Receptor-type protein tyrosine phosphatase α (RPTPα) is an important positive regulator of SRC kinase activation and a known promoter of cancer growth, fibrosis, and arthritis. The membrane domain of RPTPs comprises an extracellular region, a transmembrane helix, and two tandem intracellular catalytic domains referred to as D1 and D2. The D2 domain of RPTPs is believed to mainly play a regulatory function; however, no regulatory model has been established for RPTPα-D2 or other RPTP-D2 domains. Here, we solved the 1.8 Å resolution crystal structure of the cytoplasmic region of RPTPα, encompassing D1 and D2, trapped in a conformation that revealed a possible mechanism through which D2 can allosterically inhibit D1 activity. Using a D2-truncation RPTPα variant and mutational analysis of the D1/D2 interfaces, we show that D2 inhibits RPTPα phosphatase activity and identified a P405PFTP408 motif in D1 that mediates the inhibitory effect of D2. Expression of the gain-of-function S406A/T407A RPTPα variant in HEK293T cells enhanced SRC activation, supporting the relevance of our proposed D2-mediated regulation mechanism in cell signaling. There is emerging interest in the development of allosteric inhibitors of RPTPs but a scarcity of validated allosteric sites for RPTPs. The results of our study not only shed light on the regulatory role of RPTP-D2 domains, but also provide a potentially useful tool for the discovery of chemical probes targeting RPTPα and other RPTPs.

Protein tyrosine phosphatases (PTPs) are a large subfamily of protein phosphatases that play essential roles in signal transduction by countering the action of protein tyrosine kinases and have been demonstrated to regulate a wide range of cellular processes, including cell proliferation, differentiation, migration, cell cycle, and metabolic homeostasis (1). Classical PTPs are characterized by a ~300-amino acid conserved catalytic (PTP) domain (2, 3) and categorized into receptor- and nonreceptor-type PTPs (1, 4). The human genome encodes 20 receptor PTPs (RPTPs), classified into eight subtypes, which comprise an N-terminal extracellular domain of divergent function, sequence, and structure, a transmembrane domain, and one or two PTP domains (1, 4). Receptor-type protein tyrosine phosphatase α (RPTPα) is encoded by the PTPRA gene and, together with receptor-type protein tyrosine phosphatase ε (RPTPe), defines the R4 RPTP subtype, characterized by a short, highly glycosylated extracellular domain of unknown function, a membrane-spanning domain, and two tandem catalytic catalytic domains referred to as D1 (membrane-proximal) and D2 (membrane-distal). Despite the structural similarities between D1 and D2, most or all of the catalytic activity in D1+D2 PTPs resides on D1, whereas D2 is believed to have regulatory and substrate recognition functions (5–13). However, the mechanism of such regulatory action of the RPTP D2 has mostly remained undefined. RPTPα has been shown to be regulated by dimerization, and the observation of an inaccessible active site in the dimeric crystal structure of its isolated D1 led to the proposed “wedge” model, in which an N-terminal helix-turn-helix “wedge” motif formed by the two N-terminal helices (α1’ and α2’) of one monomer inhibits its catalytic activity of the other monomer by insertion into its active site (14, 15). It has been suggested that oxidation of the RPTPα D2 regulates phosphatase activity by modifying the relative conformation of phosphatase dimers (7). However, the canonical D1–D2 arrangement seen in several subsequent RPTP structures encompassing both domains proved incompatible with the wedge model; thus, it remains unclear whether the model applies to all RPTPs (2, 3, 16, 17).

At the core of the active site of classical PTP domains, there is a signature HCXR(S/T) motif, included in the so-called “P loop,” containing a nucleophilic cysteine and an arginine side chain responsible for coordinating the phosphate moiety and stabilizing the negative charge on the protein-substrate com-
RPTPα allosteric regulation by D2 domain

plex and the transition state (18). The same arginine is also responsible for triggering a switch from an inactive, open to an active, closed conformation of the other prominent structural element of the active site, the WPD loop, named after its conserved Trp-Pro-Asp motif, upon substrate binding (18, 19). Fig. 1A shows the location of the P and WPD loops in a previously published structure of the RPTPα D1 domain. Following substrate binding, the reaction proceeds via two irreversible catalytic steps: the formation of a phosphocysteine intermediate and its breakdown and dissociation of the phosphate group. The aspartic acid in the WPD loop acts as a general acid to facilitate the nucleophilic attack in the first step by protonating the leaving group oxygen of the substrate and as a general base in the second step by deprotonating a water molecule that attacks the phosphocysteine group to release a phosphate ion (summarized in Fig. 1B) (18). Crucially, WPD loop closure has been shown to be rate-determining for the reaction catalyzed by both the model phosphatase PTP1B and the highly active Yersinia YopH, suggesting that PTP activity can be regulated by controlling WPD loop kinetics (20) and rationalizing the inhibitory effect of ligands that prevent WPD loop closure (21).

RPTPα has been directly implicated in the regulation of a host of cellular functions, such as proliferation and survival, cell cycle arrest, fibroblast migration, neuronal migration and differentiation, and integrin signaling (22–25), and is an established tumor promoter and a cancer target. Recent studies from the Downey laboratory and our laboratory also demonstrated RPTPα’s ability to promote transforming growth factor β signaling in lung fibroblasts and cell motility via the focal adhesion kinase pathway in synovial fibroblasts. Loss of RPTPα protected mice from lung fibrosis and arthritis, suggesting additional indications for RPTPα inhibitors besides cancer (25, 26). Activation of the SRC kinase by dephosphorylation of its C-terminal inhibitory Tyr230 in many cell types has emerged as a major mechanism through which RPTPα contributes to such diverse processes (22–25).

