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3 # Reduced PTPRD expression differentially alters brain phosphotyrosine phosphoproteomic
4 # profiles of 2 and 12 month-old mice
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8 Short title: **PTPRD and brain phosphoproteomics**
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34 **Abstract**

35 The receptor type protein tyrosine phosphatase PTPRD is implicated in maturation of synapses of
36 expressing neurons, vulnerability to addictions, reward from addictive substances, vulnerability to
37 restless leg syndrome and densities of neurofibrillary pathology in Alzheimer's disease brains by a
38 variety of evidence. However, PTPRD's physiological substrates and adaptations to differences in
39 levels of PTPRD expression in brains of young and aging animals have not been explored in depth.
40 We report phosphoproteomic studies of brains of young and aged mice with different levels of PTPRD
41 expression, gene ontology studies of genes identified in this way and validation of several candidate
42 PTPRD substrates with *in vitro* assays using recombinant PTPRD phosphatase. PTPRD is well
43 positioned to modulate the extent of phosphorylation of phosphotyrosine phosphoprotein substrates,
44 including those involved in synaptic maturation and adaptation.

45

46 **Introduction**

47 The receptor type protein tyrosine phosphatase PTPRD is one of the most highly-expressed neuronal
48 protein tyrosine phosphatases (1, 2). PTPRD binds to extracellular ligands including interleukin 1
49 receptor like and accessory (IL1RL1 and IL1RAPL1), Slittrk, SALM and/or NGL proteins in ways that
50 (likely) alter activities of its intracellular protein tyrosine phosphatase and thus contribute to the
51 maturation of synapses of PTPRD-expressing neurons (1, 3).

52 Common variation in the human PTPRD gene intron 9 - 11 region exerts sizable "oligogenic"
53 influences on densities of neurofibrillary pathology in Alzheimer's disease brains, vulnerability to
54 restless leg syndrome (RLS) and levels of PTPRD mRNA in human postmortem cerebral cortex (4-6).
55 There are more modest, polygenic influences of common variation in this gene on vulnerability to
56 development of a substance use disorder, abilities to quit smoking, ability to stop use of opiates and
57 reward from psychostimulants (1, 7, 8). A rare human PTPRD knockout provides substantial
58 intellectual disability (9).

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59 Mice with constitutively-reduced PTPRD expression display reduced reward from stimulants,
60 supporting polygenic associations with addiction phenotypes (5, 10). They display disrupted sleep
61 onset, supporting associations with RLS (5, 10). Homozygous knockouts, but not heterozygotes,
62 display substantial mnemonic deficits (5, 11) in ways that fit with observations in humans. Our initial
63 observations support increased activating tyrosine phosphorylation of the tau kinases GSK3β and
64 GSK3α and greater age-related accumulation of pS202/pT205/pS208 phosphotau immunoreactivity
65 in brains of mice with reduced PTPRD expression (12). Attention to roles of altered PTPRD in aging
66 could thus add insight into potential biochemical bases for reported oligogenic associations between
67 PTPRD variants and the age-related neurofibrillary pathology found in Alzheimer's disease brains (4).

68 Phosphotyrosine phosphoproteomic studies can identify greater or less abundance of
69 phosphotyrosine phosphoproteins in brains of mice with reduced PTPRD expression. Simplistically,
70 phosphotyrosine phosphoproteins that display increased expression in knockouts and co-expression
71 with PTPRD are candidate PTPRD substrates. Proteins displaying decreased tyrosine
72 phosphorylation in PTPRD knockouts or those that are display increased phosphorylation but are not
73 co-expressed are candidates to provide adaptations to reduced PTPRD expression. Each of these
74 categories is of interest for interpreting the combined human and mouse data relevant to addiction
75 and RLS noted above. When we compare these patterns in young *vs* aged mice with constitutively-
76 altered PTPRD expression, we can assess age-related changes in both candidate PTPRD substrates
77 and in adaptations to altered PTPRD expression. These aging findings could contribute to
78 understanding PTPRD's associations with Alzheimer's disease neurofibrillary pathology.

79 We now report assessment of the abundance of tyrosine phosphorylated brain proteins in brains of 2
80 and 12 month old wildtype mice and littermates with reduced PTPRD expression. We compare
81 assessments from a complete dataset to those from prior and concurrent work with relevant *c.*
82 *elegans* and mouse phosphotyrosine phosphoproteomics (13, 14) and with data from a second
83 independent partial dataset from our laboratory. We confirm that several of the candidate PTPRD

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84 phosphotyrosine substrates identified by this work are avidly dephosphorylated by recombinant
85 PTPRD phosphatase *in vivo*. We discuss the implications and limitations of these data for
86 understanding PTPRD's physiological activities and adaptations to its modulation.
87

88 **Materials and Methods**

89 *Mice and brains:* Mice with reduced PTPRD expression and wildtype littermates were bred from
90 heterozygote x heterozygote crosses as described from mice initially generously supplied by Uetani
91 and colleagues (5, 10, 11). Mice were housed in the NMVAHCS animal facility, fed moistened food on
92 cage floors until weaning and genotyped as described (10). One mouse required sacrifice due to
93 dental issues at about 6 mos of age. Mice were sacrificed by rapid cervical dislocation at about 2 or
94 12 months of age, brains rapidly removed and split by a midsagittal razor blade cut. Our main dataset
95 comes from analyses of proteins from half brains (n = 4/genotype/age group except n = 3 for 12-
96 month old WT) that were rapidly frozen on aluminum foil placed on dry ice blocks and then rapidly
97 covered by dry ice powder and maintained at -80$^{o}$C until analyses. A second "partial" dataset was
98 obtained from brains placed into polypropylene tubes that were then floated onto liquid nitrogen in
99 ways that provided differences in time to freezing. All procedures were approved by the NMVAHCS
100 Animal care and use committee.

