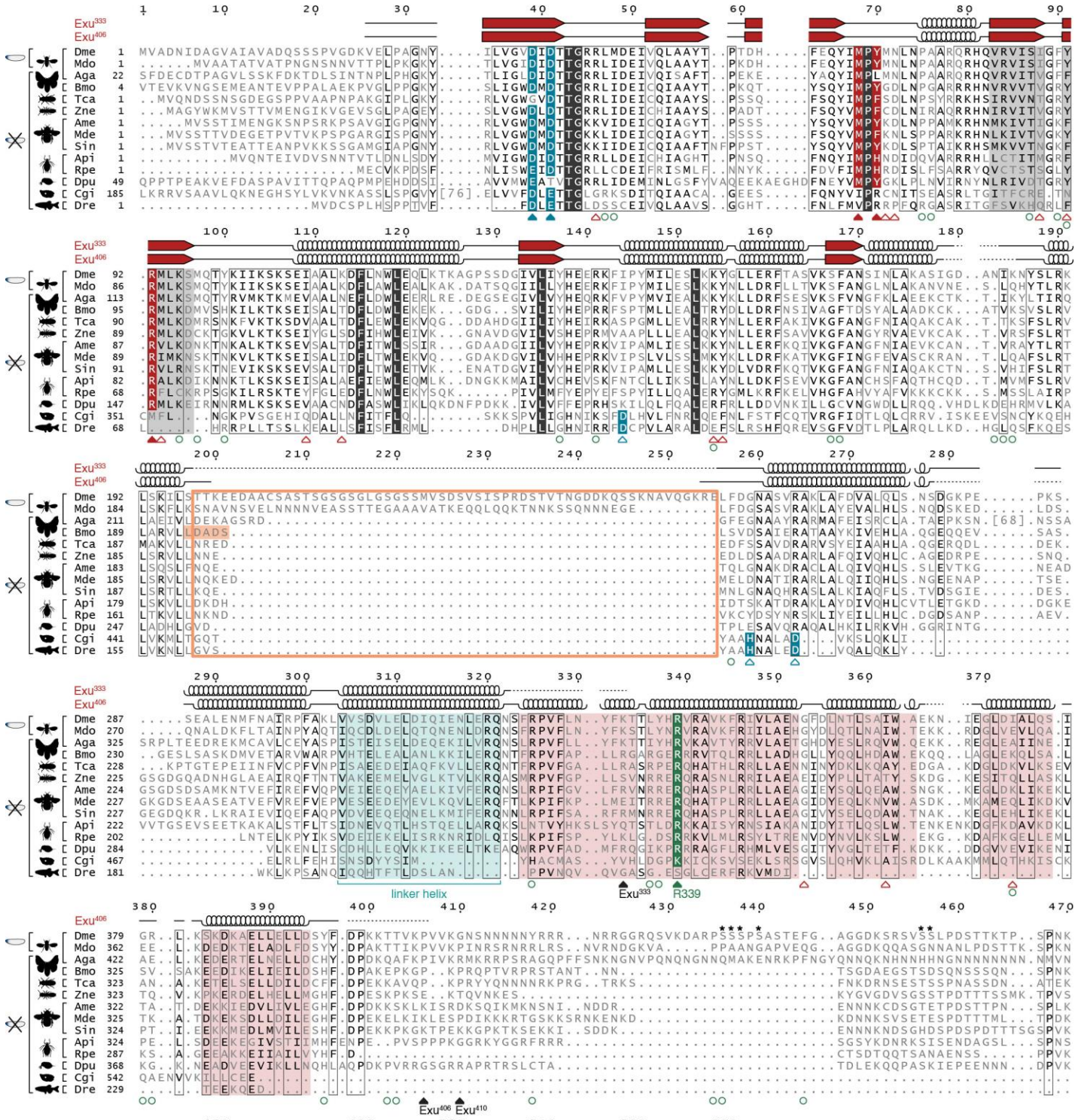
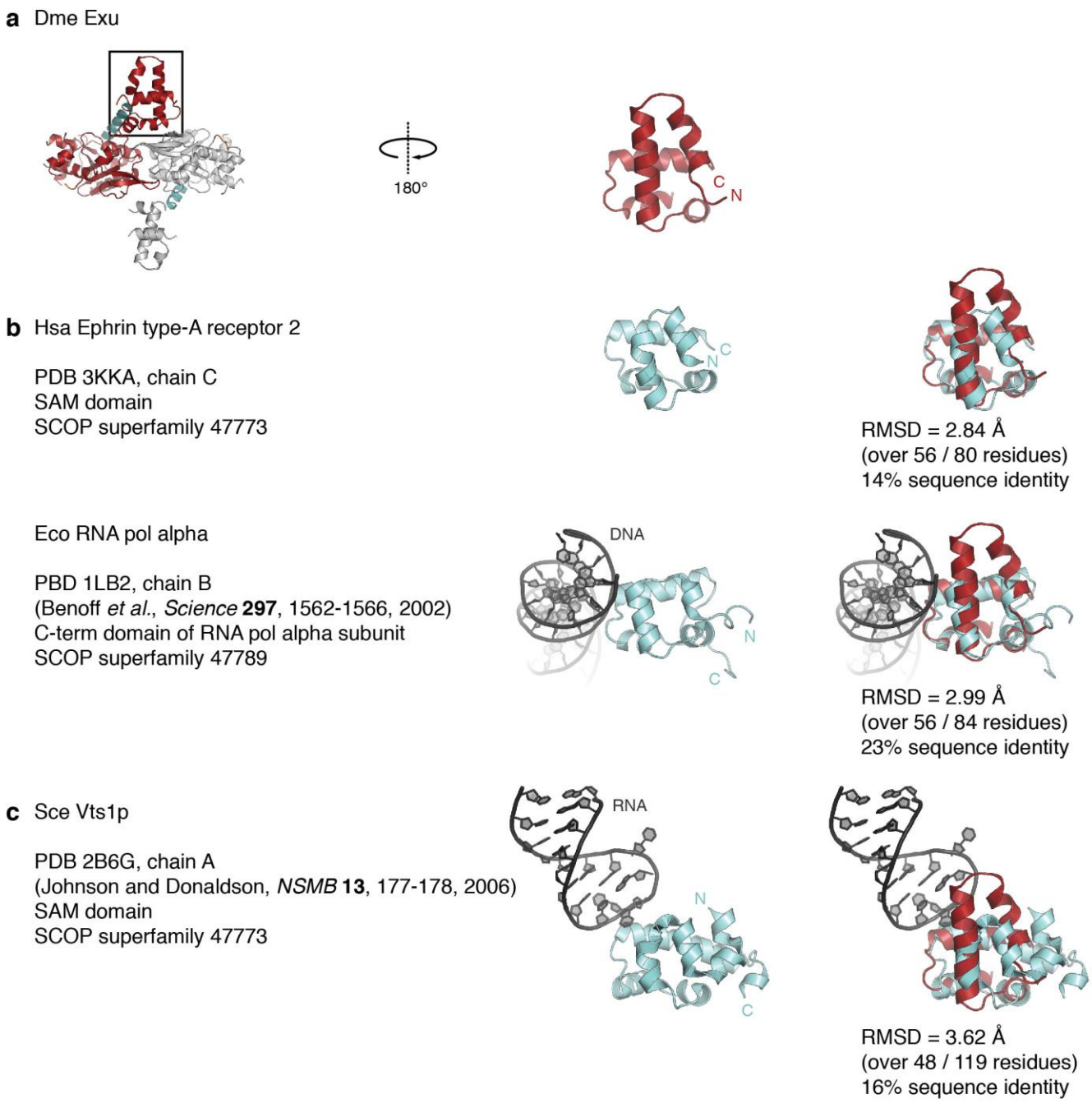

