

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Alimohammadi M, Björklund P, Hallgren Å, et al. Autoimmune polyendocrine syndrome type 1 and NALP5, a parathyroid autoantigen. *N Engl J Med* 2008;358:1018-28.

Supplemental Appendix

Full text version of the Methods

Construction and screening of cDNA library

Using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was isolated from normal human parathyroid tissue removed during thyroid surgery. Messenger-RNA was purified using Poly (A) Purist (Ambion Inc, Austin, TX, USA) and the purity and concentration of the mRNA was determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A cDNA expression library was constructed in the Zap Express vector (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The library was immunoscreened with sera (diluted of 1:2000) from APS I patients with hypoparathyroidism. Clones recognized by patient antibodies were isolated and sequenced.

Diagnosis of other APS-1 associated disease components

Hypogonadism was diagnosed by high FSH with low estradiol or testosterone. Insulin-dependent diabetes mellitus was diagnosed by measurement of fasting plasma glucose concentration and fasting C-peptide. Malabsorption was diagnosed clinically and by checking routine haematological tests. Hepatitis was diagnosed by statement of elevated levels of serum aminotransferases and histopathological findings in liver biopsy. Vitiligo and alopecia were diagnosed clinically.

PCR Expression analysis (full text version)

Real-time PCR was carried out on cDNA from human tissue using a MyiQ iCycler (Bio-Rad, Hercules, CA, USA). Human normalized multiple-tissue cDNA panels (Cat# K1421-1

Lot#2030894 and Cat#K1420-1 Lot#1100543, BD Bio Sciences, Palo Alto, CA, USA, were used and reactions were performed in a volume of 25 µl, with 300 nM of each primer and the iQ™ SYBR®Green supermix (Cat# 170-8882 Bio-Rad, Hercules, CA, USA) or QuantiTect™ SYBR®Green supermix (QIAGEN Cat# 204143) (the latter was used when measuring low copy DNA in order to reduce primer dimers) according to the manufacturer's instructions. Thermocycling conditions used an initial denaturation of 95°C for three minutes, followed by 40 cycles of 55°C for 45s and 95°C for 1 min and 55°C for 1 minute. Samples were analyzed in triplicates. After amplification, melting curve was performed to verify the specificity of the PCR reaction. The PCR analysis included a standard curve and non template negative controls. The relative amounts of the endogenous control mRNA (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) and of the gene of interest mRNA in each sample could be deduced from the standard curves.

Primer sequences

Primers were designed on the Beacon Designer® version 5.11 software

For human tissue: NALP4 5'-3': GCCTGGATTCACCTGTGTTTGG; NALP4 5'-3':

AGGTTTTGATT GCTGATGAGAGC; NALP5 5'-3':

TGTGGACTGACTTCTGATTGCTG; NALP5 5'-3': GCACAGGGTACTGCCATTTCC;

NALP6 5'-3': GTACCTGGTGGGTATG CTTCG; NALP6 5'-3':

CTGTAGTGACTGCTCGCTCAG; NALP9 5'-3': CTGATGC AGTGGTGGTGCTG;

NALP9 5'-3': TCTTCCACAGACATCAGGATCTTC; NALP10 5'-3':

