Fission yeast Duf89 and Duf8901 are cobalt/nickel-dependent phosphatase–pyrophosphatases that act via a covalent aspartyl–phosphate intermediate

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Ana M. Sanchez 1,©, Agata Jacewicz 1, and Stewart Shuman 1,*

From the 1Molecular Biology Program, Memorial Sloan Kettering Cancer Center, New York, New York, USA; 2Gerster Sloan Kettering Graduate School of Biomedical Sciences, New York, New York, USA

Edited by Wolfgang Peti

Domain of Unknown Function 89 (DUF89) proteins are metal-dependent phosphohydrolases. Exemplary DUF89 enzymes differ in their metal and phosphosubstrate preferences. Here, we interrogated the activities and structures of two DUF89 paralogs from fission yeast—Duf89 and Duf8901. We find that Duf89 and Duf8901 are cobalt/nickel-dependent phosphohydrolases adept at hydrolyzing p-nitrophenylphosphate and PPi. Crystal structures of metal-free Duf89 and Co2+-bound Duf8901 disclosed two enzyme conformations that differed with respect to the position of a three-helix module, which is either oriented away from the active site in Duf89 or forms a lid over the active site in Duf8901. Lid closure results in a 16 Å movement of Duf8901 Asp195, vis-à-vis Asp199 in Duf89, that brings Asp195 into contact with an octahedrally coordinated cobalt. Reaction of Duf8901 with BeF3−/C15Co2+ squelched an enzymatic function to the human DUF89-family protein C6orf211 as an AdoMet-dependent protein glutamate methyltransferase (3). Dennis et al. (4) subsequently clarified that human C6orf211/DUF89 is a cobalt-dependent phosphomonooesterase devoid of methyltransferase activity. Budding yeast YMR027W and human C6orf211 belong to the so-called subfamily III of DUF89 proteins, which are of similar size (~380–480 amino acids) and have a characteristic RTxK motif situated ~40 to 50 amino acids from their C termini (1).

The fission yeast Schizosaccharomyces pombe genome encodes two subfamily III DUF89 paralogs: SPCC1393.13 (referred to henceforth as Duf89) and SPAC806.04c (henceforth Duf8901). Alignment of the 442-amino acid Duf89 and 480-amino acid Duf8901 proteins highlights 312 positions of side-chain identity/similarity. Our interest in Duf89 was prompted by the finding by Henry et al. (5) that deletion of the fission yeast SPCC1393.13 gene results in elevated expression of the phosphate homeostasis gene pho1. Whereas the significance of this finding was unclear at the time, it is now known that pho1 is actively repressed during growth in phosphate-rich medium by the transcription in cis of a long noncoding (Inc) RNA from the 5′ flanking gene prt(nc-pho1) (reviewed in Ref. (6)). IncRNA 3′ processing/termination is a key control point in pho1 mRNA repression. Tandem IncRNA–mRNA transcriptional interference can be tuned by increasing or decreasing the frequency with which RNA polymerase II terminates IncRNA transcription prior to encounter with the mRNA promoter (6). Genetic manipulations that enhance or

* For correspondence: Stewart Shuman, shumans@mskcc.org.
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diminish “precocious” termination of lncRNA transcription result in derepression or hyper-repression of pho1 mRNA expression in phosphate-replete cells. In particular, lncRNA-mediated transcriptional interference is sensitive to mutation of several phosphohydrolase enzymes that act on phospho-proteins or inositol pyrophosphates (IPPs) (7, 8).

To establish a framework for understanding the physiology of DUF89 proteins in fission yeast, and to further illuminate the mechanism of the DUF89 enzyme family, we produced and purified recombinant Duf89 and Duf8901 proteins and interrogated their biochemical properties and atomic structures. We find that Duf89 and Duf8901 are cobalt/nickel-dependent phosphohydrolases with vigorous activity against p-nitrophosphosphate (a phosphomonooester). Duf89 and Duf8901 are also able to hydrolyze PPi (a phosphoanhydride) in the presence of cobalt or nickel. Human DUF89 also has cobalt/nickel-dependent pyrophosphatase activity. We present crystal structures of fission yeast Duf89 and Duf8901 that highlight two enzyme conformations that differ with respect to the position of a three-helix module, which is either oriented away from the active-site pocket in Duf89—PO4 (open state) or forms a lid over the active-site pocket in Duf8901—Co2+ (closed state). We demonstrate that Duf89 reacts with BeCl2 and NaF in the presence of divalent cations Co2+, Ni2+, or Zn2+ to form covalent Duf8901-(Asp248)–BeF3 (closed state).

Results

Recombinant S. pombe Duf89 is a cobalt/nickel-dependent phosphohydrolase

We produced recombinant wildtype Duf89 in Escherichia coli as a His6Smt3 fusion and isolated it from a soluble bacterial extract by Ni-affinity chromatography. The tag was removed by treatment with the Smt3 protease Ulp1, and Duf89 was recovered free of His6Smt3 after a second round of Ni-affinity chromatography (Fig. 1A). To interrogate Duf89 enzymatic function, we tested its activity with the generic phosphomonoesterase substrate p-nitrophosphosphate. Hydrolysis of p-nitrophosphosphate liberates p-nitrophenol, which is quantified by its absorbance at 410 nm. We found that Duf89 readily converted 10 mM p-nitrophosphosphate into p-nitrophenol during a 30 min reaction at 37 °C in the presence of 0.5 mM nickel or cobalt (Fig. 1C). Zinc, manganese, magnesium, and cadmium were progressively less effective than nickel or cobalt in supporting phosphomonoesterase activity; copper and calcium were inactive (Fig. 1C). In parallel, we tested Duf89 for phosphodiesterase activity with the generic substrate bis-p-nitrophenylphosphate. No release of p-nitrophenol was detected in reactions containing 0.5 mM nickel, cobalt, zinc, magnesium, manganese, cadmium, or copper. Subsequent characterization of the p-nitrophosphomonoesterase activity of Duf89 was performed using 0.5 mM cobalt as the metal cofactor.

Duf89 phosphatase was optimal at pH 6.0 to 6.5; activity declined gradually as the pH was increased to 9.5 and more steeply when pH was lowered to 4.5 (Fig. 1D). The extent of p-nitrophosphophosphate hydrolysis during a 30 min reaction increased steadily with input Duf89 up to 20 pmol and continued to increase with a shallower slope in the range of 20 to 120 pmol (Fig. 1E). No hydrolysis of p-nitrophosphomonoesterase was detectable during a 30 min reaction with cobalt in the absence of Duf89 (Fig. 1F). From the slope of the Duf89 titration curve in the steeper initial phase, we calculated a specific activity of 8.36 nmol of p-nitrophenol formed per pmol of Duf89, which translates into an estimated turnover number of 4.6 s−1. Duf89 activity during a 5 min reaction under steady-state conditions (0.6% of substrate consumed) displayed a hyperbolic dependence on p-nitrophosphomonoester concentration (Fig. 1F). Fitting the data to the Michaelis–Menten equation yielded $K_m$ and $k_{cat}$ values of 11.0 ± 2.0 mM and 9.72 ± 1.13 s−1, respectively ($R^2$ = 0.9811). Gel filtration of Duf89 through a Superdex-200 column showed that the phosphatase activity profile coincided with that of the Duf89 polypeptide (Fig. S1); the Duf89 peak elution volume was consistent with it being a monomer in solution.

Effect of mutations of predicted Duf89 metal-binding residues

A primary structure alignment of S. pombe Duf89 (442 amino acids) and S. cerevisiae YMR027W (470 amino acids) highlighted 237 positions of amino acid side-chain identity/similarity (Fig. S2A). Fig. S2B shows a stereo view of the active site of YMR027W, occupied by two phosphate anions and a manganese ion (1; Protein Data Bank [PDB] ID: 5F13). The amino acids of YMR027W that engage the phosphates and the metal cofactor are conserved in S. pombe Duf89 as Asp252, Asn253, Asp290, Arg371, and Lys405 (shaded in cyan in Fig. S2A). To affirm that the observed phosphatase activity inhered to the recombinant Duf89 protein, we produced and purified two mutated versions in which the predicted metal-binding active-site amino acids Asp252 and Asp290 were replaced singly by alanine (Fig. 1A). Parallel assays of the wildtype and mutant Duf89s showed that activity was abolished by the D252A and D290A mutations (Fig. 1B).