Despite the fact that RPTPα and several other PTPs have been validated as potential drug targets, development of orthosteric small-molecule inhibitors of PTPs has proven problematic due to both the charged nature and the high structural conservation of the active site (27, 28). Recently, non-active site inhibitors have been identified for SHP2 and PTP1B, two medically relevant PTPs for which allosteric regulation mechanisms have been uncovered and characterized (21, 29–32). The promise of noncompetitive inhibitors of PTPs has ignited interest in the discovery of novel allosteric mechanisms for the regulation of RPTP activity; however, currently, the allosteric regulation of RPTP-D1 domains remains mostly undefined. For PTP1B, evidence is mounting for the presence of an extended allosteric network centered on helix α7, whose position is determined by subtle conformational shifts in its surrounding structural elements (α3, α6, and L11), which in turn affects WPD loop closure (29–31) via a 185PESP188 motif (405PFTPGS in RPTPα and consensus sequence PX(X)(P/A) in D1 + D2 RPTPs; see Fig. S1) located at the C-terminal end of the WPD loop.

Here, to explore the possible allosteric regulation of RPTPα-D1, we report the first crystal structure of the cytoplasmic domain of RPTPα (D1D2). Through kinetic analysis and site-directed mutagenesis, we demonstrate that D2 is able to inhibit D1 catalytic activity. Our data suggest D2-mediated restriction of D1-WPD loop function, and we validate our resulting inhibition model in HEK293T cells. Our model helps unravel the physiological function of RPTP-D2 domains and could pave the way to future discovery of allosteric inhibitors for RPTPα and potentially other D1 + D2 RPTPs.

**Results**

**Crystal structure of the tandem catalytic domains of RPTPα**

We crystallized a large C-terminal cytoplasmic region of RPTPα, comprising D1 and D2 (residues 202–793, hereafter referred to as RPTPαWT) and solved its crystal structure by molecular replacement to a resolution of 1.8 Å (Table 1). The protein crystalizes as a monomer, consistent with previous reports (3) as well as size-exclusion data from the present work (not shown), with two molecules per asymmetric unit (chains A and B). The final model contains residues 206–793 (chain A) and 207–789 with gaps at 226–227 and 515–520 (chain B) with 1529 solvent molecules and 57% solvent content and is in good agreement with the published structures of the isolated D1 and D2 domains of RPTPα (PDB accession codes 1YFO and 1Y15 (14, 33)) with maximum root mean square deviation (r.m.s.d., all atoms) of 0.74 and 0.86 Å for D1 and D2, respectively. Ambiguous electron density was seen for residues 221–234 in PTD loop (not shown), with two molecules per asymmetric unit (chains A and B). The promise of noncompetitive inhibitors of PTPs has ignited interest in the discovery of novel allosteric mechanisms for the regulation of RPTP activity; however, currently, the allosteric regulation of RPTP-D1 domains remains mostly undefined. For PTP1B, evidence is mounting for the presence of an extended allosteric network centered on helix α7, whose position is determined by subtle conformational shifts in its surrounding structural elements (α3, α6, and L11), which in turn affects WPD loop closure (29–31) via a 185PESP188 motif (405PFTPGS in RPTPα and consensus sequence PX(X)(P/A) in D1 + D2 RPTPs; see Fig. S1) located at the C-terminal end of the WPD loop.

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**Table 1**

| Parameter | Value |
|-----------|-------|
| Data collection | Resolution (Å) 30–1.80 (1.83–1.80) |
| Wavelength (Å) | 0.9774 |
| Space group | P2₁,2 |
| Unit cell a, b, c (Å) | 112.1, 136.4, 104.7 |
| α, β, γ (degrees) | 90, 90, 90 |
| Unique reflections | 148,737 (7290) |
| Completeness | 100 (100) |
| Multiplicity | 6.2 (5.7) |
| I/σ(I) | 10.6 (1.9) |
| Rmerge | 0.037 (0.401) |
| CC₁₀₀ | 0.998 (0.751) |
| Wilson B factor (Å²) | 23.0 |

**Refinement**

| Parameter | Value |
|-----------|-------|
| Resolution (Å) | 30–1.80 (1.85–1.80) |
| Reflections used | 141,157 (10,297) |
| Rmerge/Rmerge | 0.155/0.191 (0.261/0.286) |
| Non-H protein atoms | 9682 |
| Solvent atoms | 1529 |
| Average B factor (Å²) | 38.2 |
| Protein main/side chain A | 30.8/39.2 |
| Protein main/side chain B | 34.8/43.0 |
| Solvent | 45.9 |
| r.m.s.d. bond lengths (Å) | 0.006 |
| r.m.s.d. bond angles (degrees) | 1.48 |
| Ramachandran plot | |
| Allowed (%) | 97.0 |
| Disallowed (%) | 2.8 |

**Values shown in parentheses are for the highest-resolution shell.**
ary structure elements in relation to primary and tertiary structure are shown in Fig. S1.

