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Supplementary Materials for

Structure of *Saccharomyces cerevisiae* Rtr1 reveals an active site for an atypical phosphatase

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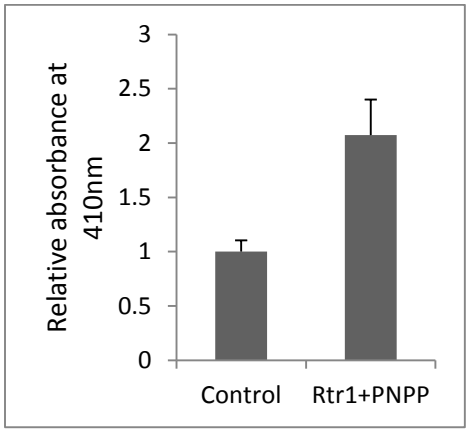


Figure S1: Activity toward *p*NPP requires prolonged incubation with *S. cerevisiae* Rtr1. Truncated $Rtr1_{1\text{-}213}$ at 28 µM concentration was incubated with 50 mM *p*NPP for 16 hours. The OD was measured at 410 nm. The values indicate the average of three independent experiments performed in duplicate and the standard deviation is shown as error bars.

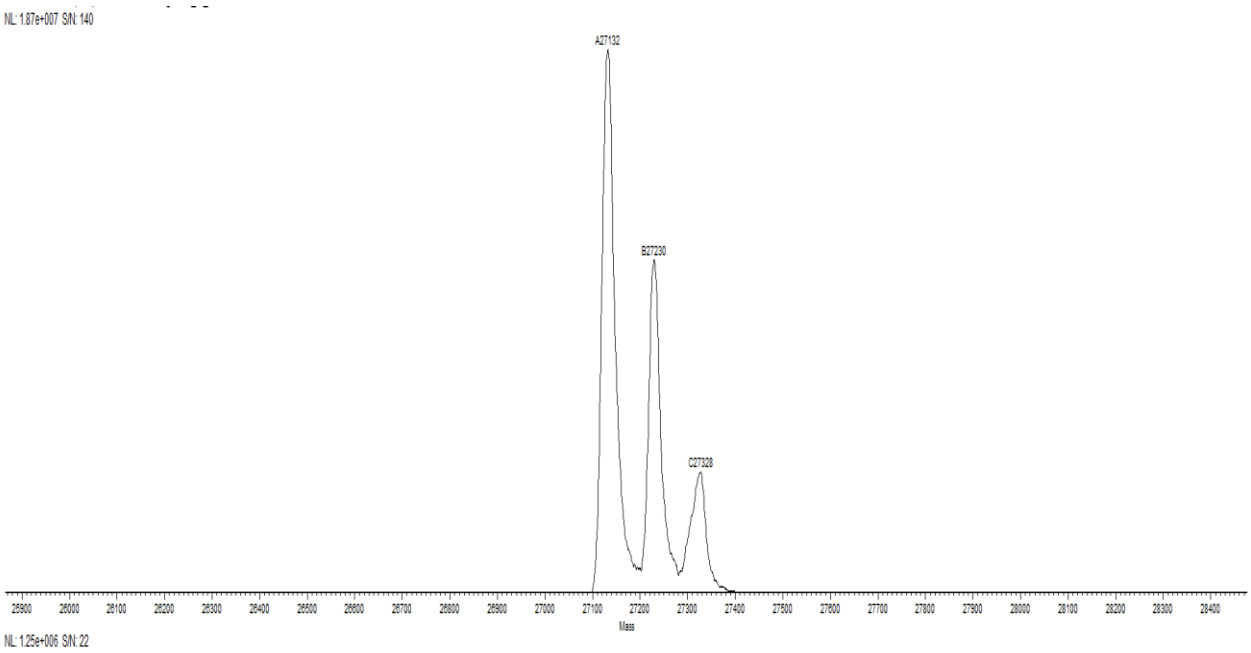


Figure S2: Electrospray ionization mass spectrometry analysis of $Rtr1_{1–213}$ in a buffer containing phosphate. Shown are the zoomed-in images of the deconvoluted peak of the proteins, which shows as a triplet peak. The left peak of 27,132 Da corresponding to $Rtr1_{1\text{-}213}$ (theoretical MW of 27,129 Da). The middle peak is 96 Da heavier than $Rtr1_{1\text{-}213}$, which is consistent with the noncovalent association of a molecule of inorganic phosphate. The right peak is another 96 Da heavier than the middle peak, implying a second phosphate associated with $Rtr1_{1\text{-}213}$.

