Supporting Information

Regulatory Mechanism for the Transmembrane Receptor that Mediates Bidirectional Vitamin A Transport

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www.pnas.org/cgi/doi/10.1073/pnas.1918540117

Methods:

Purification of STRA6 from bovine RPE cells and mass spectrometry analysis: We used immunoaffinity purification to purify STRA6 from native RPE cells. Among the several polyclonal antibodies that we raised against synthetic peptides from bovine STRA6 in rabbits, only one HS19-G4414 was determined to be effective in purifying STRA6 from native RPE cells. HS19-G4414 antibody was affinity purified with the antigen peptide fragment coupled to Affi-Gel (Bio-Rad). The purified antibody was conjugated to cyanogen bromide-activated agarose to make affinity matrices for STRA6 purification. Membrane proteins were extracted from the RPE cells using the solubilization buffer (1% [w/v] Brij-35 in PBS) supplemented with cOmplete protease inhibitor cocktail (Sigma) at the concentration recommended by the manufacture. The mixture was passed through a fine Hamilton syringe 10 times and rotated at 4°C for 45 min to maximize solubilization of membrane proteins. Unsolubilized membranes were spun down at 16,000 g for 30 min at 4°C. The HS19-G4414 matrix was mixed with the solubilized protein mixture and incubated overnight at 4°C with gentle rotation. The HS19-G4414 matrix was then pelleted by low speed centrifugation (100g for 3 min at 4^{o}C) and washed 3 times (10 min each) using the wash buffer (0.2% Brig-35 in PBS). Bound proteins were eluted with the elution buffer (0.1 M Glycine-HCl, pH 2.3, 0.2% (w/v) Brij-35). Eluted proteins were mixed with NuPAGE SDS Sample Loading Buffer (ThermoFisher Scientific), incubated at room temperature for 10 min and separated in a NuPAGE pre-cast 4-12% polyacrylamide gradient gel (ThermoFisher Scientific). The gel running buffer was NuPAGE MOPS SDS Running Buffer (ThermoFisher Scientific). The SDS-PAGE gel was stained with SYPRO-Ruby (Molecular Probes/ThermoFisher Scientific). Bands were excised and subjected to standard in-gel trypsin digestion and analyzed on an ion trap mass spectrometer (LTQ Velos, Thermo Scientific). A portion of the same samples were run on a Laemmli SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane (PALL) for western blot analysis.

Fluorescence resonance energy transfer (FRET) test in live cells. HEK-293T cells were cultured on microscopy sides and co-transfected with plasmids coding for STRA6-C-CFP and YFP-N-calmodulin. Forty-eight hours post transfection, the slides were placed in a perfusion chamber for observation under a fluorescent microscope. The quantitative visual field was selected by software, excitation wavelength was set at 320 nm and emission intensities of the visual field were recorded at 470 and 530 nm every 5 seconds. Cells on the slide were alternatively perfused with PBS/EDTA and HBSS (Hank's Balanced Salt Solution containing 2

mM calcium). FRET effect was measured by the ratio of the fluorescence intensity at 530 nm over 470 nm. Control HEK-293T cells were transfected with STRA6-C-CFP and non-tagged calmodulin.

Production and purification of holo-RBP, apo-RBP and ^{3}H-retinol/RBP. Holo-RBP, apo-RBP and ^{3}H-retinol/RBP were produced as described previously (1, 2). In brief, expression of 6XHis-tagged RBP from a pET3a vector was induced in E.coli (BL-21) with 1 mM IPTG. The bacteria were lysed and inclusion bodies were extracted. The inclusion bodies were solubilized overnight at RT with denaturing solution containing 5 M guanidine hydrochloride and 10 mM DTT. The refolding solution containing 0.3 mM cystine, 3.0 mM cysteine, 1 mM EDTA and 0.1 mM retinol was added in the darkroom and renaturation was allowed by stirring for 5 hours. The renatured His-RBP was purified by Ni-NTA (Qiagen) column. Further purification was achieved by passing through a weak anion ion exchange column (Eprogen AX-300). Apo-RBP was produced by repeated extraction of holo-RBP with an equal volume of heptane. ^{3}H-retinol/RBP was produced by incubation of 10 μg apo-RBP with 150 pmol retinol (3 μCi) at room temperature overnight. Radiolabeled holo-RBP was bound to Ni-NTA resin and the resin was washed extensively with 10 mM imidazole in PBS. ^{3}H-retinol/RBP was eluted with 100 mM imidazole in PBS.

