# Supplementary Appendix

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

Supplement to: Yu CC, et al. Abatacept is a treatment for B7-1-positive proteinuric kidney disease

**Supplementary Clinical information**

Abatacept treatment for the four patients with recurrent FSGS (Patients 1-4) was approved by the University of Miami Institutional Review Board. The patient with primary FSGS (Patient 5) was treated with abatacept[1] in compliance with Partners Healthcare and Massachusetts General Hospital Institutional Review Board policies.

Patients 1-4

Induction immunosuppression for Patients 1 and 2 was with anti-CD25 (daclizumab 1 mg/kg x 2 doses), thymoglobulin (1mg/kg x 5 doses) and a single dose of rituximab (375 mg/m$^2$)[2]. Maintenance immunosuppression was with tacrolimus (levels 5-7 ng/ml), mycophenolate mofetil (MMF) 500 mg BID and corticosteroids. Induction immunosuppression for Patients 3 and 4 was with thymoglobulin (1mg/kg x 5 doses) and basiliximab (10 mg/kg x two doses), and a single dose of rituximab (375 mg/m$^2$). Maintenance immunosuppression was with tacrolimus (levels 5-7 ng/ml), MMF 125 to 250 mg BID and corticosteroids. A transplant ureteral stent was placed that exited through the bladder and anterior abdominal wall in order to monitor UPC of the transplanted kidney as needed, to specifically differentiate the degree of proteinuria from the native/previous kidney transplants.

Patient 5

The patient has preserved renal function. Her medications include Enalapril 10 mg twice a day, Simvastatin 40 mg daily, and Furosemide daily, titrated to severity of edema. She has no known allergies.

**Supplementary Experimental Data**

**Quantitative analysis of B7-1 and CTLA4-Ig effects on podocyte migration (Figure 3)**

This section provides detailed quantitative information about the results displayed in Figure 3 of the manuscript. Stable overexpression of B7-1 but not B7-1Δtail or empty vector control (pcDNA) increases directed podocyte migration, which is blocked by CTLA4-Ig. Similar to CTLA4-Ig, B7-1Δtail expression prevents LPS induced podocyte migration (Fig. 3C). The quantitative analysis showed the following results (see Fig. 3F, left): (n = 4 - 12; migrating cells: 53.4 ± 7.23 for pcDNA (control); 142.20 ± 12.03 for pcDNA + LPS; 146.25 ± 12.71 for B7-1; 154.50 ± 26.02 for B7-1 + LPS; 69.00 ± 7.62 for B7-1 + CTLA4-Ig; 75.25 ± 10.90 for B7-1 + LPS + CTLA4-Ig; 40.25 ± 11.97 for B7-1Δtail; 50.38 ± 12.66 for B7-1Δtail + LPS; Bonferroni's Multiple Comparison Test, *P < 0.0001).* Gene silencing of B7-1 or blockade with CTLA4-Ig blocks LPS induced motility (Fig. 3D). The quantitative analysis showed the following results (see Fig. 3F, middle): n = 4- 12; migrating cells: 53.33 ± 7.21 for wild type (con); 143.17 ± 27.61 for wild type + LPS; 54.20 ± 8.26 for scrambled; 139.60 ± 16.55 for scrambled + LPS; 56.00 ± 11.36 for B7-1KD; 50.38 ± 11.39 for B7-1KD + LPS; 56.50 ± 14.89 for wild type + CTLA4-Ig; 46.75 ± 5.91 for wild type + LPS + CTLA4-Ig; n = 4-8, (Bonferroni's Multiple Comparison Test, *P < 0.0001).* Gene silencing of B7-1 or CTLA4-Ig revert hypermotility of $\alpha 3^{-/-}$ podocytes to wild type levels (Fig. 3E). The quantitative analysis showed the following results (see Fig. 3F, right): n = 4-8, migrating cells: 156.00 ± 27.02 for $\alpha 3^{-/-}$; 188.00 ± 22.84 for $\alpha 3^{-/-}$/scrambled; 73.63 ± 12.68 for $\alpha 3^{-/-}$/B7-1KD; 61.50 ± 10.72 for $\alpha 3^{-/-}$ + CTLA4-Ig; (Bonferroni's Multiple Comparison Test, *P < 0.0001).*