Exu333
Exu406
Dme
Mdo
Aga
Bmo
Tca
Zne
Ame
Mde
Sin
Api
Rpe
Dpu
Cgi
Dre
linker helix
Exu333
R339
Exu406
Exu406
Exu410

Supplementary Figure 1

Alignment of Exu homologs.

Sequences used to generate the alignment include: [Insecta, order Diptera] *D. melanogaster* (Dme), *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. virilis*, *D. mojavensis*, *D. willistoni*, *D. pseudoobscura pseudoobscura*, *D. persimilis*, *D. grimshawi*, *D. miranda*, *D. affinis*, *B. cucurbitae*, *B. dorsalis*, *C. capitata*, *M. domestica* (Mdo), *A. gambiae* (Aga), *A. sinensis*, *A. darlingi*, *C. quinquefasciatus*, *A. aegypti*; [Lepidoptera] *B. mori* (Bmo), *D. plexippus*, *P. aegeria*; [Coleoptera] *D. ponderosae*, *T. castaneum* (Tca); [Phthiraptera] *P. humanus corporis*; [Isoptera] *Z. nevadensis* (Zne), *M. rotundata*; [Hymenoptera] *B. terrestris*, *B. impatiens*, *A. mellifera* (Ame), *A. florea*, *N. vitripennis*, *M. demolitor* (Mde), *S. invicta* (Sin), *C. biroi*, *C. floridanus*, *A. echinatior*, *H. saltator*; [Hemiptera] *A. pisum* (Api), *D. citri*, *R. pedestris* (Rpe); [Crustacea] *D. pulex* (Dpu); [Mollusca] *C. gigas* (Cgi); [Chordata] *D. rerio* (Dre). Numbering refers to the Dme sequence. Only the suborder Cyclorrapha (Diptera) has a *Bicoid* gene.

