Supporting Material

Materials and Methods

Construction, expression and purification of hADAR2-D

The expression vector encoding hADAR2-D was constructed by PCR amplification from a hADAR2 cDNA template and ligated into the vector YEpTOP2PGAL1 as described previously (*S1*). This construct encodes an N-terminal 10-histidine tag, a TEV protease recognition sequence, and residues 299-701 of hADAR2. The protein was expressed in *S. cerevisiae* and purified using nickel chelating, heparin ion exchange, and gel filtration chromatography, as described for other N-terminal truncations of hADAR2 (*S1*). The purified protein was dialyzed into buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM 2-mercaptoethanol, and 5% glycerol) and concentrated to 10 mg/ml.

RNA preparation and deamination assays

Duplex RNA encoding sense and antisense sequences of the chloramphenicol acetyl transferase gene (CAT duplex) was internally labeled during *in vitro* transcription with α-^{32}P ATP, and gel purified as previously described (*S2*). The *in vitro* editing assay was performed by incubating purified protein and CAT duplex RNA under conditions described previously (*S1*), with concentrations described in the legend to Figure S1.

Crystallization and Data Collection

hADAR2-D was crystallized by vapor diffusion at 21°C by mixing 2 μl of protein solution with 2 μl of well solution (0.1 M Bis-Tris pH 6.8, 0.2 M $(NH_4)_2SO_4$, 24% PEG 3350, and 7.5% glycerol). After 10 days, crystals were mounted in nylon loops and

cryocooled by plunging into liquid nitrogen. All data were collected at 100 K using a Rigaku R-AXIS IV image plate detector mounted on a rotating anode source. Data were integrated and scaled using the programs DENZO and SCALEPACK (*S3*). The crystals belonged to space group $P2_12_12_1$ and contained two molecules in the asymmetric unit.

Multiple Isomorphous replacement, model building and refinement

Isomorphous heavy atom derivatives were prepared by soaking crystals at room temperature in well solutions containing either 0.5 mM thimerosal ($C_9H_9HgO_2SNa$) or 0.5 mM mercury acetate ($Hg[C_2H_3O_2]_2$) for 1 hour, or 20 mM sodium tungstate (Na_2WO_4) for 3 hours. Data were collected and processed as above, and scaled against the native data set. Heavy atom sites were located by inspection of Patterson functions. Most crystallographic computing used programs from the CCP4 suite (*S4*). Phases from the thimerosal and mercury acetate derivatives were calculated using SOLVE (*S5*), and used to locate two tungstate clusters (each containing the same arrangement of twelve tungsten atoms) in a difference Fourier map (Figure S2). MIR phases from the three derivatives were calculated using SOLVE (figure of merit 0.75) and generated an easily interpretable electron density map after density modification by solvent flattening (42% solvent) using the program RESOLVE (*S6*). An initial model was built by RESOLVE (*S6*), and manually completed using the XFIT program in the XTALVIEW software package (*S7*). The model was refined using REFMAC5 (*S8*), using refinement parameters for the IP_6 ligand that were generated using the SKETCHER program in CCP4 (*S4*). It is possible that some of the solvent molecules currently assigned as water are other species, such as sodium ions, although this is not apparent from the electron density or inspection

of coordination geometry. Figures were generated using the PyMOL molecular graphics system (*S9*) and Figure 3B was initially made using the program LIGPLOT (*S10*) then modified using CHEMDRAW (CambridgeSoft, Cambridge, MA).

Yeast Strains and Extract preparation

hADAR2 was expressed in the strain BCY123 as described previously (*S1*). The *IPK1* gene in this strain was replaced with the kanamycin resistance gene (*ipk1Δ::KanMX*) using a PCR-based gene targeting method (*S11*). The knockout was confirmed by the ability to grow in the presence of the antibiotic G418, and PCR of the genomic DNA. The new strain (BCY123-*ipk1*Δ) was also used to express hADAR2 as described above. Cells were harvested by centrifugation, resuspended in buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 20% glycerol and 1 mM 2-mercaptoethanol) and lysed by three consecutive passes through a French pressure cell at 20,000 psi. The lysate was centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant (S100 extract) was stored in aliquots at -80°C until use in the editing assays.

For the ADAT1 activity assays, the yeast strain BY4743 (wild-type; *S12*) and strains with the same genetic background but having the *IPK1* gene or the *KCS1* gene replaced with *KanMX* (*ipk1Δ::KanMX, kcs1Δ::KanMX)*, were obtained from Research Genetics (Huntsville, AL). The strains were grown in YPD, harvested by centrifugation, and S100 extract was prepared as described above.

RNA preparation

For the in vitro editing assays, RNA substrates were prepared using the splint ligation method of Moore and Sharp (*S13*). The R/G 27-mer RNA used for the hADAR2 editing assays was prepared as described (*S14*). The tRNAs were chemically synthesized in two halves. For labeling A34, the two RNAs spanned residues 1-33 (the 5' half) and 34-76 (the 3' half); for labeling A37, the two RNAs spanned residues 1-36 (the 5' half) and 37-76 (the 3' half). The 3' halves were phosphorylated (at either A34 or A37) with ^{32}P using polynucleotide kinase, and annealed, along with their respective 5' partner, to a DNA "splint" of complementary sequence to the 76-nucleotide tRNA^{ala}. The RNAs were then ligated using T4 DNA ligase, the DNA splint was digested with RQ1 DNase (Promega, Madison, WI), and the RNA purified on a 12% denaturing polyacrylamide gel.