Fig. 1C shows our D1 + D2 structure compared with RPTPα’s closest homolog, RPTPε (PDB code 2JJD). An extensive network of interdomain interactions (summarized in Fig. S2), seen in both molecules in the asymmetric unit with only minor differences, provides stability to the typical architecture seen in other currently available tandem domain RPTP structures (2, 3, 16, 17). The previously described interactions between residues in strands β9 and β10 and helix α3 of D1 and the β2-β3 loop...
helix α5 in D2 are also seen in our structure, albeit with differences due to the moderate sequence conservation across PTP subfamilies. The linker, of sequence 496GDTE499, is constrained to adopt the same conformation as in LAR, RPTPσ, RPTPγ, and CD45: a network of direct or water-mediated hydrogen bonds connects the main-chain oxygen of Gly496, the O of Thr498, the side chains of Glu491 on D1 α6, and those of Asp750 and Gln753 on D2 α5, whereas Asp497 and Gln499 form salt bridges with Lys516 in D1 α3 and Arg742 in D2 α4. The D2 loop between β2 and β3 contributes a backbone hydrogen bond between Tyr598 and Val667 in the β9-β10 turn, whereas Nζ of Lys591 contacts Oγ of Thr597, and the main-chain oxygen of Thr597 (molecule A) and Nη of Arg599 contact the main-chain oxygen of Val665 (molecule B). The interface is further stabilized by shape and charge complementarity (shape complementarity (S) = 0.76 and 0.70 for D1 and D2), as evidenced by several van der Waals and electrostatic interactions. In addition, the PXXP motif (405PFTP408 in RPTPα) in D1 interacts tightly with D2 residues in α4 and the region between α1 and β3, as shown in Fig. 1D. Phe406 closely contacts the side chains of Gln682 through the face of its phenylalanine ring and Asp386 in an anion–π pair through its edge, at C–C and C–O distances of 3.3 and 3.6 Å, respectively, whereas the side chains of Phe636, Leu760, and Ser757 approach van der Waals distances of 3.8, 4.0, and 4.1 Å. Thr407 contributes a water-mediated interaction with the side chains of Gln753, Lys756, and Ser757. Fig. 1E shows the same region of the molecule in its 2Fo – Fc map contoured at 1.5σ. A 2Fo – Fc map around the linker region is shown in Fig. S3. The total surface area buried at the D1/D2 interface, as calculated using the PISA (“Protein interfaces, surfaces, and assemblies”) service at the European Bioinformatics Institute (SCCR_015749) (34), is 1390 and 1350 Å2 for molecules A and B, indicating a weak interaction that is not predicted to be stable in the absence of covalent attachment.

When we compared our structure with that of RPTPε (PDB code 2JJD, chains A–F(3)) using DynDom (35), we noticed a 14–19° rotation of D2 with respect to D1 around an axis running roughly along the main interdomain interaction interface from the linker region to the β9-β10 turn. This rotation brings the WPD loop and helix α3 of RPTPα in closer contact with D2 (Fig. 1C and Movie S1). For comparison, rotation angles of 2–9°, often with major components perpendicular to the same axis, were calculated between pairs of chains in 2JJD. Because residues at the D1/D2 interface and linker region are highly conserved between RPTPα and RPTPε (Fig. 1F), we speculated that the observed differences might reflect a degree of interdomain flexibility constrained as distinct rigid conformations by the crystal packing.

D2 regulates the catalytic activity of RPTPα

It has been observed that nearly all RPTPα phosphatase activity resides at the D1 active site with little contribution from the D2 active site (9, 36). We confirmed this observation in the context of our tandem D1D2 construct by mutating the active cysteine to serine in D1 (C433S, RPTPαD1CS) or D2 (C723S, RPTPαD2CS) and showing that D2 has negligible phosphatase activity on 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as substrate (data not shown). Despite D2’s modest direct contribution to the enzymatic activity, the observation that, in the crystal structure of RPTPα, residues in D2 come in close contact and restrict the conformational freedom of the crucial PXXP motif suggests that D2 might be able to directly regulate the activity of D1. Based on our structural information (see Fig. S4), the WPD loop freedom appears to be limited when the tandem domains are in the closed state; thus, we formulated a model in which RPTPα activity is turned on and off by switching between open and closed interdomain conformations, respectively. To test our model, we first compared the phosphatase activities of RPTPαWT and the RPTPαD2 mutant (residues 202–503) using DiFMUP as substrate (Fig. 2). RPTPαD2 exhibited a turnover number (kcat) ~1.7 times that of RPTPαWT. Increased substrate conversion rate in the absence of D2 is consistent with its potential involvement in an inhibitory mechanism that regulates RPTPα WT phosphatase activity. The effect of the AD2 and other RPTPα mutations on kcat/Km will be discussed below.

Mutational analysis of the regulation of D1 activity by D2

We next sought to investigate the specific roles of residues in the RPTPα 405PFTP408 motif (here called PFTP), corresponding to 185PEPS188 in RPTPβ and PXX(P/A) in other RPTPs (Fig. 1F), in the D2-mediated RPTPα inhibition mechanism. We generated the RPTPαFATA and RPTPαD2 FATA mutants by substituting Ala for Phe406 and Thr407 in RPTPαWT and RPTPαD2, respectively, to assess the effect of the intermolecular interaction between the PFTP motif and the D2 interface. As observed following the deletion of D2, the reaction rate of RPTPαFATA was elevated (~1.8-fold) compared with RPTPαWT and similar (~1.1-fold increase) to that of RPTPαD2, whereas RPTPαD2 FATA only showed a modest (~1.2-fold) increase in kinetic activity compared with RPTPαD2 (Fig. 2). Similarly, a ~1.5-fold increase in activity toward DiFMUP was observed when we mutated Gln761 or Phe682, two D2 residues involved in the interaction with Phe406, to alanine, generating the mutants RPTPαQ761A and RPTPαP683A, respectively (Fig. 3). These data suggest that the PFTP motif exerts a D2-dependent inhibitory influence upon RPTPα-D1, although a small intrinsic activating effect of the FATA mutation on D1 activity is also measurable. These results point to a critical role of the D1-PFTP motif in the mechanism of phosphatase activity inhibition by D2.