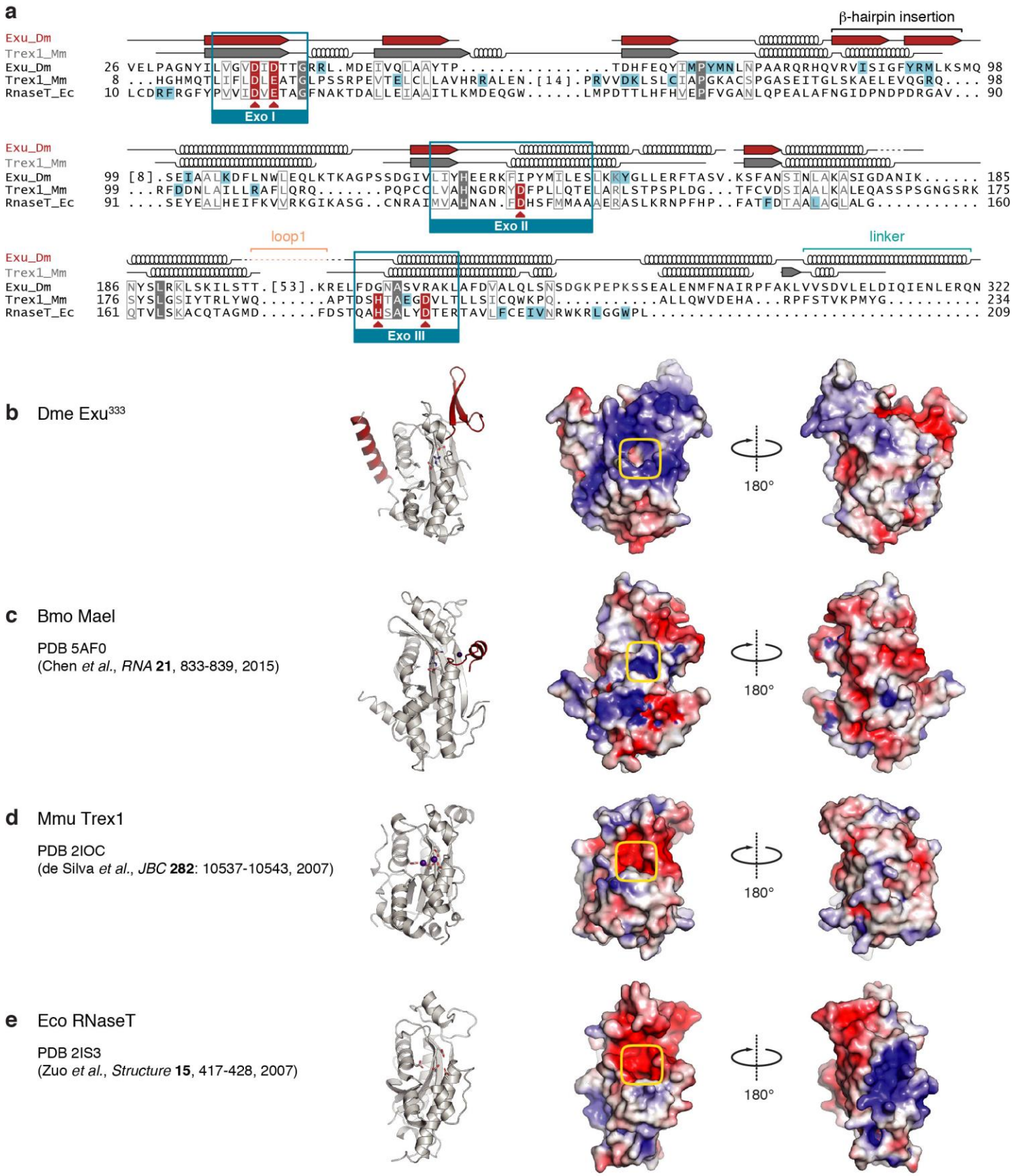
The secondary structure of Dme Exu^{333} and Exu^{406} is schematized above the alignment (red arrows: β-sheets; helices: α-helices; residues not ordered in the structure are shown as dotted lines; gaps indicate regions for which no structural information is available). Residues involved in Dme Exu dimerization: empty red arrows; residues confirmed by mutagenesis: filled red arrows; conserved residues are highlighted in red. Non conserved loop1 is framed by a orange box; the corresponding residues in Bmo Exu (used to replace the loop in the Exu406 construct used for crystallization), are highlighted in the same color. Catalytic residues in the exonuclease domain: empty blue arrows; mutated residues: filled blue arrows; conserved catalytic residues are highlighted in blue. Residues cross-linked to RNA: empty green circles; Arg339: filled green arrow. Black arrows indicate the boundaries of the Exu^{333}, Exu^{406} and Exu^{410} constructs. The phosphorylation sites identified in Dme Exu (Riechmann and Ephrussi, *Development*. **131**, 5897-5907, 2004) are marked by asterisks. The β-hairpin insertion is shaded in grey; the linker between the Exo-like and the SAM-like domains in light teal; the helices of the SAM-like domain in light red. The alignment was generated with MUSCLE (Edgar, *Nucleic Acids Res.* **32**, 1792-1797, 2004), visualized with ESPript (Robert and Gouet, *Nucleic Acids Res.* **42**, W320-W324, 2014) and edited in Adobe Illustrator.