101 *Phosphotyrosine phosphoproteomic analyses:* We used mass spectrographic analyses and
102 quantitation of immunoprecipitated tryptic phosphotyrosine phosphopeptides extracted from frozen
103 mouse half brains as described (15). Briefly, frozen half brains were sonicated in urea lysis buffer,
104 sonicated, centrifuged, reduced with DTT, and alkylated with iodoacetamide. 15 mg protein from each
105 sample was digested with trypsin and purified over C18 columns for enrichment using
106 phosphotyrosine pY-1000 motif antibodies #8803 (Cell Signaling, Danvers, Mass). Enriched peptides
107 were purified over C18 STAGE tips (16) and subjected to LC-MS/MS analyses. Replicate injections of
108 each sample were run non-sequentially. Phosphopeptides were eluted using 90-minute linear

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109 gradients of acetonitrile in 0.125% formic acid delivered at 280 nL/min. Tandem mass spectra were
110 collected in a data-dependent manner using a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass
111 spectrometer, a top-twenty MS/MS method, a dynamic repeat count of one and a repeat duration of
112 30 sec. Real time recalibration of mass error was performed using lock mass (17) with a singly
113 charged polysiloxane ion m/z = 371.101237.

114 MS/MS spectra were evaluated using Comet and the Core platform (17-19). Files were searched
115 against the SwissProt *Mus musculus* FASTA database. A mass accuracy of +/-5 ppm was used for
116 precursor ions and 0.02 daltons for product ions. At least one tryptic (K- or R-containing) terminus
117 was required per peptide and up to four mis-cleavages allowed. Cysteine carboxamidomethylation
118 was specified as a static modification. Methionine oxidation and phosphorylation on serine, threonine
119 and/ or tyrosine residues were allowed as variable modifications. Reverse decoy databases were
120 included for all searches to estimate false discovery rates (FDR) and filtered using Core's linear
121 discriminant module with a 1.0% FDR. Peptides were filtered for the presence of a tyrosine
122 phosphorylated residue (strict motif) or serine/threonine phosphorylated residue within 2 amino acids
123 of a tyrosine (lax motif). Quantitative results were generated using Skyline (20) to extract the
124 integrated peak areas of the corresponding peptide assignments. Accuracy of quantitative data was
125 ensured by manual review in Skyline and/or in the ion chromatogram files.

126 *Phosphatase/dephosphorylation assays:* Recombinant PTPRD phosphatase protein (> 95% purity)
127 was produced in *E Coli* from His-tagged constructs as described (21)(22). We synthesized human
128 actinβ_1 KCDVDIRKDL[pY]ANTVLSGGTT, actinβ_2 IVRDIKEKLC[pY]VALDFEQEMA, actinβ_3
129 GDGVTHTVPI[pY]EGYALPHAIL, cofilin 1 GDVGQTVDDP[pY]ATFVKMLPDK and dock4
130 LGLDLVPRKE[pY]AMVDPEDISI phosphopeptides and compared these data to a positive control
131 END[pY]INASL (Promega) phosphopeptide studied in the same experiments.

132 Orthophosphate release assays (Promega V2471) used Malachite green and molybdate with
133 spectrophotometric detection of liberated free orthophosphate from test phosphopeptides compared

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134 to control/comparison phosphopeptides with assessments for the times indicated. Reactions were
135 carried out in half-area 96-well plates with three wells dedicated for each time point. To each
136 experimental well, we added a mixture of 18 µL of ultrapure water, 25 µL of running buffer (43.4 µM
137 HEPES (pH 7.4), 2.2 mM dithiothreitol, 0.44% acetylated bovine serum albumin, 22.2 mM NaCl, 4.4
138 mM EDTA) and 1 µL of a 10mM DMSO solution of the desired peptide. 50 µL of molybdate dye
139 mixture was added at t =0, followed by 5 µL of a 1:100 dilution of enzyme in dilution buffer (22.9 mM
140 pH 7.4 HEPES, 1% acetylated bovine serum albumin, 4.6 mM dithiothreitol). In control experiments,
141 we added enzyme that was heated to 100C for 20 min, cooled and added to reactions similarly. Other
142 wells were initiated *via* the addition of 5 µL of the diluted enzyme mixture @ t=0 and terminated at the
143 desired timepoints by addition of 50 µL of the dye solution. Wells were read @ 605 nm using a
144 Spectromax spectrophotometer (Molecular Devices, San Jose CA).

145 *Data analyses:* We used data from the phosphopeptides whose abundance was most different and
146 statistically-significant (nominal) to identify genes presented tables in the body of the paper, for gene-
147 based analyses of overlap with other datasets (using hypergeometric tests) and for tests of
148 overrepresentation among Gene ontology terms. We used 1.5 or 2 fold cutoffs for genes in tables and
149 $p < 10^{-8}$ or $< 10^{-7}$ for gene ontology terms following manual inspection of the datasets. Ambiguities in
150 phosphopeptide assignment to genes are maintained in these tables.

151 Corresponding phosphopeptide-level datasets are included in the Supplement. Coexpression data
152 come from Allen brain institute mouse cortex/hippocampus single cell RNA seq datasets.

153

154 **Results**

155 *Phosphopeptides identified:* We identified 1835 discrete phosphopeptides corresponding to 813
156 genes or groups of genes that were immunoprecipitated using phosphotyrosine antibodies and
157 identified by these approaches.