ATGAACCTGTTGGAACCTTGTGGAC; NALP10 5'-3': GTATCTGCCATTGACTCCT

GCTTC; NALP11 5'-3': AATATTGGGCTAGAAGAGTGCATG; NALP11 5'-3': GTGTT

TTGTTGGTGGTAAGAACAG; GAPDH 5'-3': AGGGCTGCTTTTAACTCTGGTAA

GAPDH 3'-5': CATATTGGAACATGTAAACCATGTAGTT

Generation of antiserum to NALP5 and immunoblot analysis

Primary antibodies were raised against human NALP5 by immunization of rabbits with a keyhole limpet hemocyanin conjugated peptide (KLH-CKSLSLAGNKVTDQG). The chosen sequence (aa 897-910) displays a high homology to bovine NALP5. The antiserum obtained was subsequently affinity purified on a peptide column (Innovagen AB, Lund, Sweden). The specificity of the serum was checked by immunoblotting including inhibition of the antiserum's reactivity in the presence of the peptide used for immunization. Immunoblot evaluation of NALP5 antiserum was done on one gram of bovine parathyroid tissue or $>10^5$ human parathyroid cells were lysed in 1 ml CytoBuster™ protein extraction reagent (cat#74004 lot N51407, Novagen®, Darmstadt, Germany) supplemented with Complete Mini Protease inhibitor Cocktail™ (Roche, Mannheim, Germany). 500 µl of the lysate was diluted 1:1 with Laemmli Sample Buffer (cat# 161-0737, Bio-Rad laboratories Hercules, CA) supplemented with 50 mM dithiothreitol and denatured at 95°C for 2 minutes and separated on a 5% Criterion SDS-PAGE Tris-HCl gel (cat#345-0019, Bio-Rad laboratories Hercules, CA) and transferred onto Nitrocellulose membrane (cat#162-0167 Bio-Rad laboratories Hercules, CA). Running and transfer conditions according to the manufacturer's instructions (Bio-Rad). The blot was blocked with 5% dry milk for 30 minutes and incubated with the primary antibody (1:400-1:2000) at 4°C overnight. After washing, bound primary antibodies were detected by a HRP conjugated secondary antibody using an ECL kit (Cat#RPN2132 GE Healthcare) according to the manufacturer's instruction.

Immunohistochemistry

Human parathyroid tissue specimen removed during thyroid surgery, bovine parathyroid gland and bovine ovaries (obtained from the slaughterhouse) were fixed in PBS with 4% paraformaldehyde over night followed by paraffin embedding. Four μm sections were deparaffinized and microwave treated in citrate buffer as standard protocol. Non-specific bindings were blocked by pre-exposure of the sections for 30 minutes with PBS containing 10% normal goat serum. The slides were then incubated with the NALP5 antiserum (dilution 1:4000) or patient sera (dilution 1:8000) overnight at $+4^{\circ}\text{C}$ followed by washing and incubation with biotinylated secondary goat anti human or rabbit immunoglobulin antibody (Vector laboratories, Burlingame, CA 94010) for 30 minutes. Peroxidase conjugation was performed using the VECTASTAIN[®] ABC system (PK-6100 Vector laboratories, Burlingame, CA 94010) and the staining reaction used ChemMate[™] DAKO Envision[™] Detection kit (Dakocytomation, Glostrup, Denmark). The specificity of the immunostaining was tested in control slides by omission of the primary antibody, by using pre-immunization serum from the rabbit in which the antiserum was raised, and by blocking the primary antibody through preincubation with the peptide used for the immunization in concentrations of 10-100 nmol/ml.

Parathyroid cell culture and metabolic ³⁵S radiolabelling of parathyroid cells

Bovine parathyroid glands were collected immediately after slaughter. Fat and connective tissue was removed, and the glands were minced with scissors. Cell suspensions were prepared by digestion in 1 mg/ml collagenase (Sigma, St Louis, Missouri, USA), 0.05 mg/ml DNase I, 1.5% bovine serum albumin and 1.25 mM Ca^{2+} . After digestion in a shaking incubator for 120 min, the suspensions were filtered through nylon mesh (125 μm) and

exposed to 1 mM EGTA in 25 mM HEPES buffer (pH 7.4) containing 142 mM NaCl and 6.7 mM KCl. Debris and dead cells were removed by centrifugation through 25 and 75% standard isotonic Percoll (GE Healthcare). Cell viability, as determined by the Trypan blue exclusion test, exceeded 95%. Cells were cultured for 4 h in DMEM/Ham's F-12, 1 mM total calcium, 5% fetal bovine serum, 15 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine and 1% non-essential amino acids. Freshly isolated cells (10^7) were precultured for 30 min in methionine-free RPMI 1640. The medium was changed to methionine-free RPMI1640 containing 5% dialyzed fetal bovine serum and 0.5 mCi ^{35}S methionine (GE Healthcare) followed by incubation for 6h. The medium was removed and cells were washed 2 times with ice cold RPMI 1640 medium and 6 times with ice cold PBS. Cells were lysed on ice in a 20mM Tris-HCl buffer (pH 7.4) containing 0.15 NaCl, 1% Triton X-100, 1 mM phenylmethyl sulfonylfluoride and 1% Trasylol. Insoluble material was removed by centrifugation at 100 000 x g for 20 min at +4°C.