Duf89 activity is inhibited by phosphate

Budding yeast YMR027W crystals with two phosphate ions in the active site were grown by Huang et al. (1) using a precipitant/reservoir solution containing 0.5 M phosphate. The presence of two phosphates in a subfamily III DUF89 protein raises the following questions regarding fission yeast Duf89: (i) Does it act on polyphosphorylated substrates? (ii) Is...
Duf89 inhibited by free phosphate? To address the latter issue, we supplemented the standard reaction mixtures containing 10 mM \( p \)-nitrophenylphosphate with increasing concentrations of sodium phosphate (pH 6.5) up to 100 mM. Two sets of control reactions were supplemented with the equivalent concentrations of sodium chloride or sodium sulfate. Phosphate elicited a concentration-dependent inhibition of Duf89 phosphatase activity, by 47% at 5 mM phosphate compared with the no phosphate control, and by 69%, 82%, 91%, and 96% at 10, 20, 50, and 100 mM phosphate, respectively (Fig. 2). These results suggest that orthophosphate (a reaction product) binds to the active site as well as, or better than, the \( p \)-nitrophenylphosphate substrate. By contrast, sulfate and chloride had relatively little impact, even up to 100 mM, at which point, activity was diminished by only 29% (chloride) or 30% (sulfate) compared with the unsupplemented control (Fig. 2).

\[ \text{Duf89 hydrolyzes inorganic pyrophosphate (PPI)} \]

Duf89 (0.1 \( \mu \)M) was reacted for 60 min with 1 mM PPi, in the presence of a panel of divalent cations at a concentration of 0.5 mM. The formation of P1 product was determined via colorimetric assay using the Malachite Green reagent. Duf89 catalyzed optimal hydrolysis of PPi to P1 in the presence of cobalt or nickel (870–1150 pmol of P1 formed or 435–575 pmol of PPi hydrolyzed, per pmol of Duf89, and increasing concentrations of P-nitrophosphophosphate as specified on the x-axis were incubated at 37 °C for 5 min. P-Nitrophosphenol production is plotted as a function of substrate concentration, and the data were fit to the Michaelis–Menten equation in Prism. All data plotted in B–F are the averages of three independent experiments ± SEM. Duf89, Domain of Unknown Function 89.
Pyrophosphatase activity displayed a hyperbolic dependence on PPi concentration (Fig. 5D). Fitting the data to the Michaelis–Menten equation yielded a $K_m$ value of 1.15 ± 0.16 mM PPi and an apparent $k_{cat}$ of 31.9 ± 1.7 min⁻¹ ($R$ squared = 0.966) with respect to moles of PPi consumed per enzyme. That the apparent $K_m$ of Duf89 for PPi was 10-fold lower than its $K_m$ for the generic phosphomonoester $p$-nitrophosphophosphate hints that a pyrophosphorylated metabolite might be a relevant substrate for Duf89 in fission yeast. We suspect that the lower turnover number for the Duf89 pyrophosphatase versus $p$-nitrophosphophosphate reactions reflects slower dissociation of the phosphate leaving group of pyrophosphate from the active site versus more rapid dissociation of the $p$-nitrophosphophosphate leaving group of $p$-nitrophosphophosphate.

**Fission yeast Duf8901 is a phosphatase/pyrophosphatase paralog of Duf89**

We produced and purified recombinant Duf8901 protein (Fig. 4A). Duf8901 hydrolyzed 10 mM $p$-nitrophosphophosphate to $p$-nitrophosphophosphate in the presence of 0.5 mM nickel or cobalt (Fig. 4B). Zinc, manganese, magnesium, and cadmium were progressively less effective than nickel or cobalt in supporting phosphomonoesterase activity; copper and calcium were inactive (Fig. 4B). The extent of $p$-nitrophosphophosphate formation in a 30 min reaction with 0.5 mM cobalt was proportional to input Duf8901 (Fig. 4C). No hydrolysis of $p$-nitrophosphophosphate was detectable during a 30 min reaction with cobalt in the absence of Duf8901 (Fig. 4C). We calculated a specific activity of 2.3 nmol of $p$-nitrophosphophosphate formed per pmol of Duf8901, which translates into an estimated turnover number of 1.28 s⁻¹. Duf8901 activity during a 5 min reaction under steady-state conditions (<6% of substrate consumed) displayed a hyperbolic dependence on $p$-nitrophosphophosphate concentration (Fig. 4D). Fitting the data to the Michaelis–Menten equation yielded $K_m$ and $k_{cat}$ values of 6.48 ± 1.2 mM and 2.89 ± 0.29 s⁻¹, respectively ($R$ squared = 0.969). Duf8901 hydrolysis of 10 mM $p$-nitrophosphophosphate was inhibited in a concentration-dependent manner by phosphate, with an IC₅₀ of ~10 mM phosphate and IC₅₀ of ~75 mM phosphate (Fig. 4E). Sulfate and chloride had comparatively little effect, even up to 100 mM, at which point Duf8901 activity was diminished by 20% (chloride) or 47% (sulfate) compared with the unsupplemented control (Fig. 4E).

Duf8901 also catalyzed cobalt-dependent and nickel-dependent hydrolysis of PPi (Fig. 4G). Zinc and magnesium were less effective as metal cofactors; manganese, calcium, cadmium, and copper were ineffective. The inorganic pyrophosphatase activity of Duf8901 in the presence of cobalt, calculated from the slope of the titration curve (Fig. 4F), was 58.3 pmol of phosphate released (i.e., 29.2 pmol of PPi hydrolyzed) per pmol of Duf8901, which translates into an estimated turnover number of 0.49 min⁻¹. Thus, whereas the $p$-nitrophosphophosphate specific activity of Duf8901 was 28% of that observed for Duf89 assayed under the same conditions, the inorganic pyrophosphatase activity of Duf8901 was 7% of that observed for Duf89 assayed under the same conditions. We surmise that Duf89 is a more vigorous pyrophosphatase enzyme than is its Duf8901 paralog.

**Human DUF89 is a cobalt/nickel-dependent pyrophosphatase**

The human C6orf211 gene encodes a 441-amino acid polypeptide that shares 201 positions of side-chain identity/similarity with fission yeast Duf89. In light of our findings here that *S. pombe* DUF89s have pyrophosphatase activity, we queried if human DUF89 protein is also competent for pyrophosphate hydrolysis. We produced recombinant human DUF89 in *E. coli* and purified it via sequential Ni-affinity chromatography, His₁₀Smt3 tag cleavage, and His₁₀Smt3 tag-depletion steps (Fig. S3A). We first affirmed that human DUF89 displayed metal-dependent phosphomonoesterase activity against $p$-nitrophosphophosphate, with cobalt and nickel as the preferred cofactors (Fig. S3B). Human DUF89 hydrolyzed 74.5 pmol of $p$-nitrophosphophosphate per 20 pmol of enzyme during a 30 min reaction in the presence of cobalt, which translates into an estimated turnover number of 2.1 s⁻¹ (i.e., ~45% as active as fission yeast Duf89 assayed under the same conditions). A distinctive feature of human DUF89 versus fission yeast Duf89 is that zinc is not an effective cofactor for $p$-nitrophosphophosphate hydrolysis by the human enzyme (compare Figs. S3B and 1C). Here, we found that human DUF89 also hydrolyzed PPi, with optimal activity in the presence of cobalt or nickel (Fig. S3C). Human DUF89 hydrolyzed 2540 pmol of pyrophosphate per 10 pmol of enzyme during a 60 min reaction with cobalt, which translates into an estimated turnover number of 4.2 min⁻¹ (i.e., ~58% as active as fission yeast Duf89 assayed under the same conditions).