## Methods

### Immunofluorescence microscopy for B7-1 in native human renal biopsies

Archival frozen renal biopsy cores were obtained by the diagnostic renal pathology service at Brigham and Women's Hospital as approved by BWH Institutional Review Board Protocol Number 2011P00269, and were analyzed by immunofluorescence microscopy. Diagnosis, patient gender, age and proteinuria are detailed in Tables 1, 2. Frozen tissues were cut in 4 μm sections and air-dried 10 minutes at room temperature. Sections were washed in PBS 10 minutes and then fixed in 95% ethanol for 10 minutes. Sections were washed in PBS twice for 5 minutes each and incubated with a goat anti-human B7-1/CD80 antibody (R&D Systems) at 1:200 dilution for 45 minutes at room temperature, washed with PBS for 10 minutes, followed by incubation with donkey anti-goat Alexa Fluor488 secondary antibody (Invitrogen) at 1:200 dilution for 35 minutes at room temperature. The sections were washed with PBS and mounted with fluorescent mounting medium. Images were taken at fixed manual exposure (200 ms) with an Olympus BX53 fluorescence microscope, and processed using Adobe Photoshop software. Immunofluorescence staining intensity was scored by a blinded renal pathologist (A.W.) from 0-4+.

### Podocyte B7-1 expression in allograft biopsies of Patients 1 and 3

Pre- and post-reperfusion allograft biopsies were examined for B7-1 expression by immunofluorescence microscopy. The pre-perfusion biopsies were taken within 30 min of clamping the renal artery and vein of the donor kidney. The post-reperfusion biopsies were taken approximately 2 hours later. Frozen sections were incubated with a goat anti-human B7-1/CD80 antibody (R&D Systems), as above, and mouse monoclonal anti-synaptopodin[3], followed by exposure to Alexa-488 secondary donkey anti-goat and Alexa-647 anti-mouse antibodies (Invitrogen). Image acquisition was performed on a Leica SP5 inverted confocal microscope.

**Cell culture and transfection and fluorescence microcopy**

HEK293 cells[4], wild type podocytes[5], $\alpha3^{-/-}$ podocytes[6,7] and K562 cells[8] were cultured as described before. GFP-fusion proteins were analyzed by direct fluorescence microscopy in living cells or after fixation and processing for immunocytochemistry[4,6,7]. B7-1 full-length and truncated cDNAs (B7-1ΔEC, B7-1Δtail and B7-1-tail) were cloned into pEGFP-N1 (BD Biosciences Clontech) and verified by DNA sequencing. Transient transfection of HEK293 cells and podocytes was done as described before[4]. For the generation of stable podocyte cell lines, wild type podocytes were transfected with 2 μg of pcDNA3.1/Zeo-B7-1-FL or pcDNA3.1/Zeo-B7-1ΔT plasmids using FuGENE6 following the manufacturer's instructions. A pcDNA3.1/Zeo empty plasmid served as control. Two days after transfection, 400 μg/ml zeocin was added to the culture media for selection. Single clones were picked, and B7-1 protein abundance was assessed by Western blotting using anti-B7-1 (Epitomics) at 1:1000. For immunofluorescence microscopy of cultured podocytes, hamster anti-total β1 integrin (BioLegend), and rat anti-mouse monoclonal antibody 9EG7 against activated mouse β1 integrin[9] (BD Biosciences) were used at 1:100. Confocal microscopy and image processing were performed as before[10].

**FACS analysis of β1 integrin activation in podocytes**

Podocytes were transfected with GFP (control), and GFP-tagged B7-1 constructs (full-length, Δtail or tail). At 48 h after transfection, fluorescence labeling was performed as described before[11] with some modifications[12]. Cells were harvested and suspended in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) containing 5% BSA with 0.1% sodium azide for 30 min at 4 °C. For each experiment $10^6$ cells were incubated on ice for 30 min with primary antibody (9EG7) against mouse activated β1 integrin[9] at 1:100 in TBS. Cells were washed twice with ice-cold TBS, incubated on ice for 20 min with allophycocyanin-labeled goat anti-rat IgG (Molecular Probes, Invitrogen) at 1:200 and washed twice with ice-cold TBS. The cells were subjected to FACS

analysis (FACSCalibur CellQuest Pro Software; Becton Dickinson) as described before[12]. All experiments were run in triplicates.