For D2 to be able to inhibit the phosphatase activity of D1 in response to a change of their relative orientation in solution, RPTPαWT must exist as an equilibrium between at least two (closed/open) and possibly a continuum of intermediate states. To further validate our model, we therefore introduced attractive or repulsive charges into D1 and D2, generating mutants RPTPαD1−/D2+ and RPTPαD1+/D2− to shift the equilibrium toward a more closed or open structure. We chose residues in α1’ of D1 and residues following α2’ in D2 as the gap between these surfaces widens by 3–4 Å in the open state seen in RPTPε versus the closed state in RPTPα, whereas they are still sufficiently removed from the active site and the WPD loop to minimize any direct influence on the catalytic rate constants. For the D1− mutation, the sequence 220EINRRMA227 was replaced with EIDERMED, and for the D2− and D2+ mutations,
531LTSIKIQNDK540 was replaced with LTEIEIQEDK and LTKIKIQNKK, respectively, as shown in Fig. 4A. As expected, the catalytic activity of RPTPαWT and its mutants: RPTPαFATA, RPTPαFATA, and RPTPαFATA, was significantly reduced compared with that of RPTPαD1/H11002/D2/H11001 (Fig. 4, B–D). The turnover number of RPTPαD1/H11002/D2/H11002 was more than double that of RPTPαD1/H11002/D2+. However, the difference was lost after the introduction of the FATA mutation (RPTPαD1/H11002/D2+FATA). This observation is consistent with the effect of introducing attractive and repulsive charges in the D1/D2 interface being mediated by the PFTP motif and lends further support to a regulation mechanism by interdomain flexibility in RPTPα.

**Kinetic data support inhibition of D1-WPD loop catalysis by D2**

To seek evidence in support of the involvement of the WPD loop in the allosteric regulation mediated by the PFTP motif, we took advantage of differences in the catalytic reaction mechanism between two PTP substrates, DiFMUP and the closely related 4-methylumbelliferyl phosphate (MUP; Thermo Fisher Scientific). PTP catalysis (see also Fig. 1B) proceeds via reversible substrate binding (step 1 in Reaction 1, where ES represents the substrate-bound enzyme and EP is the phosphocysteine intermediate), followed by two irreversible steps, the formation (step 2) and breakdown (step 3) of a phosphocysteine intermediate.

\[
\begin{align*}
E + S &\rightleftharpoons ES \\
ES &\rightarrow EP \\
EP &\rightarrow E + P
\end{align*}
\]

Reaction 1

In step 2, the aspartic acid in the WPD loop acts as a general acid by protonating the leaving group. In the phosphocysteine breakdown (step 3), the aspartate acts as a base to facilitate the nucleophilic attack of a water molecule that dephosphorylates the cysteine. However, because the product of DiFMUP dephosphorylation (DiFMU) has a pK_a of 4.7, due to the presence of two F substituents on the aromatic ring (37), dissociation of DiFMU is predicted not to require acid catalysis at the chosen physiological pH of 7.3. Therefore, as a consequence of the dissociative nature of the transition state (discussed in Ref. 18), the reaction rate of step 2 with DiFMUP as substrate will be relatively independent of WPD loop involvement. According to the...
Michaelis–Menten model for a two-step reaction under the quasi-steady-state approximation, $k_{\text{cat}}/K_m$ is solely dependent on the kinetics of substrate binding and the first irreversible catalytic step (18).

\[
K_m = \frac{k_3}{k_2 + k_3} \frac{k_2 + k_{-1}}{k_1} \quad \text{(Eq. 1)}
\]

\[
k_{\text{cat}} = \frac{k_2 k_3}{k_2 + k_3} \quad \text{(Eq. 2)}
\]

\[
k_{\text{cat}} = \frac{k_2 k_1}{k_2 + k_{-1}} \quad \text{(Eq. 3)}
\]

Thus, the observation that, compared with $k_{\text{cat}}/K_m$, $k_{\text{cat}}$ is exclusively or more strongly affected by mutations that abolish or relax the conformational restraints on the PFTP motif by D2 is consistent with the involvement of the WPD loop in the allostERIC regulation that we propose in our model. When we assessed RPTPαFATA-mediated dephosphorylation of MUP whose dephosphorylation product has a pK$_a$ of 7.8 (37), as a substrate at pH 6.0 (Fig. 5), we observed a significant increase of $k_{\text{cat}}/K_m$ for RPTPαFATA versus RPTPαWT. This observation is consistent with the assumptions in our structure-based hypothesis described above.