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158 *Phosphotyrosine phosphopeptides whose abundance increased with reduced PTPRD expression in*
159 *the main dataset from young mice:* We identified 101 phosphopeptides from 76 proteins (or groups of
160 related proteins sharing the same sequence) that displayed $\geq$ 1.5-fold increased abundance and
161 nominal $p < 0.05$ in 2 month old PTPRD knockout *vs* wildtype mice from our main dataset (Table I;
162 Supplement Table 1).

163 Table I: Genes encoding phosphopeptides with increased abundance in 2 mo old PTPRD knockout mice

| Fold change young KO : WT | p young KO : WT | Gene Name(s) |
|---|---|---|
| 7.9 | 0.00218 | Actb;Actg1;Actbl2*** |
| 5.9 | 0.04940 | Nyap2** |
| 5.0 | 0.02009 | Psmb6* |
| 4.2 | 0.00006 | Srsf1** |
| 4.0 | 0.00140 | Cfl1;Cfl2** |
| 4.0 | 0.02958 | Mpzl1 |
| 3.8 | 0.00001 | Ckb*** |
| 3.7 | 0.00003 | Grin2b*** |
| 3.6 | 0.00336 | Cyfip2** |
| 3.5 | 0.04939 | Fkbp5* |
| 3.4 | 0.03835 | Irs2* |
| 3.4 | 0.00130 | Caskin2** |
| 3.4 | 0.02578 | Ank2*** |
| 3.3 | 0.00005 | Dock4** |
| 3.2 | 0.00218 | Fgfr1* |
| 3.1 | 0.00117 | Crkl* |
| 3.0 | 0.00670 | Usp14* |
| 3.0 | 0.01850 | Psmd11** |
| 3.0 | 0.00009 | Hgs |
| 3.0 | 0.00292 | Mapk8;Mapk9;Mapk10*** |
| 2.9 | 0.03977 | Pura*** |
| 2.9 | 0.00180 | Epha5*** |
| 2.8 | 0.04198 | Gnao1*** |
| 2.8 | 0.04294 | Cilk1 |
| 2.8 | 0.00435 | Srcin1** |
| 2.7 | 0.02921 | App*** |
| 2.7 | 0.01346 | Mrpl58* |
| 2.7 | 0.01275 | Rpn1* |
| 2.7 | 0.00071 | Pcdhga4;Pcdha4 |
| 2.7 | 0.01675 | Tuba1a;Tuba1b;Tuba1c;Tuba3a;Tuba4a;Tuba8** |

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| | | |
|---|---|---|
| 2.7 | 0.00259 | Gprc5b* |
| 2.7 | 0.00446 | Syngap1 |
| 2.6 | 0.01242 | Gdi1;Gdi2** |
| 2.6 | 0.00658 | Cadm1*** |
| 2.6 | 0.00001 | Fbp1 |
| 2.6 | 0.00152 | Rpl31* |
| 2.6 | 0.02494 | Syt1*** |
| 2.6 | 0.00345 | Ap2b1** |
| 2.5 | 0.03793 | Atp6v1h** |
| 2.5 | 0.01381 | Npepps** |
| 2.5 | 0.04000 | Pacs1* |
| 2.4 | 0.00050 | Anxa2 |
| 2.4 | 0.02382 | Cadm2*** |
| 2.4 | 0.02550 | Rpl6*** |
| 2.4 | 0.00041 | Got1** |
| 2.4 | 0.01478 | Afap1l2 |
| 2.4 | 0.00451 | Kcnab2* |
| 2.4 | 0.03207 | Idh1 |
| 2.4 | 0.00000 | Prkacb*** |
| 2.4 | 0.01592 | Camkv** |
| 2.3 | 0.00468 | Ptprj** |
| 2.3 | 0.00014 | Ofd1 |
| 2.3 | 0.01615 | Gdi1* |
| 2.3 | 0.00189 | Kiaa0513na |
| 2.3 | 0.00170 | Nck2* |
| 2.3 | 0.00368 | Madd** |
| 2.3 | 0.00196 | Plcb1*** |
| 2.2 | 0.03739 | Mapk8;Mapk10*** |
| 2.2 | 0.00033 | Vcl* |
| 2.2 | 0.00287 | Atp1a1** |
| 2.2 | 0.00547 | Pdha1** |
| 2.1 | 0.00104 | Caskin1* |
| 2.1 | 0.00414 | Vta1 |
| 2.1 | 0.00105 | Synj1** |
| 2.1 | 0.04279 | Hk1** |
| 2.1 | 0.03279 | Rpl8*** |
| 2.0 | 0.00106 | Pafah1b1*** |
| 2.0 | 0.00303 | Ywhah*** |
| 2.0 | 0.01709 | Mydgf* |
| 2.0 | 0.00063 | Khnyn |
| 2.0 | 0.00000 | Mapk14* |
| 2.0 | 0.00370 | Uba1* |
| 2.0 | 0.00005 | Ntrk2*** |
| 2.0 | 0.01335 | Hsp90ab1*** |

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| | | |
|---|---|---|
| 2.0 | 0.00639 | Gpm6b** |
| 1.9 | 0.01621 | Crk** |

164

165 Table 1: Genes containing phosphotyrosine phosphopeptides whose abundance is increased > 1.5
166 fold (fold increase column 1) with $p < 0.05$ (nominal p value column 2) in proteins extracted from
167 brains of 2 month old PTPRD knockout mice compared to wildtype littermates. n = 4/genotype. Some
168 phosphopeptides could come from several genes, as listed. Asterix number: relative extent of
169 coexpression of the gene in the cell types that express PTPRD in mouse cerebral cortex +
170 hippocampus RNAseq data (Allen brain institute). *** substantial coexpression; ** moderate
171 coexpression; *coexpression in several cell types.