**Cell migration assays**

The role of B7-1 in podocyte migration before and after LPS treatment (50 μg/ml) was assessed in a wound-healing assay as previously described[4]. For each experiment $7x10^5$ cells per cell line were seeded overnight on type-I collagen coated 6-well plates. Each well was then scratched with a sterile 200 μl pipette tip, washed with PBS and placed into fresh medium. After 24 h, cells were fixed with 4 % paraformaldehyde and permeabilized with 0.3 % Triton X-100 in PBS and cell nuclei were stained with DAPI. Pictures were captured by phase contrast microscopy under a 10× objective on a DMI 6000B microscope (Leica) at 0 and 24 h after scratching and the number of cells that had migrated into same size square fields (marked in Supplementary Fig. 3C-E) were counted. The data shown represent the mean ± SD of 5-8 independent experiments. For pharmacologic blockade of B7-1 with CTLA4-Ig, podocytes were pretreated with 100 μg/ml CTLA4-Ig for 4 days, then followed by 50 μg/ml LPS with additional 100 μg/ml CTLA4-Ig.

**Gene silencing of B7-1**

For the gene silencing of B7-1 three target sites were selected, and the following oligonucleotides (nt) were cloned into *Bgl*II-*Hind*III (all restriction enzymes were from New England Biolabs) sites of pSUPER-Zeo[13]. The B7-1 specific insert was a 19-nt sequence corresponding to the mouse B7-1 ORF, which is separated by a 9-nt noncomplementary spacer (TTCAAGAGA) from the reverse complement of the same 19-nt sequence. A control vector (pSUPER-control) was constructed using a 19-nt scrambled sequence with no significant homology to any mammalian gene sequence. Sequences of the synthesized primers (from 5' to

3') are shown below. Insertion of the oligonucleotides was verified by DNA sequencing. The following primers were used for gene silencing of B7-1:

scr-S: GATCCCCCTACAGTAACTCCGTCACTTTCAAGAGAAGTGACGGAGTTACTGTAGTTTTTA

scr-AS: AGCTTAAAAACTACAGTAACTCCGTCACTTCTCTTGAAAGTGACGGAGTTACTGTAGGGG

273-S: GATCCCCACATGACAAAGTGGTGCTGTTCAAGAGACAGCACCACTTTGTCATGTTTTTTA

273-AS: AGCTTAAAAAACATGACAAAGTGGTGCTGTCTCTTGAACAGCACCACTTTGTCATGTGGG

372-S: GATCCCCGGAAAGAGGAACGTATGAATTCAAGAGATTCATACGTTCCTCTTTCCTTTTTA

372-AS: AGCTTAAAAAGGAAAGAGGAACGTATGAATCTCTTGAATTCATACGTTCCTCTTTCCGGG

424-S: GATCCCCAAGAAGGAAAGAGGAACGTTTCAAGAGAACGTTCCTCTTTCCTTCTTTTTTA

424-AS: AGCTTAAAAAAAGAAGGAAAGAGGAACGTTCTCTTGAAACGTTCCTCTTTCCTTCTTGGG

Wild type and $\alpha 3^{-/-}$ podocytes were transfected with 2 $\mu$g of shRNA plasmid using FuGENE6 transfection agent (Roche) according to the manufacturer's instructions. 48 hours after transfection, cells were selected with 400 $\mu$g/ml zeocin. Single clones were picked, and depletion of B7-1 protein abundance was verified by Western blotting using anti-B7-1 (Epitomics) at 1:1000.

**Immunoprecipitation and GST binding**

SDS-PAGE, Western blotting, Co-IP of FLAG-tagged and GFP-tagged fusion proteins from transfected HEK293 cells, as well as endogenous Co-IP from podocytes were done as described before[4,14]. For endogenous Co-IP studies, the cells were lysed in IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) with protease inhibitors on ice for 30 min, then centrifuged at 4°C, 13000 rpm, for 15 minutes. Anti-$\alpha$5 integrin (R&D Systems) was used at 1:500, anti-$\alpha$V integrin (eBioscience) at 1:500, anti-$\beta$1 integrin (BioLegend) at 1:1000. Co-IP of FLAG- and GFP-tagged fusion proteins from co-transfected HEK293 cells as well as GST interaction studies with purified recombinant proteins were done as described before[14].