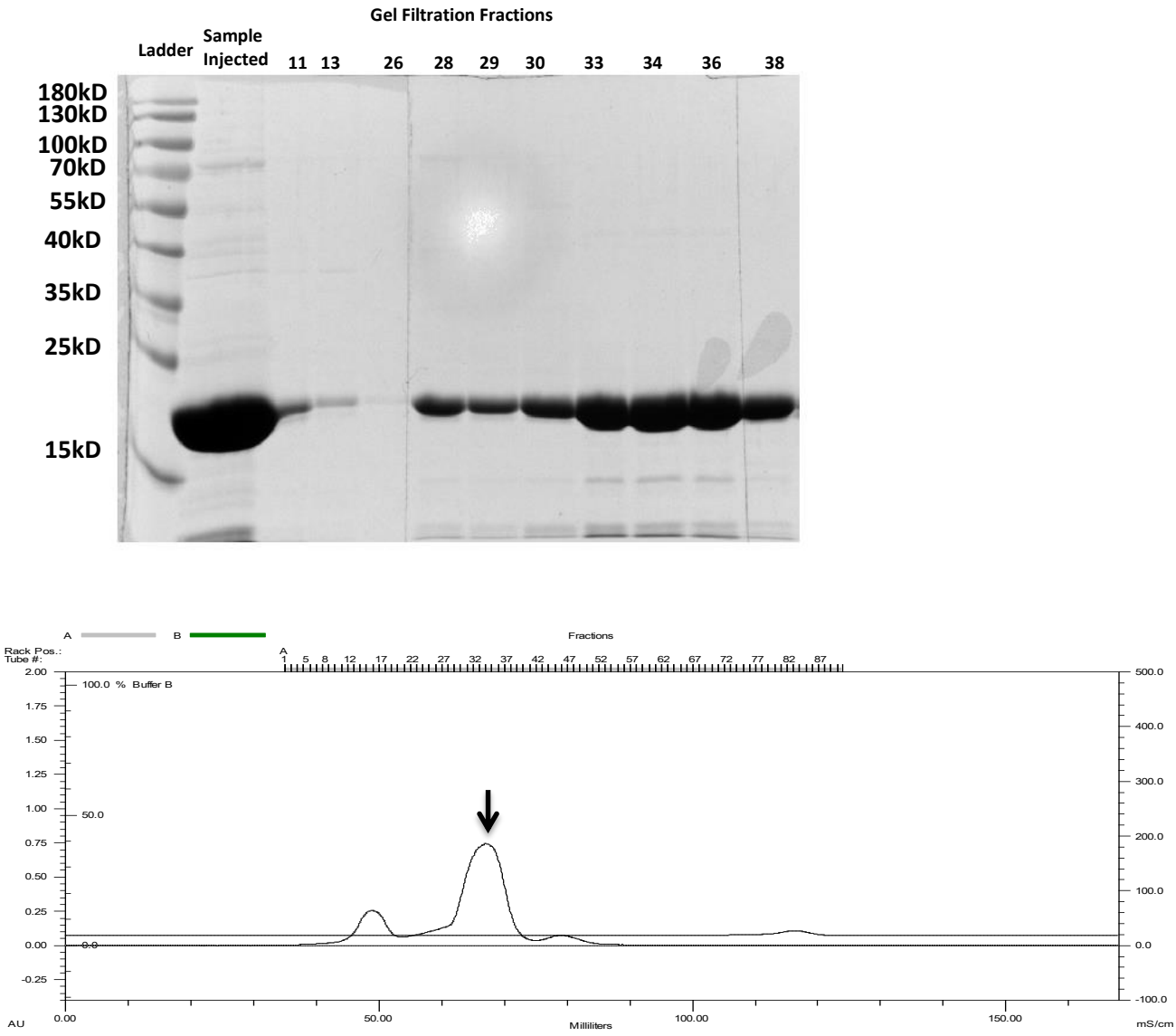
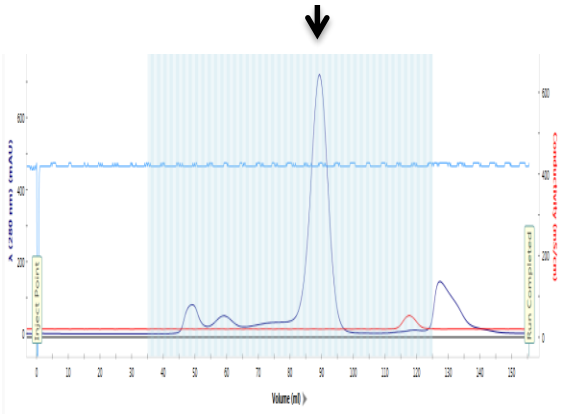
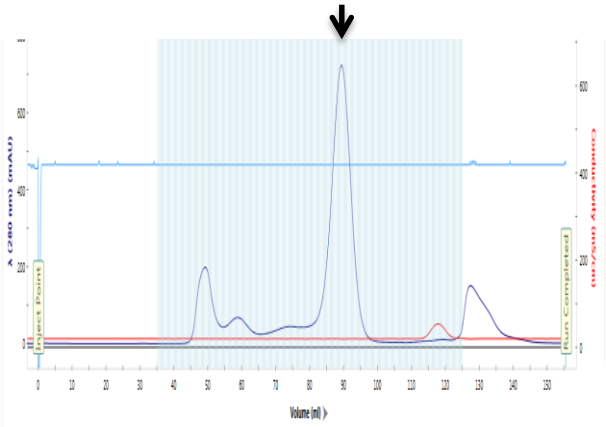