Immunoprecipitation was performed with different control sera, patient sera and rabbit antisera for 6h at +4°C and the bound immune complexes were captured by Fast Flow Protein A Sepharose followed by SDS-PAGE analysis.

Supplementary Results and Discussion

Verification of parathyroid NALP5 expression and immunoprecipitation studies

As a further verification of the expression of NALP5 protein in parathyroid cells, bovine parathyroid cells were radiolabeled with ^{35}S -methionine, lysed and used for immunoprecipitation (Supplemental Figure 3B). The lysate was used for sequential precipitation as described for Figure 3B. As control the lysate was also immunoprecipitated with the anti-aa 897-910 rabbit antiserum and a band with the expected molecular weight of 124 kDa for bovine NALP5 could be detected. Pre-incubation with serum from a patient recognizing NALP5 could remove this band (lane2), and when serum from a patient not recognizing NALP5 (lane3) or serum from a healthy control (lane 4) were used the NALP5 band remained.

Comparison of results from the NALP5 autoantibody assay with immunofluorescence data

We examined sera from 15 APS-1 patients with hypoparathyroidism and NALP5 autoantibodies, 9 APS-1 patients with hypoparathyroidism but without NALP5 autoantibodies, 4 APS-1 patients without hypoparathyroidism, 4 patients with isolated hypoparathyroidism, and 9 healthy blood donors by immunofluorescence. Positive immunostaining was seen in sera from: 11 of 15 APS-1 patients with hypoparathyroidism and NALP5 autoantibodies, 2 of 9 APS-1 patients with hypoparathyroidism and absence of NALP5 autoantibodies, 1 of 4 patients with isolated hypoparathyroidism and 0 of 4 APS-1 patients without hypoparathyroidism. None of the healthy blood donor sera stained parathyroid tissue. (Supplementary Table 2)

There is not a 100% correlation between the immunofluorescence results and the results from the NALP5 autoantibody assay using specific ³⁵S-labeled autoantigen. Our results are consistent with the known fact that detection of autoantibodies using specific autoantigen assay is a more sensitive and specific method than immunofluorescence. In addition, sera from all individuals in this experiment were diluted 1:500 to minimize non-specific staining and this means that sera with low autoantibody titres are diluted to below the limit of detection in the immunofluorescence assay. This is supported by the fact that 3 of the 4 sera with NALP5 autoantibodies using specific autoantigen assay did not show positive stain in the immunofluorescence test and these sera have the lowest autoantibody index in the group studied. Overall these results demonstrate that NALP5 is the main parathyroid autoantigen in APS-1. We can, however, of course not exclude existence of other minor autoantigens or markers in the parathyroid glands.

Supplementary tables

Supplementary table 1. Relative expression of NALP5 in different tissues

Tissue	Relativ NALP5 mRNA expression
Parathyroid gland	100.0
Ovary	1.6
Placenta	0.5
Brain	0.5
Pancreas	0.1

Relative expression of NALP5 mRNA in the tissue panel used in Figure 2A is indicated. Predominant expression of NALP5 was seen in the parathyroid gland while lower concentrations could also be found in ovaries, placenta, brain and pancreas.

Supplementary Table 2. Correlation of parathyroid immunostaining results and presence of NALP5 autoantibodies.

Patient sera	Presence of NALP5 autoantibodies	Positive staining pattern in immunofluorescence
APS-1 with hypoparathyroidism and NALP5 autoantibodies (n=15)	15/15	11/15 ¹
APS-1 with hypoparathyroidism , without NALP5 autoantibodies (n=9)	0/9	2/9 ²
Isolated hypoparathyroidism (n=4)	0/4	1 /4 ²
APS-1 without hypoparathyroidism	0/4	0/4
Healthy blood donors (n=9)	0/9	0/9

¹)The results indicate a high correlation between the immunofluorescence data and the results for results from the NALP5 autoantibody assay using the specific ³⁵S-labelled autoantigen.

²)Sera from 2 patients with hypoparathyroidism but without detectable NALP5 autoantibodies and a patient with isolated hypoparathyroidism give raise to positive staining pattern in immunofluorescence.

