SUPPLEMENTARY APPENDIX

SUPPLEMENTARY METHODS

Renal biopsy

Biopsies were processed by light, immunofluorescence, and electron microscopy using routine techniques[1]. For light microscopy, kidney biopsies were fixed in buffered formalin, dehydrated in graded alcohols, and embedded in paraffin using standard techniques. Serial 3 µm-thick sections were cut and treated with hematoxylin and eosin, Jones methenamine silver, Masson trichrome, and periodic acid-Schiff reagent. Immunofluorescence samples were transported in Michel's media, washed in buffer, and frozen in a cryostat. Sections, cut at 4 µm, were rinsed in buffer and reacted with fluorescein-tagged polyclonal rabbit anti-human antibodies to IgG, IgA, IgM, C3, C4, C1q, fibrinogen, and κ-, and λ-light chains (all from Dako, Carpenteria, CA, USA) for 1 h, rinsed, and a coverslip applied using aqueous mounting media. The stains were evaluated by standard immunofluorescence microscopy using a Leica L5 filter cube and scored on a scale of 0 to 3. PLA2R was performed on paraffin embedded sections following pronase digestion using rabbit polyclonal anti-PLA2R antibodies (Sigma-Aldrich) at a dilution of 1:50 followed by highly cross-adsorbed Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) at a dilution of 1:100 as previously described[2]. Immunoperoxidase staining for THSD7A was performed in paraffin embedded sections using rabbit polyclonal anti-THSD7A antibodies (Sigma-Aldrich) at a dilution of 1:800 as previously described[3].

LRP2 staining was performed on all renal biopsies positive for anti-brush border antibodies by serologic testing as well as 50 negative controls. The controls included 40 biopsies with evidence of granular IgG-positive tubular basement membrane deposits by immunofluorescence including 27 patients with lupus nephritis, 7 with IgG4-related disease, 6 with BK nephritis as well as 10 biopsies with kidney injury negative for tubular basement membrane deposits including 5 with nephrotoxic acute tubular injury and 5 with interstitial nephritis. The stain was performed on formalin fixed paraffin embedded tissue. Sections, cut at

3 µm, were deparaffinized and antigen retrieval was performed at 99 C. The sections were then reacted with mouse monoclonal anti-human megalin (EMD Millipore, Billerica, MA) at a dilution of 1:1000 followed by polyclonal (Rhodamine Red-X) goat anti-mouse IgG Fcγ subclass 1 (Jackson ImmunoResearch, West Grove, PA). Specificity of the antibody was established by comparing staining of the primary with a mouse monoclonal IgG1 κ isotype control (Abcam, Cambridge, MA). All cases were run with a negative control (secondary antibody only) to ensure that any positive staining was not resulting from the secondary antibody. Additionally, HEK293 cell extract containing the expressed third set of LA repeats (which contains the epitope recognized by the anti-LRP2 monoclonal antibody) can specifically block this staining in both the brush border of normal kidney as well as the tubular basement membrane deposits in an ABBA biopsy, whereas a similar cell extract but expressing a portion of the first set of LA repeats does not block (Supplementary Figure 6).

Colocalization of IgG and LRP2 (megalin) in the tubular basement membranes of patients with anti-brush border antibody disease biopsies was demonstrated using a Zeiss LSM 880 confocal laser scanning microscope with Airyscan. For this analysis, paraffin embedded sections were heat retrieved and then reacted with polyclonal (FITC-conjugated) goat anti-human IgG at a dilution of 1:100 (Kent Laboratories, Bellingham, WA) followed by staining for megalin as described above. Negative controls were performed to ensure antibody specificity by omitting primary antibodies. Additionally, ten cases of immune complex-mediated glomerulonephritis positive for IgG1 were reacted with the polyclonal goat anti-human IgG at a dilution of 1:100 (Kent Laboratories, Bellingham, WA) followed by the polyclonal (Rhodamine Red-X) goat anti-mouse IgG Fcγ subclass 1 secondary antibody. All 10 control cases showed strong positive staining for IgG (FITC) in glomeruli and were completely negative for the rhodamine signal, ensuring the absence of cross-reactivity of the goat anti-mouse IgG Fcγ subclass 1 secondary antibody with human IgG1.

The ends of the renal biopsy specimen were removed as 1 mm cubes, dehydrated using graded alcohols and embedded in epon/araldite resin. Sections 1-µm thick were cut using an ultramicrotome, stained with toluidine blue and examined with a light microscope. Thin sections were examined in a Jeol 1400 electron microscope (Jeol, Tokyo, Japan).

Indirect immunofluorescence for brush border antibodies

Patient sera was tested for anti-brush border antibodies by reacting the serum with sections of normal human kidney tissue at a dilution of 1:10 followed by polyclonal (FITC-conjugated) goat anti-human IgG at a dilution of 1:100 (Kent Laboratories, Bellingham, WA). The stain was then evaluated by standard immunofluorescence microscopy using a Leica L5 filter cube. Strong staining of the proximal tubule apical membrane was regarded as positive for the presence of brush border antibodies and was necessary for study inclusion. There was no reactivity against the tubular basement membranes of normal kidneys in any of the patients. Control sera from thirty patients in which the renal biopsy showed membranous glomerulopathy without evidence of tubular basement membrane deposits were also tested and showed no brush border reactivity. Additionally, control sera from 10 patients with Crohn's disease kindly provided by the MGH Inflammatory Bowel Center courtesy of Dr. Xavier Ramik tested negative for brush border reactivity. The IgG subclass of the brush border antibodies was determined by reacting the normal human serum at a dilution of 1:10 against normal human kidney tissue followed by mouse monoclonal anti-human IgG1, IgG2, IgG3, and IgG4 (Sigma-Aldrich, St Louis, MO) at a dilution of 1:50.

Colocalization of ABBA patient serum antibodies with LRP2 in human kidney tissue was demonstrated using a Zeiss LSM 880 confocal laser scanning microscope with Airyscan. For this analysis, the normal human kidney tissue was reacted with human serum and anti-human IgG as described above. The sections were then reacted with rabbit polyclonal anti-LRP2 (ab101011, Abcam, Cambridge, MA) followed by polyclonal (Alexa Fluor® 555- conjugated) goat anti-rabbit IgG (ab150086, Abcam). Negative controls were performed to ensure antibody specificity by omitting primary antibodies.

Preparation of human tubular extract

Cortical portions of deceased donor human kidneys that had been deemed unsuitable for transplantation (New England Organ Bank) were used immediately or snap frozen and stored in liquid nitrogen for later processing. Several samples were immediately homogenized

and frozen in RLT buffer (Qiagen) for RNA extraction. Cortical sections were minced and sieved (Fisher Scientific #140, #80, and #200 sieves) with cold phosphate buffered saline (PBS). Glomeruli and any attached proximal tubular segments were retained on the final #200 sieve, while the remaining tubular and interstitial components passed through all the sieves and were collected in PBS and harvested by centrifugation for 4 min at 2500 rpm at 4°C. The tissue pellet was washed three times with PBS and resuspended in an equal volume of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; Boston BioProducts) with 1x Protease Inhibitor Cocktail Set I (Calbiochem / EMD Chemicals, Inc.), then manually Dounce homogenized while on ice. Tubular proteins were extracted on ice for 40 min, with intermittent vortexing. RIPA-insoluble debris was removed by a 10 min centrifugation at 14,000 rpm at 4° C. Due to the presence of contaminating human IgG in this preparation, the human tubular extract was incubated for 4 h (or overnight) at 4° C with Immobilized Protein G Plus (Thermo Fisher) and the beads discarded. Thereafter, all subsequent experiments were performed with these detergent extracts of homogenized whole tubules (HTE).

Western blotting

Western blotting was performed as previously described[6] on 4-15% Mini-PROTEAN TGX gels (BioRad) with 25 µL of sample in 1x non-reducing gel loading buffer (Boston BioProducts) loaded per lane. Beta-mercaptoethanol was added to a final concentration of 3% prior to boiling when experiments called for reducing conditions. Proteins were transferred to nitrocellulose membranes following gel electrophoresis, then transiently stained with Ponceau before blocking. When needed, at the time of the Ponceau stain the membrane was cut to separate the lanes and allow the incubation with different primary or secondary antibodies. After blocking in 10% non-fat dried milk in Tris-buffered saline with 0.2% Tween20 (TBS-T) for 1h at RT, blots were incubated in patient serum diluted blocking buffer overnight at 4°C or with the appropriate primary antibody (anti-FLAG F3165 Sigma; anti-LRP2 MABS489 EMD millipore; or anti-fibronectin F0916 Sigma) for 1h at room temperature (RT). When incubated with human serum, blots were next incubated for 1h at RT in sheep anti-human IgG4 (The Binding Site; 1:3000) or sheep anti-human IgG1 (The Binding Site; 1:4000), followed by peroxidase-

conjugated donkey anti-sheep IgG (Jackson ImmunoResearch, 1:10,000) with washes in TBS-T in between incubations. When blotted with monoclonal antibody, membranes were incubated with peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 1:5,000). After incubation in chemiluminescence solution (20 mL Tris, pH 8.5; 100 µL luminol; 44 µL p-coumaric acid; and 5.5 µL hydrogen peroxide), blots were exposed to film (Denville) and developed using a Kodak X-Omat developer.

Biotinylation of brush border antigens

To identify proteins with exposed extracellular domains, a human kidney tubular fraction obtained by sieving (see above) was immediately incubated for 1 hr at 4° C with EZ-Link™ Sulfo-NHS-Biotin (Thermo Scientific) in cold PBS. The reaction was quenched by resuspending the biotinylated tubules in Tris buffered saline (TBS). After centrifugation of the cellular pellet, the biotinylated tubular fraction was extracted in RIPA buffer with added protease inhibitors as above.

