Supplementary Information for

Pin1 and WWP2 regulate *GluR2* Q/R site RNA editing by ADAR2 with opposing effects.

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Materials and Methods

Immunoblot analysis, immunoprecipitation and GST pulldown.

For protein extraction, cells were resuspended in lysis buffer (150 mM NaCl, Tris-HCl 50mM pH 7.5, 1 % NP-40, 1mM EGTA, and 10% glycerol, supplemented with the protease and phosphatase inhibitor cocktail, Complete EDTA-free, PhosSTOP, (Roche)). The insoluble material from the extracts was removed by centrifugation at 12,000 g at 4˚C for 20 min, and the supernatants were used for both immunoblot analysis and immunoprecipitation experiments. After protein quantification with Bradford Protein Assay (Bio-Rad), cell extracts or immunoprecipitated proteins were diluted in SDS sample buffer and boiled for 5 min. Proteins were electrophoresed on 4-12 % SDS-PAGE pre-cast gels (Invitrogen) and transferred onto nitrocellulose for immunoblot analysis. For immunoprecipitation, cell lysates were incubated rotating for 2h at 4˚C with 20µl 50% slurry of M2 anti-FLAG protein agarose beads (Sigma). The GST and GST-Pin1 recombinant protein were bound to Glutathione Sepharose^{TM} 4B beads (GE Health Care) as follows: 10µg of recombinant protein was incubated with 20µl of a 50% slurry of glutathione-agarose beads in 250µl of lysis buffer. Binding was performed at 4˚C for 2h in the presence of 10µg of each of the recombinant hADAR2 mutants. After 5 washes with lysis buffer (200 mM NaCl, Tris-HCl 50mM pH 7.5, 1 % NP-40 and 1mM EGTA, supplemented with protease and phosphatase inhibitor cocktail, Complete EDTA-free, PhosSTOP, (Roche)), immune complexes were electrophoresised on 4-12 % SDS-PAGE pre-cast gels and then analyzed by immunoblot. Membranes were incubated with the indicated primary antibodies: mouse α-FLAG 1:3000 (Sigma), mouse α-HA 1:1000 (Sigma), mouse α-MPM2 1:1000 (Upstate Cell Signaling) (Davis et al., 1983) mouse α-Pin1 1:500 (G-8) Santa Cruz Biotechnology, Santa Cruz, CA) rabbit α-ADAR2 1:1000 (Sigma), mouse α-GST 1:5000 dilution (Amersham Pharmacia), overnight at 4˚C, followed by an 1h incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako). Membranes were then washed and visualized with an enhanced chemiluminescence detection system (HRP ECL kit, Amersham Pharmacia Biotech or PIERCE).

λ phosphatase treatment.

For λ phosphatase treatment, phosphatase inhibitors were omitted, λ phosphatase (400 U ml^{-1}) was added to cell extracts and the reaction was performed for 2h and 2h 30min at 30°C, according to the manufacturer's instructions (New England Biolabs) before immunoprecipitation.

Cycloheximide treatment.

The protein synthesis inhibitor cycloheximide (Fluka) was added to the cells at a final concentration of 60μg/μl for the indicated time, followed by immunoblot analysis with rabbit α-ADAR2 1:1000, mouse α-tubulin 1:10000, mouse α-Pin1 1:500, mouse α-FLAG 1:3000, mouse α-GFP 1:5000.

Cell lines, transfection conditions and RNA extraction.

