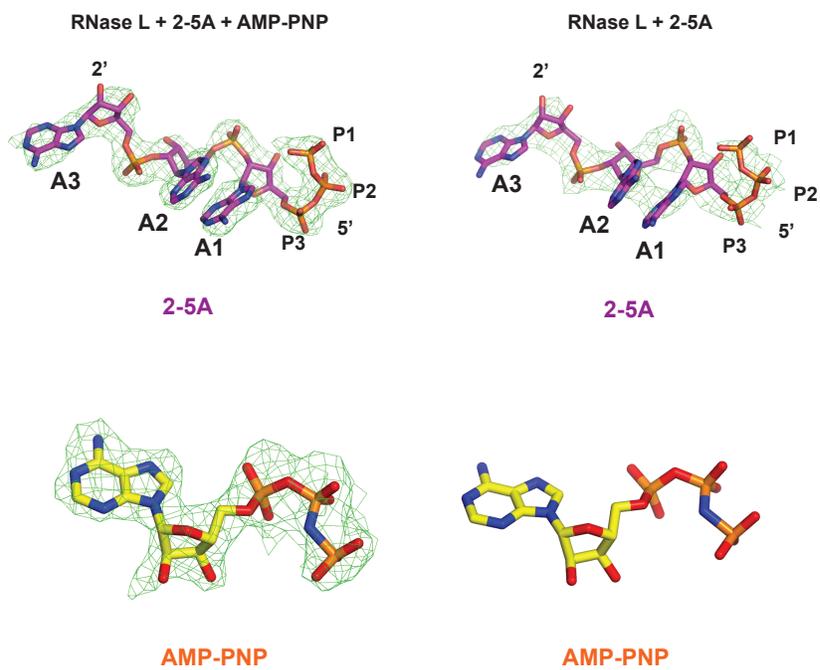
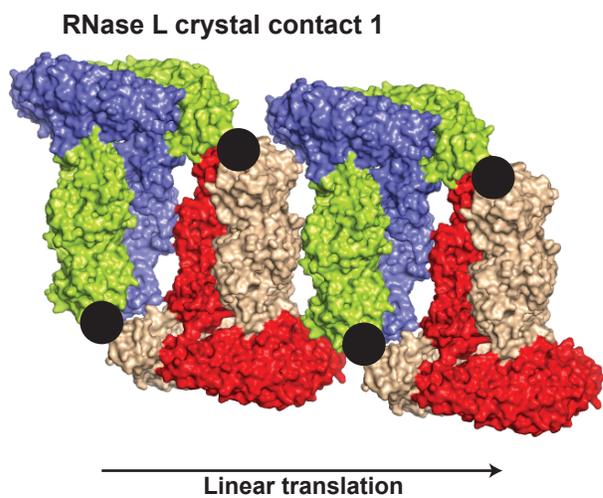