**Phorbol myristate acetate (PMA) induced cell spreading of K562 cells**

We used a previously described cell adhesion and spreading assay[8] to determine the effects of B7-1 and B7-1Δtail on integrin-mediated cell spreading. K562 cells (ATCC) constitutively express α5β1 integrin[15,16]. K562 cells stably expressing αVβ3 integrin (K562 αVβ3) were generated as described before[8]. $10^7$ cells were transfected by electroporation with 10 μg each of human pcDNA-αV and pcDNA-β3. Integrin expressing cells were selected using 500 μg/ml of G418 (Invivogen) and single cell clones expressing high levels of integrin on the cell surface were obtained using FACS and limited dilution cloning[8]. B7-1 and B7-1Δtail were introduced in these cell lines by electroporation K562 or K562 αVβ3 cells with 10 μg each of pcDNA-B7-1 and pMSCV-puro empty vector or pcDNA-B7-1Δtail and pMSCV-puro empty vector, respectively. Cells were then grown in 2 μg/ml puromycin and 500 μg/ml G418. High B7-1 expressing single cell clones were selected by limited dilution cloning and B7-1 and B7-1Δtail protein abundance was determined by FACS analysis using FITC conjugated anti-B7-1 antibody (BD Bioscience) at 1:100 (Fig. 4A, D of Supplementary Appendix). For the cell spreading assays, K562 cells expressing α5β1 were seeded on 96-well Maxisorp plates (Nunc) coated with 6 μg/ml purified recombinant fibronectin[17] overnight at 4 ºC. The K562 αVβ3 cells were seeded on plates coated with 5 μg/ml vitronectin (US Biologicals). 30,000 cells were added to each well in serum free IMDM media (Mediatech), stimulated with PMA (10 nM) to induce spreading and incubated at 37 ºC until spreading was observed[18,19]. The cells were fixed with 4% PFA in PBS and images of ten independent random fields were captured under DIC microscopy with a 20x objective using a DMI 6000B microscope (Leica). The total number of round and spread cells was manually counted in a blinded fashion and the percentage of spread cells was calculated. For B7-1 blockade with CTLA4-Ig, 384 well High Bind plates (Corning) were coated overnight with 6 μg/ml of fibronectin in PBS. K562 cells expressing α5β1, α5β1 and B7-1, or α5β1 and B7-1Δtail were incubated overnight with 100 μg/ml of CTLA4-Ig. Cells were washed twice with IMDM +

0.1% BSA. 5000 $\alpha 5\beta 1$ expressing K562 cells were added to each well of a coated 384 well plate. 100 $\mu$g/ml of CTLA4-Ig and 10 nM PMA were added to each well. Cells were allowed to adhere at 37 ºC. After 1h, the plates were inverted for an additional 2 h. Cells were fixed with 4% PFA in PBS and spreading was quantified as described above. Statistical analysis was done with Graphpad Prism 5 software.

**Supplementary Figures**

**Supplementary Figure 1 Integrin β1 binds to B7-1 via direct tail:tail interaction**

(**A**) Western blot analysis of anti-β1 immunoprecipitates developed with anti-α5, anti-αV and anti-β1 antibodies. α5 and αV integrins co-precipitated with β1 integrin in wild type and $\alpha3^{-/-}$ podocytes. IP with anti-GFP (control) served as negative control. (**B**) Confocal microscopy showed co-localization of B7-1 and vinculin in focal contacts of $\alpha3^{-/-}$ integrin podocytes (arrowheads). (**C**) Western blot analysis showing endogenous Co-IP of B7-1 and β1 integrin from $\alpha3^{-/-}$ but not from wild type podocytes. No interaction was found with anti-GFP (control) serving as negative control in the IP. (**D**) Domain structure of B7-1 (full-length) and three deletion constructs, ΔEC, Δtail and tail. TM: transmembrane domain. (**E**) To map the interaction domains between B7-1 and β1 integrin, we examined three FLAG-B7-1 constructs (full-length, ΔEC, and Δtail) for their ability to co-precipitate GFP-β1 integrin or a GFP-β1 integrin deletion protein lacking the extracellular domains (β1ΔEC). Western blot analysis of co-precipitates with anti-GFP, blotted with anti-FLAG (middle panel) or anti-GFP (upper and lower panels). We observed a specific interaction between both full-length proteins (data not shown), confirming the endogenous Co-IP results, and the interaction was preserved between B7-1ΔEC and β1ΔEC. In contrast, deletion of the cytoplasmic tail of B7-1 (B7-1Δtail) abrogated β1ΔEC binding. No interaction was found between FLAG-B7-1 and GFP-sui (control); *: IgG light chain, **: IgG heavy chain. (**F**) Left panel: Direct binding of purified FLAG-B7-1 to purified GST-β1ΔEC but not the GST control. The B7-1-β1ΔEC mixture was immunoprecipitated with anti-GST and blotted using anti-FLAG or anti-GST. Right panel: Coomassie stained SDS PAGE shows purity of purified recombinant proteins.