Assays for retinol uptake using ^{3}H-retinol/RBP. The uptake assay was performed as described previously (1, 3). In brief, transfected COS-1 cells or untransfected control cells were washed once with Hanks balanced salt solution (HBSS) and incubated in serum-free medium (SFM) 24 h after transfection. Cellular ^{3}H-retinol uptake assay from ^{3}H-retinol/RBP was performed 48 h after transfection. Cells were incubated with ^{3}H-retinol/RBP diluted in SFM for 1 hour at 37 °C. After a further HBSS wash, the cells were solubilized in 1 %Triton X-100 in PBS. ^{3}H-retinol remaining in the cells was measured with a scintillation counter. All experiments were done in triplicates.

Real-time fluorescence analysis of STRA6-mediated vitamin A transport. The real-time assay was performed as described previously with modification (3, 4). In brief, black 96-well microplates were blocked with Blocker Casein (Pierce) overnight to prevent the non-specific sticking of RBP to plastic. Adherent 293-T cells ectopically co-expressing bovine STRA6 and calmodulin were detached with PBS supplemented with 5 mM EDTA. Collected cells were spun down by low speed centrifugation and re-suspended with HBSS or PBS containing 5 mM EDTA and 50 μM BAPTA-AM and distributed into microplate wells. Fluorescent signals were read in

a POLARstar Omega (BMG Labtech) microplate reader, with the excitation wavelength set at 320 nm, and emission wavelength at 460 nm. The retinol release reaction was initialized with the addition of holo-RBP. To start the retinol loading assay, all-trans retinol (typically 1 μM final concentration) was added to the wells under dim red light before addition of apo-RBP. The plate was read every 5 mins after 10 sec of double-orbital shaking for 1-3 hours.

Acute chemical modification to study STRA6's vitamin A transport pore. Real-time analysis and biotin modification of STRA6 mutants to study the vitamin A transport pore of STRA6 was done as described (4). In brief, membrane preparations were treated with 10 mM methanethiosulfonate ethylammonium-biotin (MTSEA-biotin) for 30 min at room temperature, washed and resuspended with HBSS or PBS supplemented 5 mM EDTA before starting of the assay. The STRA6-catalyzed retinol release from holo-RBP was initialized with the addition of holo-RBP at time 0. Fluorescent signals were read in a POLARstar Omega (BMG Labtech) as described above.

HPLC analysis of Vitamin A uptake by cultured primary bovine RPE cells: Primary bovine RPE cells were cultured as described previously (5). In brief, bovine RPE cells were obtained from freshly dissected eyeballs and cultured on membrane inserts (Millicell-HA or Millicell-PCF) for two weeks to form a dense monolayer. Medium in the well under the insert (basal-lateral side of cultured RPE cells) was replaced with normal human serum (containing RBP) diluted 1 to 5 times with HBSS or PBS containing 5 mM EDTA. After 12 hours of incubation, the membrane was cleaned, cut out from the insert and treated with 2 ml of absolute ethanol containing known amounts of retinyl acetate as an internal standard. The retinoids were then extracted into hexane. The samples in hexane were dried under a flow of nitrogen and re-solubilized with 100 μl of mobile phase and analyzed on an Agilent 1100 HPLC equipped with Ultrasphere C18 column (4.6 · 250mm). The column was eluted isocratically with the mobile phase containing 70% acetonitrile, 15% methanol and 15% dichloromethane at 1.8 ml/min for 15 min. UV absorption was recorded at 325 nm. Amounts of retinol and retinyl esters in the samples were quantitated by comparing integrated peak areas of extracted retinoids against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of the internal standard.