Human cell lines used in this work were cultured in Dulbecco's modified Eagle's medium with glutamax (Invitrogen) supplemented with 10% fetal bovine serum. MEF cells were cultured in a Hypoxic incubator, 10% CO_2, 3% O_2 (Thermo Scientific HeraCell 150i) (Parrinello et al., 2003). The DNA used for transfections was purified with a Quiagen kit following the manufacturers' instructions. Cells were transfected with 1μg of expression constructs with Lipofectamine 2000 reagent (Invitrogen) and for MEF cells with Fugene 6 transfection reagent (Roche) according to the manufacturers' instructions. The final concentration of *GluR-2 B13* minigene plasmid in all experiments was 150ng. Cells were harvested 24h after transient transfection. Total RNA was extracted with Trizol reagent (Invitrogen). RNA was resuspended in RNase-free water and treated with Turbo DNA-free DNAseI beads (Ambion) according to manufacturer's instructions to avoid contamination from the untransfected minigene. cDNA synthesis was performed with First Strand cDNA kit (Invitrogen) and random-hexamer primers together with 1μg of DNAse-treated total RNA. RT–PCR was performed with primer, 5'-atggaagagaaacacaaagt-3' that anneals to exon 11 and antisense primer 5'-gaatgataggaaccttctgc-3' that anneals to intron 11 of the *GluR-2 B13* minigene (Higuchi et al., 1993). The conditions for PCRs were the following: 94 °C for 3 min for the initial denaturation, followed by 28 cycles of: 94°C for 30s, 54°C for 30s, 72°C for 45s and 72°C for 7 min for the final extension.

For experiments performed with the endogenous *GluR2* transcript, 1µg of DNAse treated total RNA was used for cDNA synthesis and RT-PCR was performed with Superscript^{TM}III One step RT-PCR System (Invitrogen) according to the manufacturer's instructions. RT–PCRs were performed with primer, 5'-atggaagagaaacacaaagt-3' and the antisense primer 5'-ttccctttggacttccgcac-3' that anneals to exon 13.

PCR products were electrophoresised on a 1.3% agarose gel and visualized by staining with ethidium bromide.

Specific RT-PCR products were gel purified (Qiaquick, Qiagen), subcloned into pGEM-TEASY vector (Promega) and sequenced. Sequences of individual clones were analysed with Seqman software (DNASTAR) and the level of A to G editing was calculated as following: % editing= (number of edited transcripts / number of sequenced transcripts) X 100.

The level of A to G editing of an RT-PCR product was calculated by measuring nucleotide peaks at the edited site as following: % editing = (height of G peak/ height of A peak + length of G peak) X 100. The measurement given is the average value of the editing level from 3 independent RT-PCR reactions.

RNAi knockdown.

siRNA or pSUPER transfections were performed in HeLa and SH-SY5S cells with Lipofectamine 2000 reagent (Invitrogen). RNAi control experiments were performed with siRNA against *GAPDH* (Dharmacon Thermo Scientific) to a final concentration of 100nM. For RNAi experiments the following smart Pool of siRNAs against human *Pin1* (LPIN1, Dharmacon Thermo Scientific) were added to the cells to a final concentration of 100nM.

Target sequence: GUGCAUUUGUACCGUGAAA

Target sequence: GAACUUUGGCUGCGAGAAU

Target sequence: CAAAUUAAGGGCAAGCGAA

Target sequence: ACAGCAAACAAGACGGAUU

The pSUPER*Pin1* and pSUPER*LacZ* were used as siRNA controls (Rustighi et al., 2009). Cells were maintained in 1000µl of Opti-MEM without antibiotics and FCS. After 8h 5% of FCS was added to the cells. After 24h, a second siRNA transfection was

performed as described above, followed by the transfection with 150ng of *GluR-2 B13* minigene. The following day, cells were harvested for RNA extractions, RT-PCR analyses and immunoblots.

Indirect Immunofluorescence.

Cells were plated on sterile cover-slips in 6-well plates at 2.5 x 10^5 cells/well and grown overnight before transient transfection of expression constructs as well as *GluR2 B13* minigene with Fugene 6 transfection reagent (Roche) used according to the manufacturers' instructions. At 24h post-transfection, cells were fixed for 15 min in 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100, 20mM HEPES, 300mM Sucrose, 50mM NaCl, 3mM $MgCl_2$ for 5 min at 4°C. After 4 washes in PBS+0.2% BSA, cells were blocked for 30 min at room temperature with PBS+0.05% Tween 20 and 5% Donkey or Goat fetal serum depending on the secondary antibody used. Primary antibodies: rabbit or mouse anti-HA antibodies (Sigma) and mouse anti-FLAG M2 (Sigma), were both diluted 1:500 in PBS+BSA 0.2% + 5% Donkey or Goat fetal serum and were incubated for 1h at 37°C. After an additional 4 washes in PBS+0.2% BSA secondary antibodies: Alexa Fluor 488, Alexa Fluor594, Donkey anti-mouse IgG and Alexa Fluor 488, Alexa Fluor594, Goat anti-mouse IgG or Goat anti-rabbit IgG (Invitrogen) were diluted at 1:2000 and added for 1h at room temperature.