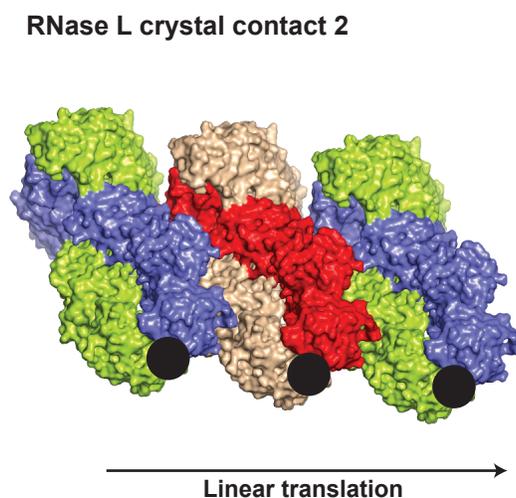
**A**



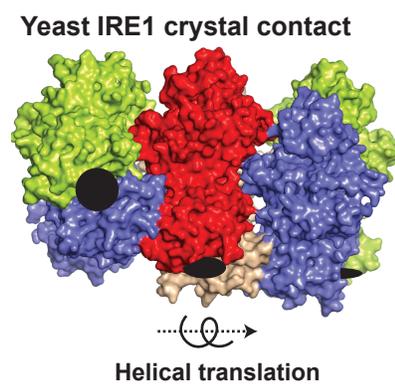