Figure S3: The purification of Rtr1$_{1–178}$ using size exclusion chromatography. In SDS-gel, the sample prior to size exclusion column purification was run on the lane next to the protein standard. The other lanes are corresponding to the different elution volume for the gel filtration profile below. After purification, the fraction 30-36 were collected and used for crystallization experiment.

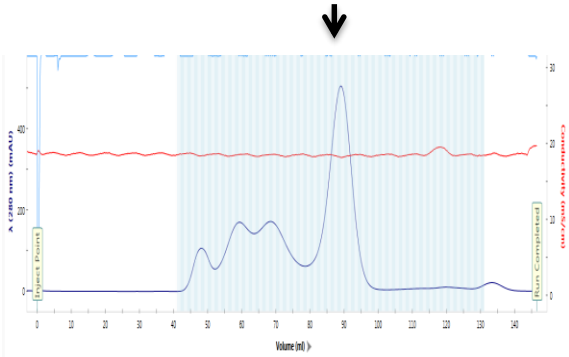
A



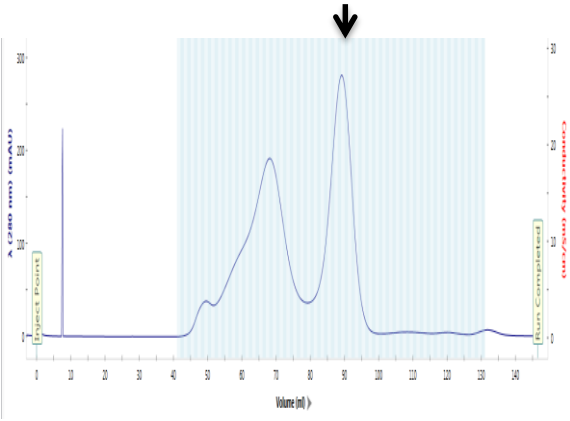
$Rtr1_{1-213}$Y105A gel filtration Profile