**Crystal structure of fission yeast Duf89**

We grew crystals from a premixed solution of Duf89 with 5 mM cobalt and 10 mM PPi. The Duf89 crystals diffracted
X-rays to a resolution of 2.0 Å, were in space group C2, and contained one Duf89 protomer in the asymmetric unit (ASU). The structure was solved as described under the Experimental procedures section and summarized in Table S1. The refined model (\(R/R_{\text{free}} = 16.0/20.0\)) comprised a continuous polypeptide from Leu3 to Asp442. A stereo view of the Duf89 tertiary structure is shown in Figure 5A. It is composed of seven \(\beta\)-strands, 21 \(\alpha\) helices, and five 310 helices, which are displayed over the amino acid sequence in Figure 6. The fold consists of a \(\beta\)-sheet (with topology \(\beta_{1}\downarrow\beta_{7}\uparrow\beta_{6}\downarrow\beta_{5}\uparrow\beta_{2}\downarrow\beta_{3}\uparrow\beta_{4}\)) flanked on both sides by helices. The fold is stabilized by a multitude of salt bridges (Arg122-Asp92, Arg131-Glu65, Arg344-Glu99, Arg371-Asp376, Arg243-Glu272, Arg246-Asp355, Arg246-Asp248, Arg331-Asp355, Arg402-Asp367, Arg149-Asp145, Arg161-Glu187, Arg161-Glu168, Lys144-Asp295, Lys144-Asp138, Lys152-Glu156, Lys365-Asp261, Lys365-Glu257, and Lys225-Glu222) and by an Arg371-Trp113 cation–\(\pi\) stack. A surface electrostatic model (Fig. 5B) highlights the presence of single phosphate anion within a deep active-site pocket that is exposed to solvent. Neither metal nor pyrophosphate (the input ligands) were evident, suggesting that the pyrophosphate substrate had been hydrolyzed in the presence of cobalt and that the metal cofactor and one of the two phosphate products had dissociated from the enzyme, abetted perhaps by chelation of the metal by the 0.1 M citrate present in the precipitant solution used for crystal growth. The phosphate is coordinated by Asp252-O\(\delta\), Glu257-O\(\varepsilon\), Thr403-O\(\gamma\), and Leu193-O (Fig. 5C).

Figure 3. Duf89 hydrolyzes PP\(_i\). A, pyrophosphatase reaction mixtures (100 µl) containing 50 mM Tris-acetate (pH 6.5), 25 mM NaCl, 1 mM (100 nmol) PP\(_i\), 0.5 mM CoCl\(_2\), and 0.1 µM (10 pmol) of wildtype or mutant Duf89 preparations were incubated at 37 °C for 60 min. The extents of phosphate production are plotted. B, metal cofactor specificity. Reaction mixtures (100 µl) containing 50 mM Tris-acetate (pH 6.5), 25 mM NaCl, 1 mM (100 nmol) PP\(_i\), 0.1 µM (10 pmol) Duf89, and varying concentrations CoCl\(_2\) or NiCl\(_2\) as specified on the x-axis were incubated at 37 °C for 60 min. Phosphate production is plotted as a function of divalent cation concentration. C, metal titration. Reaction mixtures (100 µl) containing 50 mM Tris-acetate (pH 6.5), 25 mM NaCl, 1 mM (100 nmol) PP\(_i\), 0.1 µM (10 pmol) Duf89, and varying concentrations CoCl\(_2\) or NiCl\(_2\) as specified on the x-axis were incubated at 37 °C for 60 min. Phosphate production is plotted as a function of substrate concentration, and the data were fit to the Michaelis–Menten equation in Prism. The data plotted in A–D are the averages of three independent experiments ± SEM. Duf89, Domain of Unknown Function 89.
fission yeast Duf89 amino acids Gly195 to Ala219—which includes the α10, α11, and α12 helices—is disordered in the human DUF89 structure. The segment corresponding to fission yeast Duf89 amino acids Asn196 to Gln212 is also disordered in the budding yeast DUF89 structure.

**Structure of Duf8901**

Crystals of Duf8901 preincubated with 5 mM CoCl₂ were grown by vapor diffusion against precipitant solution containing 1.8 M sodium/potassium phosphate. The crystals diffracted X-rays to a resolution of 1.9 Å, were in space group P2₁2₁2₁, and contained two Duf8901 protomers in the ASU. The B protomer comprised a continuous polypeptide from Met1 to Ala438. The A protomer was punctuated by a disordered six-amino acid surface loop (from Ile168 to Asp173). The Duf8901 secondary structure elements are displayed under the amino acid sequence in Figure 6. The primary, secondary, and tertiary structures of Duf8901 and Duf89 were highly homologous; pairwise alignment of the Duf8901 B protomer to Duf89 in Dali yielded a Z score of 53.3 and an rmsd of 1.5 Å at ≥420 Ca positions. 27 of 31 of the basic and acidic side chains that form fold-stabilizing salt bridges in the Duf89 structure are conserved in Duf8901. Figure 7A shows a stereo view of the Duf8901 fold (depicted as a cartoon model with cyan helices and magenta β-strands) superimposed on that of Duf89 (rendered as a green ribbon trace). Duf8901 contains a single cobalt ion (magenta sphere) and corresponding anomalous difference peak (green mesh contoured at 3σ) in its active site (Fig. 7B). The metal ion is coordinated by Asp248-Ο−, Asp286-Ο−, Asn249-Ο−, Asp195-Ο−, and Asp195-Ο−, and Phe283-Ο (Fig. 7B). Duf8901 Asp248, Asp286, and Asn249 are conserved amino acids, the counterparts of which coordinate a manganese ion in the budding yeast DUF89 structure and a magnesium ion in the human DUF89 structure.

The proximity of Asp195 to the metal cofactor is a distinctive feature of the Duf8901•Co²⁺ complex, insofar as the corresponding conserved aspartates in budding yeast DUF89 (Asp195) and human DUF89 (Asp195) are located within their
respective disordered segments (embracing helices 10, 11, and 12 of the fission yeast proteins) that were mentioned in the
preceding section. The counterpart of the helix-10,11 segment
(containing Asp166) is disordered in the structure of an
*Ara-
bidopsis* DUF89*/C15* Mg\(^{2+}\) complex (PDB ID: 2Q40).

The superposition of Duf8901 and Duf89 in *Figure 7A*
provides new insight by highlighting the mobility of the helix-
10,11,12 module, which is depicted as a *yellow cartoon model*
for Duf8901 and a *green cartoon model* for Duf89 and
demarcated by *arrows*. The α10,11,12 module in Duf89 is
reflected away from the active-site pocket and packs against
helices α7, α8, and α9 (*Figs. 5A* and 7A). By contrast, in
Duf8901, the module has flipped over the active-site pocket,
serving as a "lid" that closes off the active site (*Fig. 7A*). The lid
closure entails a 16 Å movement of the Duf8901 Asp195-C\(^{α}\)
vis-à-vis the corresponding Asp199-C\(^{α}\) in Duf89 (*Fig. 7A*),
thereby bringing Asp195 into the orbit of the active-site metal
ion. We envision that interaction of the conserved lid aspartate

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*Figure 5. Structure of fission yeast Duf89.*

A, stereo view of the tertiary structure of Duf89, depicted as a cartoon model with magenta β-strands, cyan α-helices, and blue 310 helices. The secondary structure elements of Duf89 are labeled according to their order in the primary structure, as shown in *Figure 6*. A phosphate ion adjacent to β5 is rendered as a stick model. B, surface electrostatic model of Duf89 generated in Pymol, looking down into the active-site pocket, in which a phosphate ion (stick model) is bound. C, stereo view of the Duf89 active site with selected amino acids depicted as stick models with beige carbons. Enzymic contacts to the phosphate are indicated by dashed lines. Omit density \(F_o-F_c\) for the phosphate is depicted as blue mesh contoured at 3σ.

Duf89, Domain of Unknown Function 89.
with the active-site metal either triggers lid closure or else stabilizes the closed conformation, which could explain why there is no phosphate anion seen in the Duf8901/C15Co2+ complex, notwithstanding the presence of 1.8 M phosphate in the crystallization solution.