**B**



**C**

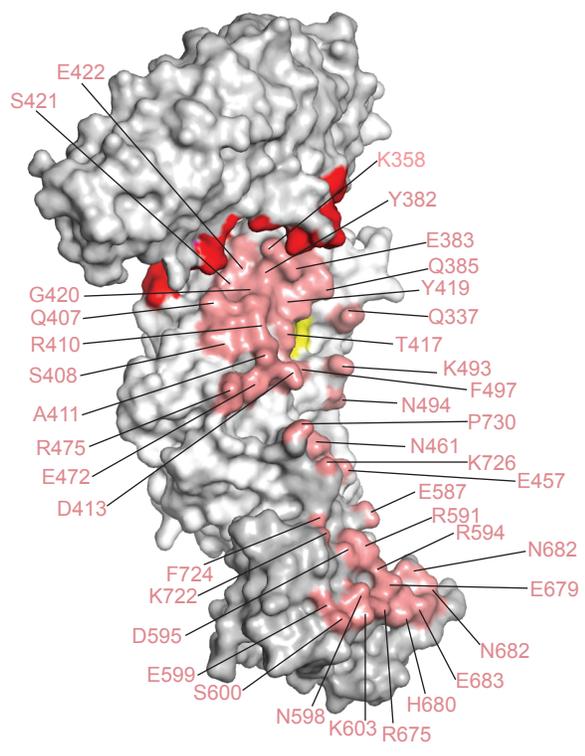
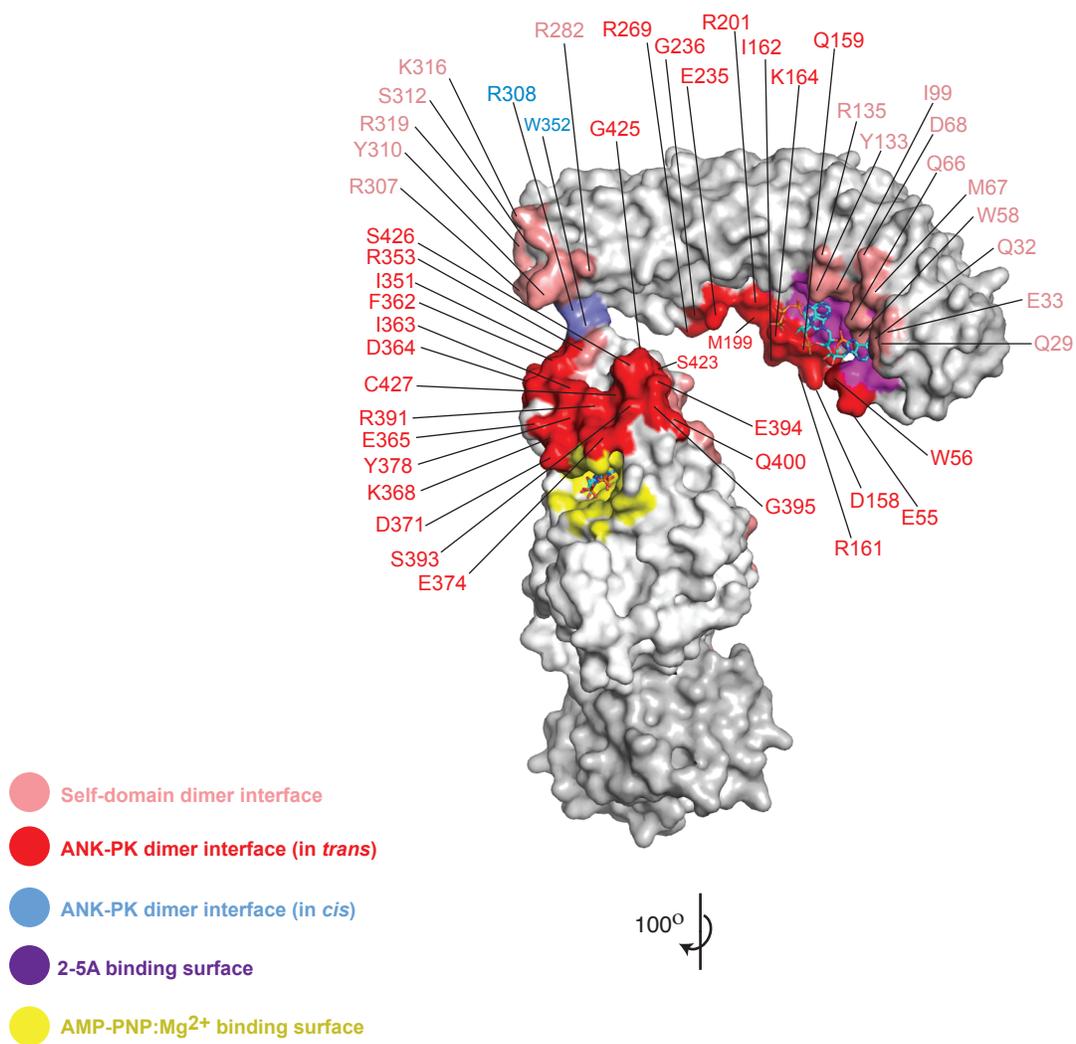