Immunoprecipitation of the brush border antigen and mass spectrometric analysis

ABBA-containing serum (or normal control serum) was incubated with either biotinylated or non-biotinylated HTE for 1h at RT. Immunoglobulin-antigen complexes were immunoprecipitated using either Protein G Plus beads (Thermo Fisher Scientific) or Capture Select IgG4 (BAC BV). Following extensive washing in PBS, the beads were boiled in non-reducing gel loading buffer and electrophoresed as described above. Blots were probed with streptavidin-peroxidase to confirm the immunoprecipitation and to localize the position of immunoprecipitated surface-biotinylated autoantigen. Based on the precise location of the immunopreciptated band, the corresponding 250-350 kDa region from an identical gel (loaded with ABBA and control IP) was excised and analyzed by mass spectrometry.

Mass spectrometry analysis

<u>Liquid Chromatography-Mass Spectrometric Analysis of Target Antigen Containing Gel Slices:</u>

Gel band analysis: SDS-PAGE gel bands containing candidate target antigens were processed for

liquid chromatography-mass spectrometric analysis separately using trypsin (Promega, Madison, WI) and Asp-N proteases as previously described[7,8]. The digest supernatant and gel piece extracts[9] were combined, lyophilized and re-dissolved in 2% acetonitrile / 0.1% formic acid prior to analysis.

LCMS data acquisition: Peptide samples were loaded onto an in house pulled (360µm OD x 100µm ID) fused silica tip needle tip packed with 12cm of Aeris Peptide XB-C18 3.6µm, 100Å material (Phenomenex, Torrance, CA, USA) using a Proxeon EASY n-LC (Thermo-Fisher Scientific, Waltham, MA, USA) UHPLC system. Peptides were eluted using a linear gradient of 2% v/v acetonitrile / 0.1% v/v formic acid to 40% v/v acetonitrile / 0.1% v/v formic acid over 80min. The sample was introduced into an LTQ-Orbitrap ELITE (Thermo-Fisher Scientific) using a Nanospray Flex source with the ion transfer capillary temperature of the mass spectrometer set at 225°C, and the spray voltage was set at 1.6kV. Data were acquired with an approach known as an Nth Order Double Play created in Xcalibur v2.2. Scan event one of the method obtained an FTMS MS1 scan (normal mass range; 240,000 resolution, full scan type, positive polarity, profile data type) for the range 300-2000m/z. Scan event two obtained ITMS MS2 scans (normal mass range, rapid scan rate, centroid data type) on up to twenty peaks that had a minimum signal threshold of 5,000 counts from scan event one. The lock mass option was enabled (0% lock mass abundance) using the 371.1012m/z polysiloxane peak as an internal calibrant standard. The assignment of peptide sequence information included the variable modification of lysine (K) with biotinylation (+226 Da) following alkylation by EZ-Link NHS-Biotin (Thermo Fisher) that would arise from the corresponding protein modification event.

Data Analysis: Data were searched as recently described[10] using proteome Discoverer v1.4.1.114 (Thermo) to direct the data analysis using Mascot v2.5.1 (Matrix Science Inc., Boston, MA, USA) and SequestHT (Thermo-Fisher Scientific) with the UniprotKB Homo sapiens reference (ver 3/9/2016) proteome canonical and isoform sequences appended with the cRAP database (ver 1/1/2012, thegpm.org). Search results were converted to .msf files and were loaded into Scaffold Q+S v4.4.5 (Proteome Software, Inc., Portland, OR, USA). The false

discovery rate for peptides was calculated using the Scaffold Local FDR algorithm. Protein probabilities were calculated using the Protein Prophet algorithm. Proteins were grouped by the Scaffold protein cluster analysis to satisfy the parsimony principle. The search results were filtered for peptide mass accuracies of 2ppm or greater for peptides of seven amino acids or longer. The results were annotated with human gene ontology information from the Gene Ontology Annotations Database (ftp.ebi.ac.uk).

Purification of LRP2 from human tubular extract

Receptor-associated protein (RAP) is a chaperone required for the proper folding and secretion of several members of the low-density lipoprotein receptor family, including LRP2. Taking advantage of the high affinity of RAP for LRP2, we were able to partially purify LRP2 from human tubular extract (HTE). Full-length human RAP protein with a C-terminal Myc-DDK(FLAG) tag (LRPAP1 (NM_002337) human ORF clone, RC211255, OriGene) was expressed in HEK293 cells and a cell extract prepared. Using beads directed against the C-terminal tag (anti-FLAG M2 Affinity Gel, Sigma), RAP was linked to the beads, followed by extensive washing with PBS. The anti-FLAG-RAP beads were then mixed with HTE and agitated at 4°C for 30 minutes. After extensive washing with PBS, RAP-bound proteins were eluted using 0.1M glycine HCl, pH 3.5 for 5 min at room temperature followed by centrifugation. The supernatant was carefully collected and equilibrated with the appropriate volume of 0.5M Tris HCl, pH7.4.

Cloning and expression of human *LRP2* (megalin)

Due to the very large size of the *LRP2* mRNA, we decided to amplify and subclone smaller portions of the gene to produce FLAG-tagged recombinant *LRP2* constructs. Total RNA was extracted from human kidney cortex using the tissue that had previously been homogenized and stored in RLT buffer (RNeasy kit, Qiagen). cDNA were generated by reverse transcription of total renal cortical RNA (Maxima H minus, Thermo Scientific) and then amplified specific fragments of the megalin coding sequence by PCR (Phusion PCR Thermo Fisher) using the following specific primers:

LRP2 cDNA region amplified (in bp)	Forward	Reverse
N 107 to 2710	5’ GGTGCAGACCTAAAGGAGCG 3’	5’ GCATAGGGCTGTCTGAGGGA 3’
N 3012 to 7640	5’ GAGGCTGGCTTCCAATCACT 3’	5’ TTCACAATGGGTACGCGGAA 3’
N 5415 to 9564	5’ GACAGATGGCACCAACAGGA 3’	5’ TCCATCTGGTTCTCGGAGGT 3’
N 9569 to N13360	5' CCTGCCGGCAAAACAGTAAC 3'	5' AAGGGAGCCGGTCCTTCTAT 3'

Then, using specific primers containing restriction enzyme sites (see table below), megalin cDNA fragments were amplified by PCR, digested by restriction enzyme Not1 and BamH1 (New England Biolabs) and inserted to the vector p3XFLAG-CMV13 (Sigma-Aldrich) by T4 DNA ligase (New England Biolabs). For the N-terminal fragment containing the megalin signal peptide sequence, the plasmid p3XFLAG-CMV14 (Sigma Aldrich) was used. The resulting constructs were transformed into competent E.coli (New England Biolabs). Construct sequences were verified by sequencing (Genewiz NJ). After extraction (Qiaprep kit, Qiagen), plasmids were transfected into HEK293 cells using calcium phosphate precipitates. At 48 h, cells were washed twice with PBS and lysed with RIPA buffer or TBS Triton X 1% EDTA 1mM plus protease inhibitors (Halt ^{TM} Protease inhibitor, Thermo Scientific) and extracted on ice for 40 mn with intermittent vortexing. Lysis-insoluble debris was removed by a 10 min centrifugation at 14,000 rpm at 4° C. Expression of the recombinant fragments was verified by running cell extracts by Western blot and detecting with an anti-FLAG mouse monoclonal antibody. Recombinant fragments were assayed using whole cell lysate or after purification by antiFLAG M2 Affinity Gel (Sigma Aldrich).

rLRP2 fragment	**Forward**	**Reverse**
aa 1-311 LA repeats 1-7	5’ATAAGAATgcggccgcAATGGATCGCGGGCCGGCAGCA3’	5’CGCggatccCAGAGTCATACTACAGTATTT3’
aa 1026-1350 LA repeats 8-15	5'ATAAGAATgcggccgcACCCACAGAGCAGTGTGGCTTA3'	5'CGCggatccCCCATTGCAAAGTGGGGACTC3'

aa 2700-3011 LA repeats 16-26	5'ATAAGAATgcggccgcTAATGGTGAACGATGTGGTGCA3'	5'CGCggatccAATGCCACAGCCTTTCTCATC3'
aa 3509-3969 LA repeats 26-36*	5'ATAAGAAT**gcggccgcc**ATGCCCATGTGCTCCAGCACC3'	5'CGC**ggatcc**TGTTCTTTCTTTTCCTTTATT3'

Cloning primer sets used to produce recombinant *LRP2* fragments. Restriction enzyme sites are indicated by small characters.

* These primers were designed to clone the entire fourth set of LA repeats, LA26 to 36, but due to the presence of an in-frame BamH1 restriction sequence naturally present in position N 11455 of *LRP2* cDNA, only the sequence encoding amino acids 3509 to 3820 was inserted in the p3XFLAG-CMV13 plasmid, corresponding to the LA repeats 26-32.

IP of the recombinant N-terminal megalin fragment

RIPA extract from HEK293 cells expressing the recombinant N-terminal LRP2 (rLRP2) fragment was first incubated with 75 µl of normal goat serum for 30 min at 4°C to occupy any potential non-specific ligand-binding sites within the rLRP2 fragment with goat IgG, which is an IgG that is not well recognized by protein A. The blocked rLRP2-containing extract was then incubated with 50 µl each of ABBA-containing or control human serum for 1 h at 4° C, followed by the addition of 50 µl of Immobilized Protein A-agarose (Santa Cruz Biotechnology) and further incubation for 2h at 4°C to pull down human IgG-antigen complexes. After washing with TBS, the immunoprecipitates were electrophoresed under reducing conditions, and Western blotted with mouse monoclonal anti-FLAG 1:5000 (Sigma-Aldrich).