Legends to Supplementary Figures:

Supplementary Figure 1

Verification of the antiserum produced against human and bovine NALP5

A) Western blot analysis on lysate from human parathyroid cells (the proteins of the lysate were separated on a 5% SDS-PAGE prior to the blot), lane 1 antiserum dilution 1:400, lane 2 antiserum in dilution 1:800, lane 3 antiserum in dilution 1:1600, lane 4 antiserum in dilution 1:1600 preincubated with 20 nmol of the peptide used for immunization.

B) Immunoprecipitation of bovine ³⁵S radiolabeled parathyroid cell lysate with NALP5 antiserum. The migration was analyzed on a 7.5% SDS-PAGE. **C)** Immunohistochemistry on bovine ovaries using NALP5 antiserum in dilution 1:2000, red arrows indicating oocytes in a primary ovarian follicle (left part) and NALP5 antiserum in dilution 1:2000 preincubated with 20 nmol of the peptide used for immunization (right part).

Supplementary Figure 2

Immunohistochemistry on paraffin embedded bovine parathyroid sections

A) Omission of primary serum (=background). **B)** Preimmunization serum in dilution 1:800. **C)** NALP5-specific rabbit antiserum in dilution 1:1600. **D)** NALP5-specific rabbit antiserum in dilution 1:1600 preincubated overnight with 20 nmol of the peptide used for immunization. The figure demonstrates parathyroid tissue staining can be induced by immunization of a rabbit with a NALP5-specific peptide (panel C). This staining can be abolished if the rabbit serum is preincubated with the peptide used for the immunization (panel D).