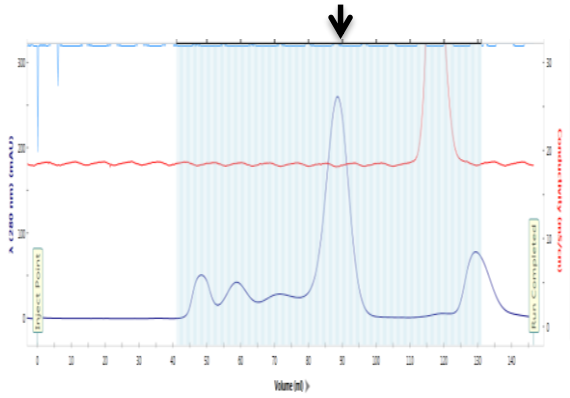
$Rtr1_{1-213}$D62A gel filtration



$Rtr1_{1-213}$D65A gel filtration Profile



$Rtr1_{1-213}$E66A gel filtration



$Rtr1_{1-213}$E66A gel filtration Profile

B

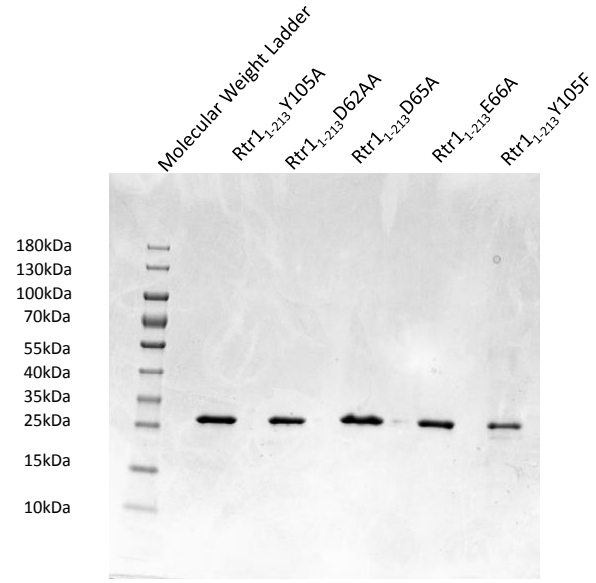


Figure S4: Gel filtration profile and SDS-PAGE electrophoresis of Rtr1$_{1–213}$ mutation variants with low in vitro activity. (**A**) Gel filtration results with arrows indicating the peaks that were collected during elution and used in SDS-PAGE gel in (**B**).