**Structure of a BeF3 mimic of a Duf8901 aspartyl–phosphate intermediate**

In the budding yeast DUF89 crystal structure in complex with phosphates and manganese (1), the octahedral metal coordination complex is filled by two aspartates and an asparagine, a phosphate oxygen (from the putative scissile phosphate), and two waters (Fig. S2B). The secondary structure elements of Duf89 and Duf8901 are displayed above and below their respective primary structures, with β-strands depicted as magenta arrows, α-helices as cyan cylinders, and 3₁₀ helices as blue cylinders. The secondary structure elements of Duf89 are labeled as in Figure 5A. Duf89, Domain of Unknown Function 89; Duf8901, Domain of Unknown Function 8901.
catalysis is characteristic of the DxDxT family of phosphohydrolase and phosphotransferase enzymes, named for the conserved active-site motif, wherein the first aspartate acts as the catalytic nucleophile. Whereas the chemical lability of an aspartyl–phosphate adduct makes it difficult to capture the phosphoenzyme intermediate, it has been possible to trap a mimetic of the intermediate by reacting DxDxT enzymes with beryllyn chloride and sodium fluoride in the presence of divalent cation, so that the enzyme forms a covalent BeF₃ adduct to the catalytic aspartate that is resistant to hydrolysis. Crystal structures have been determined for aspartyl–BeF₃ derivatives of exemplary DxDxT family members phospho-serine phosphatase (10), CTD protein phosphatases Scp1 and Fcp1 (11, 12), fungal trehalose-6-phosphate phosphatase (13), and phage T4 polynucleotide 3′-phosphatase [www.rcsb.org/structure/5UJ0].

At present, there is no experimental evidence that DUF89 enzymes form a covalent intermediate. To address this knowledge gap, we captured an analog of the covalent phosphophoenzyme intermediate by crystallizing Duf8901 that had been preincubated in the presence of BeCl₂, NaF, and CoCl₂. The structure, solved at 2.16 Å resolution, revealed a covalent bond between Asp248 Oδ₁ and the beryllium atom of BeF₃. The Asp–BeF₃ adduct adopts the tetrahedral geometry expected for the imputed aspartyl–phosphate intermediate (Fig. 8A). An anomalous difference peak corresponding to the cobalt ion is seen at the center of an octahedral coordination complex (Fig. 8A). The six metal ligands are Asp248-Oδ₂, Asp286-Oδ₁, Asn249-Oδ₂, two waters, and a fluoride atom of BeF₃ (which mimics a phosphate oxygen) (Fig. 8A). The interatomic distances between cobalt and the six metal ligands are 2.0 to 2.2 Å. The metal-bound waters are bridged to the enzyme via Asp286-Oδ₂, Asp195-Oδ₂, and Phe283-O (Fig. 8A). The enzyme makes a network of contacts to the three fluoride atoms of BeF₃ and the bridging oxygen to Asp248 (i.e., representing the four phosphate oxygens). The interatomic distances between cobalt and the six metal ligands are 2.0 to 2.2 Å. The metal-bound waters are bridged to the enzyme via Asp286-Oδ₂, Asp195-Oδ₂, and Phe283-O. The enzyme makes a network of contacts to the three fluorine atoms of BeF₃ and the bridging oxygen to Asp248 (i.e., representing the four phosphate oxygens). The fluorine/phosphate ligands in the aspartyl–BeF₃/phosphate adduct are Lys401-Nζ, Asn192-Nδ, Asn249-Nδ, and the Asp363 and Leu364 main chain amides (Fig. 8A). The interatomic distances between the enzyme and the metal-bound waters and the fluorine atoms of BeF₃ are 2.6 to 3.0 Å. A water in the active site is located 4 Å from the beryllium atom in a near-apical orientation to the bridging oxygen of Asp248 (water–Be–Oδ₁ angle of 155°) and is a plausible mimetic of a water nucleophile for hydrolysis of an aspartyl–phosphate catalytic intermediate.

**Structure-guided alanine scan of the Duf8901 active site**

We produced and purified a series of Duf8901 mutants in which amino acids Asp248, Asn249, Lys401, Asp286, and Asp195 that interact with BeF₃ or cobalt were changed singly to alanine (Fig. 8B). The Duf8901-Ala proteins were tested for
p-nitrophenylphosphatase activity in parallel with wildtype Duf8901. Activity was eliminated by the D248A, N249A, D286A, and K401A mutations (≤0.2% of wildtype) and reduced to 5% of wildtype by the D195A change (Fig. 8C).

Nickel and zinc support Asp–BeF₃ adduct formation

Because nickel and zinc are also effective cofactors for the phosphomonoesterase activity of Duf8901 (Fig. 4B), we tested their ability to replace cobalt in promoting formation of the aspartyl–BeF₃ adduct, by crystallizing Duf8901 that had been preincubated in the presence of BeCl₂, NaF, and either NiCl₂ or ZnCl₂. Diffraction data collected at the nickel and zinc absorption peak wavelengths were used to solve structures of the Duf8901-(Asp248)–BeF₃•Ni²⁺ and Duf8901-(Asp248)–BeF₃•Zn²⁺ adducts at a resolution of 2.1 Å (Table S3). The active sites of the BeF₃•Ni²⁺ and BeF₃•Zn²⁺ structures are shown in Figure 9, A and B, with anomalous difference peaks over the nickel and zinc ions contoured at 28σ and 12σ, respectively. The nickel and zinc coordination complexes and the enzymic interactions with metals and BeF₃ (Fig. 9, A and B) are virtually identical to those seen in the Duf8901-(Asp248)–BeF₃•Co²⁺ complex (Fig. 8A). This ensemble of aspartyl–BeF₃ structures with three different “active” metals fortifies the case for a covalent aspartyl–phosphate intermediate.

Replacing the aspartate nucleophile with asparagine abolishes Duf89 and Duf8901 activity

Because the Asp248 side chain in Duf8901 does double duty—with one Oδ acting as the nucleophile for covalent catalysis and the other Oδ coordinating the metal cofactor—
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(Figs. 8A and 9)—the finding that mutating Duf8901 Asp248 to alanine and Duf89 Asp252 to alanine eliminates phosphatase activity does not report directly on the essentiality of aspartate as nucleophile. To approximate this issue, we introduced asparagine in lieu of Duf8901 Asp248 and Duf89 Asp252, a conservative single-atom modification that is isosteric to aspartate, capable in principle of coordinating the metal cofactor via Oδ but unable to form a covalent phosphoenzyme via Nδ. The recombinant Duf8901 D248N and Duf89 D252N proteins were purified (Fig. 10A) and tested for p-nitrophenylphosphatase activity in parallel with wildtype Duf8901 and Duf89. The asparagine mutations effaced phosphatase activity in both cases (Fig. 10B).

Structure of a Duf8901-(Asp248)–AlF4 transition state mimetic

We captured an AlF4 analog of the transition state of the hydrolysis reaction by crystallizing Duf8901 that had been preincubated in the presence of AlCl3, NaF, and CoCl2. The structure, solved at a resolution of 1.8 Å, revealed that AlF4 adopts a square bipyramidal geometry with a water nucleophile and the Asp248-Oδ-leaving group at the apices, equidistant from the aluminum center—1.8 Å for water and 1.9 Å for Asp248-Oδ—and with a water–Al–Oδ angle of 175° (Fig. 11). The distances and geometry are consistent with an associative in-line mechanism of phosphoryl transfer. The cobalt ion (shown in Fig. 11 with its overlying anomalous difference peak contoured at 32σ) is engaged octahedrally in the bipyramidal transition state mimetic to the same six ligands (cobalt to ligand distances of 1.9–2.1 Å) as seen in the tetrahedral BeF3 adduct: Asp248-Oδ, Asp286-Oδ, Asn249-Oδ, two waters, and a fluorine atom of AlF4 (in lieu of a fluorine in BeF3) (Fig. 11). The planar fluorine atoms of AlF4 and the Asp248-Oδ-leaving group receive the same enzymic contacts from Lys401-Nζ, Asn192-Nδ, Asn249-Nδ, and the Asp363 and Leu364 main chain amides (Fig. 11; interatomic distances of 2.5–3.0 Å) as their counterparts in the BeF3 structure. A salient