172 Gene ontology (GO) annotation of this list of genes documents overrepresentation of interesting GO
173 terms with FDR-corrected $p < 10^{-4}$, including: cell junction, synapse, cytoplasm, cell projection,
174 postsynaptic density, asymmetric synapse, neuron projection, postsynaptic specialization, neuron to
175 neuron synapse and glutamatergic synapse. (Supplement Table 2). Most of these genes are
176 coexpressed with PTPRD in mouse cortical and hippocampal neuronal types defined by Allen Brain
177 Institute single cell RNAseq datasets (Table 1). Only Caskin 2, Pcda4, Tub1a, Tub1c, Tub 3a, FBP1,
178 Idh 1 and vta 1 fail to display substantial coexpression in at least one of the PTPRD-expressing
179 neuronal types described in this data.

180 The group of coexpressed genes is thus likely to contain many PTPRD substrates, though some
181 could also contain phosphotyrosines whose differential abundance could provide indirect adaptations
182 to reduced PTPRD expression.

183 *Phosphotyrosine phosphopeptides whose abundance decreases with reduced PTPRD expression in*
184 *the main dataset from young mice:* We identified 101 phosphopeptides from 40 proteins (or groups of
185 related proteins sharing the same sequence) that displayed $\geq$ 1.5-fold decreased abundance and
186 nominal $p < 0.05$ in 2 month old PTPRD knockout *vs* wildtype mice (Table II, Supplement Table 3).

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187 Table II: Genes encoding phosphopeptides with decreased abundance in 2 mo old PTPRD knockout

188 mice

| Young KO : WT | Young KO : WT | Gene Name |
|---|---|---|
| -19.5 | 0.00001 | Il1rapl1*** |
| -9.5 | 0.04435 | Shank2** |
| -9.0 | 0.01587 | Slitrk5* |
| -6.2 | 0.00472 | Gria3** |
| -4.1 | 0.00142 | Grin2a*** |
| -4.1 | 0.00356 | Syngap1* |
| -4.1 | 0.00032 | Lrrc7*** |
| -3.7 | 0.02165 | Grin2b*** |
| -3.6 | 0.00015 | Ildr2** |
| -3.5 | 0.00721 | Gja1 |
| -3.4 | 0.04394 | Ctnnd2*** |
| -3.4 | 0.00256 | Arhgef33 |
| -3.2 | 0.04114 | Dlg2*** |
| -3.1 | 0.00511 | Septin5 |
| -3.1 | 0.02536 | Caskin1* |
| -3.0 | 0.03716 | Ptk2** |
| -2.9 | 0.00094 | Ajm1 |
| -2.8 | 0.01108 | Elfn2* |
| -2.8 | 0.02941 | Pkp4* |
| -2.7 | 0.04453 | Pclo*** |
| -2.6 | 0.00219 | Kcnab2* |
| -2.5 | 0.03234 | Baiap2** |
| -2.5 | 0.01833 | Dlgap1*** |
| -2.5 | 0.04695 | Septin4* |
| -2.5 | 0.03359 | Grin2c |
| -2.3 | 0.04501 | Dlgap4** |
| -2.3 | 0.03262 | Tgfb1i1 |
| -2.2 | 0.01523 | Hcn2* |
| -2.2 | 0.00868 | Igf1r;Insr** |
| -2.2 | 0.02114 | Rapgef4*** |
| -2.1 | 0.00525 | Cacng2** |
| -2.1 | 0.00559 | Pag1* |
| -2.1 | 0.02832 | Ablim1** |
| -2.1 | 0.02474 | Adgrb1* |
| -2.1 | 0.02322 | Begain* |
| -2.1 | 0.03372 | Akr1b1 |
| -2.0 | 0.01554 | Glud1* |
| -2.0 | 0.00651 | Gab1 |
| -2.0 | 0.01905 | Nefh* |

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| -2.0 | 0.01419 | Psma2** |
|---|---|---|

189 Table 2: Genes containing phosphotyrosine phosphopeptides whose abundance is decreased > 1.5
190 fold (fold decrease column 1) with p < 0.05 (nominal p value column 2) in proteins extracted from
191 brains of 2 month old PTPRD knockout mice compared to wildtype littermates. n = 3 - 4/genotype.
192 Some phosphopeptides could come from several genes, as listed. Asterix number: relative extent of
193 coexpression of the gene in the cell types that express PTPRD in mouse cerebral cortex +
194 hippocampus RNAseq data (Allen brain institute). *** substantial coexpression; ** moderate
195 coexpression; *coexpression in several cell types

196 Gene ontology terms that are identified by this group of genes with FDR corrected p < $10^{-4}$ include:
197 cell junction, postsynapse, postsynaptic specialization, synapse, postsynaptic density, asymmetric
198 synapse, glutamatergic synapse, somatodendritic compartment, dendrite, neuron projection,
199 ionotropic glutamate receptor complex, neurotransmitter receptor complex, plasma membrane region,
200 ion channel complex and postsynaptic membrane (Supplementary Table 4).

201 These terms align with the idea that many of the adaptations to reduced PTPRD expression can be
202 found in neuronal specializations that can be postsynaptic to often-presynaptically-expressed PTPRD
203 (14). In particular, some of the most striking changes are found in candidate PTPRD ligands. The
204 largest reduction in phosphorylation is in phosphopeptides in the PTPRD ligand Il1rapl1 (23). The
205 third largest is in Slittrk5, another candidate PTPRD ligand (24). Each of these features is consistent
206 with the idea that many of the reductions in protein tyrosine phosphorylation in PTPRD knockout mice
207 could represent adaptations to PTPRD loss.