SUPPLEMENTARY RESULTS

Supplementary Tables 1-3 describe the histopathologic features noted by light microscopy and immunofluorescence analysis of the kidney biopsies from the available cases of anti-brush border associated nephropathy. All biopsies showed varying degrees of interstitial fibrosis and tubular atrophy, although the presence of interstitial inflammatory infiltrates or actual evidence of tubulitis was more limited (Supplementary Table 1). Of note, all biopsies but one showed the

presence of segmental “holes” or “craters” within the glomerular basement membrane, similar to the more global “holes” seen in membranous nephropathy. Immunofluorescence for human IgG uniformly showed evidence of IgG within the tubular basement membrane, with more variable staining of the proximal tubular brush border (which appeared to be related to the amount of proximal tubular injury). With the exception of Patient 9 (who also did not exhibit the GBM “holes”), there was evidence of segmental granular IgG deposits within the glomerular basement membrane (Supplementary Table 2). The subclass of IgG within the TBM deposits was analyzed using antibodies specific for each human IgG subclass (Supplementary Table 3). In all but one patient, IgG4 was dominant or co-dominant within the TBM deposits, and IgG1 was often present in significant amounts. Of interest, IgG3 was never identified in the TBM deposits.

Supplementary Table 1. Light microscopy features on renal biopsy

Patient	Glomeruli	Tubular epithelium	Interstitial inflammation	Tubulitis	Interstitial fibrosis	Tubular atrophy
1	Negative for proliferation	ATI	+	+	+	+
2	Negative for proliferation	ATI	-	-	++	++
3	Negative for proliferation	ATI	-	-	+	++
4	Negative for proliferation	ATI	++	+	++	++
5	Negative for proliferation	ATI	+	-	+++	+++
6	Negative for proliferation	ATI	+++	-	+++	+++
7	Negative for proliferation	ATI	++	+	+++	+++
8	Negative for proliferation	ATI	+	-	+++	+++
9	Negative for proliferation	ATI	-	-	++	++
10	Negative for proliferation	ATI	++	+	++	+

Abbreviations: ATI, acute tubular injury; GBM, glomerular basement membrane
-, none
+, mild
++, moderate
+++, severe

Supplementary Table 2: Immunofluorescence staining features of renal biopsies

Patient	PT BM granular IgG	PT BM granular C3	PT brush border IgG	GBM IgG	Bowman's capsule IgG	Granular TBM LRP2 staining
1	DP	DP	Neg	Segmental	DP	FP
2	DP	DP	FP	Segmental	DP	DP
3	DP	DP	Neg	Segmental	DP	FP
4	DP	DP	FP	Segmental	DP	FP
5	DP	DP	DP	Segmental	DP	FP
6	DP	DP	Neg	Segmental	DP	DP
7	DP	DP	Neg	Segmental	FP	FP
8	DP	DP	DP	Segmental	DP	DP
9	DP	DP	Neg	Negative	FP	FP
10	DP	DP	FP	Segmental	DP	DP

Abbreviations: BM, basement membrane; DP, diffuse positive; FP, focal positive; FS, focal segmental; GBM, glomerular basement membrane; Neg, negative; PT, proximal tubule; TBM, tubular basement membrane

Supplementary Table 3: IgG subclass of ABBA

Patient	IgG1	IgG2	IgG3	IgG4
1	+++	-	-	++
2	+	+	-	+++
3	+	+	-	+++
4	+++	-	-	+++
5	-	-	-	+++
6	+++	+	-	+++
7	+++	++	-	+++
8	-	+	-	+++
9	+	+	-	+++
10	++	-	-	+++

-, none
+, mild
++, moderate
+++, severe

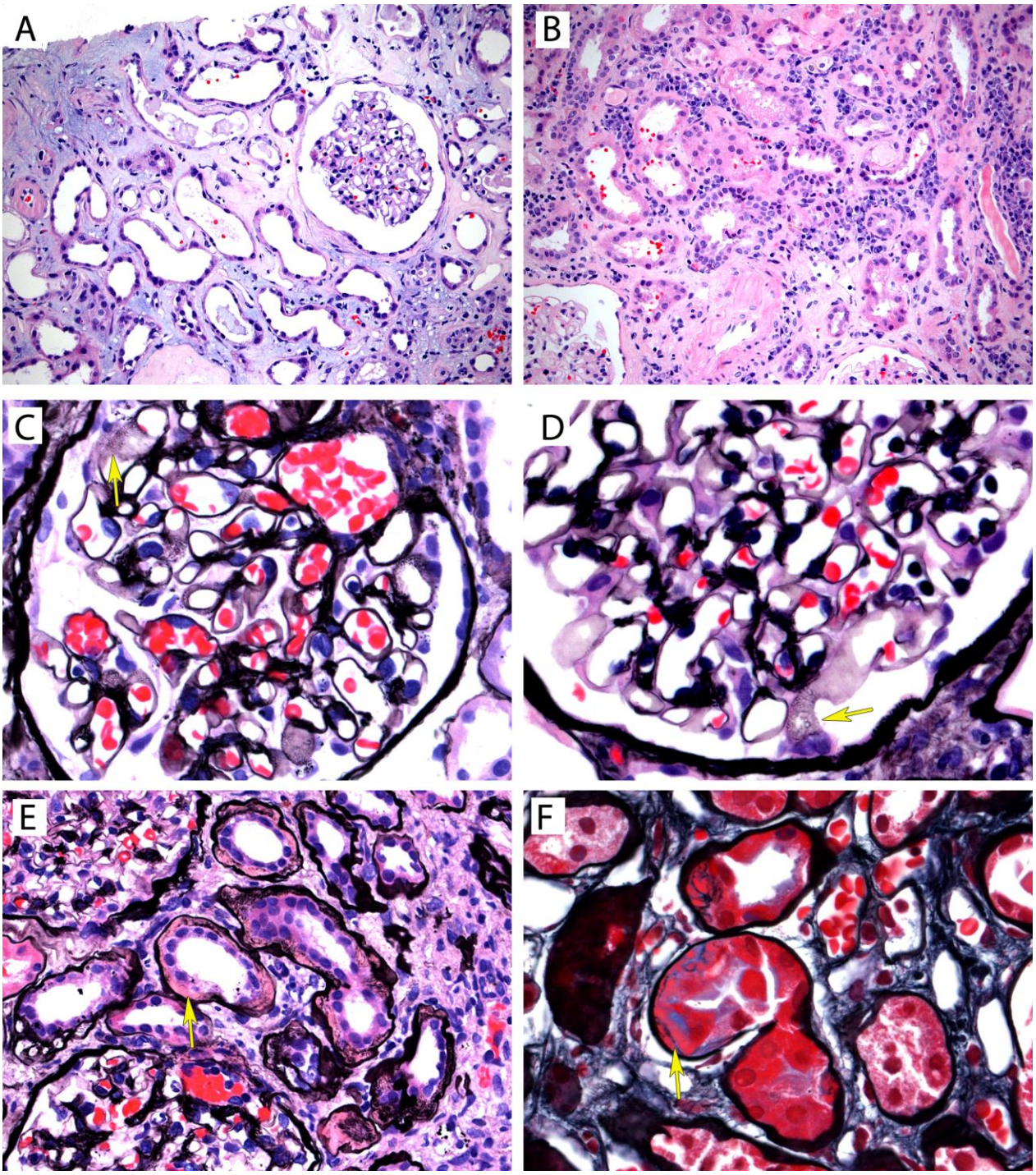
Supplementary Table 4: List of best candidate proteins for brush-border antigen from mass spectrometry analyses

Protein	Size (kDa)	Ratio ABBA+/ Control
Low-density lipoprotein receptor-related protein 2 (megalin)	517	331.3
Fibronectin, isoform 3	259	150.6
Fibrinogen, alpha chain, isoform 2	70	ABBA+ only
Proteoglycan 4, Isoform C	141	ABBA+ only

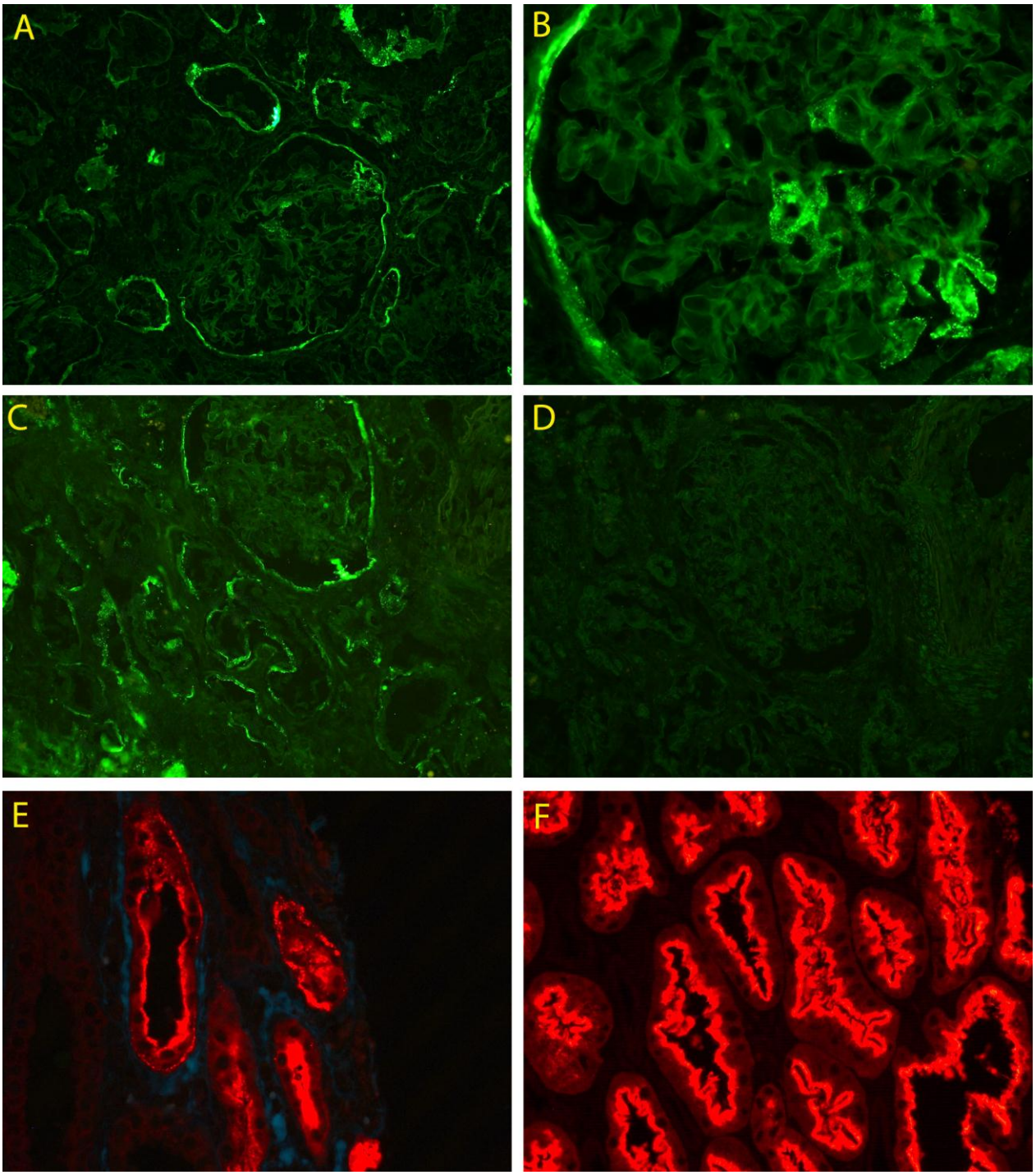
To distinguish further between the candidates for brush border antigen, we utilized a second mass spectrometric approach. In order to find the smallest immunoreactive band of this putative brush border antigen that could be used for further immunoprecipitation experiments, we conducted a partial proteolysis of the tubular extract with trypsin. The tubular extract was incubated with increasing amounts of trypsin (cell culture grade, starting concentration 0.25%) for 15 min at 37°C prior to heat denaturation and Western blotting. When immunoblotted with serum from patient #3, reactive bands are clearly observed at about 140 kDa, 120 kDa, and 95 kDa (Supplementary Figure 2, left). Upon longer exposure (120 sec; Suppl. Fig. 2, middle), an even smaller 60 kDa immunoreactive fragment was observed. A separate ABBA patient serum (Patient #5) was more reactive with this 60 kDa fragment (Suppl. Fig. 2, right), and thus both sera were used for the following immunoprecipitation analysis.