Figure 9. Structures of Duf8901-(Asp248)–BeF3•Ni2+ and Duf8901–(Asp248)–BeF3•Zn2+ adducts. Stereo view of the active site of the Duf8901-(Asp248)–BeF3•Ni2+ (A) and Duf8901–(Asp248)–BeF3•Zn2+ (B) structures with selected amino acids depicted as stick models. Nickel and zinc are rendered as green and gold spheres, respectively. Two waters in the octahedral metal coordination complex are depicted as red spheres. BeF3 is depicted with a yellow–green beryllium atom and light blue fluorine atoms. The anomalous difference peak overlying the nickel is depicted as a magenta mesh contoured at 28σ. The anomalous difference peak overlying the zinc is depicted as a green mesh contoured at 12σ. Atomic contacts of Asp–BeF3 and the metal complexes are indicated by dashed lines. A water (green sphere) adjacent to the BeF3 adduct is a plausible mimetic of the water nucleophile. BeF3, beryllium trifluoride; Duf8901, Domain of Unknown Function 8901.
**Structure and mechanism of fission yeast DUF89 phosphatases**

Figure 10. Replacing the aspartate nucleophile with asparagine abolishes Duf89 and Duf8901 activity. A, aliquots (5 μg) of purified wildtype Duf89, Duf89-D252N, wildtype Duf8901 and Duf8901-D248N were analyzed by SDS-PAGE. The Coomassie blue–stained gel is shown. The positions and sizes (kilodalton) of marker polypeptides are indicated on the left. B, phosphatase reaction mixtures (50 μl) containing 50 mM Tris-acetate (pH 6.5), 50 mM NaCl, 10 mM (500 nmol) p-nitrophenylphosphate, 0.5 mM CoCl2, and either 0.6 μM (30 pmol) of Duf89 or 1 μM (50 pmol) of Duf8901 proteins as specified were incubated at 37 °C for 30 min. The extents of p-nitrophenol production are plotted. Data are the averages of three independent experiments ± SEM, Duf89, Domain of Unknown Function 89; Duf8901, Domain of Unknown Function 8901.

Figure 11. Structure of a Duf8901(Asp248)–AlF4 transition state mimetic. Stereo view of the active site of the Duf8901(Asp248)–AlF4–OH2–Co2+ structure with selected amino acids depicted as stick models. Cobalt and two waters in the octahedral metal coordination complex are rendered as magenta and red spheres, respectively. AlF4 is depicted with a yellow aluminum atom and light blue fluorine atoms. The anomalous difference peak overlying the cobalt is depicted as a blue mesh contoured at 3σ. Atomic contacts of Asp-AlF4 and the cobalt complex are indicated by dashed lines. The water nucleophile for hydrolysis of aspartyl phosphate—situated 1.8 Å from the aluminum and almost perfectly apical to the Asp248-OH2-leaving atom—is coordinated by Asp195-Oδ and Asp363-Oδ. AlF4, aluminum fluoride; Duf8901, Domain of Unknown Function 8901.

Feature of the transition state vis-à-vis the covalent intermediate is the unambiguous assignment of the water nucleophile (red water sphere in Fig. 11 versus the “green” water spheres in Figs. 8A and 9). The candidate water surmised from the BeF3 structures has closed the distance to the aluminum (i.e., the phosphorus) in the transition state and is poised apically for catalysis. Coupled to this transition, the water nucleophile is engaged by Asp195-Oδ and Asp363-Oδ, each situated 2.9 Å from the water oxygen (Fig. 11). Whereas Asp195 was seen to coordinate the “out of position” green water in the BeF3 structures, the Asp363 side-chain carboxylate was pointing away from BeF3 at a distance of 4.7 Å from the green water. Compared with the covalent intermediate, the transition state Asp363 side chain has undergone a rotation about Ca–Cβ that results in a ~3.1 Å movement of Oδ that enables its coordination of the water nucleophile. Dual hydrogen bonds from the water nucleophile to Asp195 and Asp363 serve to orient it for attack on aspartyl phosphate and to provide for activation of the water via general base catalysis.

**Distinctive effects of duf89 and duf8901 deletion on Pho1 expression**

We constructed fission yeast duf89Δ and duf8901Δ strains as well as a duf89Δduf8901Δ double mutant. Spot testing for growth on YES agar showed that duf89Δ, duf8901Δ, and duf89Δ duf8901Δ cells grew as well as a wildtype strain (Fig. 12A). We then assayed pho1 expression during exponential growth at 30 °C in liquid culture under phosphate-replete conditions (Fig. 12B). Cell surface–associated Pho1 acid phosphatase activity (a gauge of Pho1 enzyme level that correlates with pho1 mRNA levels) was quantified by incubating suspensions of serial dilutions of the cells for 5 min with p-nitrophenylphosphate and assaying colorimetrically the formation of p-nitrophenol. The basal Pho1 activity of wildtype cells was derepressed by ninefold in duf89Δ cells (Fig. 12B), thus affirming the finding of Henry et al. (5). By contrast, the duf8901Δ allele had no effect per se on Pho1 expression and did not impact the derepression of Pho1 elicited by duf89Δ (Fig. 12B). We infer that Duf8901 and Duf89 are neither functionally equivalent nor functionally redundant with respect to vegetative growth; nor are they functionally equivalent with regard to involvement in phosphate homeostasis.

**Discussion**

DUF89-family metal-dependent phosphohydrolases are prevalent in all phylogenetic domains and presently grouped into three subfamilies (1). The DUF89 enzymes characterized...
to date display relatively vigorous activity with p-nitrophenylphosphate (vis-à-vis other substrates tested). Individual DUF89 enzymes differ in their metal cofactor and substrate preferences, as gauged by biochemical screening of exemplary members of each DUF89 subfamily for their ability to hydrolyze a panel of 95 additional phosphosubstrates, primarily low–molecular weight compounds, such as sugars, nucleotides, amino acids, and vitamin derivatives (1). It is proposed that DUF89 enzymes act as scavengers to limit the intracellular accumulation of deleterious or damaged phosphometabolites (1). However, establishing the true nature of the in vivo targets of DUF89 activity and biological contexts in which DUF89 enzymes might be important (e.g., an overt cellular phenotype elicited by DUF89 deficiency) remains challenging. Informative structures have been reported for several DUF89 proteins, including as complexes with a divalent cation—either magnesium or manganese, neither of which is optimal for activity. Nonetheless, the catalytic mechanism of DUF89 enzymes is not well understood.

The present study provides new insights into DUF89 mechanism and repertoire via characterization of two subfamily III DUF89 paralogs—Duf89 and Duf8901—from the fission yeast S. pombe. Hydrolysis of p-nitrophenylphosphate by Duf89 and Duf8901 relies on nickel or cobalt as the divalent cation cofactor. Their apparent $k_{\text{cat}}$ values (Duf89: 9.7 s$^{-1}$; Duf8901: 2.9 s$^{-1}$) are similar to, or several-fold greater than, those reported for p-nitrophenylphosphate hydrolysis by human, plant, and archaeal DUF89 proteins (1, 4). A new finding here is that Duf89 is also adept at cobalt/nickel-dependent hydrolysis of PP$_i$ ($k_{\text{cat}}$ 32 min$^{-1}$). Whereas pyrophosphatase was included in the panel of 96 substrates used by Huang et al. (1) to screen exemplary DUF89 enzymes, their report did not mention pyrophosphatase activity as does the human DUF89 homolog.