208 *Supporting studies of PTPRD phosphatase activities:* We synthesized several actin as well as cofilin
209 and dock4 phosphopeptides as candidate substrates for PTPRD's phosphatase since these were: a)
210 among those whose abundance increased most strikingly in two month old mice with reduced PTPRD
211 expression; b) products of genes that are coexpressed abundantly with PTPRD in cerebral cortical

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212 neuronal cell types and c) products of genes that are good candidates for involvement in neuronal
213 adaptations plausibly associated with PTPRD functions as a synaptic organizer (3, 25-28).

214 PTPRD phosphatase cleaved orthophosphate from each of these phosphopeptides and from our
215 END(pY)INASL at rates much greater than those (essentially zero) for control experiments using
216 boiled/inactive phosphatase. Rates for orthophosphate release ranged from almost twice those for
217 the positive control END(pY)INASL for Dock4 and actinβ_3 phosphopeptides to about 20% of the
218 positive control for the cofilin 1 phosphopeptide (Table 3). Each of these rates were significantly

219 Table III: Relative rates of orthophosphate release by recombinant PTPRD phosphatase from
220 phosphopeptides whose abundance increased in mice with reduced PTPRD expression

| | **Ratio to pyEND** | |
|---|---|---|
| | **Ratio** | **SEM** |
| **Actinβ_1** | **0.38** | **0.08** |
| **Actinβ_2** | **0.71** | **0.07** |
| **Actinβ_3** | **1.45** | **0.14** |
| **Cofilin 1** | **0.17** | **0.02** |
| **Dock4** | **1.88** | **0.14** |

221 Table 3: Relative rates of orthophosphate release from synthetic phosphopeptides corresponding to
222 candidate PTPRD substrates actin (three regions), cofilin and dock 4. Rates of orthophosphate
223 release from phosphopeptides shown were compared to rates of release from positive control
224 END(pY)INASL in the same experiment. Values are mean +/- SEM from three replicate experiments,
225 each using triplicate assays and data from four time points.

226 different from rates for experiments using boiled control phosphopeptide. These results support our
227 phosphopeptide phosphoproteomic studies, since each of the tested candidate substrates is actively
228 hydrolyzed by recombinant PTPRD phosphatase *in vitro*.

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229 *Phosphotyrosine phosphopeptides with smaller KO:WT differences in older vs younger mice:* We
230 identified 31 phosphopeptides from 24 genes that displayed > 1.5-fold smaller differences in
231 abundance with p < 0.05 nominal significance in comparisons of old knockout to old wildtype *vs*
232 young knockout to young wildtype mice (Table 4, Supplementary Table 5).

233 Table IV: Genes encoding phosphopeptides with smaller KO:WT differences in 12 vs 2 mo old
234 PTPRD knockout mice

| Difference Old-Young | Fold change Old KO : WT | Fold change Young KO : WT | p Old KO : WT | p Young KO : WT | Gene Name |
|---|---|---|---|---|---|
| -14.0 | -15.7 | -1.7 | 0.0174 | 0.03 | Grin2b |
| -7.3 | -11.4 | -4.1 | 0.0346 | 0.00 | Lrrc7 |
| -6.7 | -4.7 | 2.0 | 0.0214 | 0.33 | Shank3 |
| -4.6 | -2.6 | 1.9 | 0.0385 | 0.02 | Crk |
| -4.5 | -7.1 | -2.6 | 0.0001 | 0.21 | Ablim3 |
| -4.1 | -2.8 | 1.3 | 0.0013 | 0.66 | Grin2b |
| -4.1 | -3.0 | 1.1 | 0.0261 | 0.93 | Grin2a |
| -4.1 | -2.2 | 1.9 | 0.0431 | 0.11 | Crkl |
| -3.7 | -2.6 | 1.1 | 0.0144 | 0.90 | Clcn2 |
| -3.7 | -2.6 | 1.1 | 0.0109 | 0.76 | Ywhag |
| -3.7 | -2.1 | 1.6 | 0.0448 | 0.63 | Ablim3 |
| -3.6 | -2.4 | 1.3 | 0.0459 | 0.54 | Anxa7 |
| -3.6 | -2.1 | 1.4 | 0.0490 | 0.10 | Mapk11 |
| -3.5 | -2.4 | 1.1 | 0.0205 | 0.94 | Shisa6 |
| -3.5 | -2.5 | 1.0 | 0.0022 | 0.95 | Kcnab2 |
| -3.4 | -2.3 | 1.1 | 0.0390 | 0.96 | Arap2 |
| -3.3 | -5.1 | -1.8 | 0.0025 | 0.29 | Shank1 |
| -3.3 | -2.0 | 1.4 | 0.0297 | 0.45 | Ntm |
| -3.3 | -2.2 | 1.0 | 0.0287 | 0.93 | Cacng2 |
| -2.8 | -4.3 | -1.5 | 0.0225 | 0.41 | Grid2ip |
| -2.1 | -3.3 | -1.2 | 0.0309 | 0.67 | Slc25a4 |
| -1.7 | -3.0 | -1.2 | 0.0071 | 0.70 | Axl |
| -1.7 | -2.7 | -1.1 | 0.0096 | 0.14 | Arhgap32 |
| -1.6 | -3.2 | -1.6 | 0.0433 | 0.12 | Sirpa |

235 Table 4: Genes containing phosphotyrosine phosphopeptides whose abundance change in 12 month
236 old knockouts displays p < 0.05 and which display the greatest reductions in fold change compared to
237 comparisons between 2 month old knockouts vs wildtype mice. n = 3 - 4/genotype. Some

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238 phosphopeptides could come from products of several genes, as listed. These genes represent
239 candidates for interactions between effects of PTPRD expression and aging.