**D**

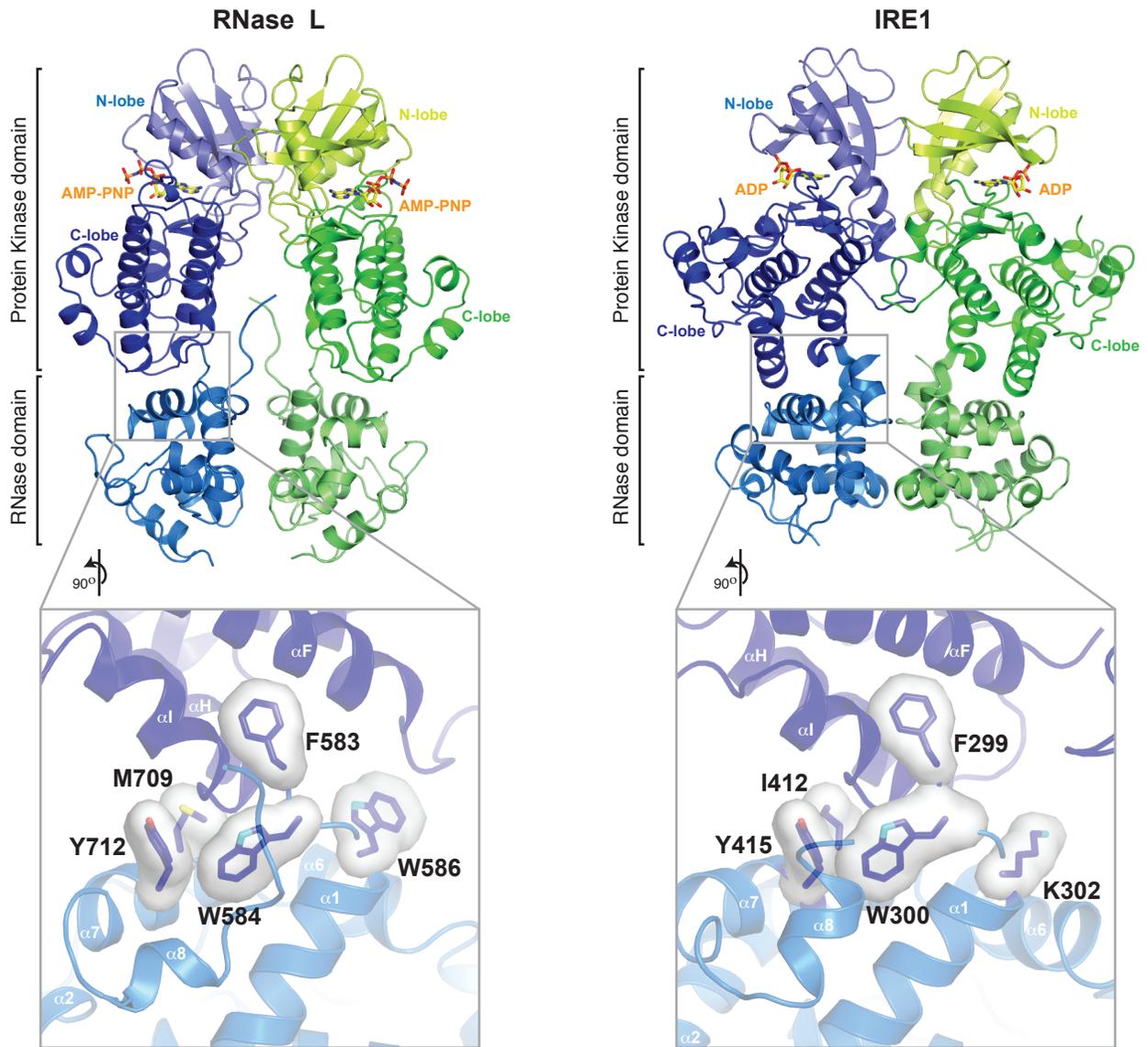
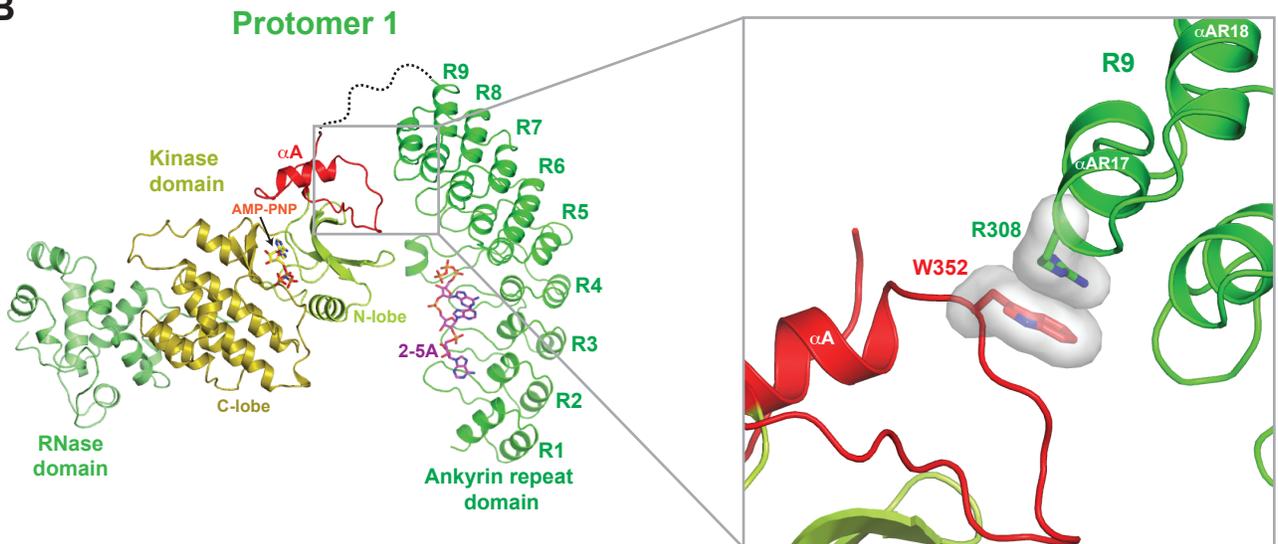