Our determination of crystal structures of the fission yeast DUF89 paralogs highlights several key points regarding DUF89 mechanism. First, we unveil a mobile structural module of subfamily III DUF89 proteins, comprising Duf89 helices 10,11,12 and the corresponding helices in Duf8901. This lid-like module is reflected away from the active-site pocket in the metal-free Duf89 structure and closes over the active-site pocket in the metal-bound Duf8901 structures. The lid closure results in a ~16 Å movement of Duf8901 Asp195 vis-à-vis the equivalent Asp199 in Duf89 that incorporates Asp195 into an octahedral cobalt coordination complex via a bridging water. We regard the closed state of the protein as being on-pathway insofar as this is the conformation seen in the cobalt-coordinated BeF$_3$ mimic of a covalent aspartyl–phosphate intermediate. Because the lid closure appears to occlude access to the active-site pocket, we infer that the open conformation seen in the Duf89 structure approximates a state permissive for entry of phosphosubstrate and metal into the active-site pocket, after which lid closure is triggered. The dynamic nature of the lid is underscored by the fact that this segment (and its counterpart of metal-binding Asp199) is disordered in the budding yeast, human, and plant DUF89 structures. In the case of the Pyrococcus DUF89 structure (PDB ID: 2G8L), the lid is visualized in an intermediate position relative to Duf89 (wide open) and Duf8901 (closed), whereby the Cα atom of Asp111 is displaced by 6.1 Å from that of Duf8901 Asp195. Presumably, the lid reopens after catalysis to allow product release. It is not clear whether the crystallization of a closed complex of Duf8901 in the presence of cobalt results because the metal promotes module closure, versus the crystallization conditions per se favoring this conformation as opposed to that of Duf89. (Note that Duf89 and Duf8901–BeF$_3$ crystallized under different conditions and their respective crystal lattices differed with respect to the number of protomers in the ASU.)

The structures of Duf8901 as an aspartyl–BeF$_3$ adduct and an aspartyl–AlF$_4$–OH$_2$ adduct instate a mechanism of covalent catalysis for this DUF89 enzyme, one that we suspect applies to all DUF89 family members, regardless of what their preferred phosphosubstrates might be. For Duf8901, we propose a two-step reaction pathway entailing: (i) nucleophilic attack of Asp248 on the phosphorus of an R–O–PO$_3$ substrate, resulting in the formation of a covalent enzyme–aspartyl–phosphate intermediate and an R–OH product and (ii) hydrolysis of the aspartyl phosphate to release P$_i$ product. Both steps likely proceed in-line via a pentacoordinate phosphorane transition state in which the nucleophile and leaving atoms occupy the apices of the trigonal bipyramid. AlF$_4$ complexes have been instrumental in obtaining structural snapshots of transition state analogs of enzymic phosphoryl transfer enzymes in which the anionic square planar AlF$_4$ mimics the trigonal planar configuration of the scissile phosphate (14). In our structure of the Duf8901–(Asp248)–AlF$_4$–OH$_2$ complex, AlF$_4$ forms a square bipyramid with a water nucleophile and the Asp248–Oδ leaving group at the apices. The water–Al and Al–Oδ distances and geometry implicate an associative in-line mechanism of phosphoryl transfer.
The Duf8901 aspartyl–BeF₃ and aspartyl–AlF₄ structures suggest that the transition state is stabilized by electrostatic and polar contacts to the nonbridging phosphate oxygens made by Lys401 and Asn249 and by the divalent cation cofactor. In principle, we can invoke general acid–base catalysis as a component of the Duf89 mechanism, whereby an enzymic general acid donates a proton to the R–OH leaving group during step 1 and a general base accepts a proton from the water nucleophile during step 2. In the case of DxDxT family phosphotransfersases that act via a covalent aspartyl–phosphate intermediate, the second Asp of the DxDxT motif is proposed to act sequentially as general acid and general base. Huang et al. (1) had suggested that Asp292 in budding yeast YMR027W (corresponding to Asp286 in Duf8901) could activate the water nucleophile during the phosphohydrolyase reaction. Based on the Duf8901-(Asp248)–AlF₄–OH₂ transition-state structure (in which water is poised for catalysis), we feel this is most unlikely, insofar as Asp286 is coordinating a metal-bridged water that is 3.8 Å from the aluminum atom and orthogonal to the leaving Asp248–O搬迁 atom of the aspartyl–AlF₄ adduct (water–Al–Asp248–O搬迁 angle of 84°). Rather, we propose that Asp195 and Asp363, acting singly or in concert, take the role of general base catalyst of the hydrolysis reaction. Asp195–O搬迁 and Asp363–O搬迁 are equidistant (2.9 Å) from the water nucleophile, in position to accept bifurcated hydrogen bonds from the water that orient the water for its attack on the scissile phosphate and allow for its deprotonation by transfer to an Asp–O搬迁. We envision that the R–O leaving group in the transition state of the first step of the Duf8901 pathway occupies the same position as the water nucleophile in the Duf8901-(Asp248)–AlF₄–OH₂ transition state, and that analogous interactions of Asp195 and Asp363 (one or both of which are protonated) with the R–O leaving atom allows for general acid catalysis of R–OH product expulsion. Asp195 and Asp363 are conserved among DUF89 family members. We show here by alanine mutagenesis that Asp195 is critical for Duf8901 p-nitrophenylphosphatase activity. Huang et al. (1) reported that alanine mutation of the counterpart of Duf8901 Asp363 in budding yeast YMR027W (Asp384) abolished p-nitrophenylphosphatase activity.

With respect to fission yeast Duf89, it is noteworthy that the site occupied by phosphate in the Duf8901•PO₄ structure is not what one would expect for either the hydrolyzed phosphate product of step 2 or (potentially) the phosphate leaving group derived by step 1 reaction with PPi. It is possible that the hydrolyzed phosphate migrates to the position after step 2 chemistry; alternatively, phosphate occupancy of this site is off pathway and occurs during Duf89 crystallization at pH 5.

Given our new appreciation that Duf8901 shares with DxDxT-family phosphotransfersases the formation of a covalent enzyme–(aspartyl)–phosphate intermediate, we queried whether there is any structural homology underlying this property by performing a pairwise Dali comparison of the Duf8901-(aspartyl)–BeF₃•Co²⁺ structure to the aspartyl–BeF₃•Mg²⁺ structure of the autonomous polynucleotide-3′-phosphatase domain (amino acids 157–300) of T4 polynucleotide kinase phosphatase www.rcsb.org/structure/5UJ0. Dali returned an alignment of the two structures with a relatively low Z score of 3.9, with an rmsd of 3.1 Å at 95 Ca positions that included only 5% amino acid identity. The fold homology embraced a five-strand parallel β-sheet (corresponding to Duf8901 B4•B3•B2•B1•B6 in the view shown in Fig. 13A) and an α-helix. In both enzymes, the aspartyl–BeF₃ adducts that define the active site are located within a loop immediately following the middle β-strand of the sheet. However, the respective BeF₃ adducts and the metals are not coincident in the structure-based alignment. (Dali aligns Duf8901 Asp248 to T4 Asp167, the second Asp in the DxDxT motif.) Moreover, the aspartyl–BeF₃ moiety is oppositely oriented with respect to the metals in Duf8901 versus T4 3′-phosphatase (Fig. 13A). To gauge the extent to which the active sites might be conserved, we superimposed them with respect to the respective BeF₃•metal complexes (Fig. 13B).

Whereas four of the six constituents of the octahedral metal complexes are spatially conserved (BeF₃•F; Asp248/165–O搬迁; Asp286/278–O搬迁; water), two are distinct. To wit: (i) the direct contact of Asn249–O搬迁 to cobalt in Duf8901 is a water-bridged contact to magnesium via Asp277 in T4 3′-phosphatase and (ii) the position of a water-engaging cobalt in Duf8901 is taken by the Asp167 main chain amide in T4 3′-phosphatase (Fig. 13B). Most notable is that none of the contacts to the phosphate oxygen equivalents of the BeF₃ adducts are conserved between Duf8901 and T4 3′-phosphatase. In particular, there are no equivalents of essential Duf8901 residues Lys401 and Asp195 in T4 3′-phosphatase. We surmise that DUF89 and DxDxT enzymes share a Rossmanoid fold β-sheet but are not descendants of a common ancestor.