Nuclei were stained with 250 ng/ml of 4',6-diamidino-2-phenylindole (DAPI) and mounted in VectaShield reagent (Vector Laboratories California, USA). Images were recorded digitally on a Zeiss Axiovert 200 inverted microscope equipped with a CCD camera (Photometrics Ltd, Tucson, AZ). Images were captured using a neofluar objective at 100X and 63X (with a numerical aperture of 1.25). Color additive filters (Andover Corporation, Salem, NH) installed in a motorized filter wheel (Ludl Electronic Products, Hawthorne, NY) were used sequentially to collect blue, green and red images, which were then superimposed to form a color image. Image capture and analysis were performed using in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA). The brightness and contrast were altered using the advanced histogram section in either IP Lab Spectrum. This was done by manually setting the minimum pixel intensities on the histogram. If necessary the gamma was altered on the histogram.

Preparation of Cytoplasmic and Nuclear Extracts.

Cells were grown in 100mm plates and expression constructs were transfected with Fugene 6 according to the manufacturer's instructions. At 24h post-transfection, cells were washed twice with PBS and resuspended for 10 min at 4°C in the lysis buffer (50mM NaCl, 10mM HEPES, 500mM Sucrose, 1mM EDTA, 0.5mM Spermidine, 0.15mM Spermine, 0.2 TritonX-100 supplemented with protease inhibitor cocktail). Cells were centrifuged at 3,300*g* for 20min at 4°C and the supernatant collected which contains the cytoplasmic proteins.

Nuclei were resuspended and lysed in hypotonic lysis buffer for 30 min at 4°C (350mM NaCl, 10mM HEPES, 25%Glycerol, 0.1mM EDTA, 0.5mM Spermidine, 0.15mM Spermine supplemented with protease inhibitor cocktail).

Extracts were centrifuged at 15,800*g* for 5min at 4°C and the supernatant (nuclear extract) collected. Nuclear and cytoplasmic fractions were quantified (Bradford Protein Assay, Bio-Rad) and analyzed by SDS-PAGE, followed by immunoblot analysis on a nitrocellulose membrane and detected with anti- mouse α-FLAG 1:3000 (Sigma). Anti-mouse tubulin 1:2000 and anti-rabbit Heterochromatin protein 1 (HP1α) (Cell Signaling Technology) 1:1000 were controls for cytoplasmic/nuclear contamination.

References

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Higuchi, M., Single, F.N., Kohler, M., Sommer, B., Sprengel, R. and Seeburg, P.H. (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. *Cell*, **75**, 1361-1370.

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Legends to Supplementary Figures.

Supplementary Figure 1. Conservation of serine, threonine and proline motifs at the amino terminus of ADAR2.

Amino acid sequence alignment of the amino terminus of ADAR2 from 11 different species. The conserved SP is high lighted in grey, STP is highlighted in black and TP is highlighted in darker grey.

Supplementary Figure 2. Silencing of Pin1 in SH-SY5Y cells to analyze editing level of the endogenous *GluR2 R/G* site.

Cell lysates of SH-SY5Y cells were analyzed by immunoblot with either anti-Pin1 (upper panel) or anti-tubulin (lower panel) to detect the level of silencing of Pin1.