Representative histopathologic images of LRP2-associated nephropathy

Additional images are provided to represent the typical features of this unique clinical entity and to demonstrate the range of interstitial inflammation associated with this disorder (Supplementary Figures 8 - 10).

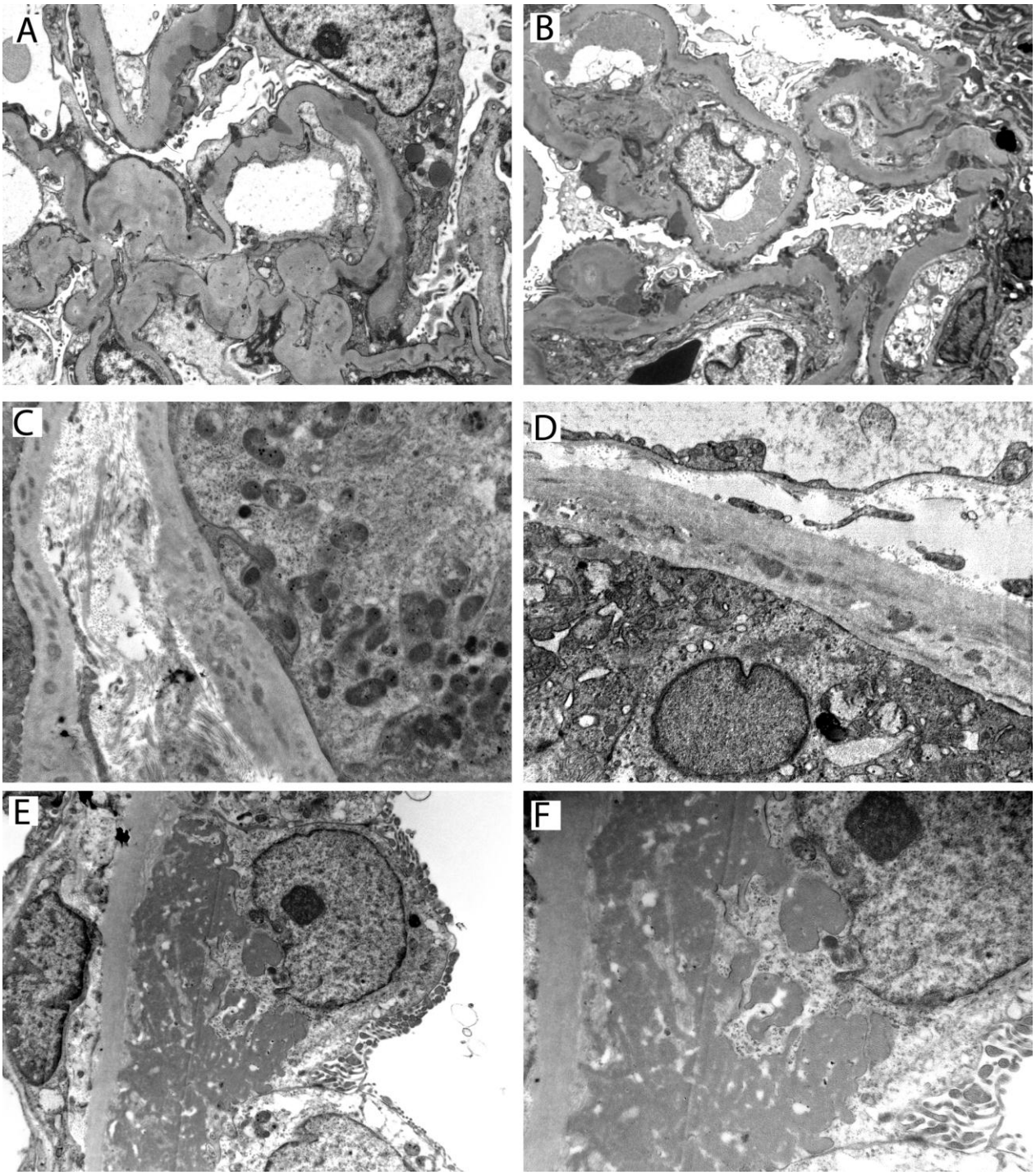


Supplementary Figure 1: Light microscopic photomicrographs demonstrating the histopathologic spectrum of LRP2-associated nephropathy. (A and B) All cases showed evidence of tubular injury with variable degrees of interstitial inflammation. Most biopsies were similar to patient #1 pictured in panel A, with interstitial edema and minimal inflammation (H&E). Others, such as patient #10 pictured in Panel B, had a patchy interstitial inflammatory infiltrate rich in plasma cells. (H&E). (C and D) Most glomeruli in biopsies from ABBA patients had evidence of segmental basement membrane 'holes' as demonstrated here in patients #10 (C) and #3 (D) (Jones methenamine silver). (E and F) There were focal tubular profiles present in some of the biopsies with deposits visible along the tubular basement membranes by light microscopy. Eosinophilic deposits are seen along apical aspect of the tubular basement membrane in this Jones methenamine silver stained section from patient #9 (E). Similarly, fuchsinophilic deposits are seen in this silver methenamine-Masson trichrome stained section along apical aspect of the tubular basement membrane in patient #2 (F).



Supplementary Figure 2: Immunofluorescence microscopy findings in LRP2-associated nephropathy. (A-E) Representative photomicrographs from patient #10. (A and B) Granular IgG staining is present along tubular basement membranes, Bowman's capsule, and segmentally within the glomerular basement membranes. C3 is positive in the same pattern as the IgG (C)

while C1q is completely negative in the tissue (D). The LRP2 stain using the mouse monoclonal antibody shows granular staining along the proximal tubular basement membranes (E). By comparison, an LRP2 stained section from a patient with lupus nephritis using the same antibody is completely negative in the proximal tubular basement membranes (F).

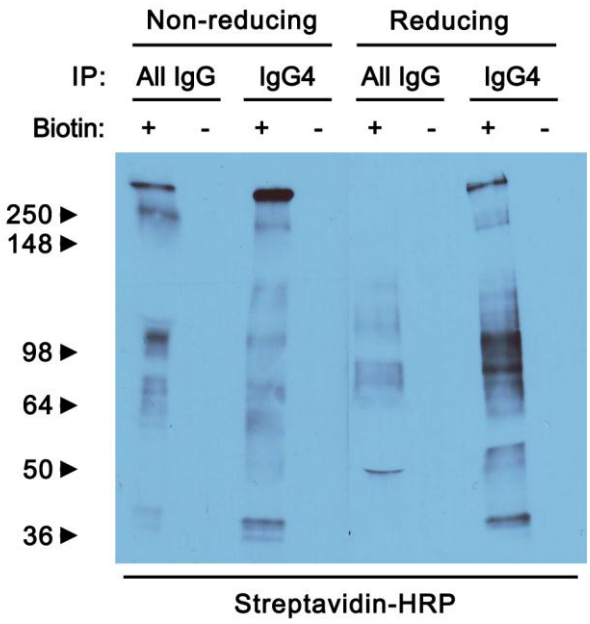


Supplementary Figure 3: Ultrastructural features of LRP2-associated nephropathy. (A and B) Glomerular basement membranes with segmental large subepithelial deposits similar to those pictured in patients #8 (A) and #1 (B) were identified in most biopsies. (C and D) Tubular basement membranes with intramembranous electron dense deposits were easily found in all cases. Examples from patient # 6 (C) and #7 (D) are shown here. (E and F) Some biopsies had

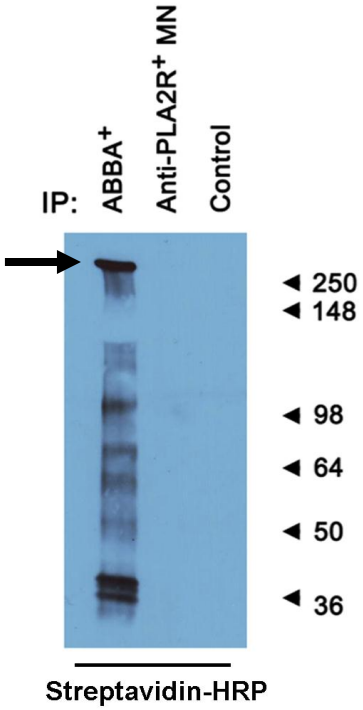
evidence of large electron dense deposits along the apical aspect of the tubular basement membrane as shown here in patient #9.