Primary RPE culture: Although primary RPE cultures take many months to establish and maintain, primary RPE culture have been an excellent model to study the cell biology of retinoid uptake and metabolism of the RPE cells (1, 6-8). Before doing functional assays, RPE cultured

on transwells were grown to reach a high transepithelial resistance as described (9-11). Characterization of the primary RPE culture has been published previously (1, 5, 9). Expression of STRA6 in the primary RPE culture is demonstrated by immunofluorescence staining of RPE cells derived from the naturally nonpigmented region of tapetum lucidum (**Figure 7A)**.

Knocking down of calmodulin in RPE. After extensive screening of numerous chemical and electroporation conditions for transfection, we developed a protocol that can achieve a high transfection rate for primary RPE cells. Electroporation of primary RPE cells was performed at 150V and 40 ms using the ECM 830 Electroporation System (*Harvard Apparatus*). Using this protocol and the pSUPER system that has been used successfully for shRNA-mediated gene knockdown (12), we further screened two shRNA constructs for each of the three calmodulin genes for a combination of 9 mixtures of shRNA constructs and identified an shRNA mixture that effectively knocked down calmodulin. Five short-hairpin sequences targeting all three bovine calmodulin genes (Table 1) were inserted into pSUPER.basic vector (OligoEngine, Seattle, WA) using BglII / HindIII or BglII / XhoI (shRNA2) restriction enzymes. Vectors were combined (shRNA mix 1 = shRNA1 + shRNA5, shRNA mix 2 = shRNA2 + shRNA3 + shRNA4, shRNA mix 3 = shRNA3 + shRNA4 + shRNA5).

	Target protein	**Target sequence**	**Forward oligo sequence**	**Reverse oligo sequence**
shRNA1	bCaM1 bCaM2	CTGACTG AAGAGCA GATTGC	GATCGCCCTGACTGAAGAGCA GATTGCTTCAAGAGAGCAATC TGCTCTTCAGTCAGTTTTTA	AGCTTAAAAACTGACTGAAGA GCAGATTGCTCTCTTGAAGCA ATCTGCTCTTCAGTCAGGGC
shRNA2	bCaM3	CGAGCTG CAGGACA TGATC	GATCTCCCGAGCTGCAGGACA TGATCTTCAAGAGAGATCATGT CCTGCAGCTCGTTTTTC	TCGAGAAAAACGAGCTGCAGG ACATGATCTCTCTTGAAGATCA TGTCCTGCAGCTCGGGA
shRNA3	bCaM1	TGACTAT GATGGCT AGAAA	GATCTCCTGACTATGATGGCT AGAAATTCAAGAGATTTCTAGC CATCATAGTCATTTTTA	AGCTTAAAAATGACTATGATGG CTAGAAATCTCTTGAATTTCTA GCCATCATAGTCAGGA
shRNA4	bCaM2	GGACATG ATTAATGA AGTA	GATCTCCGGACATGATTAATG AAGTATTCAAGAGATACTTCAT TAATCATGTCCTTTTTA	AGCTTAAAAAGGACATGATTAA TGAAGTATCTCTTGAATACTTC ATTAATCATGTCCGGA
shRNA5	bCaM3	AGGTCAA TTATGAA GAGTT	GATCTCCAGGTCAATTATGAA GAGTTTTCAAGAGAAACTCTTC ATAATTGACCTTTTTTA	AGCTTAAAAAAGGTCAATTATG AAGAGTTTCTCTTGAAAACTCT TCATAATTGACCTGGA

Live-cell RBP/STRA6 binding assay. 293T cells were transfected with plasmids expressing STRA6 and calmodulin in serum containing medium. Twenty four hours after transfection the cells were washed once with SFM and grown in SFM. Forty eight hours after transfection, cells

from one well on a 12 well plate were detached by gentle flushing with SFM, spun down and re-suspended in 200 μl of SFM with or without 1 μM of thapsigargin. One microgram of holo-RBP or apo-RBP was added to the suspended cells and incubated for 30 min. After three washes with 500 μl of SFM by pelleting cells at low speed, the cell pellets were extracted with 100 μl of 1% Trtion in PBS. Extracted proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. RBP associated with STRA6 on the surface of 293T cells was detected by Western blot analysis using an anti-RBP antibody (Accurate Chemical, Westbury, NY). Protein loading was normalized to cell number.