Supplementary Figure 3. Mis-localization of ADAR2$^{S26A/S31A/T32A}$ in HeLa cells

Immunofluorescence of HeLa cells that were transiently co-transfected with either FLAG-ADAR2 or the triple mutant FLAG-ADAR2$^{S26A/S31A/T32A}$as well as the *GluR2* minigene *B13*. Panel (i) DAPI staining of nuclei. Panel (ii) staining with anti-FLAG antibody of FLAG-ADAR2. (iii) Merge of DAPI and FLAG images. Panel (iv) DAPI staining of nuclei. Panel (v) staining with anti-FLAG antibody of FLAG-ADAR2$^{S26A/S31A/T32A}$. (vi) Merge of DAPI and FLAG images (iv) and (v). Scale bar, 10μm.

Supplementary Figure 4. Cytoplasmic localization of ADAR2$^{\Delta 4-72}$ in HeLa cells

Immunofluorescence of HeLa cells that were transiently co-transfected with HA-Pin1 and FLAG-ADAR2$^{\Delta 4-72}$. Panel (i) DAPI staining of nuclei. Panel (ii) staining of HA-Pin1 with anti-HA antibody. Panel (iii) staining of FLAG-ADAR2$^{\Delta 4-72}$ with anti-FLAG antibody. Panel (iv) merge of DAPI and FLAG-ADAR2$^{\Delta 4-72}$ images. Panel (v) merge of DAPI, HA and FLAG images. Scale bar, 10μm.

Supplementary Figure 5. Conservation of PPxY motif inADAR2.

Amino acid sequence alignment of the amino and carboxyl termini of ADAR2 from 23

different species. The conserved PPxY motif is high-lighted in red. In some species only the carboxy-terminal sequence is present in Ensembl so it is not possible to state if the motif is present at the amino terminus and this is represented with ?

Supplementary Figure 6. Position of PPLY in the ADAR2 deaminase domain. Ribbon structure of the deaminase domain of hADAR2 (Macbeth et al., 2005), yellow balls refer to the N-terminus and blue balls represent the C-terminus. The zinc atom at the active site is represented by a purple ball and IP6 is shown in orange. The PPLY motif that binds WWP2 is shown in red and is on the outside of the protein, on the opposite side of the protein to the region that is thought to interact with dsRNA.

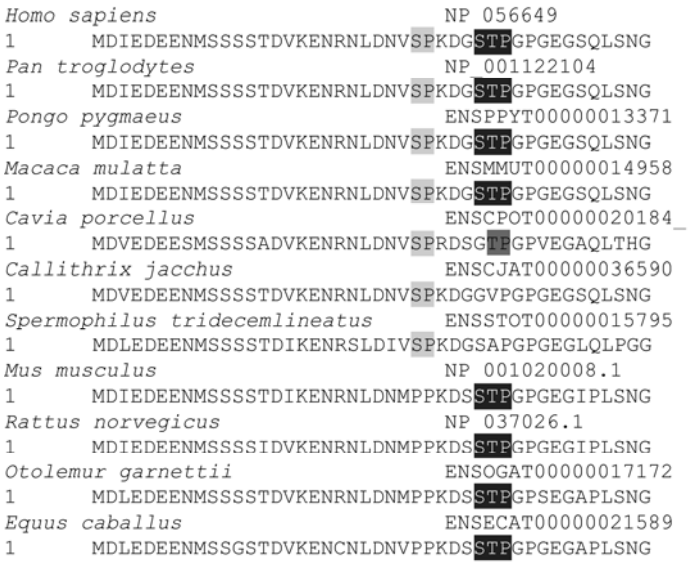
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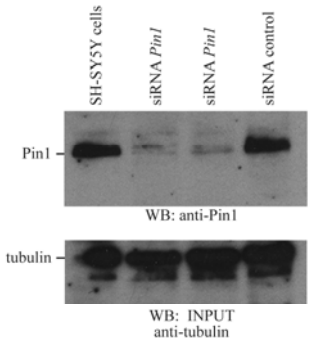
FIG. S1


SH-SY5Y cells
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siRNA Pin1
siRNA control
Pin1
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tubulin
WB: INPUT
anti-tubulin