Supplementary Figure 2

Structural homologs of the Exu SAM-like domain.

a) Cartoon representation of Exu[406] (left) and of Exu SAM-like domain structure (aa 321-397; right). **b**) A structural similarity search in PDBeFold (Krissinel and Henrick, *Acta Cryst.* **D60**, 2256-2268, 2004) with Exu SAM-like domain outputs the sterile alpha motif (SAM), with Z-score = 4.4, and the C-terminal domain of RNA polimerase alpha, with Z-score = 4.2. **c**) Yeast Vts1p is a homolog of the *Drosophila* protein Smaug, and also contains a SAM domain which is involved in RNA binding (see also **Supplementary Fig. 5**). All structures in **b**) and **c**) belong to the SAM domain-like fold (SCOP 47768). Structural alignment was done with cealign in Pymol (v 1.7.6.0).

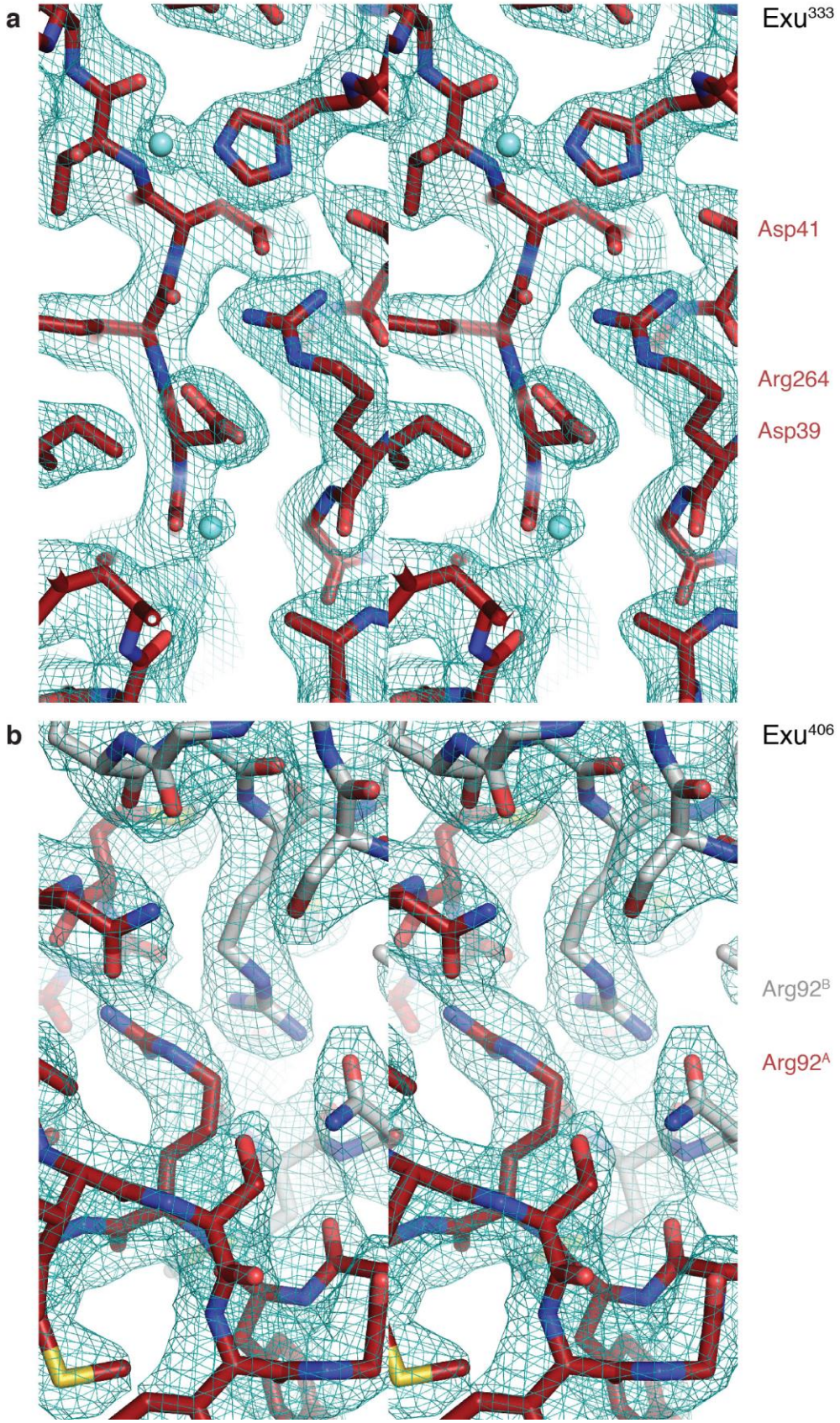

a
β-hairpin insertion
Exu_Dm
Trex1_Mm
Exu_Dm 26 VELPAGNYILVGVDIDTTGRRL.MDEIVQLAAYTP.....TDHFEQYIMPYMNLNPAARQRHQVRVISIGFYRMLKSMQ 98
Trex1_Mm 8 ...HGHMQTLIFLDLEATGLPSSRPEVTELCLLAVHRRALEN.[14].PRVVDKLSLCIAPGKACSPGASEITGLSKAELEVQGRQ.... 98
RnaseT_Ec 10 LCDRFRGFYPVVIDVETAGFNAKTDALLEIAAITLKMDEQGW.....LMPDTTLHFHVEPFVGANLQPEALAFNGIDPNDPDRGAV... 90
Exo I
Exu_Dm
Trex1_Mm
Exu_Dm 99 [8].SEIAALKDFLNWLEQLKTKAGPSSDGIVLIYHEERKFIPYMILESLKKYGLLERFTASV.KSFANSINLAKASIGDANIK..... 185
Trex1_Mm 99 ...RFDDNLAILLRAFLQRQ.....PQPCCLVAHNGDRYDFPLLQTELARLSTPSPLDG...TFCVDSIAALKALEQASSPSGNGSRK 175