240 This group of genes provided $p < 10^{-7}$ FDR-corrected significance for over-representation in GO
241 categories: including neurotransmitter receptor complex, inotropic glutamate receptor complex,
242 synapse, ion channel complex, transporter complex, postsynapse, postsynaptic density, asymmetric
243 synapse, postsynaptic specialization, postsynaptic membrane and cell junction (Supplementary Table
244 6). These localizations fit a hypothesis that aging reduces the magnitudes of a number of the synaptic
245 adaptations to loss of PTPRD.

246 *Phosphotyrosine phosphopeptides with larger KO:WT differences in older vs younger mice:* We also
247 identified 33 phosphopeptides from products of 24 genes whose abundance differences between
248 knockout and wildtype mice were > 1.5-fold larger with $p < 0.05$ in comparisons of old knockout *vs* old
249 wildtype mice (Table 5, Supplementary Table 7).

| Difference old-young | Fold change old KO : WT | Fold change Young KO : WT | p old KO : WT | p young KO : WT | Gene Name(s) |
|---|---|---|---|---|---|
| 37.8 | 26.9 | -10.9 | 0.00 | 0.29 | Dbi |
| 33.3 | -2.9 | -36.2 | 0.03 | 0.20 | Shisa6 |
| 10.1 | 8.7 | -1.5 | 0.03 | 0.65 | Camkv |
| 6.4 | 3.3 | -3.0 | 0.02 | 0.04 | Ptk2 |
| 6.1 | 2.8 | -3.3 | 0.04 | 0.00 | Grin2a |
| 4.3 | 3.0 | -1.3 | 0.02 | 0.57 | Mapk1;Mapk3 |
| 4.2 | 2.1 | -2.1 | 0.02 | 0.03 | Akr1b1 |
| 4.2 | 2.7 | -1.5 | 0.01 | 0.16 | Ptpn11 |
| 4.1 | 2.5 | -1.6 | 0.02 | 0.31 | Syngap1 |
| 3.9 | 2.4 | -1.5 | 0.03 | 0.47 | Esyt1 |
| 3.9 | 1.8 | -2.0 | 0.02 | 0.02 | Glud1 |
| 3.7 | 2.2 | -1.5 | 0.02 | 0.24 | Shc3 |
| 3.7 | 2.2 | -1.5 | 0.04 | 0.24 | Pcnx1 |
| 3.6 | 2.3 | -1.3 | 0.04 | 0.09 | Iqsec2 |
| 3.6 | 2.1 | -1.4 | 0.02 | 0.43 | Dlg4 |
| 3.4 | 2.3 | -1.1 | 0.03 | 0.78 | Dlg2 |
| 3.4 | 2.3 | -1.2 | 0.03 | 0.27 | Grin2b |
| 3.3 | 2.2 | -1.2 | 0.04 | 0.53 | Lasp1;Nebl |
| 3.3 | 2.1 | -1.1 | 0.02 | 0.65 | Bcr |

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| | | | | | |
|---|---|---|---|---|---|
| 2.8 | 4.1 | 1.3 | 0.04 | 0.61 | Copa |
| 2.8 | -16.7 | -19.5 | 0.01 | 0.00 | Il1rapl1 |
| 2.3 | -2.1 | -4.4 | 0.02 | 0.26 | Ajm1 |
| 1.9 | 3.0 | 1.1 | 0.01 | 0.96 | Pkm |
| 1.5 | 2.6 | 1.1 | 0.02 | 0.98 | Actr3 |

250 Table 5: Genes containing phosphotyrosine phosphopeptides whose abundance change in 12 month
251 old knockouts displays $p < 0.05$ and which display the greatest fold change compared to comparisons
252 between 2 month old knockouts vs wildtype mice. n = 3 - 4/genotype. Some phosphopeptides could
253 come from products of several genes, as listed. These genes represent candidates for interactions
254 between effects of PTPRD expression and aging.

255 Overrepresented $p < 10^{-6}$ GO terms supported aging influences on synaptic adaptations to loss of
256 PTPRD including: glutamatergic synapse, cell junction, postsynaptic density membrane, synapse,
257 postsynaptic specialization membrane, asymmetric synapse, postsynaptic density, neuron to neuron
258 synapse, postsynaptic membrane, ionotropic glutamate receptor complex, intrinsic component of
259 postsynaptic density membrane and neurotransmitter receptor complex (Supplementary Table 8).
260 These localizations again fit with aging influences on the synaptic adaptations to loss of PTPRD.

261 *Replications in an independent dataset:* We identified nominally-significant > 1.5 fold/ $p < 0.05$
262 changes in several of the top genes from our primary dataset in brains of independent groups of mice
263 with a different brain freezing approach. Irs2, Kcnab2 and Pdha1 provided smaller magnitude, but p <
264 0.05 increased phosphopeptide abundance in this independent dataset. There was a trend (p = 0.08,
265 hypergeometric test) toward significant overlap with the set of genes with more abundant
266 phosphopeptide abundance in the data presented in Table I.

267 There was decreased expression (> 1.5-fold, $p < 0.05$) of Il1rapl1, Slitrk5 and Dlgap1in young
268 homozygous knockouts in this dataset. The three overlapping genes whose phosphopeptide
269 abundance decreased in young knockouts (Table 2) provided $p = 10^{-5}$ (hypergeometric testing). Dlg2
270 and Grin2a were also decreased > 1.5 fold though with $p > 0.05$.

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271 *Gene dose relationships in our independent dataset:* Data comparing changes in homozygous *vs*
272 heterozygous knockouts (*vs* wildtype mice) provide information about gene dose-response
273 relationships for phosphopeptides from gene products that are most increased or decreased in
274 abundance in knockouts. Phosphopeptides that displayed increased abundance in knockouts *vs*
275 wildtype mice displayed typical gene dose-response patterns: heterozygotes displayed smaller
276 differences from wildtype and less statistical significance than homozygotes for phosphopeptide
277 products of each of the genes that displayed nominally-significant upregulation > 1.5-fold in our
278 replication dataset.