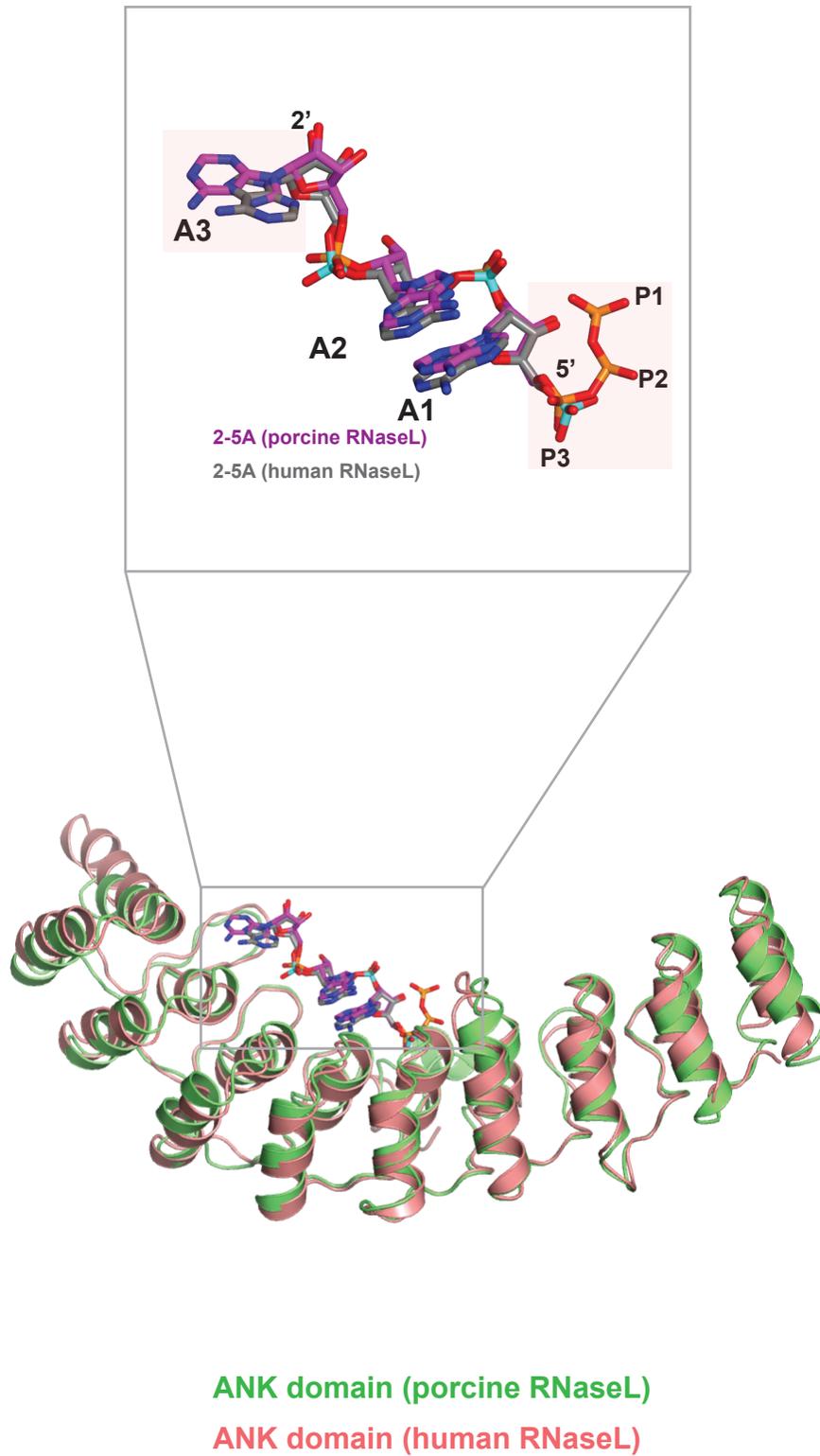
**Supplementary Figure 1**