FIG. S2

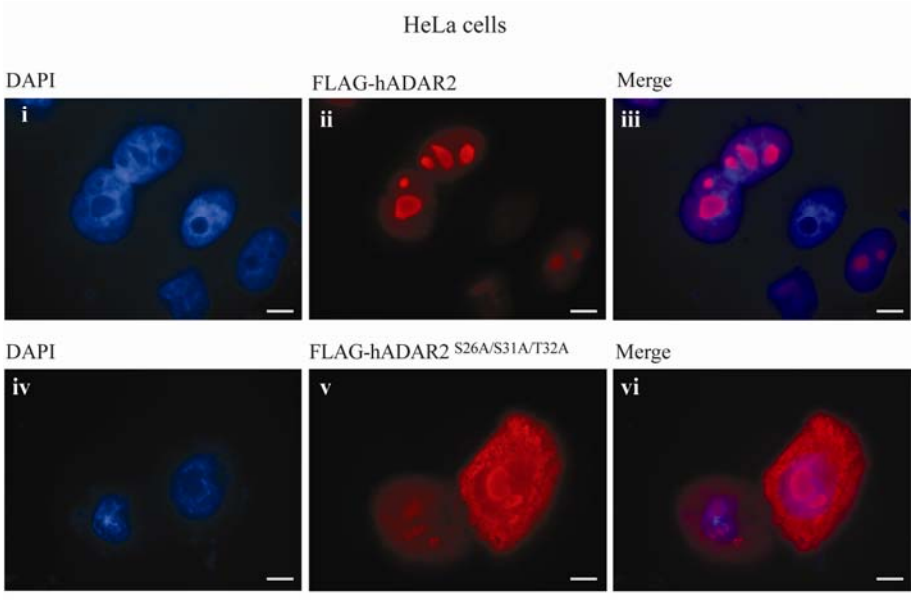
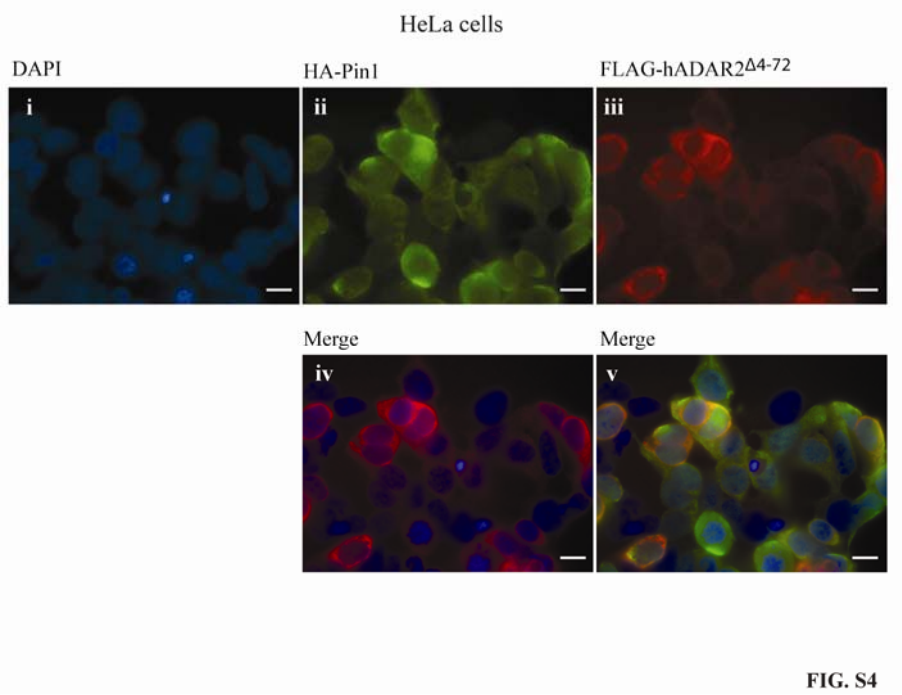