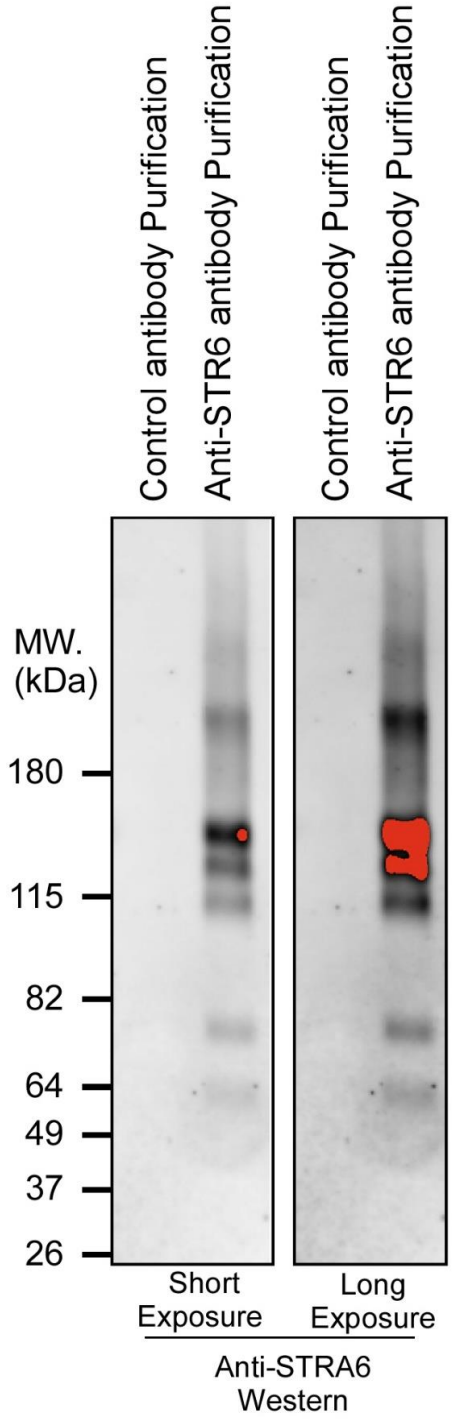

Figure S1: Western blot analysis of STRA6 purified from RPE cells freshly dissected from bovine eyes by immunoaffinity purification. The control antibody is a control IgG.