**Conservation of the PXXP-mediated inhibitory mechanism in other RPTPs**

To examine whether our hypothesized RPTPα-inhibitory mechanism is conserved in RPTPε, we introduced mutations homologous to those described above for RPTPα into RPTPε, thus generating the RPTPεFATA (F307A and T308A), RPTPεD1/D2, and RPTPεD1/D2+. Here we replaced the sequence122EIRVRSAD129 with EIDERSED for the D1/D2 mutation and the sequence432LTNVRIMKENM442 with LTKVRIMKKKM for the D2 mutation. Somewhat surprisingly, given the 70% sequence identity and surface homology between RPTPε and RPTPα within the catalytic domains (Fig. 6), all of the above-mentioned RPTPε mutants displayed similar kinetics when using DiFMUP as substrate (Fig. 6, B and C), indicating that the regulatory mechanism via the PFTP motif described for RPTPα is not echoed in RPTPε, at least under the experimental conditions employed. This observation is, however, consistent with the more open interdomain configuration, and the consequent increased distance between the PXXP motif and D2, in all
six independent molecules in the crystal structure of RPTPε (3). On the contrary, when we considered the more distantly related RPTPσ (see PTPRA and PTPRS in Fig. 1F and sequence conservation in Fig. 6D), mutation of RPTPσ120PEYP123 to PAAP (referred to as RPTPσEAYA) led to a greater than 2-fold increase in phosphatase activity compared with RPTPσWT (Fig. 6, E and F). This phenomenon was observed using both DiFMUP and MUP as substrates, mirroring what was seen with the corresponding RPTPα mutants, albeit with differences, namely a marked decrease and a smaller than expected increase in $K_m$. Whereas these differences in catalytic parameters are possibly due to electrostatic effects following the loss of a negative charge, the observed behavior of RPTPσ mutants is in agreement with the close approach between D2 and the PxP motif in the three available crystal structures of the tandem catalytic domain of RPTPσ (PDB codes 2FH7, 3SR9, and 4BPC (3, 38, 39)). Although more subtle than in our RPTPα structure, a similar interaction in RPTPσ occurs between the aromatic ring of Tyr1523 and the side chains of Glu1521 in D1 and the side chains of Thr1876 and Ser1678 in D2, respectively (3).

**Overexpression of RPTPαFATA promotes SRC activation in HEK293T cells**

Because RPTPα is known to dephosphorylate the SRC kinase on its inhibitory Tyr530, leading to increased activity and phosphorylation at the activating Tyr419 (40, 41), we sought to indirectly measure RPTPα phosphatase activity in cells by detecting phospho-SRC (Tyr419) levels in cells overexpressing RPTPα. We therefore overexpressed HA-tagged RPTPαWT and RPTPαD1H11002/D2H11002 constructs in HEK293T cells and analyzed phosphorylation of SRC by phospho-flow cytometry in populations of cells gated for expression of HA-RPTPα (Fig. 7A). Consistent with our in vitro observation of an elevated phosphatase activity of RPTPαFATA, the mean fluorescence intensity of phospho-SRC (Tyr419) was elevated in cells expressing RPTPαFATA compared with cells expressing similar levels of RPTPαWT (Fig. 7B). A similar difference was observed when comparing cells transfected with RPTPαD1H11002/D2H11002 with cells expressing RPTPαD1H11002/D2H11002 (Fig. 7C). These data provide an initial validation of our model of allosteric regulation of RPTPα in a cellular context.
**RPTPα allosteric regulation by D2 domain**

![Graph showing allosteric regulation](https://via.placeholder.com/150)

**Figure 5.** The PFTP motif limits D1 phosphatase activity by restricting WPD loop catalysis, as suggested by its effect on \( k_{cat}/K_m \). A, representative Michaelis–Menten curves showing enzyme activity of RPTP\(\alpha^{WT} \) and RPTP\(\alpha^{FATA} \) using MUP as substrate. Data points are presented as means ± S.D. B, table summarizing \( k_{cat}, K_m, k_{cat}/K_m \) of RPTP\(\alpha^{WT} \) and RPTP\(\alpha^{FATA} \) with MUP as substrate. Values are means ± S.D. C, bar graphs showing comparisons of \( k_{cat}, K_m, k_{cat}/K_m \) of RPTP\(\alpha^{WT} \) and RPTP\(\alpha^{FATA} \) with MUP as substrate. Each data point represents one individual experiment, and error bars represent S.D. A * t test was used to compare two data sets (****, \( p < 0.0001 \)).

**Discussion**

The allosteric regulation of PTP activity has gained attention in recent years in light of the increasing number of validated drug targets among PTPs (42) and the therapeutic promise offered by the discovery of noncompetitive modulators of PTP1B (14, 21, 29, 31) and SHP2 (32). It was suggested that D2 of RPTPs could regulate the activity of D1 in a manner similar to the role of helix \( \alpha 7 \) in PTP1B based on molecular architecture conservation (31). Our result shows that the presence of D2 roughly halves the activity of D1 in *in vitro* is consistent with a previous study in which p-nitrophenyl phosphate was used as substrate (9), but such a mechanism has not been demonstrated yet in detail. In this work, we describe the characterization, at the structural level, of a mechanism of D2-mediated regulation of D1 for RPTP\(\alpha \), a positive regulator of SRC that is considered a drug target for cancer, fibrosis, and rheumatoid arthritis. Our study is one of the first examples of structure-based assessment of the potential regulatory role of an RPTP-D2 on D1 activity. A recent report has described competitive inhibition of RPTP\(\beta \) by DJ001 and related compounds, which are believed to bind to the D1/D2 interface of RPTP\(\beta \) (43), an enzyme that shares with RPTP\(\alpha \) the PXXP-mediated D2 regulatory mechanism. However, the mechanism of action of DJ001 has not yet been investigated via structural biology and/or mutagenesis.