**Supplementary Figure 2 B7-1 but not B7-2 interacts with β1 and β3 integrins**

(**A**) GFP-talin-HN, GFP-B7-1 and GFP-B7-1tail co-precipitated with FLAG-β1ΔEC and FLAG-β3ΔEC from co-transfected HEK293 cells. The interaction with talin served as positive control. No interaction was found with GFP-B7-1Δtail, GFP-B7-2 or GFP-sui (control) serving as negative control. (**B**) Direct binding of purified FLAG-B7-1 to purified GST-β3ΔEC but not the GST control (left panels). Coomassie stained SDS PAGE shows the purity of recombinant proteins (right panels).

**Supplementary Figure 3 CTLA4-Ig blocks B7-1 mediated loss of β1 integrin activation in podocytes**

(**A**) Representative confocal images stained with antibodies to total β1 or activated β1 integrin of podocytes expressing empty vector (pcDNA), vector encoding B7-1 or B7-1Δtail in the absence (left panels) or presence of LPS (right panels). B7-1, but not B7-1Δtail, suppressed podocyte β1 integrin activation, but had no effect on total β1 integrin levels. CTLA4-Ig treatment of B7-1 expressing podocytes restored β1 integrin activation. Similar results were observed in the respective LPS-treated podocytes. (**B**) Gene silencing of B7-1 (B7-1kd) protected against LPS-induced loss of β1 activation. (**C**) In $\alpha 3^{-/-}$ podocytes, β1 integrin was largely absent. Gene silencing of B7-1 or CTLA4-Ig treatment restored β1 integrin activation. (**D**) Quantitative analysis of podocytes β1 integrin activation by flow cytometry. Compared to the GFP control, GFP-B7-1 (full) and GFP-B7-1-tail significantly reduced the activation of β1 integrin (% activated-β1 positive cells per GFP-positive cells: GFP: 88.96 ± 9.09 versus GFP-B7-1: 19.80 ± 7.38 versus GFP-B7-1-tail: 36.52 ± 2.18; $P < 0.001$; AVOVA). In contrast, B7-1Δtail did not suppress β1 activation (85.93 ± 2.93 %; not significant).

**Supplementary Figure 4 CTLA4-Ig rescues B7-1 mediated suppression of α5β1 integrin dependent cell spreading**

(**A**) FACS analysis of B7-1 and B7-1Δtail protein abundance in K562 cells with stable α5β1 integrin expression. Protein levels of non-transfected K562 cells are shown in gray, and K562 cells with stable expression of B7-1 or B7-1Δtail in black. (**B**) B7-1 but not B7-1Δtail reduced α5β1 dependent spreading on fibronectin. CTLA4-Ig treatment restored spreading of B7-1 expressing cells to control levels. (**C**) Quantitative analysis of B7-1 and CTLA4-Ig effects on cell spreading (n = 10-16; % spreading cells: 45.52 ± 1.27 for non-transfected control cells, 44.97 ± 1.90 for non-transfected control cells + CTLA4-Ig, 17.0 ± 0.8 for B7-1 expressing cells, 40.46 ± 1.86 for B7-1 expressing cells +CTLA4-Ig, 47.71 ± 1.67 for B7-1Δtail expressing cells, and 37.62 ± 2.36 for B7-1Δtail expressing cells + CTLA4-Ig; ****P <0.0001,* ANOVA). (**D**) FACS analysis of B7-1 and B7-1Δtail levels in K562 cells with stable αVβ3 integrin expression. Proteins levels of non-transfected K562 cells are shown in gray, and of K562 cells with stable transfection of B7-1 or B7-1Δtail in black. (**E**) Neither B7-1 nor B7-1Δtail affected spreading of αVβ3 expressing K562 cells on vitronectin. (**F**) B7-1 does not significantly change αVβ3 dependent cell spreading (n =10; % spreading cells: 15 ± 1 for non-transfected cells, 12 ± 1 for B7-1 and 11± 1 for B7-1Δtail; student's t-test, S.E.M reported). N.S., not significant.