Biotinylation and Immunoprecipitation of Brush Border Antigen

To assess if ABBA (+) serum could specifically immunoprecipitate the high molecular weight antigen identified by Western blot and to ensure that we were investigating a surface exposed antigen, we incubated serum from patient #3 with an extract of surface biotinylated HTE (EZ-link NHS-Biotin, Thermo Fisher) and then captured either total IgG (protein G beads) or the IgG4 subclass alone (Capture Select anti-human IgG4 beads). The immunoprecipitates were run by SDS-gel electrophoresis, transferred to nitrocellulose, and biotinylated products were detected using horseradish peroxidase-conjugated streptavidin. The IgG4 IP yielded a biotinylated product that retained its > 250 kDa size when run under both non-reducing and reducing conditions (Supplementary Figure 1A). This band was not seen when biotinylated extract was immunoprecipitated with sera from normal controls or from a patient with PLA2R-associated membranous nephropathy (Supplementary Figure 1B).



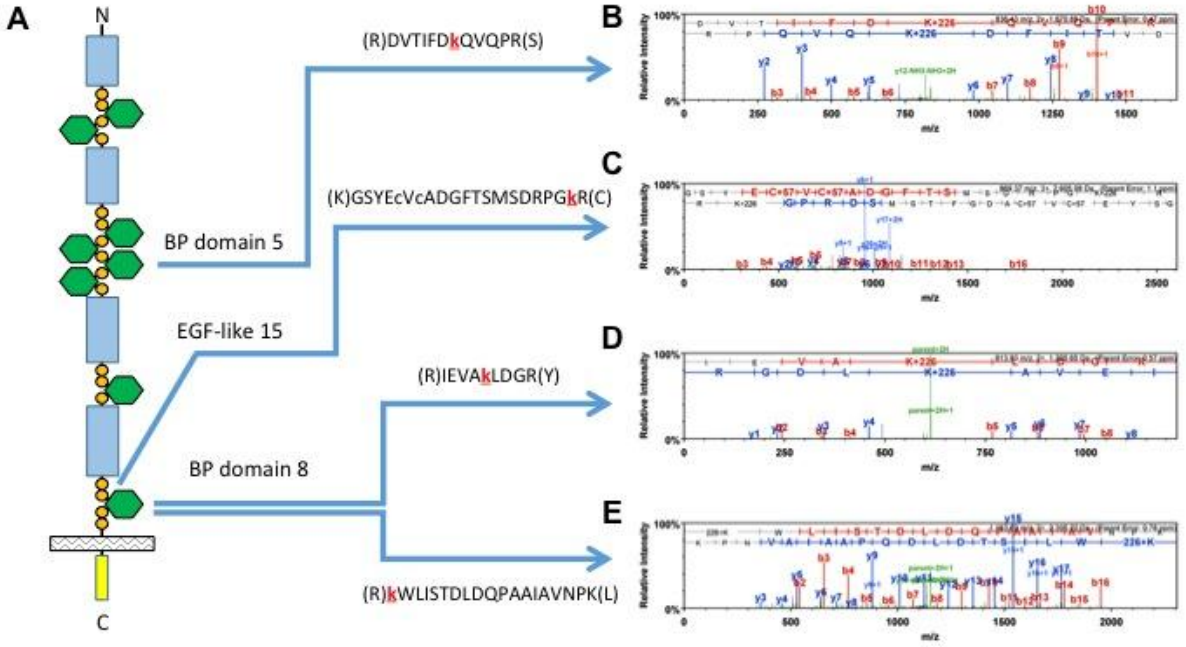
A.



B.

Supplementary Figure 4: Brush border antigen IP. Western blot with streptavidin-HRP, 1:2500. (A) Initial IP of HTE (either biotinylated or non-biotinylated) immunoprecipitated with IgG or IgG4 from ABBA-positive serum. Each IP was run in duplicate, under reducing and non-reducing conditions. (B) IP of biotinylated human tubular extract (HTE) using ABBA and control sera. Lane 1: IP with ABBA+ IgG4; the relevant band reflecting the biotinylated target antigen is indicated by arrow. Lane 2: IP with α-PLA2R+ IgG4. Lane 3: IP with normal human IgG4. The immunoprecipitation was performed using Capture Select anti-human IgG4 beads, which selectively pull down human IgG4 and associated antigens.

Under the assumption that the native, non-biotinylated antigen would be immunoprecipitated using the same protocol, and that its position would be maintained under reducing gel electrophoresis conditions, we ran identical gels with immunoprecipitates from biotinylated HTE (ABBA serum only) and from non-biotinylated HTE (ABBA and control serum). One gel was transferred to nitrocellulose and detected with streptavidin-peroxidase to localize the antigen while the identical gel was temporarily stored at 4°C. Ultimately, the gel regions from all three IP reactions corresponding to the size of the antigen under reducing conditions were excised and sent for mass spectrometric analysis. LRP2/megalin emerged as the leading candidate protein for the target of anti-brush border antibodies. In addition, mass spectrometric analysis of the immunoprecipitates using ABBA+ serum and the biotinylated human tubular extract showed evidence of biotinylated peptides from LRP2/megalin (see Supplementary Figure 3), consistent with our previous knowledge that this brush border antigen could undergo surface biotinylation.



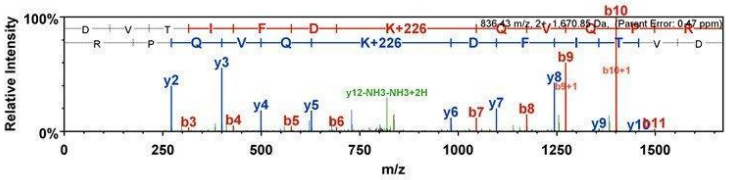
Supplementary Figure 5: Domain structure of human LRP2, localization of biotinylated LRP2 tryptic peptides, and associated MS^2 spectra. (A) LRP2 (megalin) is a single-pass type I membrane glycoprotein containing multiple protein domain structures including sets of low-density lipoprotein receptor class A (LA) repeats (blue rectangles), epidermal growth factor-like (EGF-like; orange circles), and six-bladed beta-propeller, TolB-like (6BP-TolB; green hexagons), in addition to a transmembrane region and cytoplasmic domain (yellow). Biotinylated peptides were observed from several distinct LRP2 domains including one (B) from the 5th 6BP domain, one (C) from the 15th EGF-like domain, and two (D, E) from the 8th 6BP domain. Each red 'K' in the peptide sequence denotes the lysine residue found to be covalently modified with a biotin moiety; all are present in the extracellular region of LRP2. Spectral assignment data, fragment sequence ion coverage, and spectrum error models are provided as separate figures below (Supplementary Figures 11 - 14).

Supplementary Figure 6: Amino acid sequence coverage for human LRP2_HUMAN (P98164) from immunopurified and biotinylated ABBA target antigen. Proteomic data identified 196 exclusive unique LRP2 peptides associated with 310 exclusive unique spectra resulting in a 48% LRP2 sequence coverage (yellow highlighted sequence). The observed biotinylated peptides are bolded and underlined with site specific placement of biotinylation group denoted by red font K

Supplementary Figure 7: Biotinylated peptide with sequence DVTIFDk(biotin +226 Da)QVQPR (A) assigned MS^2 spectrum. (B) Sequest HT and Mascot Server scoring statistics. (C) MS^2 fragment ion sequence coverage. (D) MS^2 spectrum error model.

LRP2/Megalin Biotinylated Peptide 1

A:

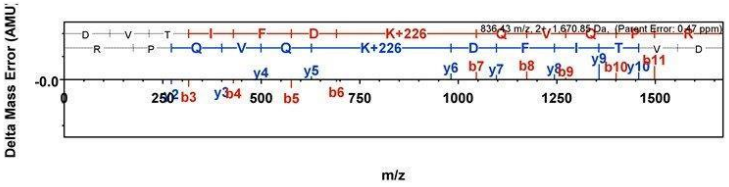


B: **Peptide** : DVTIFDkQVQPR **Protein confidence** 100% **Charge State** (z): +2 **SequestHT: Xcorr** (3.92), **δCn** (0.40) **Mascot: Ion Score** (46.71), **Identity Score** (34.02), **Delta Ion Score** (23.7) **Modification**: Biotin (K+226)

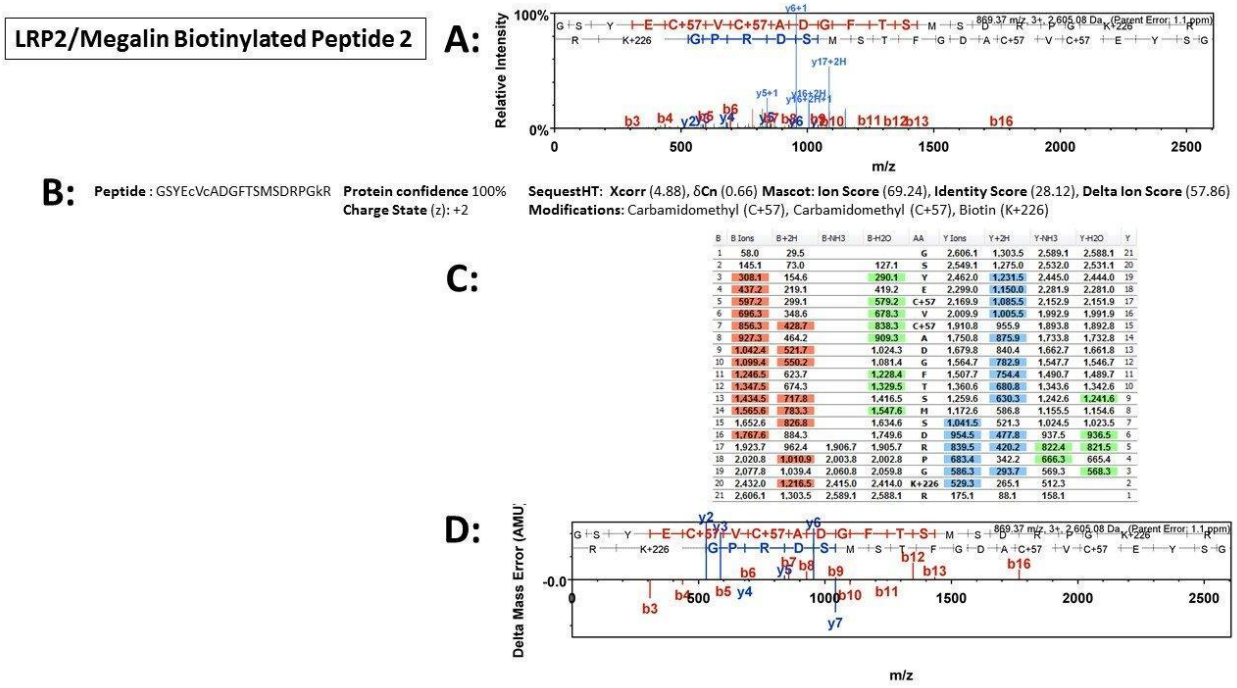
C:

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	116.0			98.0	D	1,671.9	836.4	1,654.8	1,653.8	12
2	215.1			197.1	V	1,556.8	778.9	1,539.8	1,538.8	11
3	316.2			298.1	T	1,457.8	729.4	1,440.7	1,439.7	10
4	429.2			411.2	I	1,356.7	678.9	1,339.7	1,338.7	9
5	576.3			558.3	F	1,243.6	622.3	1,226.6	1,225.6	8
6	691.3	346.2		673.3	D	1,096.6	548.8	1,079.5	1,078.5	7
7	1,045.5	523.3	1,028.5	1,027.5	K+226	981.5	491.3	964.5		6
8	1,173.6	587.3	1,156.5	1,155.6	Q	627.4		610.3		5
9	1,272.6	636.8	1,255.6	1,254.6	V	499.3		482.3		4
10	1,400.7	700.8	1,383.7	1,382.7	Q	400.2		383.2		3
11	1,497.7	749.4	1,480.7	1,479.7	P	272.2		255.1		2
12	1,671.9	836.4	1,654.8	1,653.8	R	175.1		158.1		1

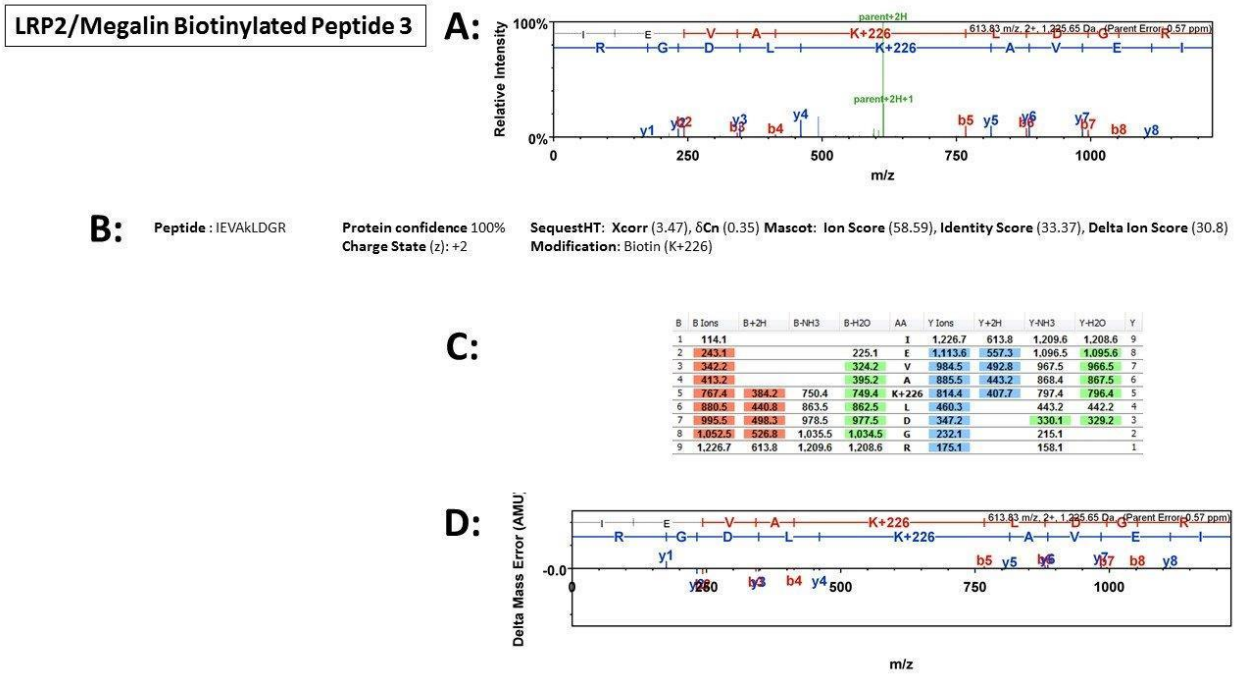
D:



Supplementary Figure 8: Biotinylated peptide with sequence GSYEc(carbamidomethyl +57Da)Vc(carbamidomethyl +57Da)ADGFTSMSDRPGk(biotin +226 Da)R (A) assigned MS^2 spectrum. (B) Sequest HT and Mascot Server scoring statistics. (C) MS^2 fragment ion sequence coverage. (D) MS^2 spectrum error model.



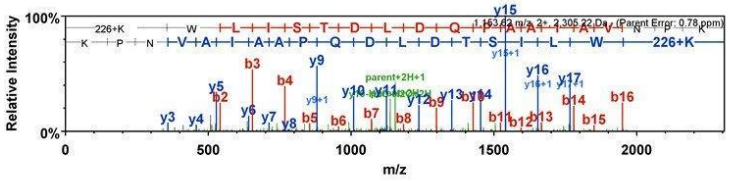
Supplementary Figure 9: Biotinylated peptide with sequence IEVAk(biotin +226 Da)LDGR (A) assigned MS^2 spectrum. (B) Sequest HT and Mascot Server scoring statistics. (C) MS^2 fragment ion sequence coverage. (D) MS^2 spectrum error model.



Supplementary Figure 10: Biotinylated peptide with sequence k(biotin +226 Da)WLISTDLDQPAAIAVNPK (A) assigned MS^2 spectrum. (B) Sequest HT and Mascot Server scoring statistics. (C) MS^2 fragment ion sequence coverage. (D) MS^2 spectrum error model.

LRP2/Megalin Biotinylated Peptide 4

A:

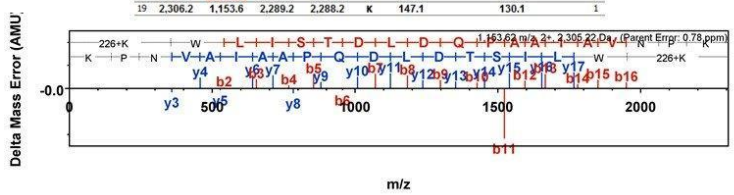


B: **Peptide :** kWLISTDLDQPAAIAVNPK **Protein confidence** 100% **SequestHT: Xcorr** (6.18), **δCn** (0.64) **Mascot: Ion Score** (109.81), **Identity Score** (32.73), **Delta Ion Score** (90.0)
Charge State (z): +2 **Modification:** Biotin (K+226)

C:

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	355.2	178.1	338.2		K+226	2,306.2	1,153.6	2,289.2	2,288.2	19
2	541.3	271.1	524.2		W	1,952.0	976.5	1,935.0	1,934.0	18
3	654.3	327.7	637.3		L	1,766.0	883.5	1,748.9	1,748.0	17
4	767.4	384.2	750.4		I	1,652.9	826.9	1,635.9	1,634.9	16
5	854.5	427.7	837.4	836.4	S	1,539.8	770.4	1,522.8	1,521.8	15
6	955.5	478.3	938.5	937.5	T	1,452.8	726.9	1,435.7	1,434.8	14
7	1,070.5	535.8	1,053.5	1,052.5	D	1,351.7	676.4	1,334.7	1,333.7	13
8	1,183.6	592.3	1,166.6	1,165.6	L	1,236.7	618.9	1,219.7	1,218.7	12
9	1,298.6	649.8	1,281.6	1,280.6	D	1,123.6	562.3	1,106.6	1,105.6	11
10	1,426.7	713.9	1,409.7	1,408.7	Q	1,008.6	504.8	991.6		10
11	1,523.8	762.4	1,506.7	1,505.7	P	880.5	440.8	863.5		9
12	1,594.8	797.9	1,577.8	1,576.8	A	783.5	392.2	766.4		8
13	1,665.8	833.4	1,648.8	1,647.8	A	712.4	356.7	695.4		7
14	1,778.9	890.0	1,761.9	1,760.9	I	641.4	321.2	624.4		6
15	1,850.0	925.5	1,832.9	1,831.9	A	528.3		511.3		5
16	1,949.0	975.0	1,932.0	1,931.0	V	457.3		440.3		4
17	2,063.1	1,032.0	2,046.0	2,045.1	N	358.2		341.2		3
18	2,160.1	1,080.6	2,143.1	2,142.1	P	244.2		227.1		2
19	2,306.2	1,153.6	2,289.2	2,288.2	K	147.1		130.1		1

D:



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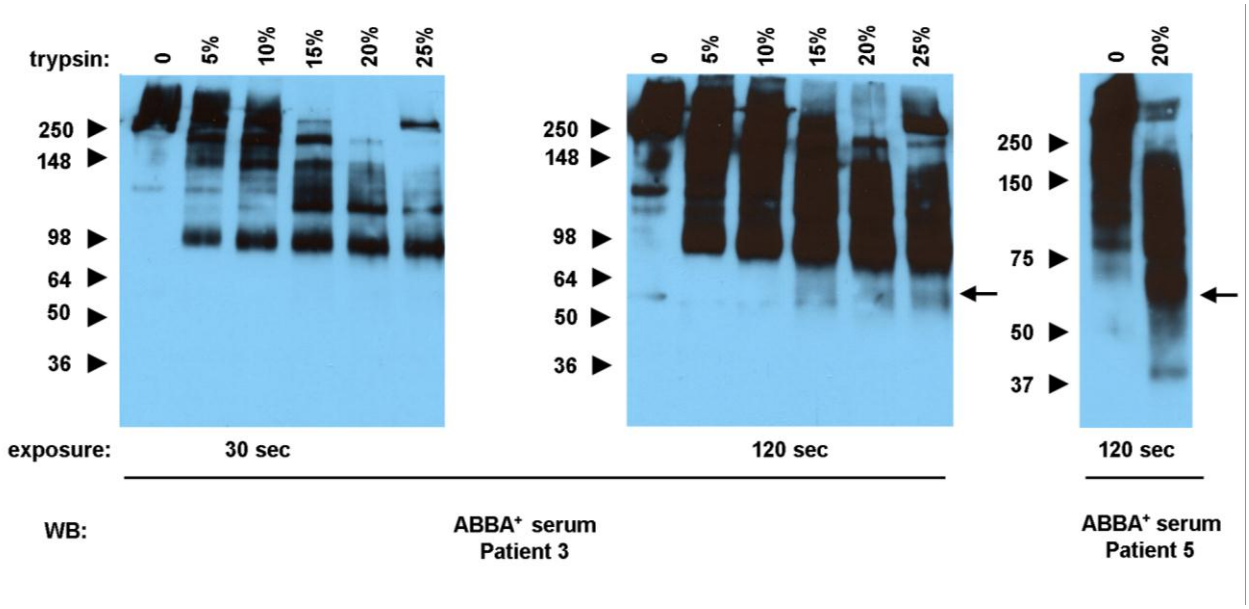


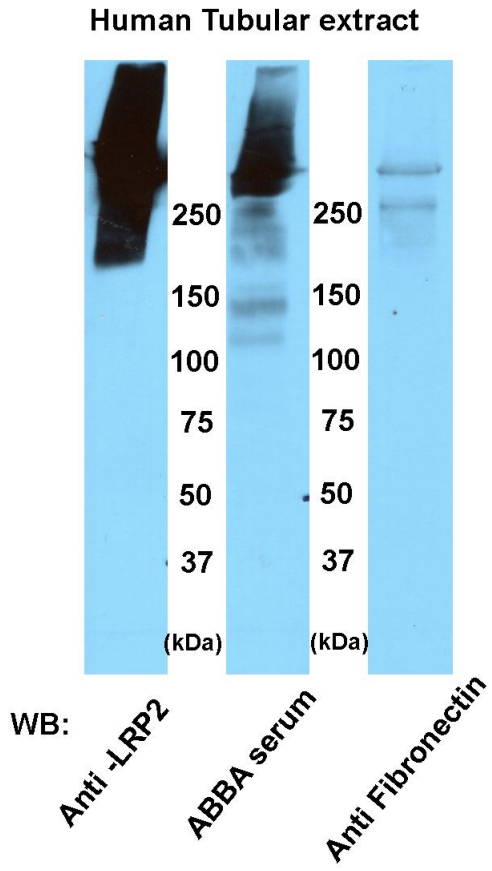
Supplementary Figure 11: Partial proteolysis of brush border antigen with trypsin. HTE proteolyzed with increasing amounts of trypsin was immunoblotted with serum from ABBA-positive patient #3 (1:100; left and middle panels) and #5 (1:200; right panel) and detected for IgG4. The arrows indicate the position of a discrete, approximately 60 kDa proteolytic fragment of the antigen that retains reactivity with both ABBA sera.

Immunoprecipitation of partially-proteolyzed HTE was carried out using serum from ABBA-positive patients #3 and #5, as well as with a control serum, using anti-human IgG4 Capture Select beads. The immunoprecipitates were gel electrophoresed under non-reducing conditions and the 60 kDa gel region cut from the three lanes, and sent for mass spectrometric analysis. Similar to the initial IP (see Supplementary Figure 4), the predominant mass spectra identified in this experiment were from LRP2 (Supplementary Figure 5), while the control IP did not contain any such spectra. It should be noted that several regions of the protein were identified, mostly within the beta-propeller and EGF-like domains, suggesting that more than one 60 kDa region of the partially-proteolyzed protein may have been immunoprecipitated by this serum. These composite results strongly suggest that the brush border autoantigen in these cases is very likely LRP2/megalin.

Supplementary Figure 12: Amino acid sequence coverage for human LRP2_HUMAN (P98164) from immunoprecipitates of partially proteolyzed HTE with ABBA-positive vs. control serum. Proteomic data identified a total 17 exclusive unique LRP2 peptides associated with a total of 18 exclusive unique spectra resulting in a total of 5% LRP2 sequence coverage (data from patient #5 represented by yellow and green highlighted sequences; single peptide from patient #3 in green).

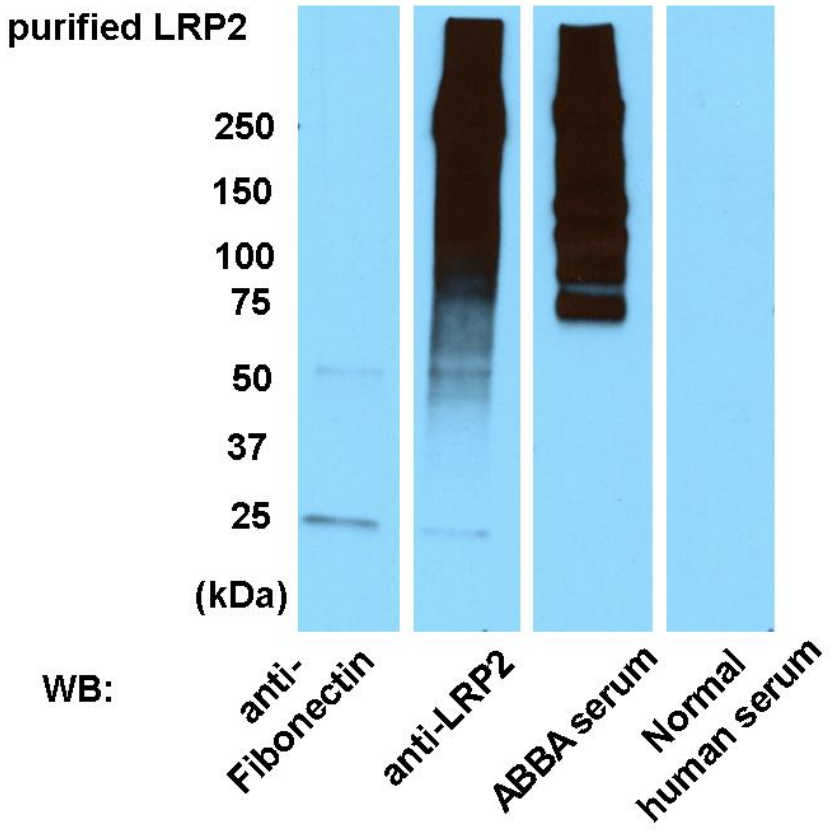
Exclusion of fibronectin as a target antigen in ABBA disease

Fibronectin isoform 3 (predicted molecular mass 259 kDa) was enriched 151-fold in the IP from ABBA patient #3 compared to that of a control IP (Supplementary Table 4). Even though fibronectin was not detected in the second mass spectrometry analysis that used trypsin proteolyzed tubular extract as starting material (Supplemental Figure 5, above), it was nevertheless important to exclude fibronectin as a target antigen. We used several methodologies, described below, to determine if the autoantibodies in ABBA disease specifically recognize human fibronectin.



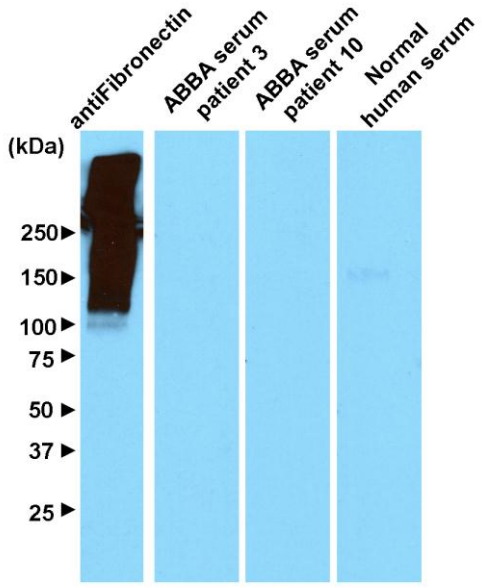
Supplementary Figure 13: ABBA serum recognizes high molecular weight bands on a Western blot of tubular extract in a pattern consistent with those produced by anti-LRP2 and not by anti-fibronectin. HTE was run under non-reducing conditions in all lanes and blotted with and a mouse anti-human LRP2 mAb (EMD Millipore; dilution 1:1000), human ABBA serum (dilution

1:50) followed by sheep anti-human IgG1 (dilution 1:4000), or a mouse anti human fibronectin mAb (Sigma; dilution 1:250). The additional bands in the 100-150 kDa region detected by human ABBA IgG1 likely represent degradation fragments of LRP2 that are detected by one or more human anti-LRP2 autoantibodies and do not contain the specific epitope in the C-terminal half of the protein that is recognized by the anti-LRP2 mAb. In contrast to the extended, high molecular weight region recognized by anti-LRP2 and ABBA serum, fibronectin, as detected by the anti-human fibronectin mAb, appears as a 250kDa band, with an additional upper band that seems to result from disulfide bond-dependent interactions, since this upper band disappears under reducing condition (not shown).