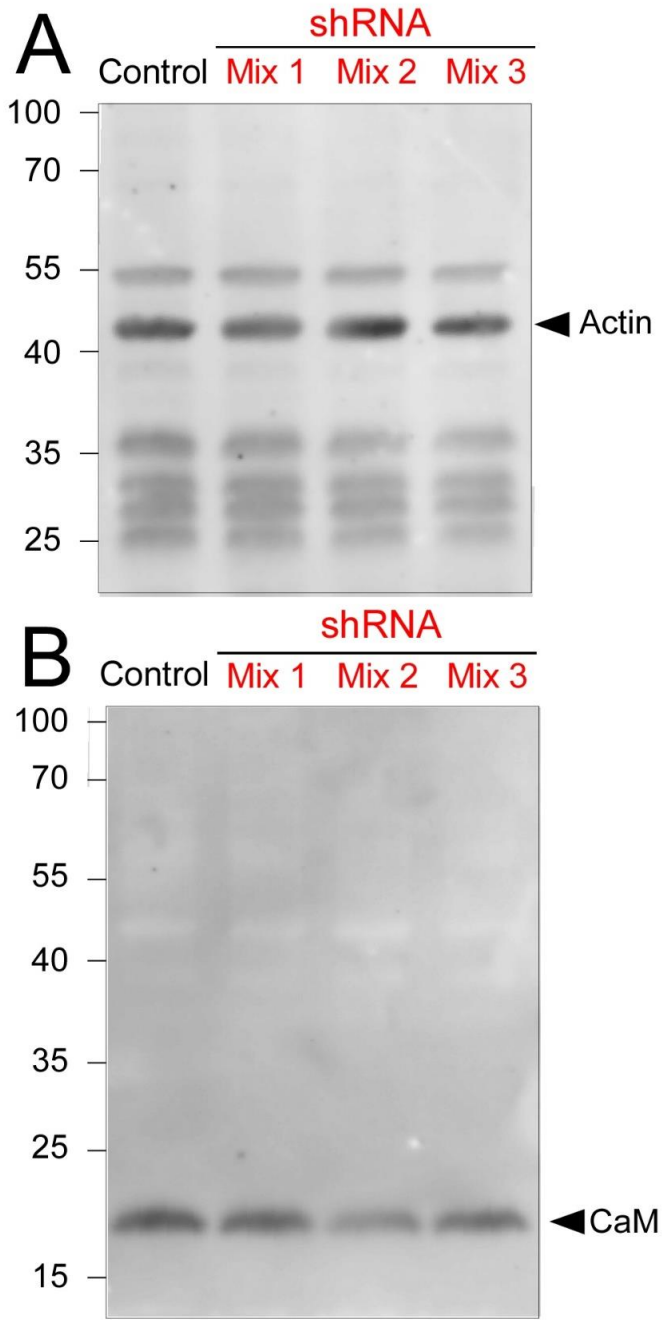

Figure S2: The original Western blots of Figure 4C. Molecular weight markers (in kDa) are shown on the left of each gel. **A.** The ant-actin Western blot. In addition to the actin band of the predicted MW (arrowhead), a few other bands were recognized by this antibody. All these bands showed equal loading. **B.** The anti-calmodulin Western blot.