We also queried the relatedness of Duf8901 to Salmonella CheY, a bacterial signal transduction protein and phosphohydrolase that has been captured structurally as a covalent CheY–(aspartyl)–BeF₃ adduct (15). Phosphorylation of the CheY A57 side chain by kinase CheA dictates the direction of bacterial flagellar rotation. CheY phosphorylation is short lived because CheY itself catalyzes aspartyl–phosphate hydrolysis. Dali returned an alignment of Duf8901 and Salmonella CheY structures with an Z score of 4.0 (rmsd of 3.5 Å at 83 Ca positions) that included 11% amino acid identity. The fold homology embraced a four-strand parallel β-sheet (corresponding to Duf8901 β3•β2•β5•β6) and an α-helix. However, the respective aspartyl–BeF₃ adducts that define the active site are located on different β-strands of the sheet (Fig. S4A). Superposition of the structures with respect to their Asp–BeF₃•metal complexes showed that the active-site architectures are dissimilar (Fig. S4B). Other than sharing a Rossmanoid fold, we conclude that Duf8901 and CheY are not derived from a common ancestral enzyme.

Whereas our present biochemical and structural studies of the fission yeast DUF89 paralogs do not reveal their physiological substrates (or the basis for substrate recognition), the distinct effects of their respective gene deletions on the expression of the phosphate homeostasis enzyme Pho1 would suggest that Duf89 and Duf8901 act on different phosphosubstrates in vivo, such that only Duf89 deletion derepresses Pho1 expression in phosphate-replete cells. Recent studies have shown that fission yeast Pho1 expression is subject to regulation.
by the IPP signaling molecule IP8, whereby inactivation of pyrophosphatase enzymes that hydrolyze IP8 leads to Pho1 derepression (8). Moreover, too much IP8 is cytotoxic to fission yeast (16). Pho1 is also derepressed when fission yeast cells are starved for thiamine (the precursor of the essential enzyme cofactor thiamine pyrophosphate) or by mutations in enzymes involved in thiamine metabolism (17, 18). Thus, we can speculate that Duf89 might aid in limiting the accumulation of potentially harmful metabolites in the IPP or thiamine pathways.

**Experimental procedures**

*Recombinant* *S. pombe* *Duf89*

The ORF encoding full-length Duf89 was PCR amplified from *S. pombe* complementary DNA (cDNA) with primers that introduced a BamHI site immediately flanking the start codon and a XhoI site downstream of the stop codon. The PCR product was digested with BamHI and XhoI and inserted between the BamHI and XhoI sites of pET28b-His10Smt3 to generate a T7 RNA polymerase–based expression plasmid encoding the Duf89 polypeptide fused to an N-terminal His10Smt3 tag. Single mutations D252A, D290A, and D252N were introduced into the expression plasmid by two-stage overlap extension PCR with mutagenic primers. All plasmid inserts were sequenced to exclude the presence of unwanted mutations. Wildtype and mutant pET28b-His10Smt3-Duf89 plasmids were transfected into *E. coli* BL21(DE3) cells. Cultures (1 l) amplified from single kanamycin-resistant transformants were grown at 37 °C in Terrific Broth containing 50 μg/ml kanamycin until the absorbance reached 0.75 to 0.8 at 600 nm. The cultures were chilled on ice for 1 h, adjusted to 2.2% (w/v) ethanol and 0.5 mM IPTG, and then incubated for 17.5 h at 17 °C with constant shaking. Cells were harvested by centrifugation and stored at −80 °C. All subsequent steps were
performed at 4 °C. Cells were thawed and resuspended in 25 ml of buffer L (50 mM Tris–HCl [pH 7.5], 500 mM NaCl, 25 mM imidazole, and 10% glycerol). Lysozyme was added to a concentration of 1 mg/ml. After incubation for 30 min, the lysate was sonicated, and the insoluble material was removed by centrifugation at 14,000 rpm for 30 min. The supernatant was mixed for 1.5 h with 2 ml of Ni–nitrilotriacetic acid agarose resin (Qiagen) that had been equilibrated with buffer L. The resin was recovered by centrifugation and washed twice with 30 ml of buffer L. The resin was centrifuged again, resuspended in 20 ml of buffer L, and poured into a column. After washing the column with 20 ml of buffer L, the bound material was eluted with 6 ml of buffer L containing 300 mM imidazole. The polypeptide compositions of the flow-through and eluate fractions were monitored by SDS-PAGE. The 300 mM imidazole eluate fractions containing His10-Smt3-Duf89 were supplemented with Smt3-specific protease Ulp1 (Ulp1/His10-Smt3-Duf89 ratio of 1:425 [w/w]) and then dialyzed overnight against 2 l of buffer L. The dialysates were mixed for 1.5 h with 2 ml of Ni–nitrilotriacetic acid agarose resin that had been equilibrated with buffer L. Tag-free Duf89 proteins were recovered in the flow-through fractions. Protein concentrations were determined with Bio-Rad dye reagent using bovine serum albumin as the standard. The yields of wildtype Duf89, Duf89-D252A, Duf89-D290A, and Duf89-D280N were 24, 20, 19, and 26 mg per liter of bacterial culture, respectively.

For protein crystallization, the tag-free Duf89 preparation was concentrated by centrifugal ultrafiltration (Amicon Ultra-15; 10 kDa cutoff) to 21 mg/ml (in 2 ml) and then further purified by gel filtration through a 125 ml 16/60 HiLoad Superdex 200 column (GE Healthcare) equilibrated in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl at a flow rate of 0.5 ml/min while collecting fractions of 2 ml. The peak Duf89 fractions were pooled and concentrated by centrifugal ultrafiltration (Amicon Ultra-15; 30 kDa cutoff) to 20 mg/ml. Protein concentration was determined from the absorbance measured at 280 nm with a Nanodrop spectrophotometer (Thermo Scientific) applying a molar extinction coefficient of 83,685 M⁻¹/cm, as calculated by Protparam.

Recombinant human DUF89

A synthetic cDNA clone for the C6orf211 ORF, codon optimized for expression in *E. coli* and containing a BamHI site immediately flanking the start codon and a Xhol site downstream of the stop codon, was purchased from Genscript. The ORF was excised with BamHI and Xhol and inserted between the BamHI and Xhol sites of pET28b-His10-Smt3. Recombinant human DUF89 was produced in *E. coli* BL21(DE3) and purified from a soluble extract of a culture of 1 l as described previously for *S. pombe* Duf89. The yield of tag-free human DUF89 was 8 mg.

Hydrolysis of p-nitrophenylphosphate

Reaction mixtures (50 μl) containing 50 mM Tris-acetate (pH 6.5), 50 mM NaCl, 10 mM (500 nmol) p-nitrophenylphosphate, 0.5 mM divalent cation, and DUF89 proteins as specified were incubated at 37 °C. The reactions were quenched by adding 5 μl of 100 mM EDTA and then 0.95 ml of 1 M Na2CO3. Release of p-nitrophenol was determined by measuring absorbance at 410 nm and interpolating the value to a p-nitrophenol standard curve.

Hydrolysis of PPi

Reaction mixtures (100 μl) containing 50 mM Tris-acetate (pH 6.5), 25 mM NaCl, 1 mM (100 nmol) sodium pyrophosphate, 0.5 mM divalent cation, and DUF89 proteins as specified were incubated at 37 °C. The reactions were quenched by adding 1 ml of Malachite Green reagent (Enzo Life Sciences) followed by an incubation of 20 min at room temperature. Phosphate release was determined by measuring absorbance at 620 nm and interpolating the value to a phosphate standard curve.

Protein crystallization

**Duf8901·Co2+ complex**

A solution containing 0.3 mM Duf8901, 5 mM CoCl2, 19 mM Tris–HCl (pH 7.5), and 142 mM NaCl was preincubated on ice for 15 min. Aliquots (2 μl) were then mixed...
with 2 μl of precipitant solution containing 1.8 M sodium phosphate monobasic monohydrate/potassium phosphate dibasic (pH 8.2).

Duf8901-BeF₃•Co²⁺ complex

A solution containing 0.3 mM Duf8901, 5 mM CoCl₂, 5 mM BeCl₂, 15 mM NaF, 15 mM Tris–HCl (pH 7.5), and 112 mM NaCl was preincubated on ice for 1 h. Aliquots (2 μl) were then mixed with 2 μl of precipitant solution containing 1.8 M sodium phosphate monobasic monohydrate/potassium phosphate dibasic (pH 8.0). Duf8901 crystals were grown by sitting drop vapor diffusion at room temperature. Crystals grew over 1 to 2 days and were harvested 1 day later. The crystals were cryoprotected with 100% paraffin oil and then flash-frozen in liquid nitrogen.