To assay editing in vivo, total RNA was prepared from the S100 extract by extraction with Trizol^{TM} reagent (Invitrogen) as per the manufacturers instructions. The RNA was precipitated with isopropanol and treated with the restriction enzyme Cac8I (New England BioLabs, Ipswich, MA), followed by RQ1 DNase (Promega) to digest any remaining DNA. The RNA was extracted with phenol, precipitated with ethanol, and quantified. tRNA^{ala} was amplified by RT-PCR, using primers specific for the tRNA^{ala} sequence and the PCR product was sequenced (see below).

RT-PCR was used to measure amounts of the ADAT1 and hADAR2 mRNAs expressed in the *ipk1*Δ strains relative to those messages produced in the wild-type strain. Total RNA was prepared as described above, except after RQ1 DNase treatment, the restriction digest step was omitted and the RNA was further purified using an RNeasy^{TM} kit (Qiagen, Valencia, CA) according to the manufacturers instructions. The RNA was

reverse transcribed with Superscript^{TM} II reverse transcriptase (Invitrogen, Carlsbad, CA) using an oligo-dT primer. A region of the cDNA (947-1175 of the ADAT1 gene, 2050-2324 of hADAR2 gene) was amplified by quantitative real-time PCR using a Roche Light Cycler with SYBR-Green fluorescent NTPs and gene specific primers. RT-PCR product amounts were normalized to that of a housekeeping gene (GAPDH) for quantitation.

Editing assays

hADAR2 concentrations in extracts prepared from BCY123 or BCY-*ipk1*Δ were quantified by western blotting. The blot was probed with a penta-His primary antibody (Qiagen) and alkaline phosphatase conjugated anti-mouse secondary antibody (Sigma-Aldrich, St. Louis, MO). Protein bands were detected using a fluorescent alkaline phosphatase ECF substrate (Amersham, Piscataway, NJ). The blot was scanned on a PhosphorImager, and the hADAR2 bands were quantified after generating a standard concentration curve of known amounts of a purified protein (the histidine tagged R_2D truncation of hADAR2, *S1*). Editing of the R/G site RNA by hADAR2 in vitro was performed as previously described (*S1*). Briefly, 5 nM RNA was reacted with increasing concentrations of hADAR2 present in yeast extracts (final concentrations of hADAR2 are indicated in Fig. 4A) for 1 hour at 30°C.

Yeast extracts for editing tRNA in vitro were prepared as described above and quantified by Bradford assay. 5 nM of A34 or A37 labeled $tRNA^{ala}$ was reacted with 10^{-3}, 10^{-2}, 0.1, 2.9, 5.8 μg/μl total protein for 1 hour at 30°C. IP_6 or IS_6 (concentrations noted in the legend to Fig. 4A; Sigma-Aldrich) were incubated with 0.1 μg/μl extract for

15 min at 30°C before adding 5 nM A37 labeled $tRNA^{ala}$. Reactions were completed as described above.

To observe editing in vivo, total RNA was prepared from the yeast S100 extracts (see above) and the tRNA was amplified by RT-PCR using gene specific primers. The PCR product was sequenced using a ^{32}P-labeled primer that hybridized to the non-template strand, and the Sequenase™ PCR product sequencing kit (USB, Cleveland, OH.) according to the manufacturers instructions. The sequencing products were separated on an 8% denaturing polyacrylamide gel. The gel was dried and exposed to a PhosphorImager plate overnight.