RnaseT_Ec 91 ....SEYEALHEIFKVVRKGIKASG..CNRAIMVAHNAN.FDHSFMMAAAERASLKRNPFHP..FATFDTALAGLALG..... 160
Exo II
loop1
linker
Exu_Dm
Trex1_Mm
Exu_Dm 186 NYSLRKLSKILSTT.[53].KRELFDGNASVRAKLAFDVALQLSNSDGKPEPKSSEALENMFNAIRPFAKLVVSDVLELDIQIENLERQN 322
Trex1_Mm 176 SYSLGSIYTRLYWQ.....APTDSHTAEGDVLTLLSICQWKPQ.....ALLQWVDEHA...RPFSTVKPMYG..... 234
RnaseT_Ec 161 QTVLSKACQTAGMD.....FDSTQAHSALYDTERTAVLFCEIVNRWKRLGGWPL..... 209
Exo III
b Dme Exu333
180°
c Bmo Mael
PDB 5AF0
(Chen et al., RNA 21, 833-839, 2015)
180°
d Mmu Trex1
PDB 2IOC
(de Silva et al., JBC 282: 10537-10543, 2007)
180°
e Eco RNaseT
PDB 2IS3
(Zuo et al., Structure 15, 417-428, 2007)
180°

Supplementary Figure 3

Comparison of DEDD exo- and pseudonucleases.

a) Structure-based sequence alignment of the EXO and EXO-like domains of *Drosophila* Exu (Exu_Dm) and of structurally similar 3'-5' DEDD exonucleases, mouse Trex1 (Trex1_Mm) and *E. coli* RNaseT (RNaseT_Ec). Secondary structure elements are shown above the sequences, in red for Exu and in dark gray for Trex1. Conserved residues are highlighted in dark gray. Blue boxes indicate EXO signature motifs, while signature catalytic residues are marked in red. Brackets indicate protein-specific insertions that are hidden for clarity. The positions of Exu β-hairpin insertion, loop1 and linker are indicated. For each protein, residues involved in homodimerization are highlighted in light blue.