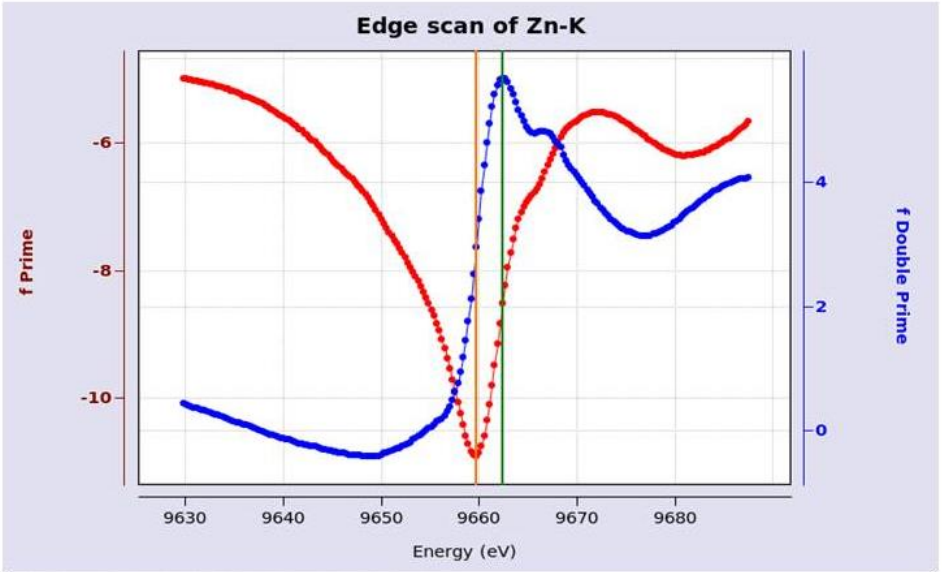


Figure S5: The presence of zinc in Rtr1$_{1–178}$ crystals. Analysis of the anomalous absorption edge at 9662.43eV in synchrotron confirmed the identity of the metal ion (zinc) in Rtr1 crystals.

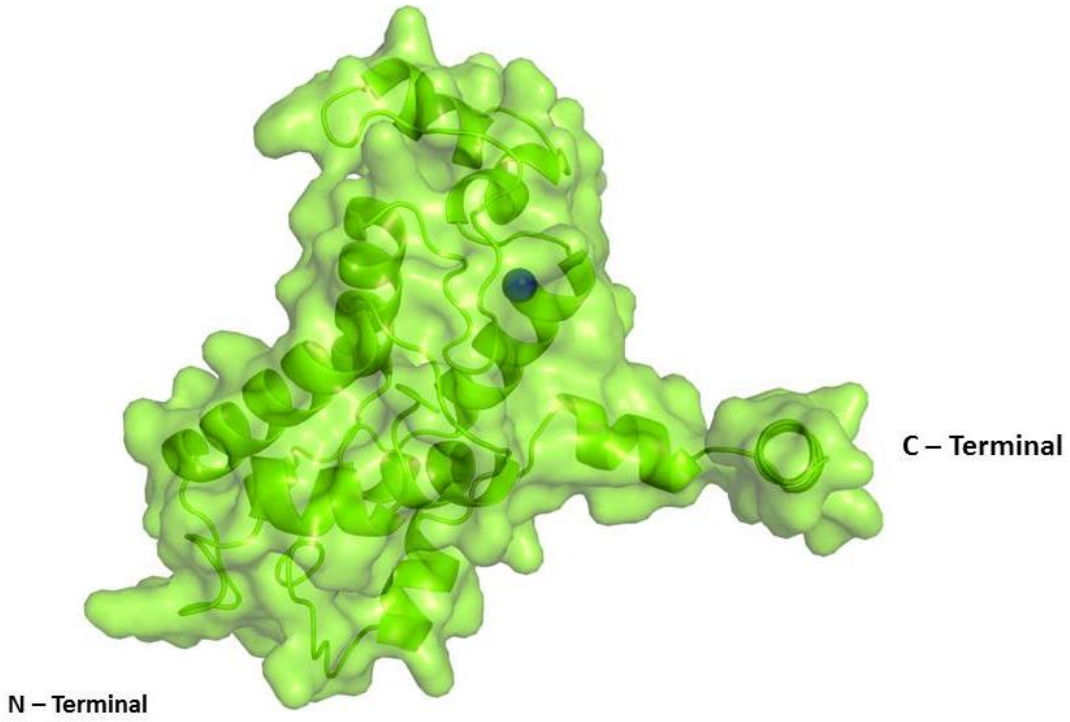


Figure S6: Surface representation of *S. cerevisiae* Rtr1$_{1–178}$. One molecule of Rtr1$_{1–178}$ from the asymmetric unit. The blue ball represents a zinc ion.