Supporting Figures

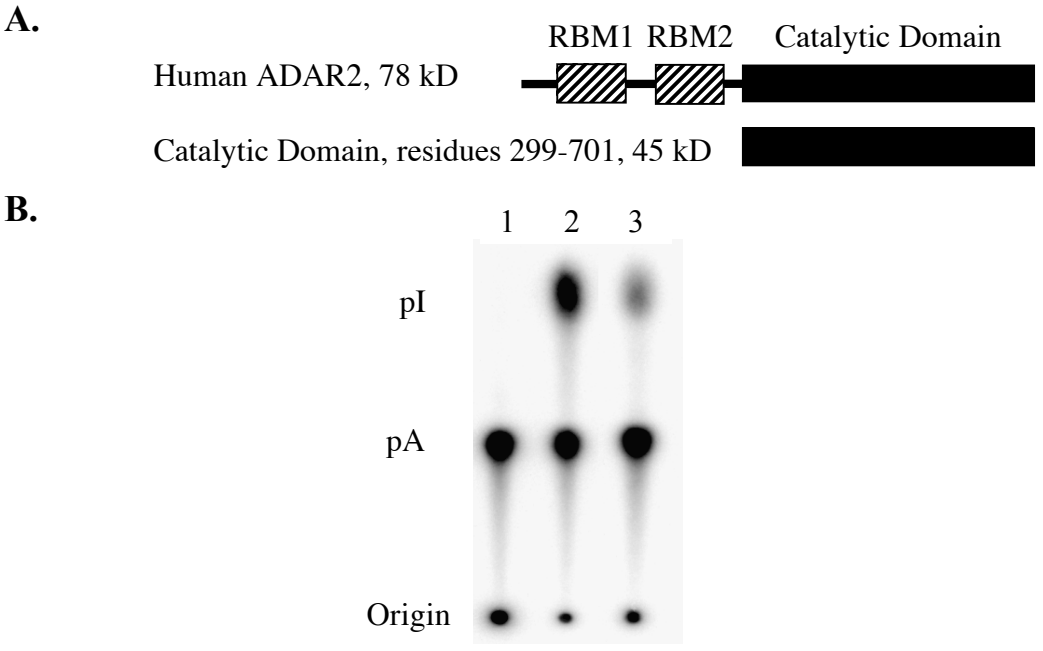


Figure S1. (A) Domain structure of hADAR2 and the catalytic domain construct used in this study. Striped rectangles, dsRBMs; filled rectangle, catalytic domain. **(B)** *In vitro* deamination assay. 10 nM ^{32}P-A-labeled dsRNA was incubated with 100 nM of full-

length hADAR2 or hADAR2-D for 1 hour. Reacted dsRNA was treated with P1 nuclease, the resulting 5'-NMPs separated by thin-layer chromatography, and the plate exposed to a phosphorimage screen. Lane 1, no protein control; Lane 2, hADAR2 treated dsRNA; Lane 3, hADAR2-D treated RNA. Origin, 5'AMP and 5'IMP spots are indicated.

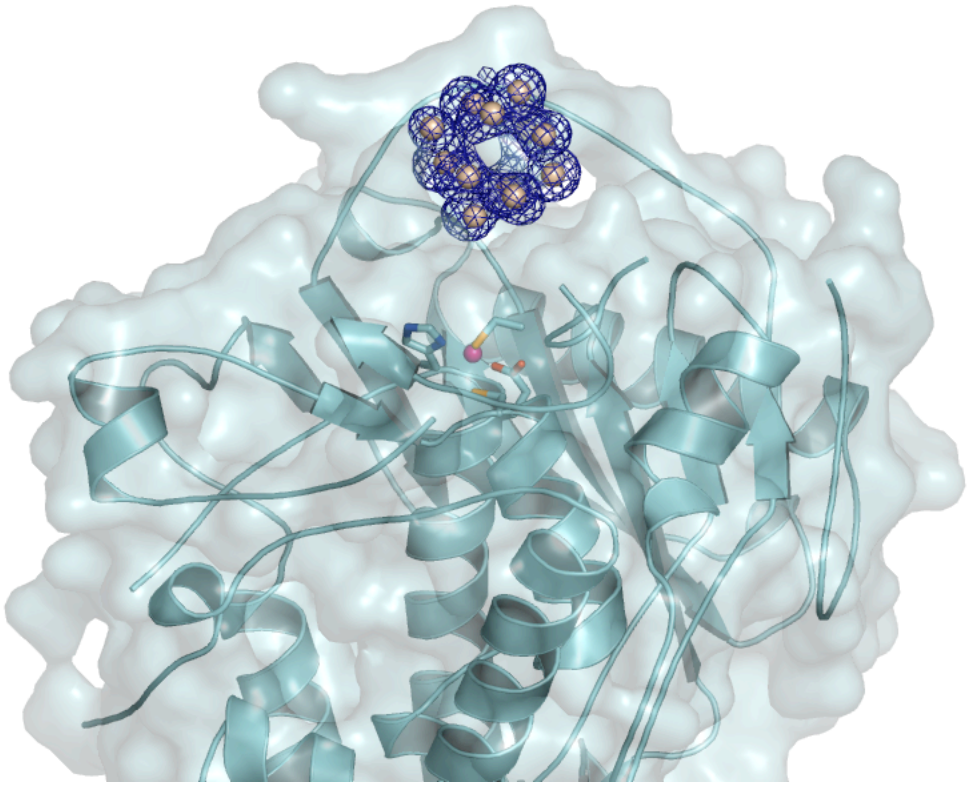


Figure S2. The electron density of a tungstate cluster containing 12 tungsten atoms observed in the heavy atom derivative (the tungsten atoms are modeled as grey spheres). The cluster is bound to a positively charged region on the surface near the active site of hADAR2-D (8 Å from the zinc, pink sphere). The density was calculated using ($F_{tungstate}$-F_{native}) with protein phases computed from the thimerosal derivative, and is contoured at 4σ.