Supplementary Figure 3

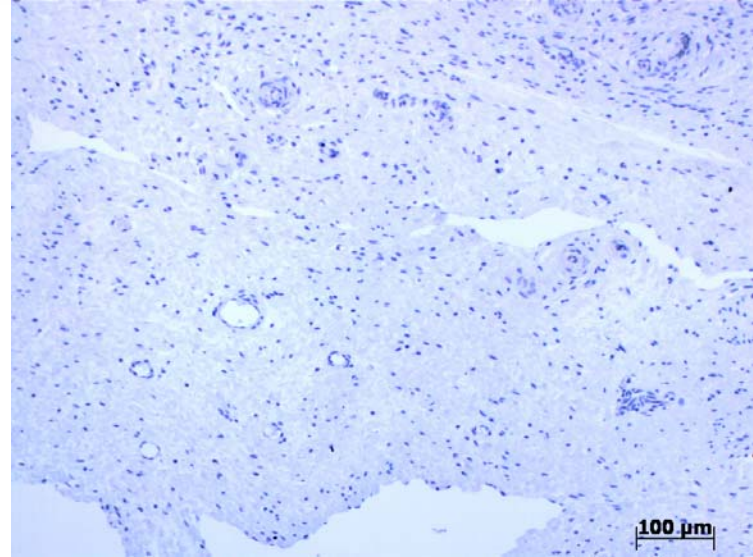
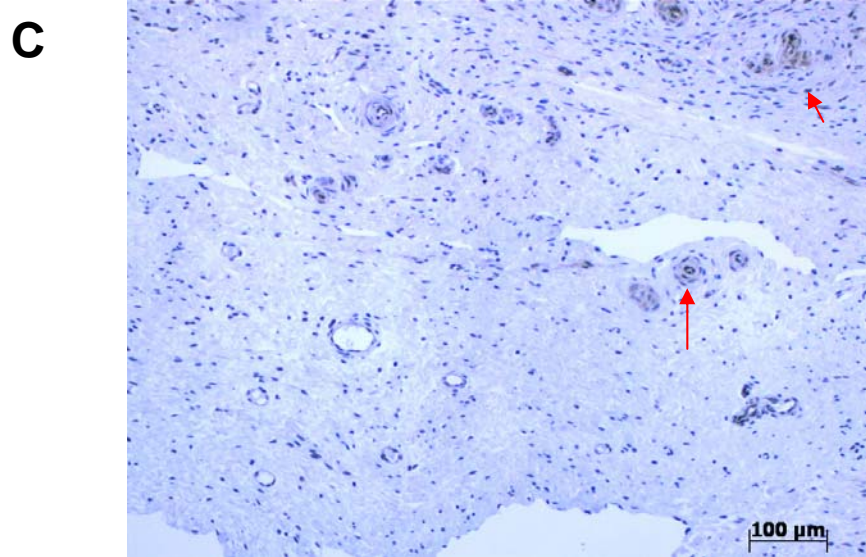
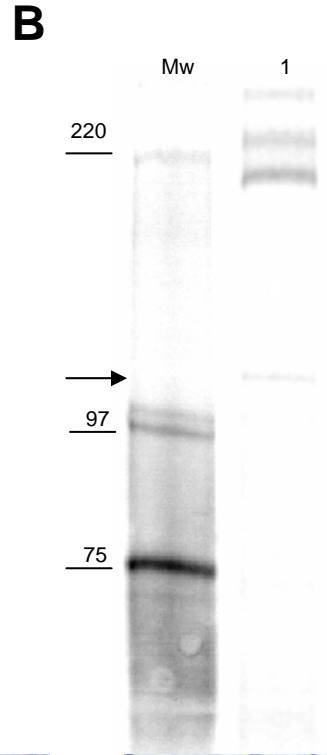
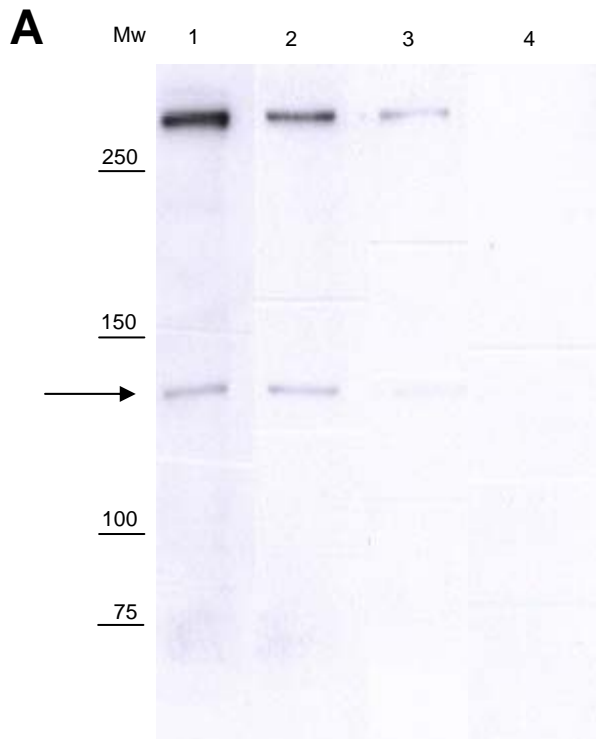
Specificity check of patients' autoantibodies by immunoprecipitation studies

A) Immunoprecipitation of in vitro transcribed and translated ^{35}S radiolabeled human NALP5 clone, encoding exons 5-14 of NALP5, isolated from the parathyroid cDNA library. Immunoprecipitates were captured by Protein-A Sepharose and evaluated on a 10% SDS-PAGE followed by autoradiography. Lane 1: immunoprecipitation with 2.5 μl of NALP5-specific antiserum, lanes 2-3: immunoprecipitation with 2.5 μl of sera from two different APS-1 patients with hypoparathyroidism and reactivity against NALP5, lanes 4-5 immunoprecipitation with 2.5 μl of sera from APS-1 patients without reactivity to NALP5, and lanes 6-7 immunoprecipitation with equal amount of healthy blood donor sera. The figure demonstrates that the NALP5 clone is bound by NALP5-specific antiserum and autoantibodies in sera from APS-1 patients with hypoparathyroidism (lanes 1-3) but not by the control sera lanes (4-7).