**Supplementary Figure 5 B7-1 disrupts binding of talin to β1 but not to β3 integrin**

(**A**) Endogenous talin co-precipitated with activated β1 integrin in wild type but not in $\alpha 3^{-/-}$ podocytes. IP with anti-GFP (control) served as negative control. (**B**) FLAG-B7-1 did not bind to GFP-talin-HN (HN: head N-terminal domain; left lane). Co-expression of GFP-B7-1-tail but not GFP-B7-1Δtail blocked the interaction of GFP-talin-HN with FLAG-β1 integrin (β1ΔEC) in co-transfected HEK293 cells. Instead, GFP-B7-1-tail co-precipitated with FLAG-β1ΔEC. No

interaction was found between FLAG-B7-1 and GFP-talin-HN. (**C**) Upper panel: immobilized GST-β1ΔEC but not the GST control directly bound to purified FLAG-talin-HN. In the presence of increasing amounts of FLAG-B7-1ΔEC, the binding of talin-HN to GST-β1ΔEC was gradually lost, whereas binding of B7-1 to β1 integrin could now be detected. Lower panel: Coomassie stained SDS PAGE analysis shows purity of recombinant proteins. (**D**) Co-expression of GFP-B7-1-tail did not block the interaction of GFP-talin-HN with FLAG-β3ΔEC in triple transfected HEK293 cells. Instead, both GFP-B7-1-tail and GFP-talin-HN co-precipitated with FLAG-β3ΔEC. No binding was found with GFP-sui (control).