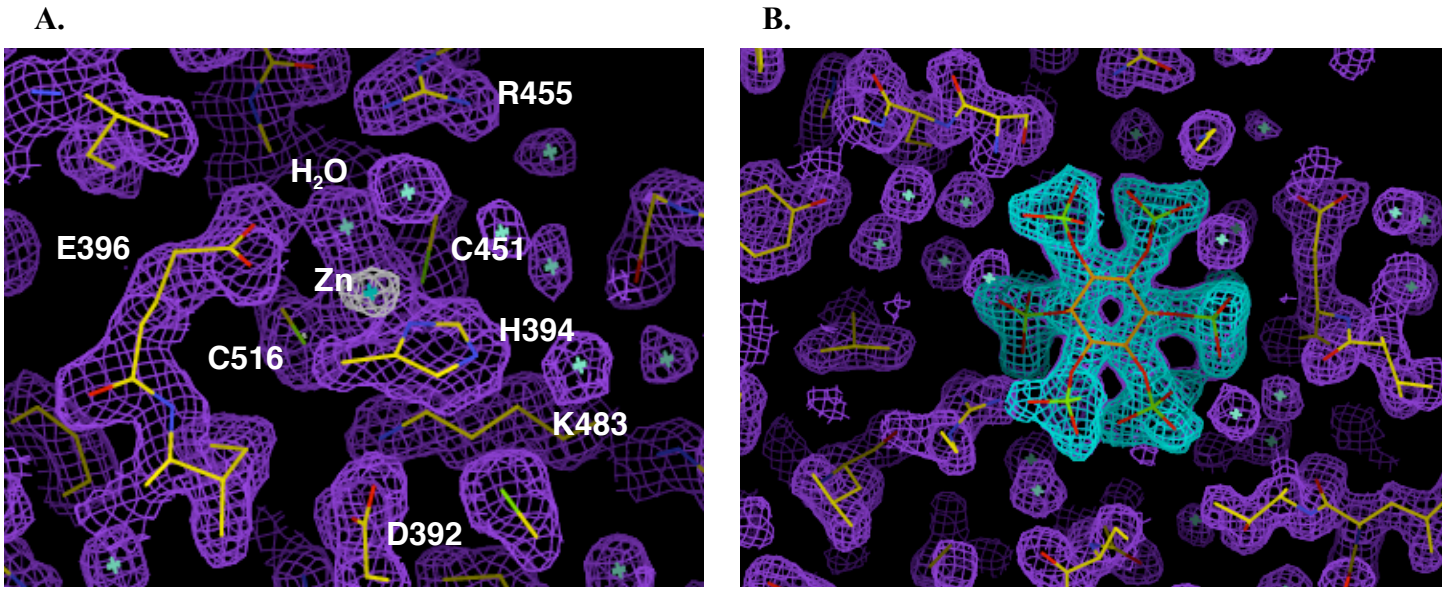


Figure S3. (A) $2F_{obs}$-F_{calc} electron density map (contoured at 1σ, purple or 9σ, white) of the region surrounding the active site Zn. **(B)** $2F_{obs}$-F_{calc} electron density map (purple) contoured at 1σ and F_{obs}-F_{calc} electron density map contoured at 3σ (cyan) calculated prior to inclusion of IP_6 in the model.

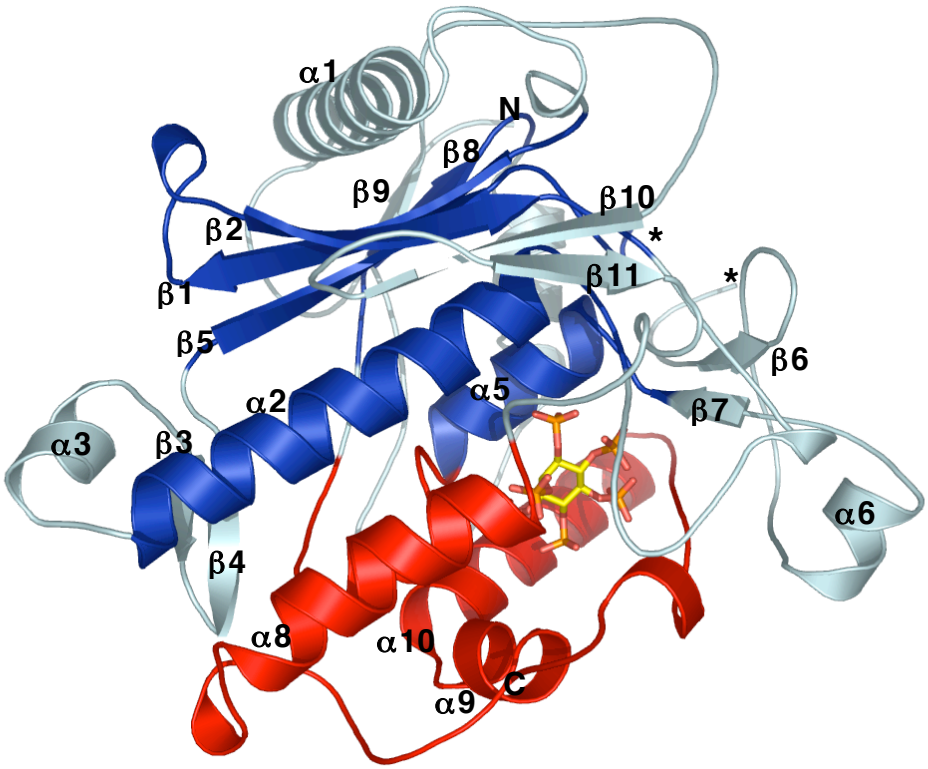


Figure S4. Another view of the ribbon model of hADAR2-D. The protein is rotated 90° about the vertical axis from Fig. 1B of the main text. IP_6 is shown as sticks. The active site zinc atom is obscured by β11. The color scheme is the same as Figure 1B of the main text. Ends of the disordered segment (residues 462-473) are indicated with asterisks (the beginning of the disordered region is obcured by β10).