The regulatory mechanism described in our study could be relevant to the discovery of inhibitors of RPTP\(\alpha \) and likely other RPTPs, if compounds that are able to stabilize the closed interdomain conformation of the RPTPs can be identified. Although a small inhibitory effect of D2 persists in our RPTP\(\alpha^{FATA} \) mutant—possibly due to residual D1-D2 interaction (Fig. 2)—we speculate that RPTP\(\alpha^{FATA} \) could become a useful tool for counter-screening RPTP\(\alpha \) inhibitors and selectively identifying those that act through the above-mentioned mechanism. However, a caveat of targeting the D1/D2 interface for drug discovery purposes is its relatively high level of conservation between different RPTPs. With regard to this point, the apparent lack of conservation of the D2-mediated regulation mechanism between RPTP\(\alpha \) and RPTP\(\varepsilon \) in *in vitro* is intriguing and unexpected based on the high conservation between these two members of the R4 subtype. Further investigations are warranted to understand the biochemical mechanisms underlying such divergent behavior, for example by assessing the relative weight of local nonconserved residues versus global molecular dynamic differences.

A limitation of our study is the fact that, whereas the geometry we observe in our crystal structure is fully closed with respect to the D1/D2 interface, we have no information about the equilibrium that likely exists in solution between open and closed populations. We were unable to reliably measure RPTP\(\alpha \) catalytic activity in the crystal itself or to generate mutants that would lock the closed state in solution by Cys-Cys cross-linking (data not shown). As a result, we could not achieve any assessment of whether any residual activity is present in the closed form of the enzyme. These considerations are relevant to the exploitation of the D1/D2 interface for discovery of noncompetitive inhibitors because the activity of the closed form would likely reflect the highest *in vitro* inhibition achievable. Similarly, we originally modeled the mechanism of allosteric D2-mediated regulation of RPTP\(\alpha \) D1 based on the observation of a downward movement of the P\(\rightarrow \)P motif in concert with WPD loop closure (20) and the conservation of the crucial PTP1B residue Pro\(185 \) involved in the so-called CH/\( \pi \) switch to stabilize the closed WPD loop conformation (30). However, no structure of RPTP\(\alpha \) with a closed WPD loop is available at this time, and the role of the CH/\( \pi \) switch has not been formally demonstrated for PTP\(\beta \) other than PTP1B. Therefore, as the D2 can affect catalytic activity by destabilizing the closed WPD conformation or by slowing down its dynamics, we cannot distinguish between these two possibilities on the basis of our kinetic analysis alone.

Mutagenesis of the PXXP motif and of D1/D2 interface residues of recombinant RPTP\(\alpha \)-D1D2 and of RPTP\(\alpha \) overexpressed in HEK293T cells led to a similar gain-of-function behavior. Despite the limitations related to overexpressing RPTP\(\alpha \) in these experiments, these data provide preliminary evidence that our model of D2-mediated regulation of D1 activity via the PXXP motif is valid for full-length RPTP\(\alpha \) expressed in a cellular context. However, we cannot speculate further about the physiological relevance of the allosteric mechanism in question for regulation of cell signaling by RPTP\(\alpha \). There is no known post-translational regulation of the D1/D2 interface of RPTP\(\alpha \) and RPTP\(\varepsilon \) and/or ligands of their ectodomains.
RPTPα allostERIC regulation by D2 domain

Thus, we currently have a limited ability to investigate whether the mechanism might be subjected to physiological regulation and/or contributes to the modulation of RPTPα activity in response to cell stimuli. Interestingly, some of the weakest and/or ambiguous electron densities, suggestive of a possible protein-protein interaction area, are found at amino acids 221–334, around the α1’–α2’ turn in D1, and 534–542, C-terminal to α2’ in D2, roughly coincident with the blue- and red-colored regions in Fig. 4. We speculate that simultaneous binding of a ligand to both patches or its interaction with the 40-residue linker connecting D1 to the transmembrane helix could tether together or force apart D1 and D2 and modulate catalytic activity. Given the role D2 can play in substrate recognition (10, 12, 13), an intriguing possibility is that D2 regulates phosphatase activity by simultaneous substrate binding and enzyme activation.

An area worthy of future investigation is the potential relationship between the allosteric mechanism described here and the dimerization of RPTPα. Two mechanisms have been proposed to mediate outside-in signaling of RPTPs involving dimerization of their intracellular domains, namely the aforementioned “wedge” hypothesis and a head-to-toe dimer in dimerization of their intracellular domains, namely the aforementioned "wedge" hypothesis and a head-to-toe dimer. An investigation of these mechanisms might be subjected to physiological regulation (5, 6) or represent a step toward unraveling the allosteric regulation of RPTPs. The latter is one of the least understood areas of PTP biology but is potentially relevant to enable the drugging of these enzymes for the treatment of multiple human diseases.