HeLa cells
DAPI
FLAG-hADAR2
Merge
i
ii
iii
DAPI
FLAG-hADAR2 S26A/S31A/T32A
Merge
iv
v
vi

FIG. S3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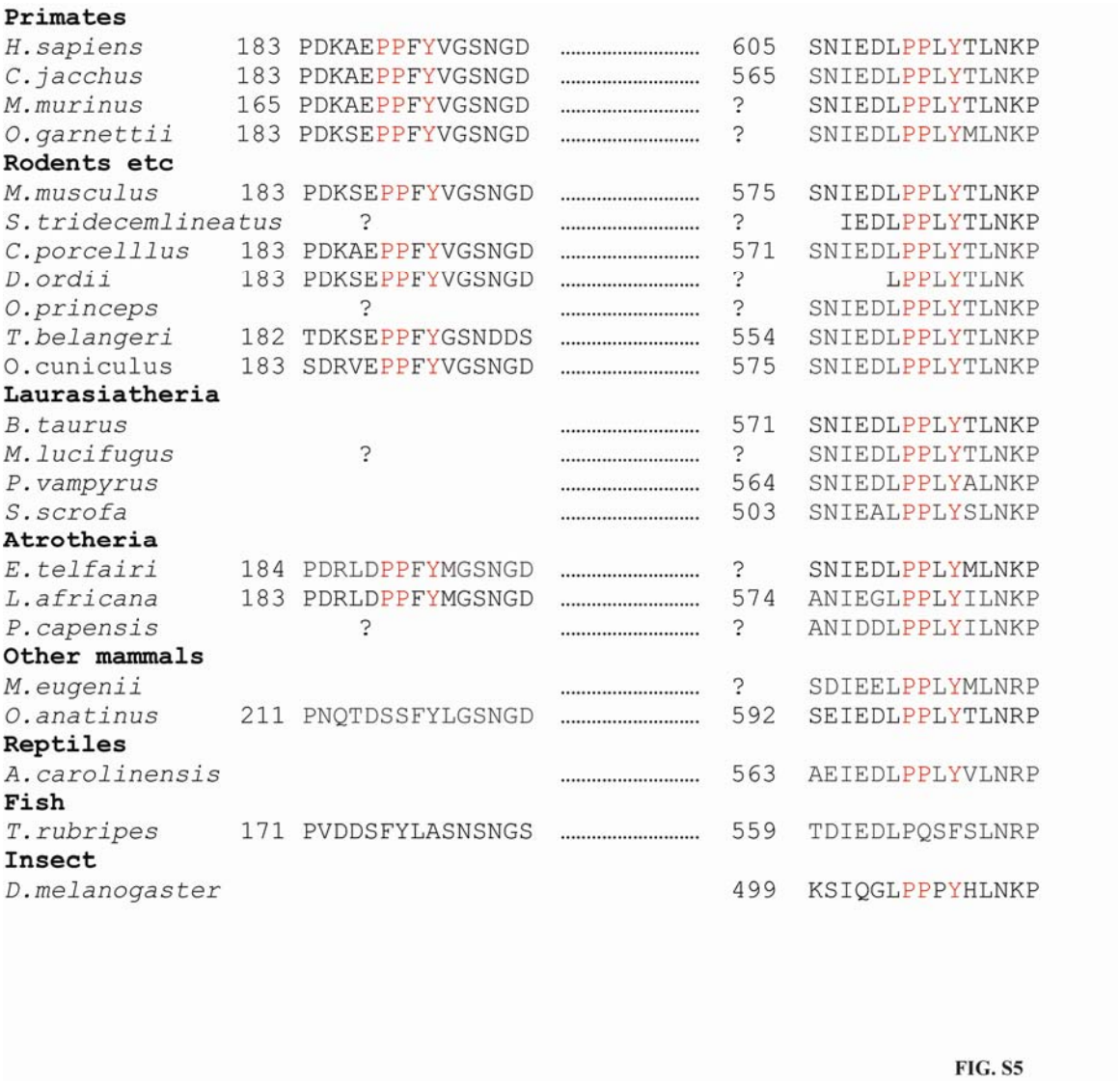
DAPI
HA-Pin1
FLAG-hADAR2Δ4-72
i
ii
iii
Merge
Merge
iv
v