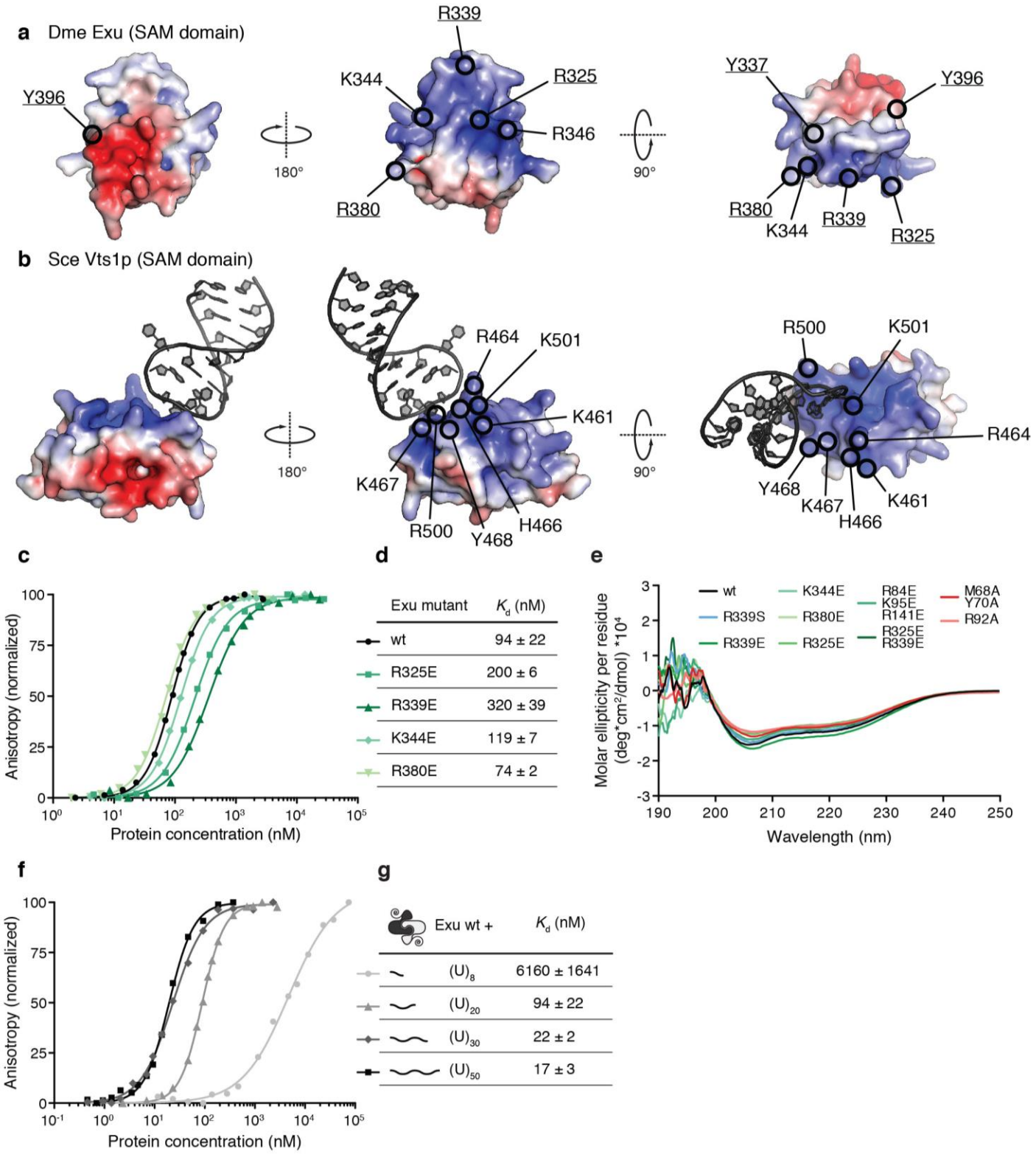
b-e) Cartoon (left) and surface (middle and right) representation of the indicated protein structures. Exu^{333} (**b**) and Trex1 (**d**) are in the same orientation as in **Fig. 2a, b**. Residues in the (pseudo-)catalytic site are shown as sticks; ions (Zn^{2+} in (**c**) and Mn^{2+} in (**d**)) are represented as spheres. Protein-specific features are highlighted in red: linker helix and β-hairpin insertion in Exu (**b**); Zn^{2+} coordinating extension in Maelstrom (Mael) (**c**). Surface is colored according to the electrostatic charge, with the (pseudo)-catalytic site boxed in yellow. Structural alignment was done with cealign in Pymol (v 1.7.6.0). Scale of electrostatic charge distribution is the same for all domains (-5 to +5 kT/e).


a
Exu333
Asp41
Arg264
Asp39
b
Exu406
Arg92B
Arg92A

Supplementary Figure 4

Quality of the electron density.

Stereo view of the electrondensity of the 2Fo-DFc maps for Exu^{333} (**a**) and Exu^{406} (**b**) structures after refinement. The structures are shown in similar views as in **Fig. 2d** and **Fig. 3b**, respectively. Monomer A is colored red, monomer B gray, the electrondensity visualized as a teal mesh contoured at 1σ. Water molecules are represented as light blue spheres.