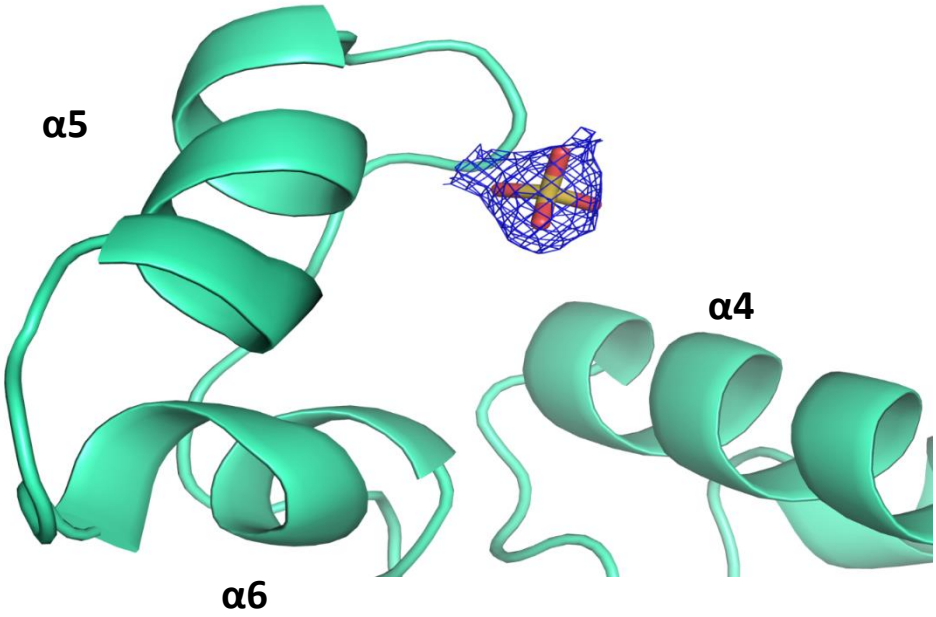


Figure S7: The electron density of sulfate molecule at site 1 in the Rtr1$_{1–178}$ structure. The blue mesh represents the 2FoFc electron density contoured to 1 σ. A sulfate ion from crystallization mother liquor is built into the density with good fit.