Duf8901-BeF₃•Zn²⁺ complex

A solution containing 0.3 mM Duf8901, 2 mM ZnCl₂, 5 mM BeCl₂, 15 mM NaF, 15 mM Tris (pH 7.5), and 112 mM NaCl was preincubated on ice for 1 h. Aliquots (2 μl) were then mixed with 2 μl of precipitant solution containing 1.8 M sodium phosphate monobasic monohydrate/potassium phosphate dibasic (pH 8.0). Crystals were grown by sitting drop vapor diffusion at room temperature. Crystals grew over 3 days and were harvested 7 days later. Crystals were cryoprotected with 25% sucrose and then flash-frozen in liquid nitrogen.

Duf8901-Co²⁺ structure

Two sets of diffraction data from a single crystal of Duf8901 grown in the presence of Co were collected at a wavelength of 0.9792 Å at beamline 24-ID-E. The crystal diffracted to 2.75 Å and belonged to space group P2₂₁2₁ with unit cell dimensions consistent with two or three protomers per ASU, assuming solvent contents of 60% or 40%, respectively. The data were combined, and the structure was determined by molecular replacement (MR) in PHENIX.PHASER using a homology model of Duf89 protein generated in Phyre2 (23) as a search ensemble. PHENIX.PHASER found 1 MR solution with translation function Z (TFZ) and log-likelihood gain (LLG) scores of 24.2 and 480.8, respectively, and two copies of Duf8901 protein in the ASU. A partially refined model was then used to solve a structure of a better-diffracting Duf8901 crystal. Diffraction data at 1.90 Å resolution (collected at a wavelength of 0.9792 Å, henceforward called a native dataset) and 2.16 Å resolution (collected at a wavelength of 1.5895 Å, corresponding to the energy of K-edge absorption for Co²⁺; henceforward called an anomalous dataset) were obtained from a single crystal of Duf8901 at beamline 24-ID-C. Phases were determined by MR in combination with single-wavelength anomalous dispersion (MR-SAD) in PHENIX.PHASER by using the anomalous dataset with a resolution cutoff of 4 Å and the partially refined 2.75 Å Duf8901 model (devoid of heteroatoms) as a search ensemble. A calculated Duf8901 substructure contained two cobalt atoms, with figure of merit (FOM) and LLG scores of 0.506 and 83.5, respectively. Following density modification in PHENIX.RE-SOLVE, the initial 2.16 Å model was built, containing two molecules of Duf8901 protein per ASU. The model was subsequently subjected to three macrocycles of refinement, including refinement of the anomalous groups, without imposing non-crystallographic symmetry (NCS) restraints. The resolution was then extended to 1.90 Å by refinement against the native dataset. The Duf8901-CO²⁺ complex was iteratively rebuilt in PHENIX.REFINEM in the presence of experimental phase restraints and included translation libration screw (TLS) B-factor refinement (14 TLS groups). The final model refined to Rwork/Rfree of 16.4/19.6, with 98.2% of the 871 amino acid residues in the favored regions of the Ramachandran plot and only a single outlier.
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**Duf8901–BeF₃•Co²⁺ structure**

Diffraction data for a single crystal of Duf8901 that had been preincubated with cobalt, BeCl₂, and NaF were collected at 1.6050 Å wavelength (cobalt K-edge) at beamline 24-ID-C. The crystal diffracted to 1.99 Å and belonged to space group C2 with unit cell dimensions consistent with one protomer per ASU, assuming a solvent content of 52%. Initial structure was obtained by MR using Duf8901–BeF₃•Ni²⁺ structure (protein chain A without heteroatoms) as a search model in PHENIX.PHASER suite (TFZ = 69.1; LLG = 5034.2). MR-SAD phases were calculated by combining the MR model from PHASER with anomalous data from this crystal at 2.1 Å resolution cutoff in PHENIX.AUTOSOL (FOM = 0.436). The model contained one Duf8901 molecule per ASU and two cobalt atoms. Phases were optimized by density modification and used in subsequent rounds of iterative refinement in the PHENIX.REFINE suite. Experimental phase restraints and TLS B-factor refinement (11 TLS groups) were applied during refinement. The final model refined to $R_{work}/R_{free}$ of 15.6/18.2, with 98.2% of the 439 amino acids in favored regions of the Ramachandran plot and 0.5% outliers.

**Duf8901–AlF₃•Co²⁺ structure**

Diffraction data from a single crystal of Duf8901 that had been incubated with cobalt, AlCl₃ and NaF were collected at 1.5895 Å at beamline 24-ID-C. The crystal diffracted to 1.39 Å and belonged to space group C2 with unit cell dimensions consistent with one protomer per ASU, assuming a solvent content of 53%. The Duf89 structure was determined by MR in PHENIX.-PHASER using the partially refined 2.75 Å Duf8901 structure (protein only) as a search model. PHENIX.PHASER found 1 MR solution with TFZ and LLG scores of 24.4 and 793.1, respectively, and one Duf89 protomer in the ASU. The 2.0 Å structure was iteratively optimized in PHENIX.REFINE with TLS B-factor refinement (three TLS groups), interspersed with manual building in COOT. The final model refined to $R_{work}/R_{free}$ of 16.0/20.0, with 97.7% of the 440 amino acids in favored regions of the Ramachandran plot and only a single outlier.

**Deletion of duf89 and duf8901**

PCR amplification and standard cloning methods were used to construct plasmids in which the duf89 gene from nucleotides +5 to +1412 (relative to the translational start codon +1) and the duf8901 gene from +1 to +1333 were replaced by a hygMX and a kanMX antibiotic resistance cassette, respectively. The duf89 and duf8901 disruption cassettes were excised from the plasmids and transfected into diploid S. pombe cells. Hygromycin-resistant and G418-resistant transformants were selected and analyzed by Southern blotting to confirm correct integration at one of the duf89 loci (thereby deleting amino acids 2–435 of the 442-amino acid Duf89 protein) or one of the duf8901 loci (deleting amino acids 1–438 of the 438-amino acid Duf8901 protein). Hygromycin-resistant duf89Δ and G418-resistant duf8901Δ
haploids were isolated upon sporulation of the heterozygous diploids. Mating and sporulation of the duf89Δ and duf8901Δ strains yielded hygromycin/G418-resistant duf89Δ duf8901Δ double mutants.

Cell-surface acid phosphatase activity

Cells were grown at 30 °C in YES medium. Aliquots of exponentially growing cultures were harvested, washed, and resuspended in water. To quantify acid phosphatase activity, reaction mixtures (200 µl) containing 100 mM sodium acetate (pH 4.2), 10 mM p-nitrophenylphosphate, and serial dilutions of cells (ranging from 0.02 to 0.1 at an absorbance at 600 nm units) were incubated for 5 min at 30 °C. The reactions were quenched by addition of 1 ml of 1 M sodium carbonate, the absorbance of the supernatant at 410 nm was measured. Acid phosphatase activity is expressed as the ratio of absorbance at 410 nm (p-nitrophenol production) to absorbance at 600 nm (cells). The data are averages (±SEM) of at least three assays using cells from three independent cultures.

Data availability

The coordinates for the refined models of Duf89, Duf8901–Co2+, Duf8901–BeF3–Co2+, Duf8901–BeF3–Ni2+, Duf8901–BeF3–Zn2+, and Duf8901–AlF3–Co2+ have been deposited in the RCSB protein structure databank under PDB ID codes 7T7N, 7T7K, 7T7O, 7U1V, 7U1X, and 7U1Y. All other data are contained within the article and supporting information.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: AlF4, aluminum fluoride; ASU, asymmetric unit; BeF3, beryllium trifluoride; cDNA, complementary DNA; DUF89, Domain of Unknown Function 89; FOM, figure of merit; LLG, log-likelihood gain; Inc, long noncoding RNA; MR, molecular replacement; NCS, noncrystallographic symmetry; SAD, single-wavelength anomalous dispersion; TFZ, translation function Z; TLS, translation libration screw.

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