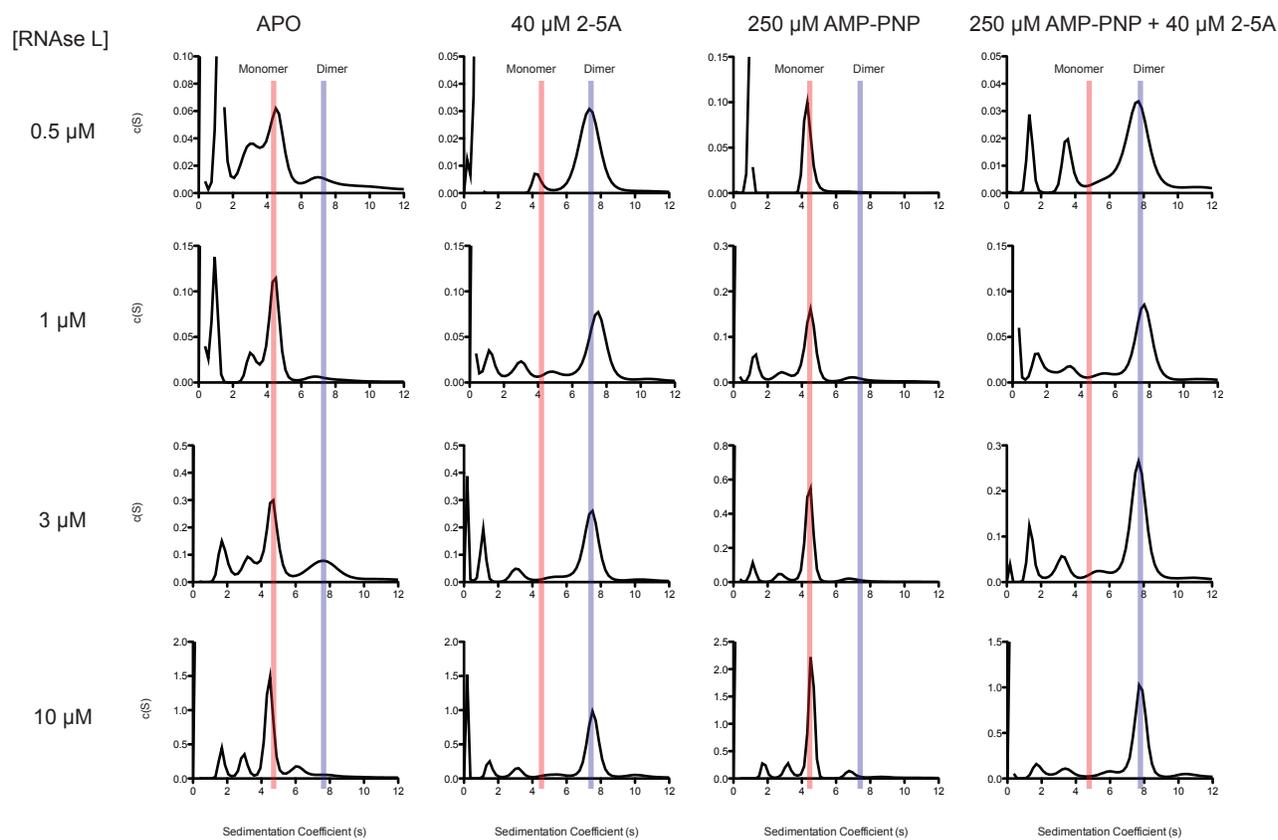
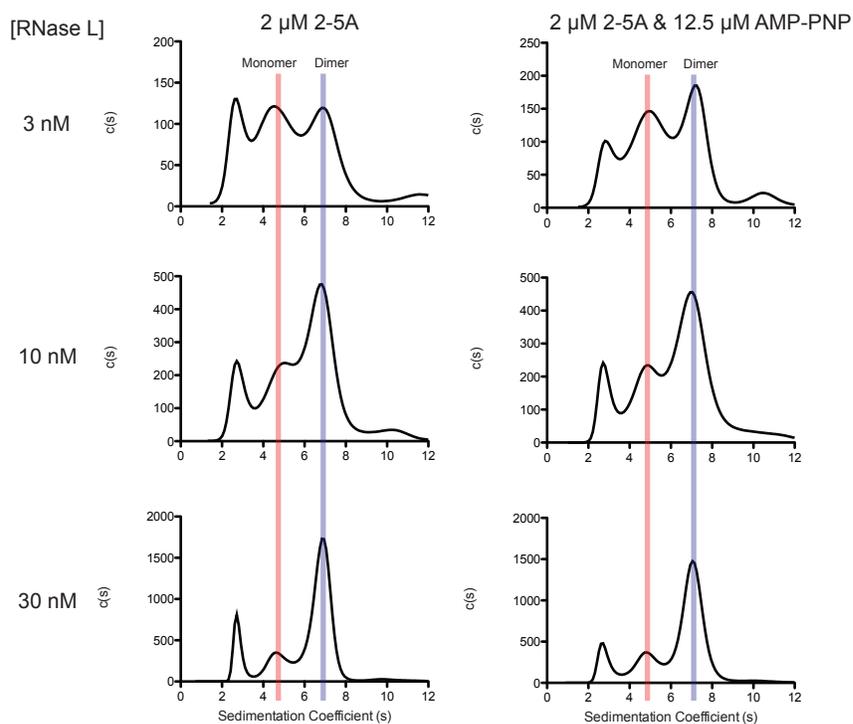