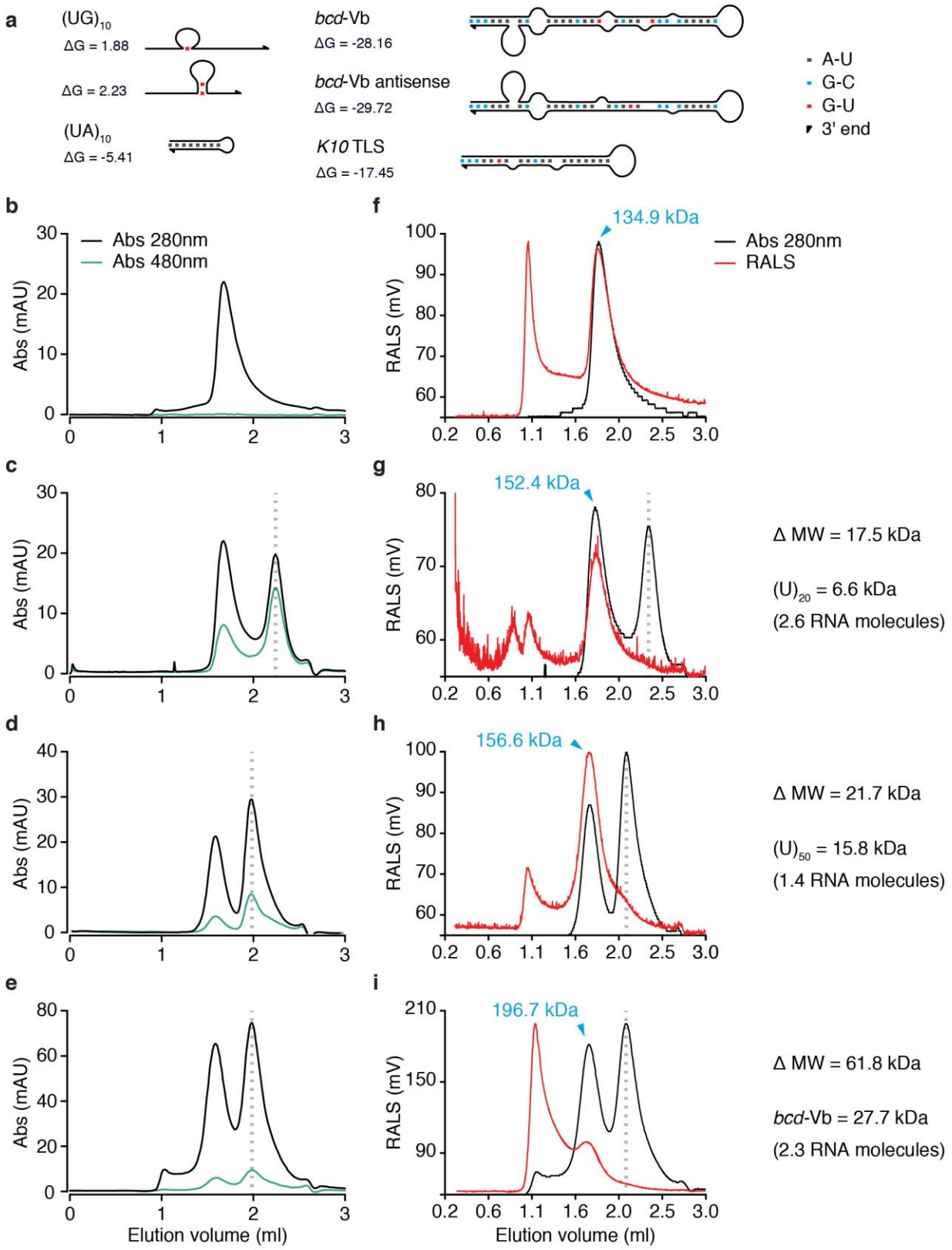


Supplementary Figure 5

Exu and Vts1p SAM domains use different surfaces for RNA binding.

a-b) Surface rendering of electrostatic charges of Exu SAM-like (**a**) and yeast Vts1p (**b**; PDB 2B6G, chain A) domain structure (see also **Supplementary Fig. 2**). **a**) Exu residues cross-linking with RNA (**Fig. 5**) are underlined. **b**) Vts1p residues shown to interact directly with the RNA (Aviv *et al.*, *NSMB* **13**, 168-176, 2006; Johnson and Donaldson, *NSMB* **13**, 177-178, 2006; Oberstrass *et al.*, *NSMB* **13**, 160-167, 2006) are indicated. Structural alignment was done with cealign in Pymol (v 1.7.6.0). Scale of electrostatic charge distribution is the same for all domains (-5 to +5 kT/e).

c-d, f-g) A constant amount of 5'-fluorescein labelled oligo was incubated with increasing concentrations of recombinantly purified Exu wt or mutant. The fluorescence anisotropy data were fitted to the Hill equation to obtain the dissociation constant (K_d); mean K_d and standard deviation from three independent experiments are reported in tables (**d, g**). **c, f**) Data from a representative fluorescence anisotropy measurement, with the best fit plotted as a solid line. **c, d**) FA measurements of SAM-like domain mutants with $(U)_{20}$. **f, g**) Affinity of Exu for oligo(U) RNAs of increasing length. **e**) Circular Dichroism (CD) spectra of Exu wt and the indicated mutants.