FIG. S4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C.jacchus 183 PDKAEPPFYVGSNGD ..... 565 SNIEDLPPLYTLNKP
M.murinus 165 PDKAEPPFYVGSNGD ..... ? SNIEDLPPLYTLNKP
O.garnettii 183 PDKSEPPFYVGSNGD ..... ? SNIEDLPPLYMLNKP
Rodents etc
M.musculus 183 PDKSEPPFYVGSNGD ..... 575 SNIEDLPPLYTLNKP
S.tridecemlineatus ? ..... ? IEDLPPLYTLNKP
C.porcelllus 183 PDKAEPPFYVGSNGD ..... 571 SNIEDLPPLYTLNKP
D.ordii 183 PDKSEPPFYVGSNGD ..... ? LPPLYTLNK
O.princeps ? ..... ? SNIEDLPPLYTLNKP
T.belangeri 182 TDKSEPPFYGSNDDS ..... 554 SNIEDLPPLYTLNKP
O.cuniculus 183 SDRVEPPFYVGSNGD ..... 575 SNIEDLPPLYTLNKP
Laurasiatheria
B.taurus ..... 571 SNIEDLPPLYTLNKP
M.lucifugus ? ..... ? SNIEDLPPLYTLNKP
P.vampyrus ..... 564 SNIEDLPPLYALNKP
S.scrofa ..... 503 SNIEALPPLYSLNKP
Atrotheria
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L.africana 183 PDRLDPPFYMGSNGD ..... 574 ANIEGLPPLYILNKP
P.capensis ? ..... ? ANIDDLPPLYILNKP
Other mammals
M.eugenii ..... ? SDIEELPPLYMLNRP
O.anatinus 211 PNQTDSSFYLGSNGD ..... 592 SEIEDLPPLYTLNRP
Reptiles
A.carolinensis ..... 563 AEIEDLPPLYVLNRP
Fish
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Insect
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FIG. S5

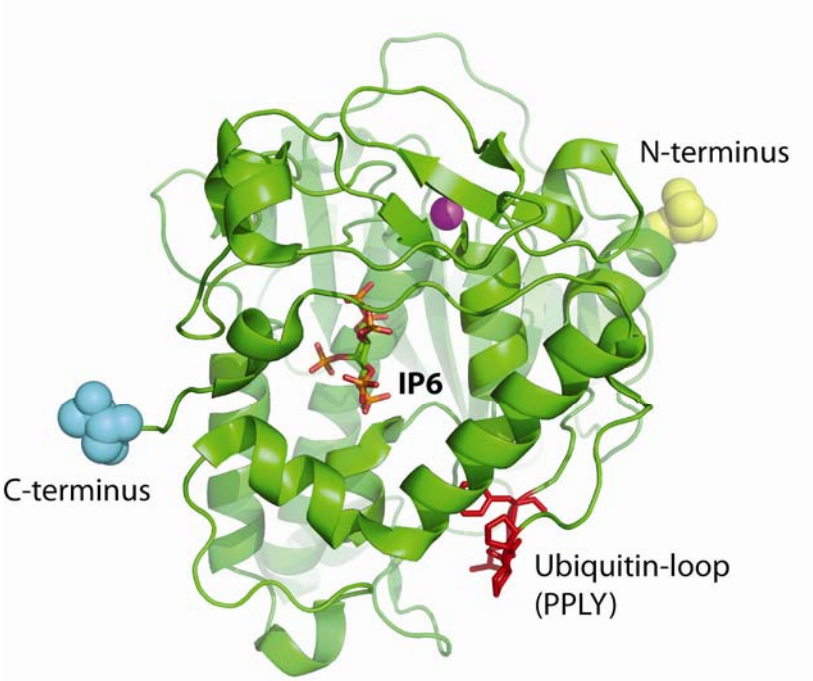

FIG. S6