Supplementary Figure 14: ABBA serum, but not anti-fibronectin, recognizes LRP2 that has been partially purified using receptor-associated protein (RAP). RAP coupled to anti-FLAG beads was used to partially purify human LRP2 from tubular extract, and the eluate from these beads was run by gel electrophoresis. Due to the trace amounts of the mouse anti-FLAG mAb that became uncoupled from the beads and appeared in the eluate, the product was run under reducing conditions in the first two lanes to reduce mouse IgG to its heavy and light chains (seen as faint bands near 50 and 25 kDa). The eluate was run under non-reducing conditions for blotting with human serum, due to the reduction sensitivity of ABBA sera for their target antigen. Western blotting of the partially purified human LRP2 yields a strong high molecular weight signal, as expected, with the anti-LRP2 mAb (EMD Millipore; dilution 1:1000), whereas there is no signal using the anti-fibronectin mAb (Sigma; dilution 1:250). Western blotting with human ABBA serum (1:50) followed by detection of IgG4 (The Binding Site, 1:3000) reveals high molecular weight bands very similar to those detected by anti-LRP2. Control serum under indentical conditions does not detect any proteins in the RAP partially-purified product.

Additionally, we have purchased human fibronectin protein (R&D Systems, catalog # 1918-FN) and Western blotted 0.5 µg of the protein per lane with anti-fibronectin mAb (Sigma), ABBA serum from patients 3 and 10, and a control serum. Only the anti-fibronectin mAb recognized human fibronectin; ABBA and control sera were not reactive.



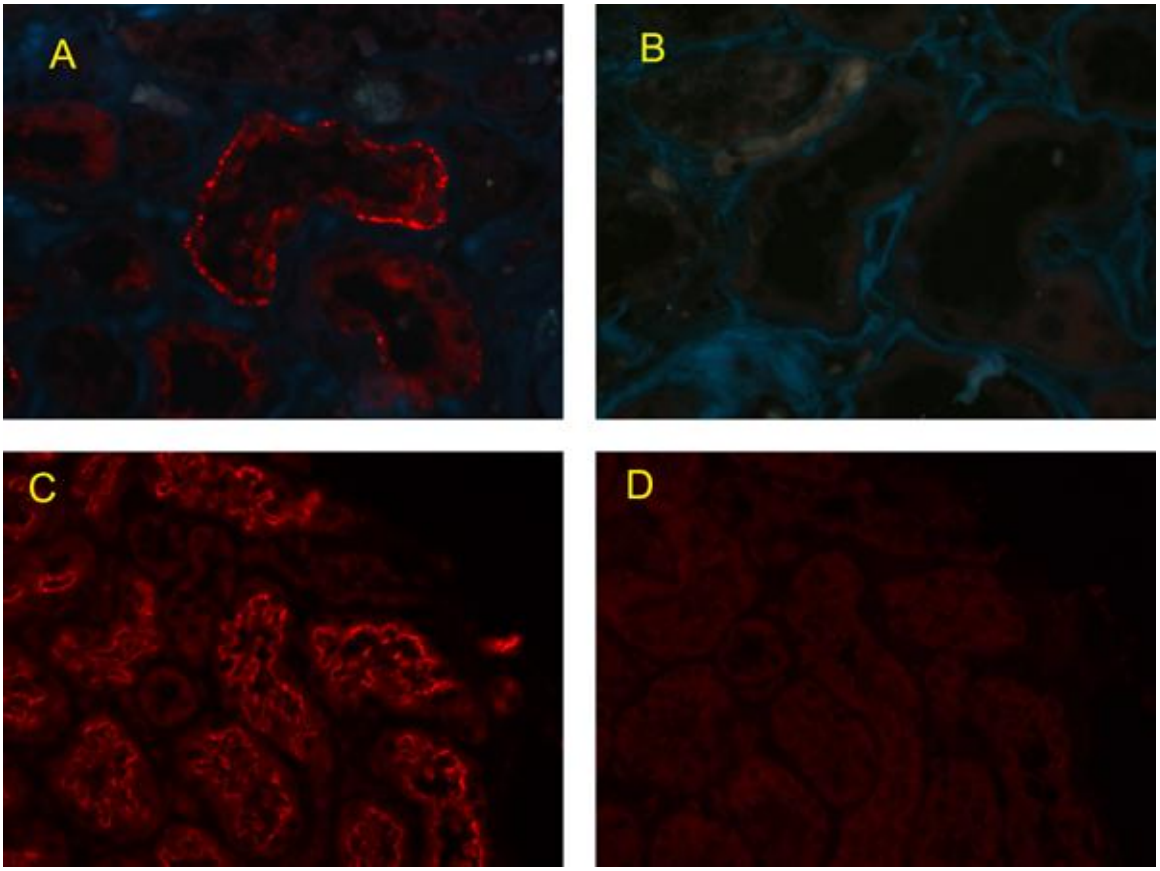
Supplementary Figure 15: ABBA sera do not recognize human fibronectin by Western blot.

0.5 µg per lane of human fibronectin was electrophoresed under non-reducing conditions and immunoblotted with anti-human fibronectin (Sigma, F0916), ABBA sera (patients 3 and 10), or normal control human serum. Neither of the ABBA sera is reactive with fibronectin.

These experiments lead us to believe that the fibronectin detected by mass spectrometry after immunoprecipitation is a co-precipitate and is not the primary ABBA antigen.

Colocalization of megalin and IgG antibodies in patients with ABBA

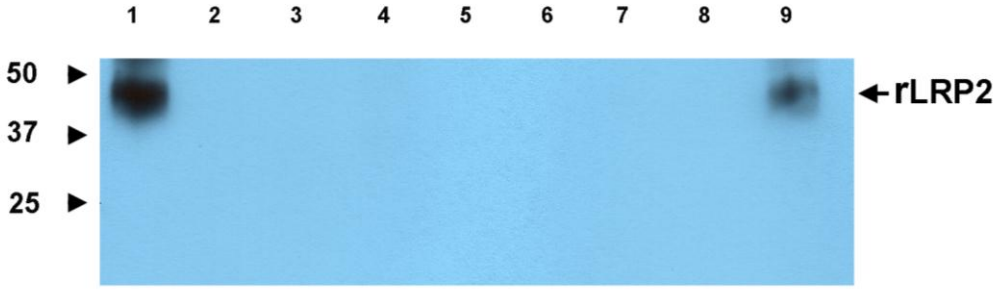
Two commercial antibodies targeting different epitopes of human megalin protein (C-terminal and internal) were used for co-localization experiments. A polyclonal rabbit antibody raised against the C-terminal portion of the human megalin protein (ab101011, Abcam, Cambridge, MA; targeting amino acids 4447 – 4655 of human LRP2) showed strong positive staining along the brush border of proximal tubules in sections from normal kidney, which precisely colocalized with the staining pattern of human sera from patients with ABBA (Figure 3). However, this particular commercial antibody was unable to recognize the antigen within the immune deposits in the tubular basement membranes of biopsy specimens from patients with this disorder. However, a mouse monoclonal antibody (MABS489, EMD Millipore, Billerica, MA) directed against an internal portion of the protein (aa 2825-2955) within the third set of LA repeats showed positive granular staining in the tubular basement membrane deposits in all 10 ABBA cases (Supplementary Table 2). As shown through confocal analysis of kidney sections from patient 5 with ABBA (Figure 3 in main article), megalin colocalizes with IgG in the tubular basement membrane deposits. An isotype control antibody was negative in tubular basement membrane deposits (not shown). The tubular basement membrane staining pattern was unique to cases with ABBA as the megalin stain was completely negative in the tubular basement membranes from all 40 control samples that had positive tubular basement membrane deposits as well as 10 additional control cases without tubular basement membrane deposits. We further assessed the specificity of the monoclonal anti-LRP2 by showing that pre-incubating the antibody with cell extracts expressing the third set of LA repeats from human LRP2 could completely abolish the signal within TBM deposits and the brush border, while pre-incubation with cell extracts expressing a portion of the first set of LA repeats did not affect the signal (Supplementary Figure 6).



Supplementary Figure 16: LRP2 staining in TBM deposits and brush border is specifically blocked by third set of LA repeats in LRP2. (A) Mouse monoclonal anti-LRP2 antibody shows granular tubular basement membrane staining in a renal biopsy from a patient with anti-brush border antibody disease when admixed with HEK293 cell extract expressing a portion of the first set of LRP2 LA repeats. (B) The tubular basement membrane staining is blocked when similar cell extract expressing the third set of LA repeats (which contains the epitope recognized by the mAb) is present. (C and D) Similarly, normal kidney shows staining of the proximal tubule brush border when the LRP2 antibody is admixed with cell extract expressing the first set of LA repeats (C) but is blocked by cell extract expressing the third set of LA repeats (D).

Expression of recombinant LRP2 in HEK293 cells

All four ligand binding domains of LRP2/megalin, in full or in part, were successfully expressed in HEK293 cells with C-terminal 3XFLAG tags: aa 1-311, containing 7 LDL receptor class A (LA) repeats; aa 1026-1350, containing 8 LA repeats; aa 2700-3111, containing 10 LA repeats; and aa 3509-3820, containing the first 7 of 11 LA repeats. Expression was verified by running cell extracts by Western blot and detecting with an anti-FLAG mouse monoclonal antibody. The third set of LA repeats contains the epitope recognized by the commercial anti-human LRP2 monoclonal antibody, and was detected by this antibody on Western blot (not shown). The N-terminal fragment was specifically recognized by $ABBA^{+}$ sera but not by control sera (Supplementary Figure 7). Control sera were also not reactive other recombinant fragments $rLRP2_{(LA8-15)}$ and $rLRP2_{(LA16-25)}$.



Supplementary Figure 17: ABBA+ sera (Lanes 1 and 9) recognize the recombinant N-terminal LRP2 fragment whereas sera from patients with other kidney diseases such as membranous nephropathy or membranous lupus nephritis (lanes 2 to 8) do not. An extract from HEK293 cells expressing the N-terminal set of seven LA repeats from human LRP2 was run in all lanes.

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Kidney Int 2017;91:501-11.