279 Gene dose-response relationships for decreases were not as simple. Decreases in both IL1RAPL1
280 and SLITRK5 phosphopeptide abundance *vs* wildypype mice were smaller and did not reach nominal
281 significance in heterozygotes, although the much larger declines found in homozygous knockouts
282 reached high levels of significance, as noted above. By contrast, the declines in Dlg2, Dlgap1 and
283 Grin2a phosphopeptide abundance were larger and reached higher levels of statistical significance
284 for differences from wildtype in heterozygotes *vs* homozygotes.

285 **Discussion**

286 The current results document differences in brain tyrosine phosphorylation that define groups of
287 candidate PTPRD substrates, sets of candidate adaptations to loss of PTPRD activity and groups of
288 potential aging effects on these candidate substrates and adaptations. We discuss the strengths and
289 limitations of these results and the ways in which they point toward important roles for PTPRD
290 activity. We note pathways whereby these changes could contribute to human and mouse model
291 associations between PTPRD variation and addictions, RLS and Alzheimer's disease
292 pathophysiologies. We map the place that these brain phosphotyrosine phosphoproteomic results
293 assume in the study of complex phosphorylation and dephosphorylation events in the brain.

294 The genes (or groups of genes) corresponding to phosphopeptides that display both ≥ 1.5-fold
295 increased abundance and nominal $p < 0.05$ in young PTPRD knockout *vs* wildtype mice form an

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296 interesting group. This group of genes contains many more whose products are expressed in
297 presynaptic localizations (where much of PTPRD is expressed (14)) than expected by chance. Allen
298 brain institute data supports colocalization of moderate to relatively high levels of expression of 45%
299 of these genes in many of the neuronal subtypes that also express PTPRD (Table 1). Although it is
300 possible that some of these increases in tyrosine phosphopeptide abundance come from adaptations
301 to loss of PTPRD, this group of genes, overall, is likely to provide many candidate PTPRD substrates.

302 There is significant, but imperfect overlap with other relevant datasets. There is significant overlap (11
303 genes, hypergeometric $p = 10^{-9}$) between the gene products identified in our studies and the 59
304 genes/groups of genes whose tyrosine phosphorylation was reported increased by > 1.5 fold ($p <$
305 $0.05$) in studies of a different strain of PTPRD knockout mice that were reported while our work was in
306 progress (14). There is also overlap with genes identified with increased tyrosine phosphorylation in
307 *C. elegans* with deletion of the ptp-3 gene that corresponds to PTPRD, PTPRS and PTPRF (6 genes,
308 hypergeometric $p = 9 \times 10^{-5}$) (13).

309 In other work we have identified 1.3-fold increases of abundance of a phosphotyrosine
310 phosphopeptide with sequence shared between GSK3β and GSK3α in Western analyses of proteins
311 from PTPRD knockout mice. This was one of the phosphopeptides increased in *C. elegans* ptp-3
312 knockouts (13). The more modest magnitude of our Western results could explain why it was not
313 detected in our phosphoproteomic datasets with > 1.5-fold thresholds.

314 Actin, cofilin and dock 4 phosphopeptides identified as candidate substrates for PTPRD's
315 phosphatase in the phosphoproteomics datasets developed herein are each actual substrates for
316 recombinant PTPRD phosphatase *in vitro*. Rates for orthophosphate release from these
317 phosphopeptides range from brisk to very brisk, with release from the actinβ_3 peptide almost 50%
318 greater and that from the dock4 phosphopeptide almost double the brisk rate of release from the
319 active positive control END(pY)INASL. Each of the tested candidate substrates is thus actually

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320 hydrolyzed by recombinant PTPRD phosphatase *in vitro,* adding substantially to our overall
321 confidence in the phosphoproteomic datasets.

322 There is also significant overall support for the set of phosphotyrosine phosphoproteins that display
323 less abundance in 2 month old mice with reduced PTPRD expression. Allen brain institute data
324 supports colocalization of moderate to relatively high levels of expression of 47% of these genes in
325 many of the neuronal subtypes that also express PTPRD. There is significant overlap (8 genes,
326 hypergeometric $p < 10^{-9}$) between the gene products identified in our studies and the 43
327 genes/groups of genes whose tyrosine phosphorylation was reported decreased by > 1.5 fold ($p <$
328 $0.05$) in studies of a different strain of PTPRD knockout mice reported while our work was in progress
329 (14). There is thus significant support for the validity of our set of downregulated genes as well as the
330 GO links to postsynaptic locations that fit with adaptive roles for these reductions in phosphotyrosine
331 phosphoprotein abundance. While several of these phosphopeptides have functional annotations in
332 one of the best phosphopeptide annotation databases, future studies will be required to identify roles
333 for most of these phosphotyrosines in regulating function. Roles in regulating the activities of products
334 of the IL1RAPL1 and SLITRK 4 and 5 genes seem especially merited due to their roles as likely
335 PTPRD ligands. In future work, we will assess possible contributions of changes in overall IL1RAPL1,
336 SLITRK4 and SLITRK5 gene expression to these changes in abundance of the corresponding
337 phosphotyrosine phosphopeptides.