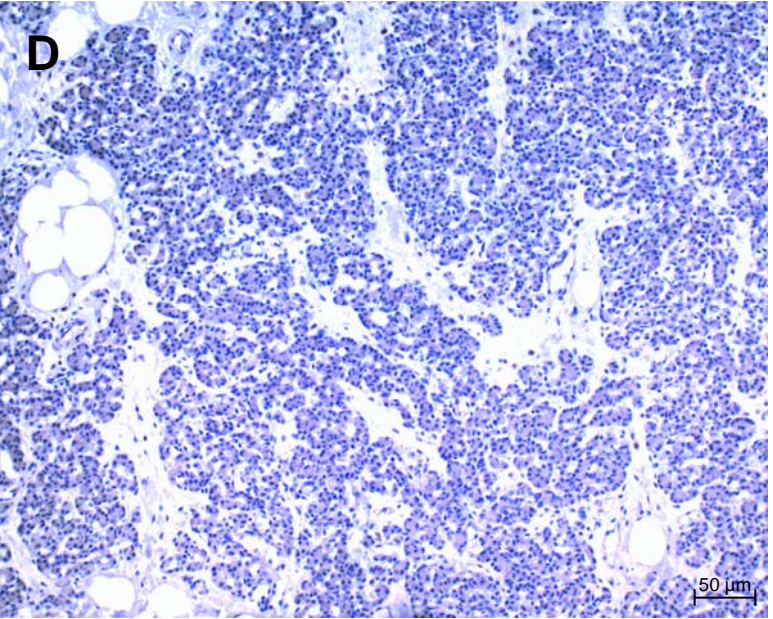
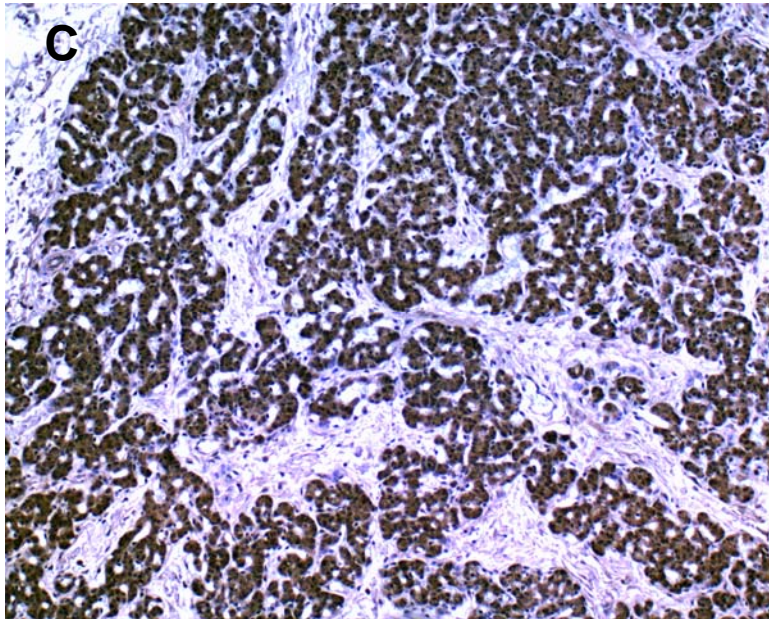
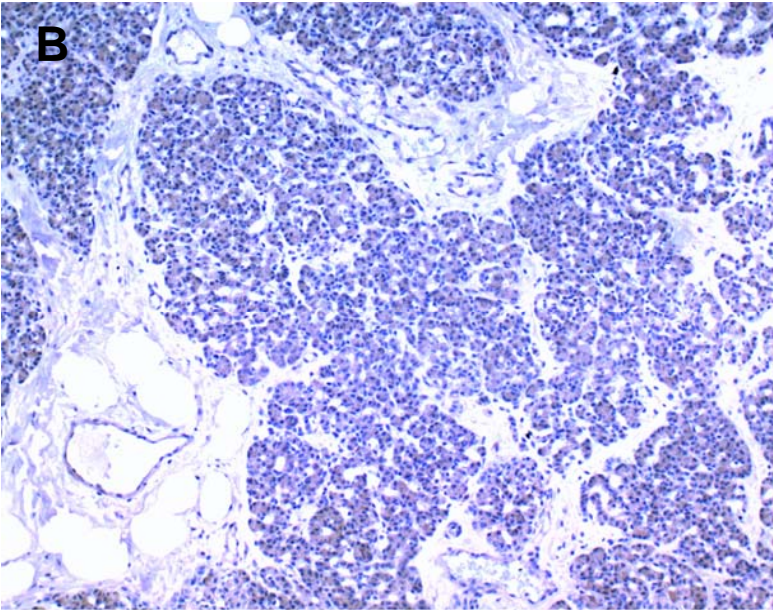
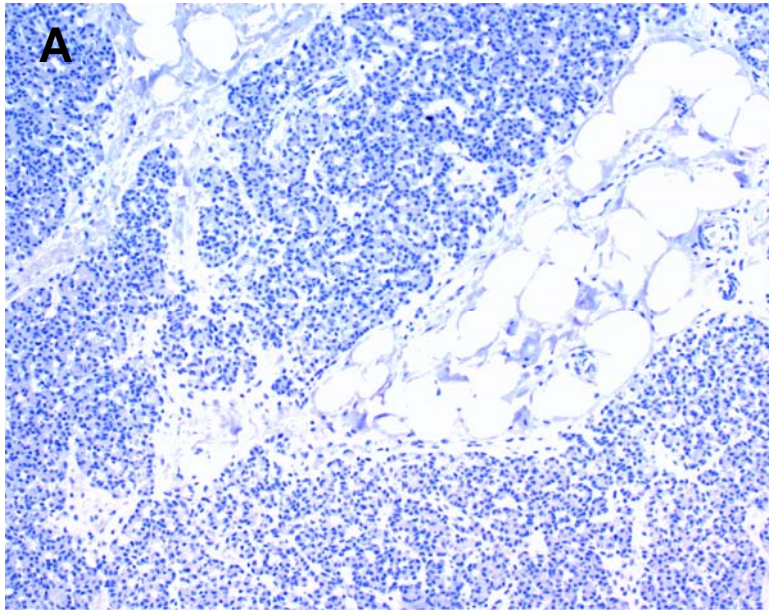
B) Sequential immunoprecipitation on cell lysates from of ^{35}S -methionine labeled bovine parathyroid cells. First immunoprecipitation step: Lane 1, no patient serum or NALP5-antiserum added; lane 2, serum from an APS-1 patient with reactivity against NALP5; lane 3, serum from an APS-1 patient without reactivity against NALP5; lane 4, serum from a healthy blood donor. The immunoprecipitates were captured by addition of protein-A Sepharose and removed by centrifugation. The remaining supernatants were subjected to a new immunoprecipitation by a NALP5-specific rabbit antiserum. The immunoprecipitates were evaluated on a 5% SDS PAGE followed by autoradiography. The figure demonstrates that bovine NALP5 can be depleted by serum from patients with NALP5 autoantibodies (lane 2), but not with sera from individuals without NALP5 autoantibodies (lanes 3-4). Lane 1 serves as control in which the total amount of bovine NALP5 remains after the first