Experimental procedures

Cloning, mutagenesis, protein expression, and purification

For protein expression, a codon-optimized ORF encoding a cytoplasmic fragment of human RPTPs (residues 202–793) encompassing the tandem catalytic domains (GenScript) was subcloned into the Ncol/Xhol site of pET28a (Novagen) and expressed in Escherichia coli BL21(DE3) as a C-terminal six-histidine fusion. Bacterial cultures were grown at 37 °C and induced with isopropyl-1-thio-β-d-galactopyranoside at room temperature for >12 h. After recovery from the soluble fraction of the cell extract, RPTPα was purified by nickel-nitriolatric acid affinity chromatography (Qiagen), anion exchange on a POROS 20 HQ column (Thermo Fisher Scientific), and size-exclusion chromatography (ENrich SEC 650, Bio-Rad). Typical yields were 20 mg of purified protein per liter of culture. For RPTPα and RPTPβ, the corresponding DNA fragments (residues 104–699 and 1331–1916, respectively) were amplified from a mouse full-length cDNA and cloned into the Ncol/Xhol site of pET28a and the Ndel/Xhol site of pet26b (Novagen), respectively. All mutants were generated by standard site-directed mutagenesis techniques and purified following the same procedures. For eukaryotic expression, a plasmid encoding full-length mouse RPTPα with an HA epitope tag at the C terminus in pCDNA3.1(+) at restriction sites AflIII and Xhol was purchased from GenScript. Mutants were generated by standard site-directed mutagenesis techniques. All mutations were confirmed by DNA sequencing. All primers used were synthesized by Integrated DNA Technologies.

Crystallization and X-ray crystallography

Crystals of RPTPα 202–793 grew in 3–7 days by sitting- or hanging-drop vapor diffusion against a buffer containing 6% PEG 20000, 100 mM MES, pH 6, 10 mM DTT at a protein concentration of 6 mg/ml. Crystals belonged to space group P2_1_2_1 with unit cell parameters a = 112.1 Å, b = 136.4 Å, c = 140.7 Å and 2 molecules/asymmetric unit. A complete native data set to 1.8 Å resolution was collected at 100 K using 25% glycerol as cryoprotectant from a single crystal at beamline 5.0.1 of the DLS.

Figure 6. The activity-modulating effect of D2 is present in RPTPα but not RPTPβ. A, sequence conservation between RPTPα and RPTPβ mapped on the RPTPα solvent-accessible surface. Identical residues are in yellow, and nonidentical residues are in dark cyan. B, top, representative Michaelis–Menten curves showing enzyme activity of RPTPα and mutants RPTPαA1α-A1α, RPTPαD1-D2, and RPTPαD1-D2 using DIFMUP as a substrate. Data points are presented as means ± S.D. Bottom, table summarizing kcat, KM, and kcat/KM from the same experiment set. Data were calculated by averaging six individual experiments, each including three technical replicates, and are reported as averages ± S.D. C, bar graphs showing kcat, KM, and kcat/KM for each of the RPTPα and its mutants above. Each data point represents one individual experiment for which Michaelis–Menten parameters were calculated by averaging three technical replicates. A one-way ANOVA test was used to calculate means (****, p < 0.0001; ***, p < 0.001; **, p < 0.01), and D, the sequence conservation between RPTPα and RPTPβ mapped on the RPTPα solvent-accessible surface as in A, E, top, representative Michaelis–Menten curves showing enzyme activity of RPTPα and mutants RPTPαA1α-A1α using DIFMUP as substrate. Data points are presented as means ± S.D. Middle, table summarizing kcat, KM, and kcat/KM for the same experiment set. Data were calculated by averaging six individual experiments, each including three technical replicates. Bottom, bar graphs showing kcat, KM, and kcat/KM for each of the mutants above. Each data point represents one individual experiment for which Michaelis–Menten parameters were calculated by averaging three technical replicates, and error bars represent S.D. An F test was used to compare two data sets (****, p < 0.0001; ***, p < 0.001; **, p < 0.01).
Figure 7. HEK293T cells overexpressing RPTPαFATA display higher phosphorylation of SRC Tyr419 than HEK293T cells with similar overexpression of RPTPαWT. HEK293T cells transfected with HA-tagged RPTPαWT versus RPTPαFATA or with RPTPαD1/D2 versus RPTPαD1/D2 expression constructs were stained with anti-HA Alexa 488–conjugated antibody and anti-pY419 SRC Alexa 647–conjugated antibody and analyzed by flow cytometry. A, representative gating strategy: live single cells with expression of RPTPα constructs over the maximum fluorescence of nontransfected cells were gated, and Alexa 488 (anti-HA), Alexa 647 (anti-phospho-SRC (Tyr419)), and the ratio of Alexa 647/Alexa 488 were assessed and plotted on histograms. The Kolmogorov–Smirnov test was used for comparing constructs within each data set (e.g., *** p < 0.001). B and C, comparison of the ratio of Alexa 647/Alexa 488 fluorescence between RPTPαWT and RPTPαFATA-expressing (B) and between RPTPαD1/D2-expressing (C) cells. Panels are representative of seven independent experiments with similar results. In the graphs, Alexa 647/Alexa 488 mean fluorescence intensity (MFI) of RPTPαWT and RPTPαD1/D2 were normalized to 1 compared with RPTPαFATA and RPTPαD1/D2, respectively. Each data point represents one of seven independent experiments. Black points indicate a significant difference (p < 0.05 by Kolmogorov–Smirnov test) within each data set. The Mann–Whitney test was used (**, p < 0.01; ***, p < 0.001).
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Advanced Light Source and processed using imosflm (45). The structure was solved by molecular replacement in PHASER (46) using the structure of mouse RPTPα 202–503 (PDB code 1YFO (14)) for both tandem domains, followed by initial automatic model rebuilding/refinement with Buccaneer (47) and manual model building in Coot (48) and refinement with refmac (49). TLS refinement was used with one TLS group per PTP domain. Data quality was analyzed with Rampage (50). S values were calculated with the CCP4 program SC (51) with default input parameters. Data collection and refinement statistics are summarized in Table 1. Molecular graphics objects were generated with Chimera (52).