338 There is evidence for both attenuation and increases in the changes in noted in young mice when 12
339 month-old knockout data is compared to data from wildtype littermate mice aged in parallel in the
340 same facility. The genes whose products display less phosphotyrosine phosphoprotein abundance
341 and those whose products display more phosphotyrosine phosphoprotein abundance in the older
342 mice each provide candidates for interactions between age and level of PTPRD expression. From this
343 group, we thus have candidates to participate in aging interactions with levels of PTPRD expression.
344 Aging x level of expression changes are, in turn, candidates to contribute to the accumulation of

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345 phosphotau immunoreactivity that we have observed in aged mice with reduced PTPRD expression.
346 This group also provides candidates to participate in the interactions between aging and altered
347 PTPRD expression that are implied by the human associations between Alzheimer's disease
348 neurofibrillary pathology and intron 10 PTPRD genomic markers that are near those that we have
349 associated with levels of PTPRD expression (4, 5).

350 There are limitations of these data. Similar sequences surrounding phosphotyrosines provide
351 ambiguity concerning which exact actin, tubulin, MAP kinase, CAM kinase, disc-large or other gene
352 products actually display increased abundance in mice with reduced PTPRD expression. Our results
353 do not separate differences in levels of expression of genes whose phosphotyrosine phosphopeptide
354 abundance that we monitor here from differences in the extent of tyrosine phosphorylation of these
355 proteins. The wealth of information about the existence of tyrosine phosphorylation sites and their
356 phosphorylation patterns has grown much faster than our understanding of the physiological or
357 regulatory roles that many of these tyrosine phosphorylations provide. The apparent lower sensitivity
358 of our datasets using a different freezing method, and thus the lower overlap of these independent
359 results with our primary dataset, underscores the exquisite sensitivity of these phosphoproteomic
360 analyses to the speed of tissue freezing.

361 Phosphotyrosine phosphoproteomic methods have been used to aid identification of tyrosine
362 phosphorylated phosphoproteins whose abundance is changed in cells or tissues with changes in
363 activity of other tyrosine phosphatases including products of the PTPRB (29), PTPRG (30), PTPN11
364 (31, 32) and PTP4A3 (33) genes. However, the relatively novelty of this area is highlighted in a recent
365 review (34) that observes that only 3% of the phosphorylation sites annotated even in one of the
366 most-updated databases (PhosphoSitePlus (35)) have a corresponding experimentally-validated
367 human kinase. Our work thus adds to a modest but growing number of approaches to identification of
368 protein tyrosine phosphatase substrates and adaptive changes to altered tyrosine phosphatase
369 activities using this approach.

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370 Human association datasets and mouse model results have motivated us to identify the first PTPRD
371 phosphatase inhibitor lead compound, 7-BIA(10), and to find that quercetin and related flavanols
372 provide the first PTPRD positive allosteric modulators/lead compounds (36). Transient
373 pharmacological PTPRD phosphatase inhibition with 7-BIA leads to increases in brain pYGSK3β and
374 pYGSK3α immunoreactivity that are about 2/3 the magnitude of changes induced by chronic genetic
375 reductions in PTPRD expression in heterozygous knockout mice (37). The current phosphoproteomic
376 datasets from knockout mice can thus provide a template for studies seeking effects of transient
377 pharmacological modulation of brain PTPRD activities in ways that could improve understanding of
378 PTPRD-associated pathophysiological disease processes in addiction, RLS and neurofibrillary
379 pathologies, This type of work can aid more precise targeting of improved therapeutics to these
380 pathophysiological mechanisms.

381

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388 “Compounds for treating tauopathies and Restless Leg Syndrome and methods of using and
389 screening for the same” that covers work with PTPRD positive allosteric modulators . GRU serves
390 without compensation on the Medical and Scientific Advisory board of the Restless Leg Syndrome
391 Foundation. MPS is an employee of Cell Signaling Technology, Inc, Danvers, Massachusetts which
392 offers commercial phosphoproteomics services.

393 **References**

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471

472 **Supplementary Table Legends**

473 Supplementary Table 1: Phosphotyrosine phosphopeptides and related genes whose abundance is
474 increased > 1.5 fold with $p < 0.05$ in proteins extracted from brains of 2 month old PTPRD knockout
475 mice compared to wildtype littermates. n = 4/genotype. Some phosphopeptides could come from
476 several genes, as listed.

477 Supplementary Table 2: Gene ontology (cellular component) terms overrepresented ($p < 10^{-4}$) in the
478 list of genes in Table 1.

479 Supplementary Table 3: Phosphotyrosine phosphopeptides and related genes whose abundance is
480 decreased > 1.5 fold with $p < 0.05$ in proteins extracted from brains of 2 month old PTPRD knockout

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481 mice compared to wildtype littermates. n = 4/genotype. Some phosphopeptides could come from
482 several genes, as listed.

483 Supplementary Table 4: Gene ontology (cellular component) terms overrepresented ($p < 10^{-4}$) in the
484 list of genes in Table 2.

485 Supplementary Table 5: Phosphotyrosine phosphopeptides and related genes whose abundance is
486 decreased > 1.5 fold more in old knockout *vs* wildtpe comparisons than in young knockout vs wildtype
487 comparisons (and which display $p < 0.05$ in old knockout/wildtype comparisons. n = 3 - 4/
488 genotype/age group. Some phosphopeptides could come from several genes, as listed.

489 Supplementary Table 6: Gene ontology (cellular component) terms overrepresented ($p < 10^{-5}$) in the
490 list of genes in Table 4.

491 Supplementary Table 7: Phosphotyrosine phosphopeptides and related genes whose abundance is
492 increased > 1.5 fold more in old knockout *vs* wildtpe comparisons than in young knockout vs wildtype
493 comparisons (and which display $p < 0.05$ in old knockout/wildtype comparisons. n = 3 - 4/
494 genotype/age group. Some phosphopeptides could come from several genes, as listed.

495 Supplementary Table 6: Gene ontology (cellular component) terms overrepresented ($p < 2 \times 10^{-6}$) in
496 the list of genes in Table 5.