibiotic containing agar plates. Colonies were inoculated in 5 mL of LB medium containing antibiotic and incubated overnight at 37 °C. Next day, the starter culture was reinoculated into 250 mL of LB medium and incubated at 37 °C until the OD reached 0.6. The expression of protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C. The cells were pelleted down by centrifugation at 6000 rpm for 5 min at 4 °C. Cell pellet was resuspended in 1× NETN lysis buffer containing protease inhibitors and incubated on ice for 20 min. The cell suspension was sonicated (20 amplitude, 30 s on and 45 s off for a period of 3 min) to complete cell lysis. Cell debris was removed by centrifuging at 13000 rpm at 4 °C for 10 min. Cell lysates were incubated with either glutathione sepharose (for GST tagged proteins) or dextran sepharose beads (for MBP tagged proteins) for 2 h at 4 °C. Beads were washed 4 times with NETN lysis buffer. GST tagged proteins were eluted using buffer containing 20 mM glutathione. MBP tagged proteins were eluted using buffer containing 10 mM maltose.

In Vitro Phosphatase Assay

Bacterially purified substrates Mob1a were phosphorylated *in vitro* using HEK293T cell lysate. Then dephosphorylation reaction was carried out in dephosphorylation buffer (20 mM Tris-HCL pH 7.4, 150 mM Nacl, 5 mM imidazole, 10 mm Mncl2and 1 mM DTT) at 30 °C for 2 h with 5 μg of bacterially purified phosphatase. The released phosphate was detected using the Malachite Green Assay Kit (Cayman) by measuring the absorbance at 620 nm.

Live Cell Imaging

HeLa cells expressing control and PTPN5 shRNA were arrested by using thymidine (2 mM) for 16 h. Later the cells were released from thymidine arrest using fresh medium and then image acquisition with 1 min interval was performed with a Nikon A1R microscope equipped with a 40× objective.

Cell Cycle Analysis

Cells were stained with propidium iodide (50 μg/mL) in citrate buffer for 30 min at 37 °C, and cell cycle analysis was carried out by using flow cytometer (BD Accuri C6 flow cytometer).

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00065.

Three supporting figures as described in the text (PDF)
List of all tyrosine phosphatases used in this study (XLSX)
Raw list of interacting proteins identified in the purification of all tyrosine phosphatases and controls (XLSX)
List of high confident interactions (HCIs) associated with tyrosine phosphatases derived after data filteration (XLSX)
List of cellular pathways and processes enriched by KEGG analysis (XLSX)
List of protein complexes identified with individual tyrosine phosphatase through COMPLEAT database search (XLSX)
List of tyrosine phosphatases and the interactors linked to human diseases identified in this study (XLSX)
List of tyrosine phosphatases and the interactors linked to human cancers identified in this study (XLSX)
List of primers used in the study (XLSX)

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Author Contributions

S.M. and P.K. designed the experiments, analyzed the data, and wrote the manuscript. P.K., P.M., K.R.C., V.J.S., S.R.S., and N.R.K. participated in the phosphatase library cloning, purification, and proteomic analysis. P.M. developed PTPN5-Mob1A functional data. R.R.H. and H.A.N. provided help during bioinformatic analysis.

Notes

The authors declare no competing financial interest.

Additional mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[33] partner repository with the data set identifier PXD006698. Submission details: Project Name, Tyrosine phosphatase interaction network; Project Accession, PXD006698, Reviewer account details: Username, reviewer12074@ebi.ac.uk; Password: YoXG6ID4.

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