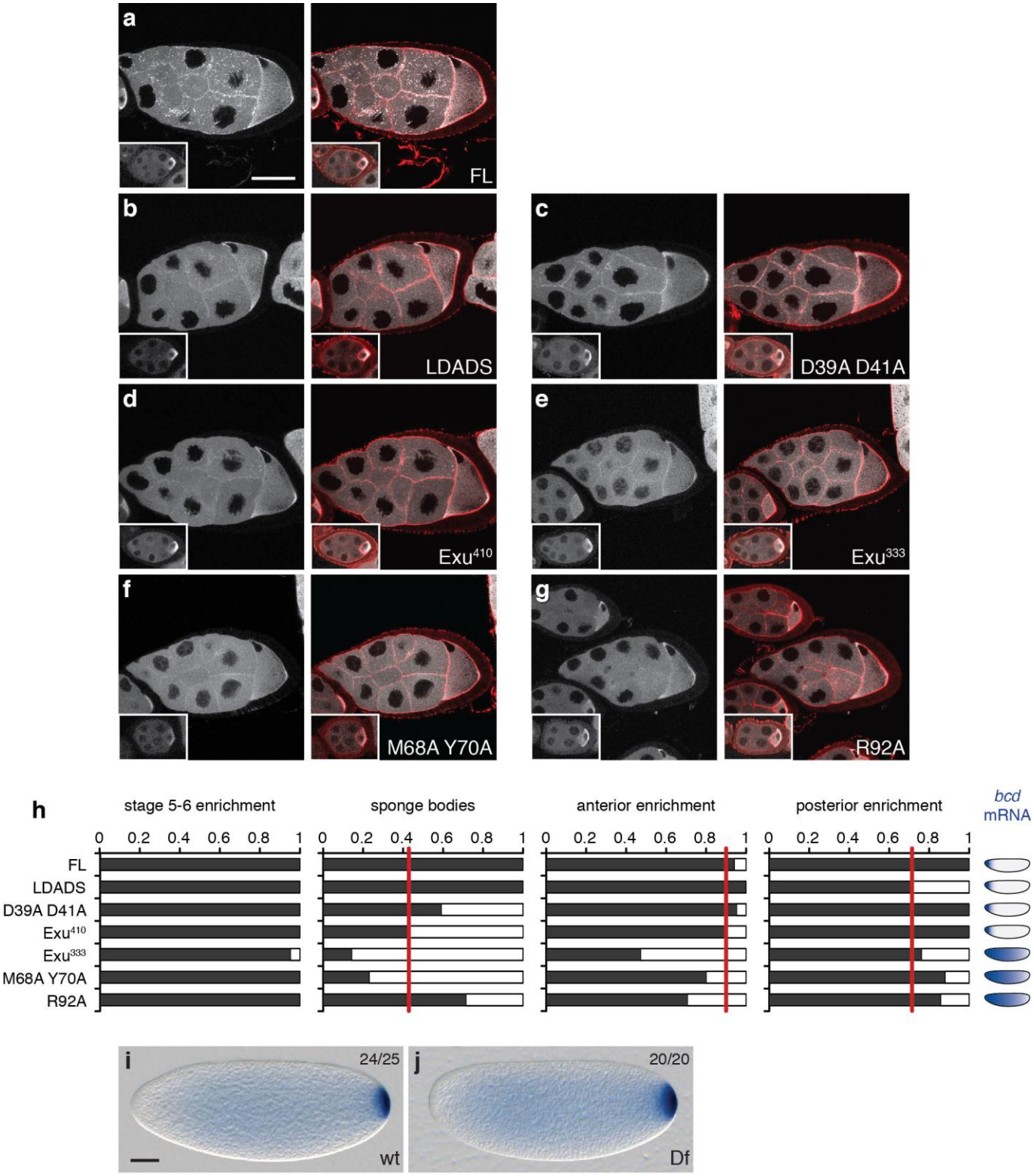


Supplementary Figure 6

Exu can bind two RNA molecules.

a) Oligonucleotides used in this study predicted to have a secondary structure, using the mFold server (Zuker, *Nucleic Acids Res.* **31**, 3406-3415, 2003). Numbers indicate the ΔG (in kcal/mol) at 22°C.

b-e) SEC profiles of purified Exu alone (**b**) or pre-incubated with fluorescein-conjugated $(U)_{20}$ (**c**), $(U)_{50}$ (**d**) or *bcd*-Vb (**e**). The elution volume of the free RNA is marked by a dotted line. **f-i**) Static light scattering profiles of the samples in (**b-e**). For each plot, the calculated molecular weight (MW) at the peak is indicated in blue; the difference in molecular weight (Δ MW) between the Exu-RNA complex and Exu alone is indicated on the right, together with the MW of the corresponding fluorescein-conjugated oligonucleotide. RALS = Right Angle Light Scattering.



Supplementary Figure 7

Localization of Venus-tagged Exu transgenes.

a-g) Fluorescence microscopy images of fixed *Drosophila* egg chambers at stage 5-6 (small box) or stage 9. The localization of Venus-

tagged Exu wt (**a**) and mutants (**b-g**) is shown in gray (left image); the merged images (right) show Venus-Exu in gray and Rhodamine-Phalloidin in red. **h**) For each genotype, at least 20 egg chambers were scored for the presence (black) or absence (white) of the following characteristics: enrichment of Venus-tagged Exu in the oocyte at early stages (1st panel from left); enrichment in sponge bodies in the nurse cells at stage 9 (2nd panel); enrichment at the anterior pole of the oocyte at stage 9 (3rd panel); enrichment at the posterior pole of the oocyte at stage 9 (4th panel). The rightmost column schematizes the localization of *bcd* mRNA in early embryos of the corresponding genotype (as in **Fig. 7**). Red lines mark the lowest percentage of egg chambers having the indicated characteristic amongst the Exu constructs which rescue *bcd* mRNA localization.

i-j) *oskar* (*osk*) *in situ* hybridization of *Drosophila* early embryos (0-2 h). Numbers at the top right corner indicate the number of embryos displaying the illustrated phenotype vs the total number of embryos examined. The genotype of each embryo is reported at the bottom of the image: **i**) wt; **j**) *Df(2R)exu1/exu*^{VL} (Df). *osk* localization is not impaired in embryos lacking Exu. Scale bars: 50 μm.