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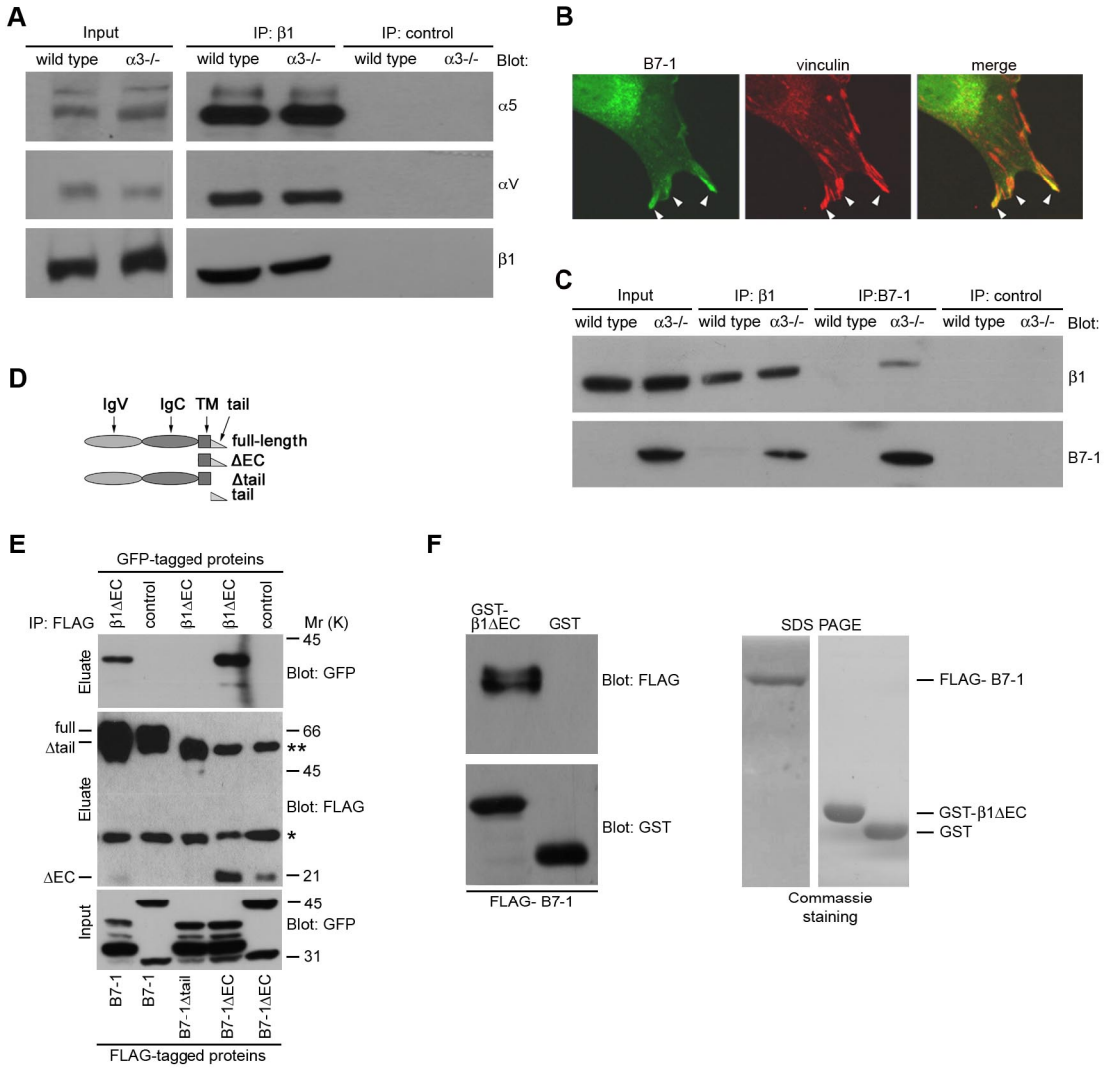