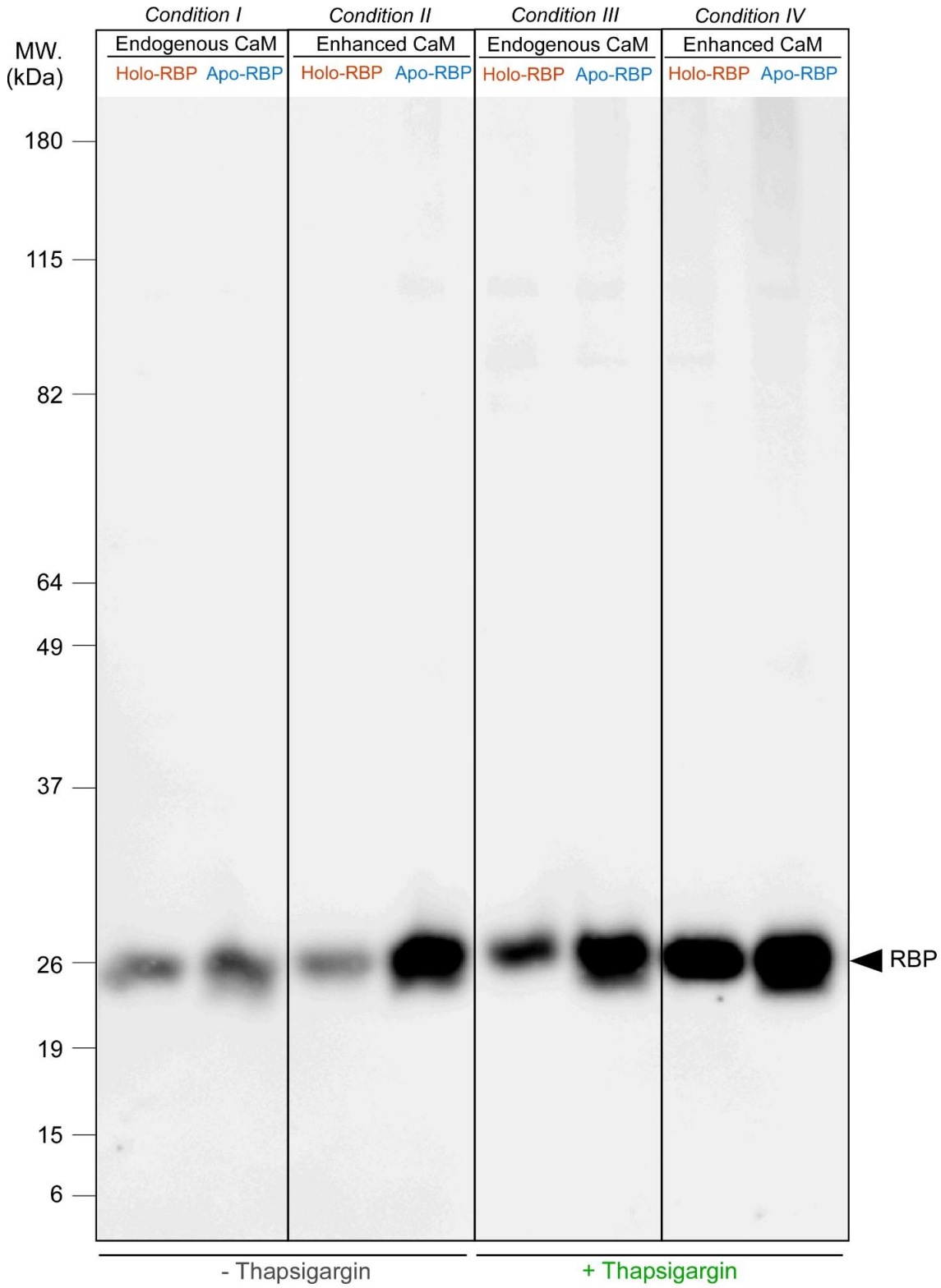

Figure S3: The original Western blots of Figure 5A. Molecular weight markers (in kDa) are shown on the left.

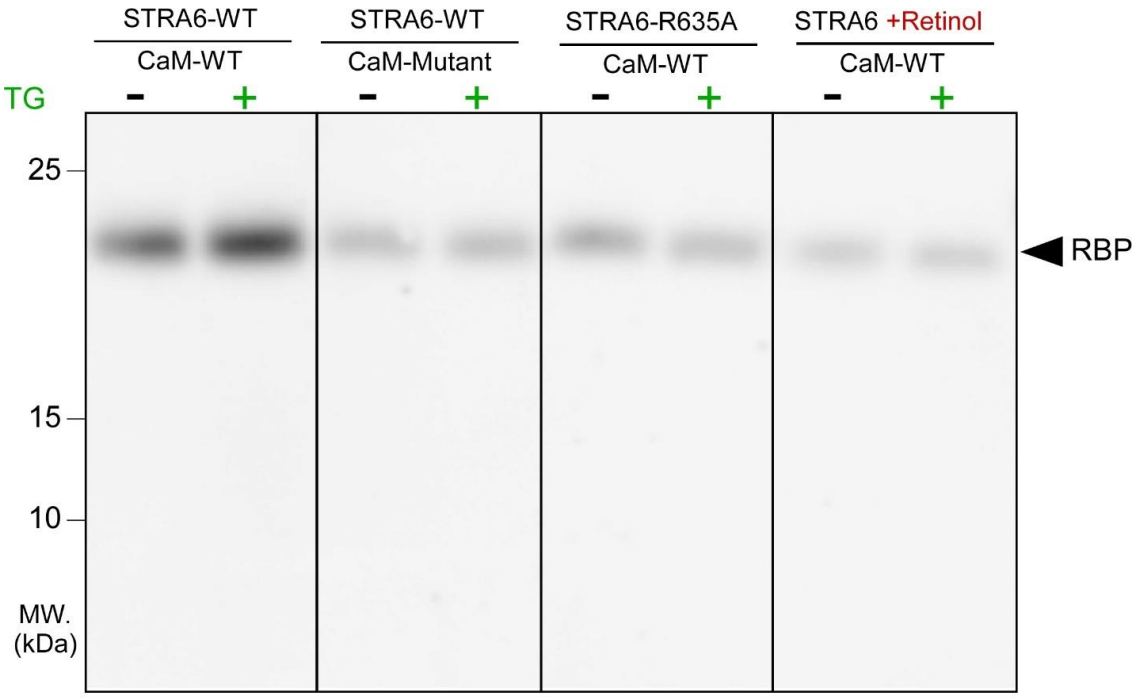

Figure S4: The original Western blots of Figure 5B. Molecular weight markers (in kDa) are shown on the left of each gel.

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