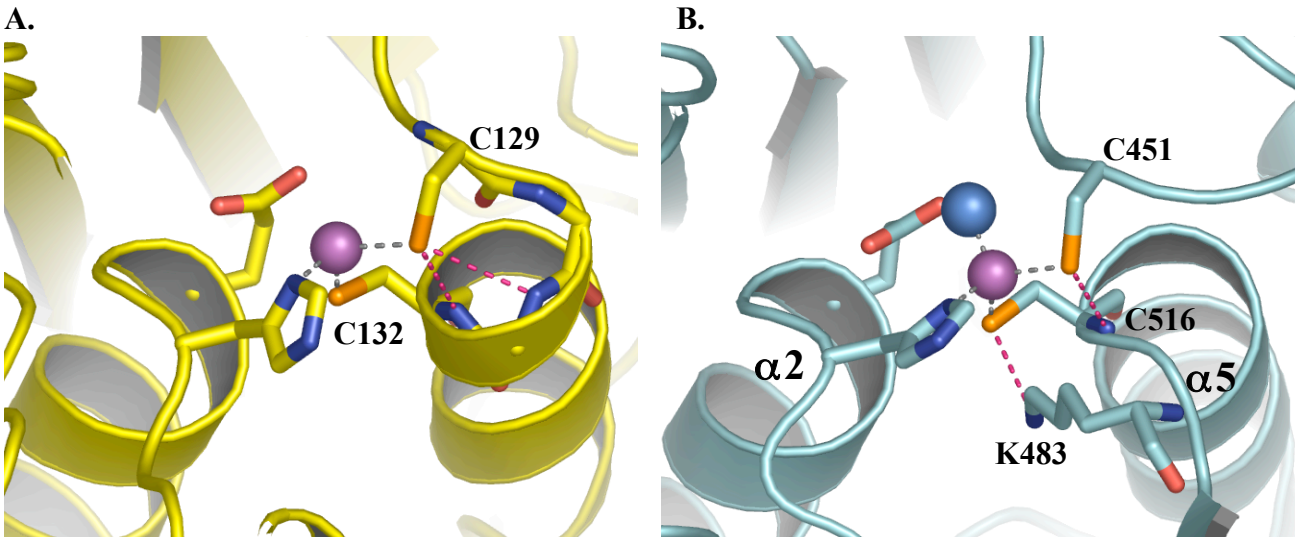


Figure S5. **(A)** The zinc coordinating environment of cytidine deaminase (*S15*). Zinc ion (magenta sphere), nucleophilic water (blue sphere), zinc coordinating bonds (grey dashed lines), hydrogen bonds (pink dashed lines). The thiolate of C129 forms hydrogen bonds with the backbone NH groups of residues H131 and C132 located at the N-terminus of the helix. These interactions are expected to reduce the negative character of the C129 thiolate, and thus increase the positive potential of the zinc ion. This may modulate catalysis by lowering the pKa of the coordinated water molecule that attacks the substrate C4 center of cytidine (*S16*). C132 is not within hydrogen bonding distance to any other residues. (**B)** The zinc-coordinating environment of hADAR2. A 64-residue loop separates C451 from C516, and the N-terminus of helix α5 is therefore less extended than in cytidine deaminase. The thiolate of C451 only forms one hydrogen bond with an amide NH (of C516). A consequent increase in water molecule pKa may be offset by a hydrogen-bonding interaction seen between the thiolate of C516 and the side chain of K483. The equivalent C132 of cytidine deaminase does not participate in hydrogen bonding interactions, whereas K483 is invariant in ADARs.

Table S1. Crystallographic Data

	Native	Thimerosal	Mercury Acetate	Sodium Tungstate
a (Å)	52.2	52.3	52.6	52.1
b (Å)	121.4	121.7	121.8	120.8
c (Å)	127.5	127.6	127.8	127.4
Resolution (Å)	20-1.7 (1.76-1.7)	20-2.7 (2.8-2.7)	20-2.7 (2.8-2.7)	20-2.6 (2.69-2.6)
# total reflections	388,243	228,747	54,141	120,441
# unique reflections	82,849	22,696	20,785	25,089
Completeness (%)	92.3 (62.5)	100 (100)	89.7 (87.9)	99.5 (99.8)
Mosaicity (˚)	0.5	0.6	0.7	0.7
I/σ(I)	35.2 (5.0)	21.2 (9.3)	10.9 (4.6)	8.7 (2.9)
R_{merge} [a]	3.9 (25.9)	13.4 (26.9)	9.2 (19.4)	11.2 (26.8)