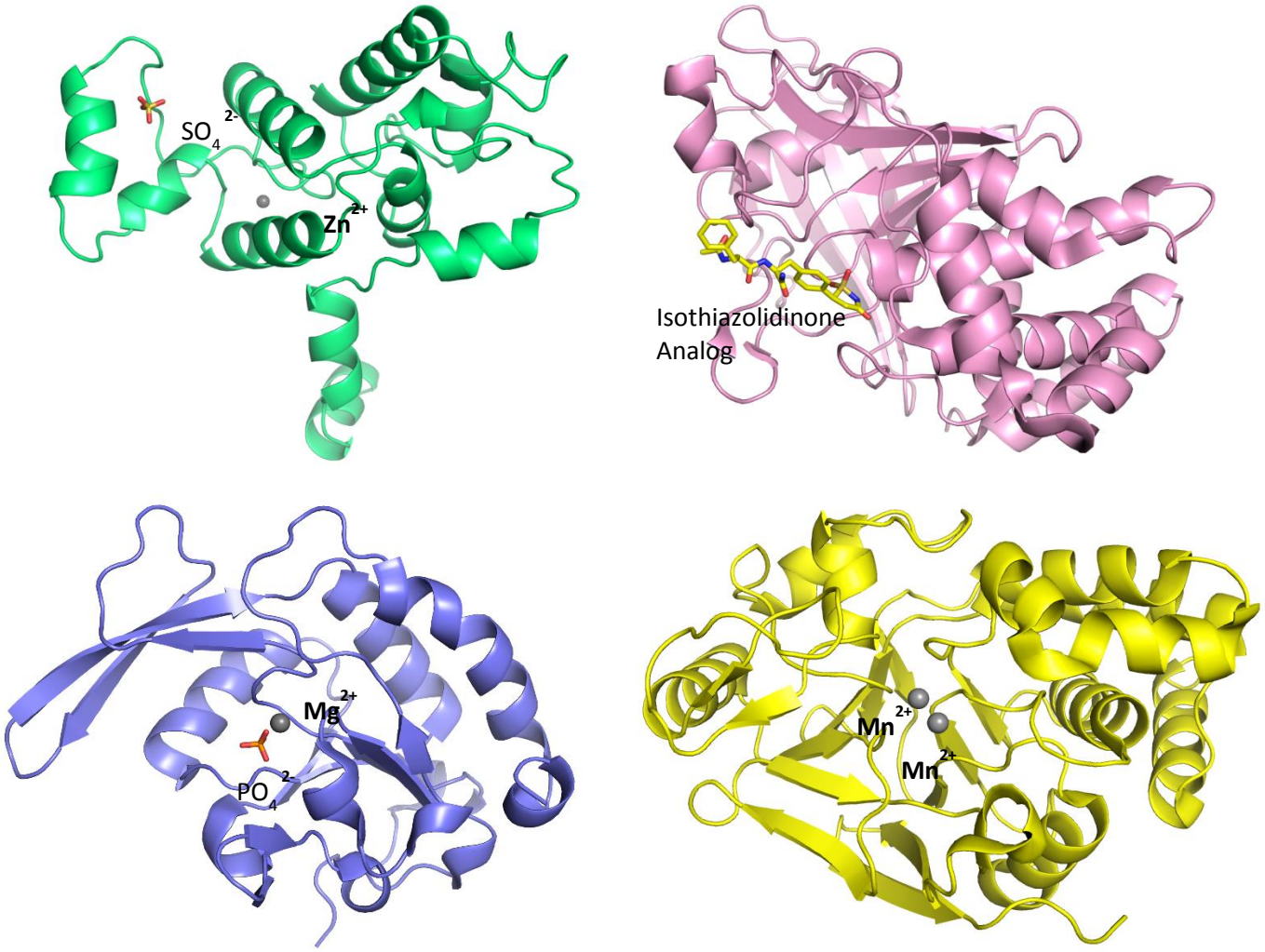


Figure S8: Comparison of protein folding in different phosphatases families. *S. cerevisiae* $Rtr1_{1\text{-}178}$ is color green with zinc ion shown as a gray sphere and sulfate ion in site 1 shown in stick representation. Cysteine-based phosphatase PTP1b (PDB ID: 2CM7) is pink with stick representation of bound inhibitor. HAD phosphatase Scp1 (PDB ID: 3L0C) is blue with Mg^{2+} ion shown as gray sphere and phosphate shown in stick representation. The catalytic domain of the metal-dependent phosphatase PP2A (PDB ID: 2NYM) is yellow with two Mn^{2+} ions shown as gray spheres.