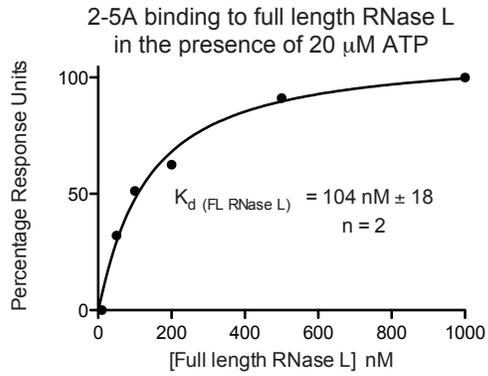
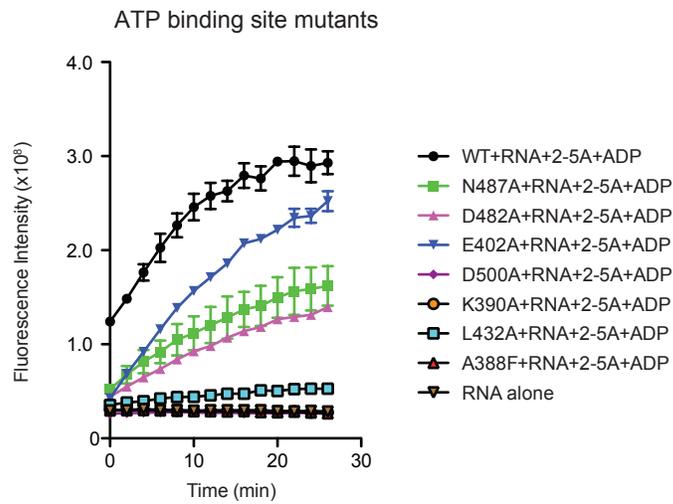
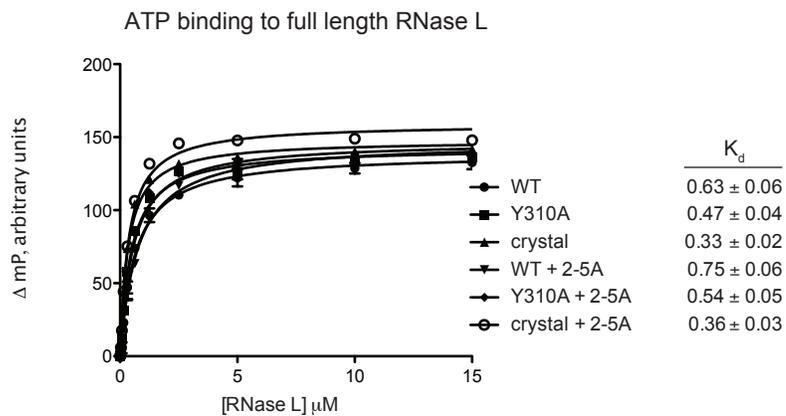
**Supplementary Figure 3**

**A****B****Supplementary Figure 4**



**Supplementary Figure 5**

**A****B****Supplementary Figure 6**

**A****B****C****Supplementary Figure 7**

## SUPPLEMENTARY FIGURE LEGENDS

### **Figure S1. Representative electron density maps and crystal contacts. Related to Figure 1.**

(A) Unbiased  $F_o-F_c$  electron density maps (contoured at  $2.5\sigma$ ) centered on the 2-5A (**top panels**) and AMP-PNP (**bottom panels**) ligand binding sites. Maps reflect an atomic model prior to introduction of ligands into the refinement process. Ligands from the final model are shown for comparison. In the absence of electron density for ADP in the ADP free RNase L crystal structure, ADP was not modeled.

(B & C) Packing contacts of RNase L dimers present in the crystal lattice. Protomers within each 2-5A induced dimer are coloured blue and green or red and beige with RNase active sites highlighted by black circle. Inter-dimer contact area =  $603 \text{ \AA}^2$  and  $1718 \text{ \AA}^2$  for (B) and (C), respectively.

(D) Crystal packing contacts of yeast IRE1 dimers implicated in polymer formation (PDBID 3FBV). Protomers within each back-to-back dimer are coloured blue and green or red and beige with RNase active sites highlighted by black circle. Inter-dimer contact area =  $2714 \text{ \AA}^2$ .