Numbers in parenthesis are for the high-resolution bin

[a] $R_{merge} = 100x\Sigma|I-<I>|/\Sigma I$

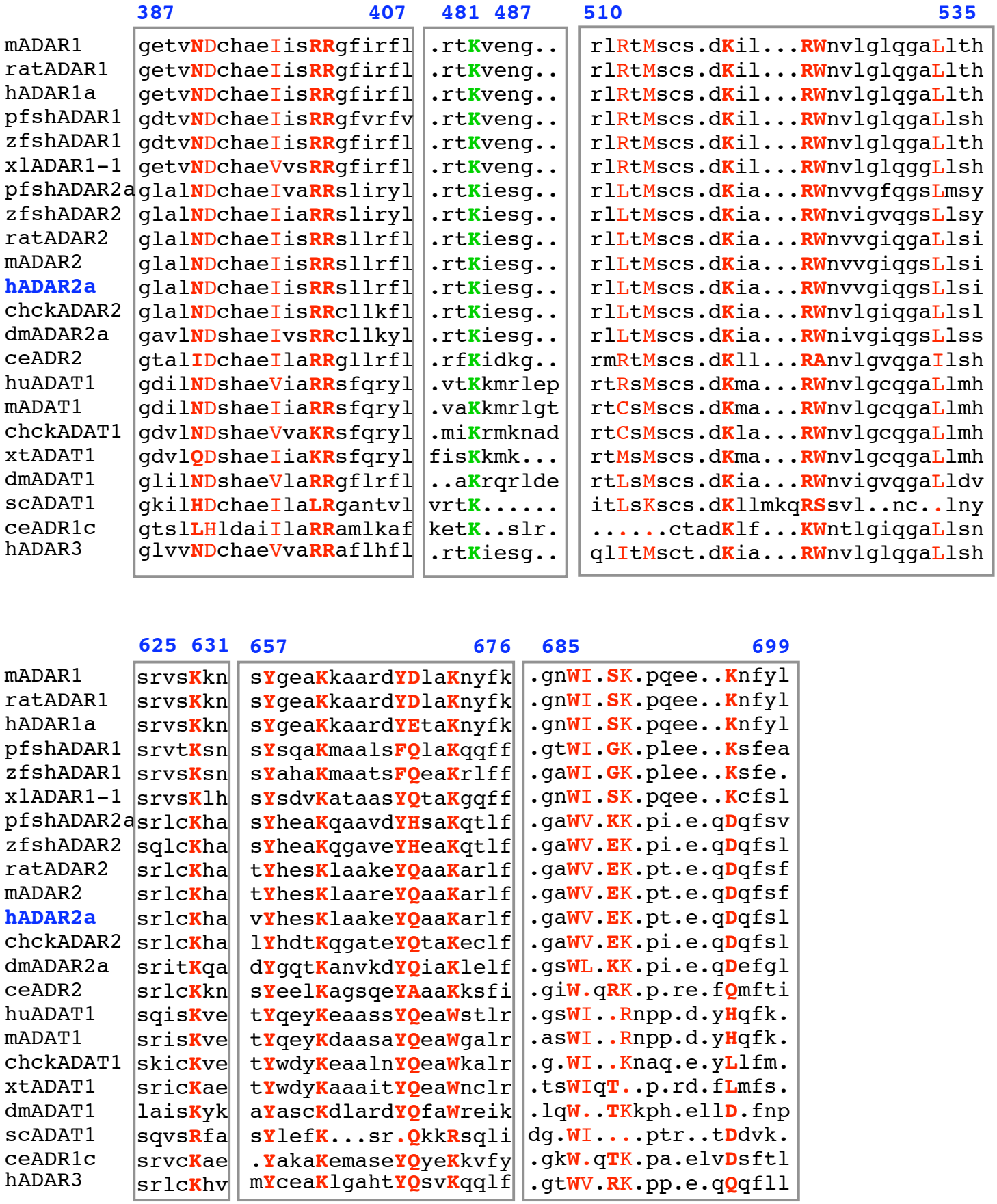
Table S2. Refinement statistics

R_{cryst} (%) [a]	17.4
R_{free} (%) [b]	20.7
# Protein residues modeled	741
# Solvent molecules	670
RMSD bonds (Å)	0.015
RMSD angles (˚)	1.554
ϕ, ψ Most favored (%)	92.7
ϕ, ψ Additional Allowed (%)	6.4
<B> Overall (Å^2)	19.9
<B> Main Chain (Å^2)	17.8
<B> Side Chains (Å^2)	20.2
<B> Water (Å^2)	28.0
<B> IP_6 (Å^2)	14.6

[a] $R_{cryst} = 100x\Sigma||F_{obs}|-k|F_{calc}||/\Sigma|F_{obs}|$

[b] R_{free} = The R_{factor} against 5% of the data removed prior to refinement

Stereochemistry was analyzed using PROCHECK (*S17*)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dmADAT1 glilNDshaeVlaRRgflrfl ..aKrqrlde rtLsMscs.dKia...RWnvigvqgaLldv
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ceADR1c gtslLHldaiIlaRRamlkaf ketK..slr. .....ctadKlf...KWntlgiqgaLlsn
hADAR3 glvvNDchaeVvaRRaflhfl .rtKiesg.. qlItMsct.dKia...RWnvlglqgaLlsh
625 631 657 676 685 699
mADAR1 srvsKkn sYgeaKkaardYDlaKnyfk .gnWI.SK.pqee..Knfyl
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hADAR3 srlcKhv mYceaKlgahtYQsvKqqlf .gtWV.RK.pp.e.qQqfll