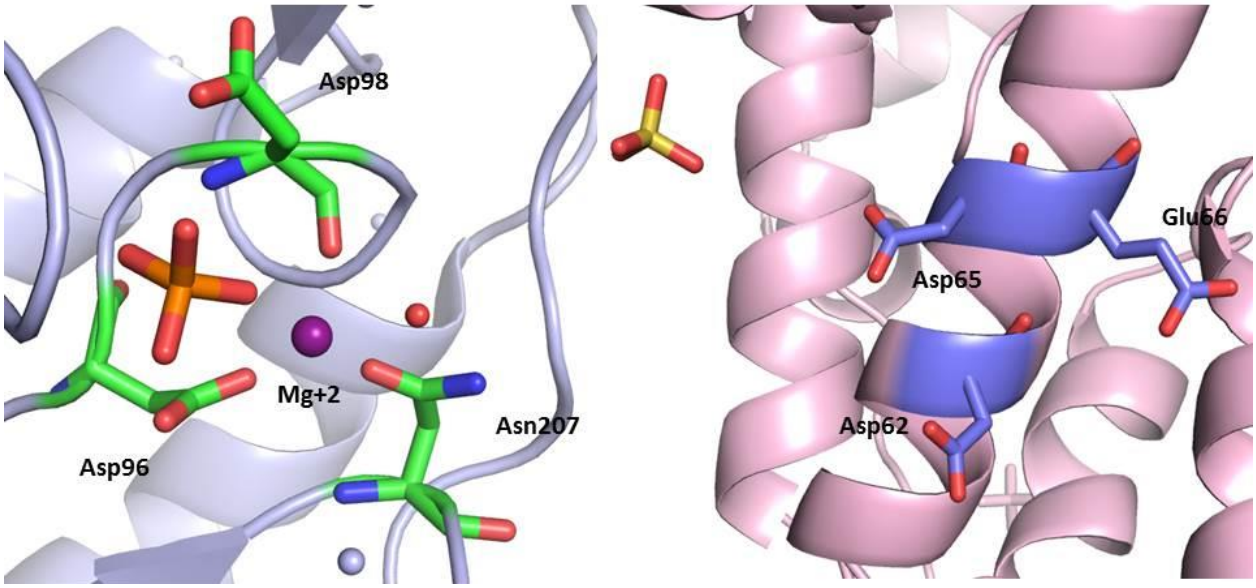


Figure S9: Comparison of the active site of Scp1 and Rtr1. Left shows the DXDX motif in Scp1 (PDB ID: 2GHT), which mediates phosphoryl transfer. Right shows Asp^{62}, Asp^{65}, and Glu^{66} (DXXDE) in Rtr1, which are important for phosphoryl transfer.

Table S1. Analysis of stability and activity of Rtr1 and the mutants. NA, not applicable. Molecular weight of full-length Rtr1 is 26239 Da, $Rtr1_{1-213}$ is 24779 Da, and $Rtr1_{1-178}$ is 20853 Da.

Protein	**Expressed in *E. coli***	**Detected as a single band of appropriate size by SDS-PAGE**	**Eluted at appropriate volume in gel filtration chromatography**	**Activity relative to that of $Rtr1_{1-213}$**
Full-length Rtr1	Yes	Yes	Yes	32.3%
Rtr_{1-129}	No	NA	NA	NA
Rtr_{1-137}	No	NA	NA	NA
Rtr_{1-167}	No	NA	NA	NA
Rtr_{1-178}	Yes	Yes	Yes	35.8%
Rtr_{1-213}	Yes	Yes	Yes	100%
$Rtr1_{1-213}$ C73A	No	NA	NA	NA
$Rtr1_{1-213}$ C78A	No	NA	NA	NA
$Rtr1_{1-213}$ C112A	No	NA	NA	NA
$Rtr1_{1-213}$ H116A	No	NA	NA	NA
$Rtr1_{1-213}$ D62A	Yes	Yes	Yes	6.3%
$Rtr1_{1-213}$ D65A	Yes	Yes	Yes	32.8%
$Rtr1_{1-213}$ E66A	Yes	Yes	Yes	5.5%
$Rtr1_{1-213}$ Y105A	Yes	Yes	Yes	2.4%
$Rtr1_{1-213}$ F130A	Yes	Yes	Yes	78%
$Rtr1_{1-213}$ Y105F	Yes	Yes	Yes	9.5%
$Rtr1_{1-213}$ R86A	Yes	Yes	Yes	25.2%
$Rtr1_{1-213}$ K97A	Yes	Yes	Yes	28.4%
$Rtr1_{1-213}$H110A	Yes	Yes	Yes	14.3%
$Rtr1_{1-213}$ R86AK97A	Yes	Yes	Yes	19.2%
$Rtr1_{1-213}$ R86AK97AH110A	Yes	Yes	Yes	6.0%

Table S2. Analysis of stability and activity of RPAP2. NA, not applicable. Molecular weight of $RPAP2_{32-274}$ is 27,821 Da; molecular weight of $RPAP2_{32-345}$ is 35,696 Da.

Protein		**Expressed in *E. coli***	**Detected as a single band of appropriate size by SDS-PAGE**	**Eluted at appropriate volume in gel filtration chromatography**	**Activity relative to that of** $RPAP2_{32-274}$
$RPAP2_{1-345}$		No	NA	NA	NA
$RPAP2_{32-274}$		Yes	Yes	Yes	100%
$RPAP2_{32-345}$		Yes	Yes	Yes	34.3%
$RPAP2_{32-274}$Y127A		Yes	Yes	Yes	Not detected