immunoprecipitation step. The arrow indicates the expected molecular weight for bovine NALP5 (121 kDa).

Supplementary Figure 1

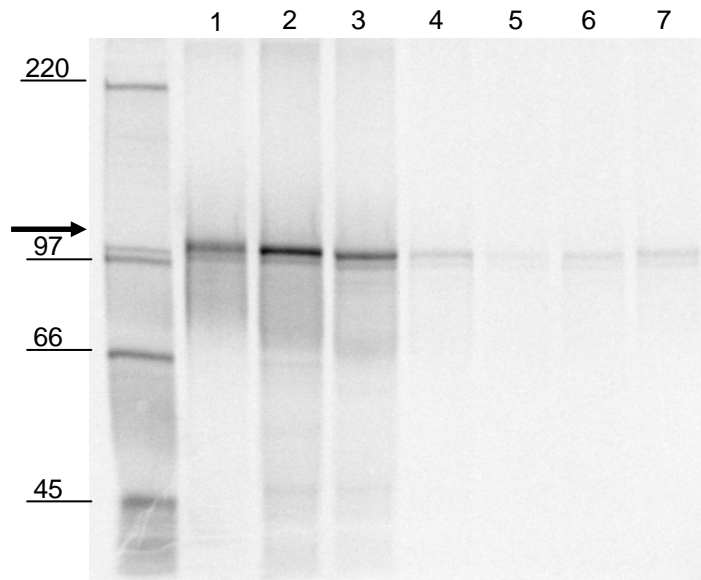


Supplementary Figure 2



Supplementary Figure 3

A



B