### **Figure S2. Sequence alignment of RNase L orthologues and Ire1. Related to Figures 1, 2 and 3.**

Structure based multiple sequence alignment of RNase L sequences from *Sus scrofa* (porcine), *H. sapiens* (human), *M. musculus* (mouse), *B. taurus* (cattle), *G. gallus* (chicken) and the dual PK domain – RNase domain module of *S. cerevisiae* (yeast) Ire1 (PDBID 2RIO). The ANK domain, PK domain, and RNase domain regions are highlighted by blue, tan and pink backgrounds. Residues invariant or highly conserved across the RNase L orthologues are shaded black and grey respectively. The secondary structure of *S. scrofa* RNase L is drawn above the alignment. Disordered regions are depicted as dashed lines. Non-canonical features of the pseudokinase domain are highlighted by coloring the secondary structure in red. Conserved RNase domain catalytic residues are colored in purple and key protein kinase catalytic motifs are indicated by magenta-bordered boxes. 2-5A—interacting residues are indicated by stars and AMP-PNP—interacting residues are indicated by grey triangles. Cross-protomer contacts (calculated by AREAIMOL) are indicated by filled boxes colored in green (ANK-PK contacts) and in orange (ANK-ANK, PK-PK or RNase-RNase contacts) boxes above the alignment. Dashed lines represent not modeled disordered regions of the crystal structure.

### **Figure S3. Contact surfaces between two RNase L protomers. Related to Figure 3.**

Surface representation of a single RNase L protomer with dimer interface residues highlighted. Surfaces of atom pairs closer than  $5.9 \text{ \AA}$  are colored as indicated. AMP-PNP and 2-5A are shown as sticks.

### **Figure S4. Structure analysis of RNase L. Related to Figure 4.**

(A) Ribbons comparison of the back-to-back dimer configuration of the dual pseudo PK domain – RNase domain module of RNase L and IRE1. A detailed representation of the

contiguous hydrophobic core linking the protein kinase and RNase domains is shown in the zoom in views below.

**(B)** Ribbons representation of the tenuous contact between ANK and PK domains within each protomer of a higher order dimer. A detailed representation of interacting residues is shown in the zoom in view to the right.

**Figure S5. Structure comparison of ANK domains and 2-5A ligands from isolated ANK and full length RNase L structures. Related to Figure 4.**

Cartoon representation of superimposed ANK domains of porcine RNase L and isolated human ANK domain structure (PDBID 4G8L) with bound 2-5A. Bound 2-5A ligands are shown in the zoom in view at top. 2-5A bound to human RNase L (ANK domain alone) is depicted as sticks with grey colored carbons and cyan colored phosphates. 2-5A bound to porcine RNase L is depicted as sticks with magenta colored carbons and orange colored phosphates.

**Figure S6. Influence of ligand binding on the oligomerization status of WT porcine RNase L. Related to Figure 5.** Analytical ultracentrifugation analyses were performed on **(A)** WT RNase L using refractive index detection or **(B)** fluorescein labeled WT RNase L using fluorescence detection at the indicated protein concentrations with the indicated concentrations of ligands.

**Figure S7. In vitro functional characterization of RNase L. Related to Figure 5.**

**(A)** Binding of 2-5A to full length RNase L in the presence of 20  $\mu\text{M}$  ADP assessed by surface plasmon resonance. Normalized response levels for specific binding of RNase L to immobilized biotinylated 2-5A were plotted against increasing protein concentration. Reported  $K_d$  is the average of two independent experiments ( $\pm$  s.e.m). Displayed results are representative of two independent experiments.

**(B)** RNase activity profiles for wild type RNase L and the indicated ATP binding site mutants monitored in the presence of 2-5A activator and 50  $\mu\text{M}$  ATP using a FRET pair labeled RNA substrate. Displayed results are representative of two independent experiments measured in duplicate ( $\pm$  s.e.m.).

**(C)** Binding of the fluorescently labeled ATP analogue (BODIPY FL ATP- $\gamma$ -S) to full length RNase L, a Tyr310Ala mutant, and the crystallization construct (crystal) in the presence and absence of 3  $\mu\text{M}$  2-5A activator assessed by monitoring the fluorescence polarization signal in the presence of increasing concentrations of protein. Displayed results are representative of two independent experiments measured in duplicate ( $\pm$  s.e.m).