Figure S6. Alignments for regions surrounding the residues that coordinate IP_6 in hADAR2 are shown, with hADAR2 numbering indicated in blue. IP_6 coordinating residues for backbone interactions (red capitals) and side chain interactions (bold, red capitals) are indicated. The alignment was prepared with the entire catalytic domain as input, using GCG software (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA). Pileup, using default parameters, was used to align the following sequences (accession number; amino acids aligned, start-end): ADAR1, human (NM_001111; 833-1226), mouse (AF052506; 756-1152), rat (U18942; 779-1175), Xenopus laevis (U88065; 879-1270), pufferfish (AAF69764; 790-1194), zebrafish (NM_131596; 952-1382); ADAR2, human (NM_001112; 317-701), rat (NP_037026; 317-711), mouse (AF403106; 317-701), chicken (AF403119; 318-701), pufferfish (AF533143; 309-694), zebrafish (AF403113; 305-689), *D. melanogaster* (AF208535; 252-632), *C. elegans* (AF051275; 113-495); the entire open-reading frame for ADAT1, human (AF125188), mouse (NM_013925), chicken (NM_001012779), *Xenopus tropicalis* (CR762003), *D. melanogaster* (AF192530). This multiple sequence alignment was used as input to the program HmmerBuild to create a profile hidden Markov model (HMM) of the consensus (global alignment setting). The HMM file was then used with the program HmmerAlign to add the following to the alignment *S. cerevisiae* ADAT1, (AJ007297; 11-401), *C. elegans adr-1c* (AY150815; 617-964) and human ADAR3 (AF034837; 355-739). The alignment was optimized by selective manual manipulation using the program Seaview (*S18*). Manual manipulation was guided by alignments of the subgroups alone. Sequences diverge considerably in the region surrounding K483; the alignment shown was chosen because the conserved lysine of various subfamilies is aligned with K483 of hADAR2.

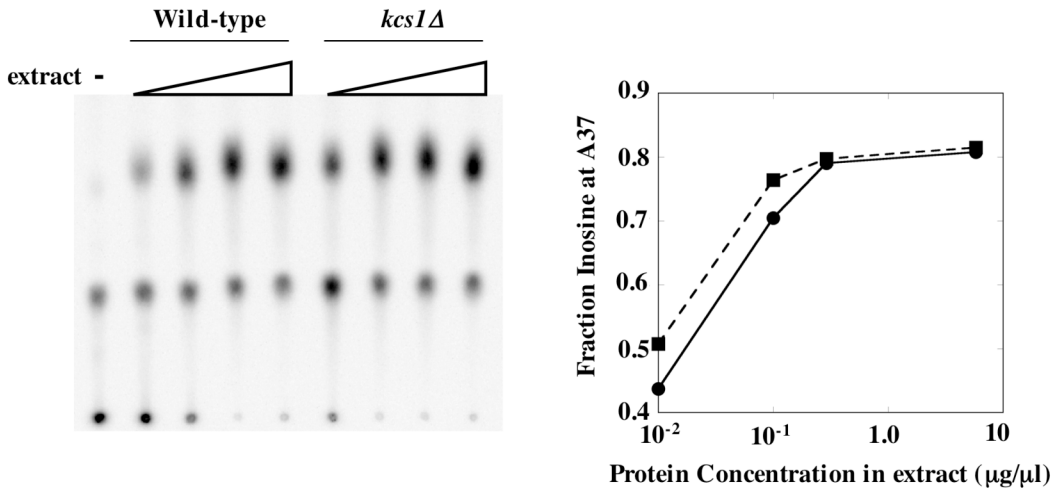

Figure S7. To rule out an ADAT1 requirement for IP_7, which is downstream of IP_6 in the inositol polyphosphate synthesis pathway, we assayed editing of A37 of $tRNA^{ala}$ by ADAT1 in extracts prepared from yeast unable to produce IP_7. The *KCS1* gene product phosphorylates IP_6 to form IP_7. ADAT1 from a *kcs1*Δ strain edits A37 with equal efficiency as ADAT1 from a wild-type strain, suggesting, along with the data from the *ipk1Δ* strain, that IP_6 is the required factor for editing activity. Left panel, TLC assay of tRNAala-A37 editing by ADAT1 in wild-type extracts or *kcs1*Δ extracts. Right panel, quantitation of inosine product as a function of wild-type or *kcs1*Δ extract concentration. Dashed line, ADAT1 activity in *kcs1*Δ extract; solid line, activity in wild-type extract.