**Phosphatase activity assays**

For assays using DiFMUP as a substrate, the protein was diluted to 2 nM in buffer 2xT1 (100 mM Tris, pH 7.3, 20 mM DTT, 0.02% Triton X-100), and various concentrations (800, 400, 200, 80, 40, 20, 8, and 0 μM) of DiFMUP (Invitrogen) were prepared in 8% DMSO. 25 μl of protein solution and 25 μl of DiFMUP solution were mixed on a 96-well solid black polystyrene microplate (Corning), and DiFMU formation was continuously monitored by measuring its fluorescence at wavelengths of 358 nm (excitation) and 455 nm (emission) on a Tecan Infinite M1000 plate reader for 20 min. To convert fluorescence readings into DiFMU formation rates, a series of calibration curves were generated by combining known amounts of DiFMU and DiFMUP in assay buffer to yield total concentrations equal to each initial DiFMUP concentration in the assay. A calibration factor was calculated for each DiFMU + DiFMUP concentration as the slope of the fluorescence versus DiFMU concentration. Initial reaction rates were fitted to a Michaelis–Menten equation of

\[ V = \frac{K_{cat}[enzyme][S]}{K_m + [S]} \]

by GraphPad Prism 8. For assays using MUP as a substrate, the protein was diluted to 25 nM in buffer 2xT2 (100 mM Bis-Tris methane, pH 6.0, 20 mM DTT, 0.02% Triton X-100), and various concentrations (16, 8, 4, 1.6, 0.8, 0.4, 0.16, and 0 μM) of MUP (free acid, Biotium) buffered at pH 6.0 were prepared in 8% DMSO. 12.5 μl of protein solution and 12.5 μl of MUP solution were mixed on a Corning 96-well solid black polystyrene microplate at 0, 2, 4, 6, 8, and 10 min, and all reactions were stopped by adding 25 μl of 1 M NaOH followed by fluorescence measurement with wavelengths of 358 nm (excitation) and 455 nm (emission) on a Tecan Infinite M1000 plate reader. Fluorescence readings were converted into 7-hydroxy-4-methyl coumarin (Acros) formation rates using a similar procedure as described above for DiFMUP/DiFMU. Initial reaction rates were fitted to a Michaelis–Menten equation,

\[ V = \frac{k_{cat}[enzyme][S]}{K_m + [S]} \]

by GraphPad Prism 8.

**Transient transfection of HEK293T cells**

HEK293T cells were cultured in Corning Dulbecco’s modified Eagle’s medium (with 4.5 g/liter glucose, l-glutamine, sodium pyruvate) with 10% fetal bovine serum (BioFluid Technologies) at 37 °C and 5% CO₂. Transient transfections were carried out in standard 6-well cell culture plates (Corning) with 2.5 μg of plasmid using three different transfection reagents, linear polyethyleneimine (Sigma), FuGENE (Promega), or Lipofectamine 3000 (Thermo Fisher Scientific), in Opti-MEM medium (Gibco). The medium was changed to 10% FBS-containing medium after 12 h, and the cells were harvested after 48 h.

**Flow cytometry**

Transfected HEK293T cells were trypsinized and suspended in FACS buffer (2.5% FBS, 1 mM EDTA, 0.01% NaN₃). Cells were stained with fixable viability dye eFluor™ 780 (eBioscience/Thermo Fisher Scientific) to select live cells. Cells were then incubated with Fc block (BD Pharmingen) and stained with anti-HA antibodies conjugated with Alexa Fluor 488 (CST) for 1 h, followed by another staining with mouse anti-SRC (pY419; clone K98-37) antibodies conjugated with Alexa Fluor 647 (BD Biosciences) for 1 h. Cells were analyzed on a Bio-Rad ZE5 cell analyzer, and collected data were analyzed using FlowJo software (Tree Star, Inc.).

**Statistical analysis**

The statistical analyses utilized for each experiment are reported in the figure legends. Nonparametric stats were used unless data were assessed as fitting a normal distribution by Shapiro–Wilks test. The significance of one-way ANOVA tests was corrected for multiple comparisons using the Tukey test.

**Data availability**

All data, except for when indicated as “data not shown,” are contained within the paper. Structural coordinates and diffraction data have been deposited in the Protein Data Bank with code 6UZT. All data described in the article, including raw data and data indicated as “data not shown,” will be made available to any qualified investigator upon request. Please E-mail Nunzio Bottini (nbottini@health.ucsd.edu) for access to any data or Eugenio Santelli (esantelli@health.ucsd.edu) for access to structural data.

**Author contributions**—E. S. and N. B. conceptualization; N. B. resources; Y. W., S. Y., E. S., and N. B. data curation; Y. W., S. Y., K. W., E. S., and N. B. formal analysis; S. Y., E. S., and N. B. supervision; N. B. funding acquisition; Y. W., S. Y., and N. B. validation; Y. W., K. W., and E. S. investigation; Y. W. and E. S. visualization; Y. W., S. Y., M. N. S., S. M. S., E. S., and N. B. methodology; Y. W., E. S., and N. B. writing—original draft; E. S. and N. B. project administration; Y. W., S. Y., K. W., M. N. S., S. M. S., E. S., and N. B. writing—review and editing.

**Acknowledgments**—We thank the staff at the Advanced Light Source at Lawrence Berkeley National Laboratory for assistance with remote X-ray data collection. We thank Dr. Ari Elson (Weizmann Institute) for generously sharing the RPTPα-encoding plasmid.

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