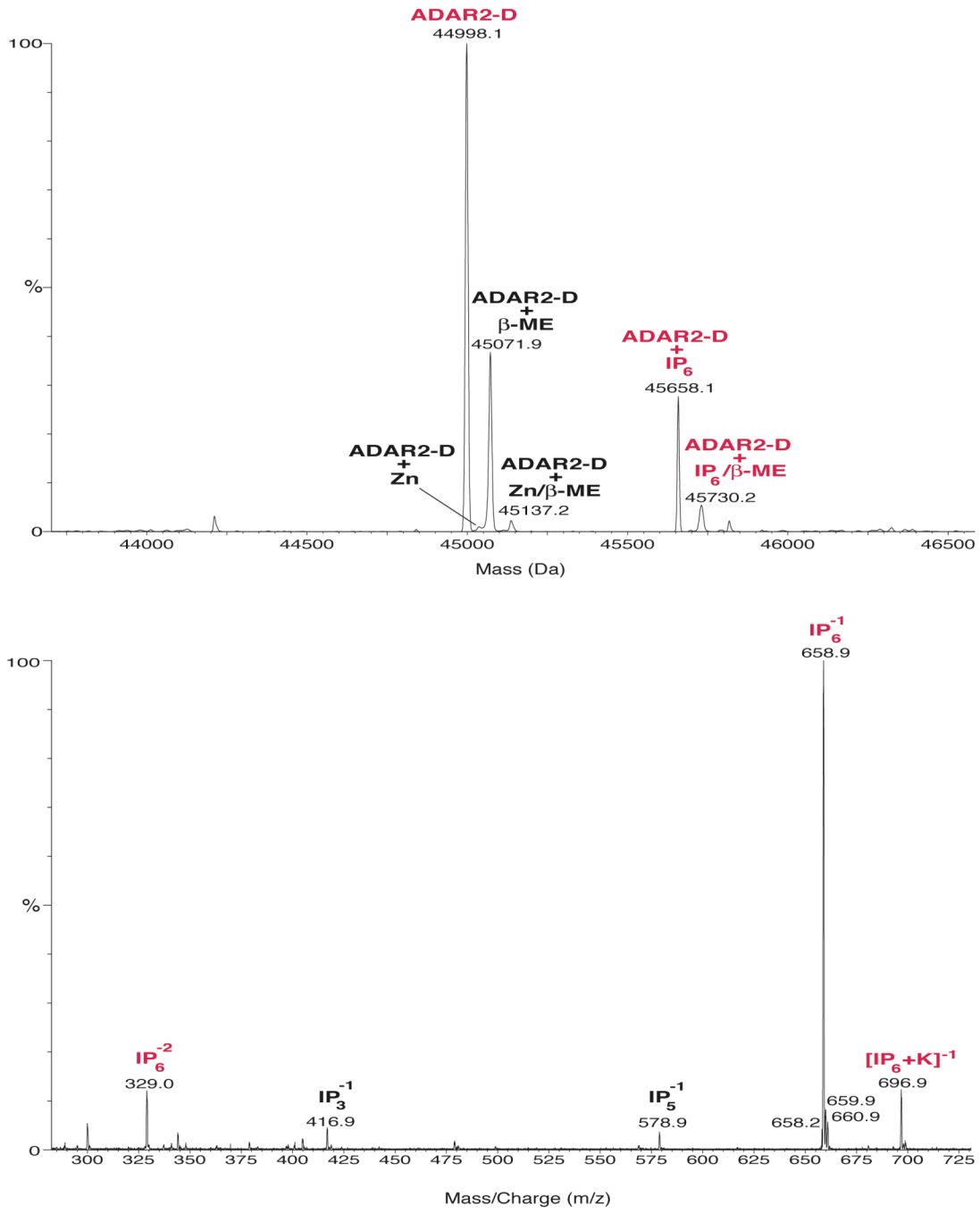

Figure S8. Electrospray mass spectrometry analysis of ADAR2-D and IP_6. Upper panel, molecular mass spectrum of native ADAR2-D. The sample was purified as described above, except Triton X-100 was not included in the lysis buffer. After purification, the protein was dialyzed against water and any remaining buffer was exchanged with water

using a Micron-10 centrifugal concentrator (Millipore, Billerica, MA). 5% acetonitrile (final concentration) was added to the sample before (+) ion electrospray data were collected in the 600-1400 m/z mass range. The spray voltage was 2.8 kV and cone voltage was 40 eV. The data were processed into a "molecular mass spectrum" using MAXENT software (Micromass, Beverly, MA). The ADAR2-D (44998.1 Da, observed; 44997.1 Da, calculated) peak and ADAR2-D peaks containing IP_6 (660 Da MW, i.e. neutral molecule) are labeled red. The 45071.9 Da and 45730.2 Da peaks are likely an oxidized cysteine/β-mecaptoethanol (β-ME) derivative (in the crystal structure, the thiol group of C634 exists in 2 conformations on the protein surface, and appears to be oxidized). In addition, the 45137.2 peak may contain zinc (65.4 Da). Lower panel, mass spectrum of inositol hexakisphosphate (dipotassium salt) purchased from Sigma (St. Louis, MO). The compound was dissolved in water (final concentration 10 mM) diluted with acetonitrile and triethylamine (final concentrations 50% and 3%, respectively) and analyzed by electrospray in (-) ion mode. The peaks corresponding to the -1 and -2 charge states as well as a potassium salt of IP_6 are labeled red. IP_3 and IP_5 contaminants/hydrolysis products are also labeled.

Supporting References

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