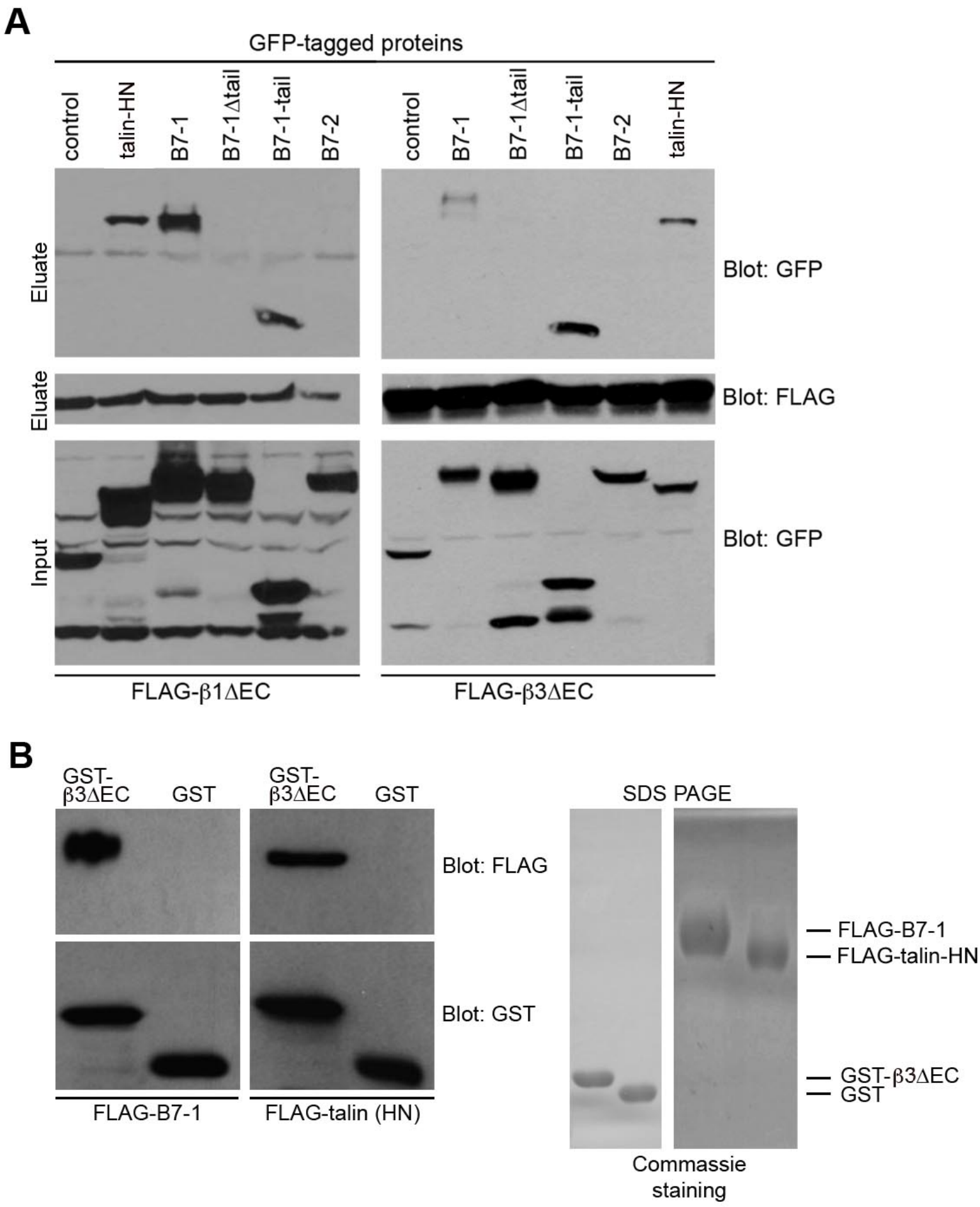
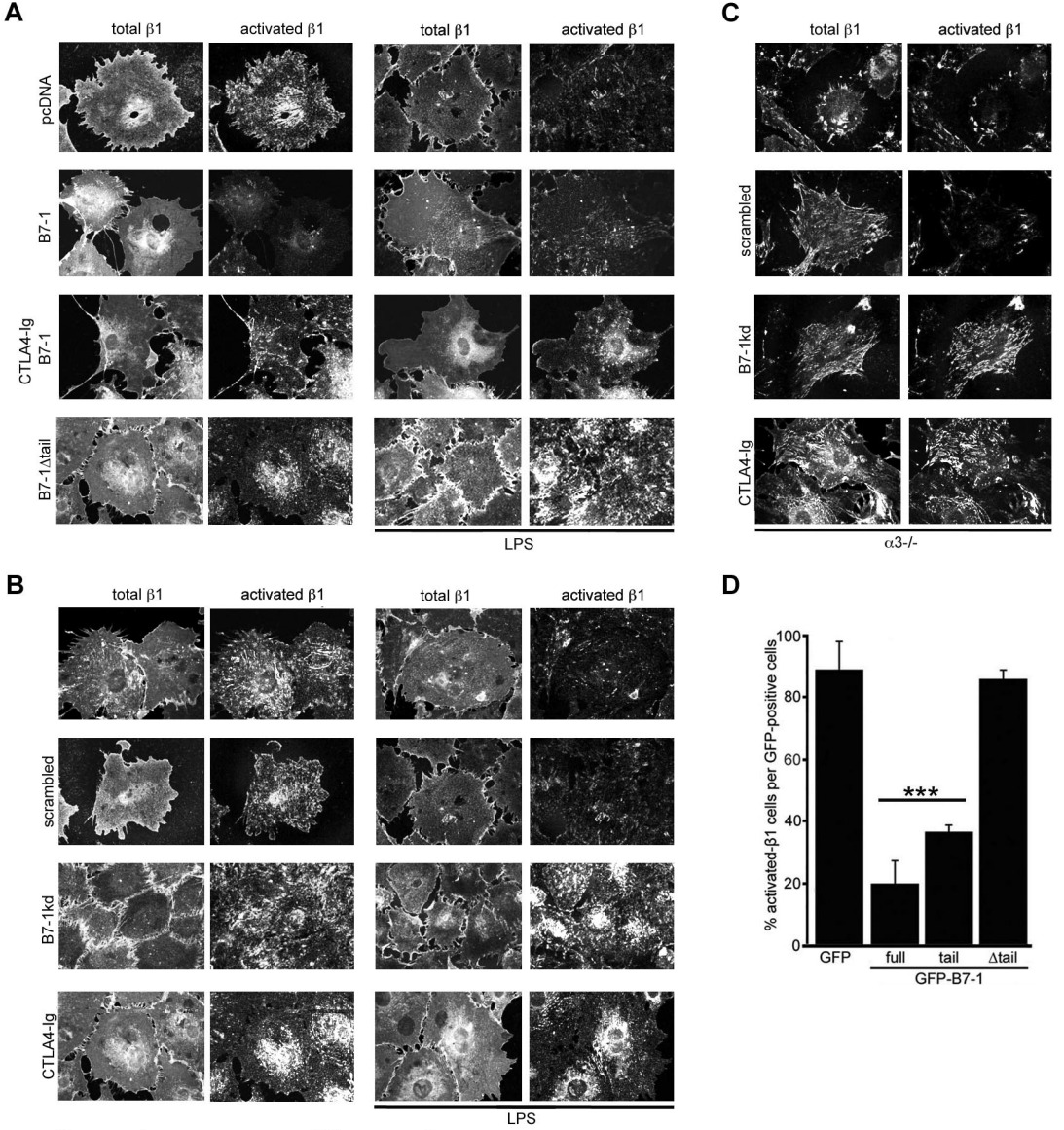


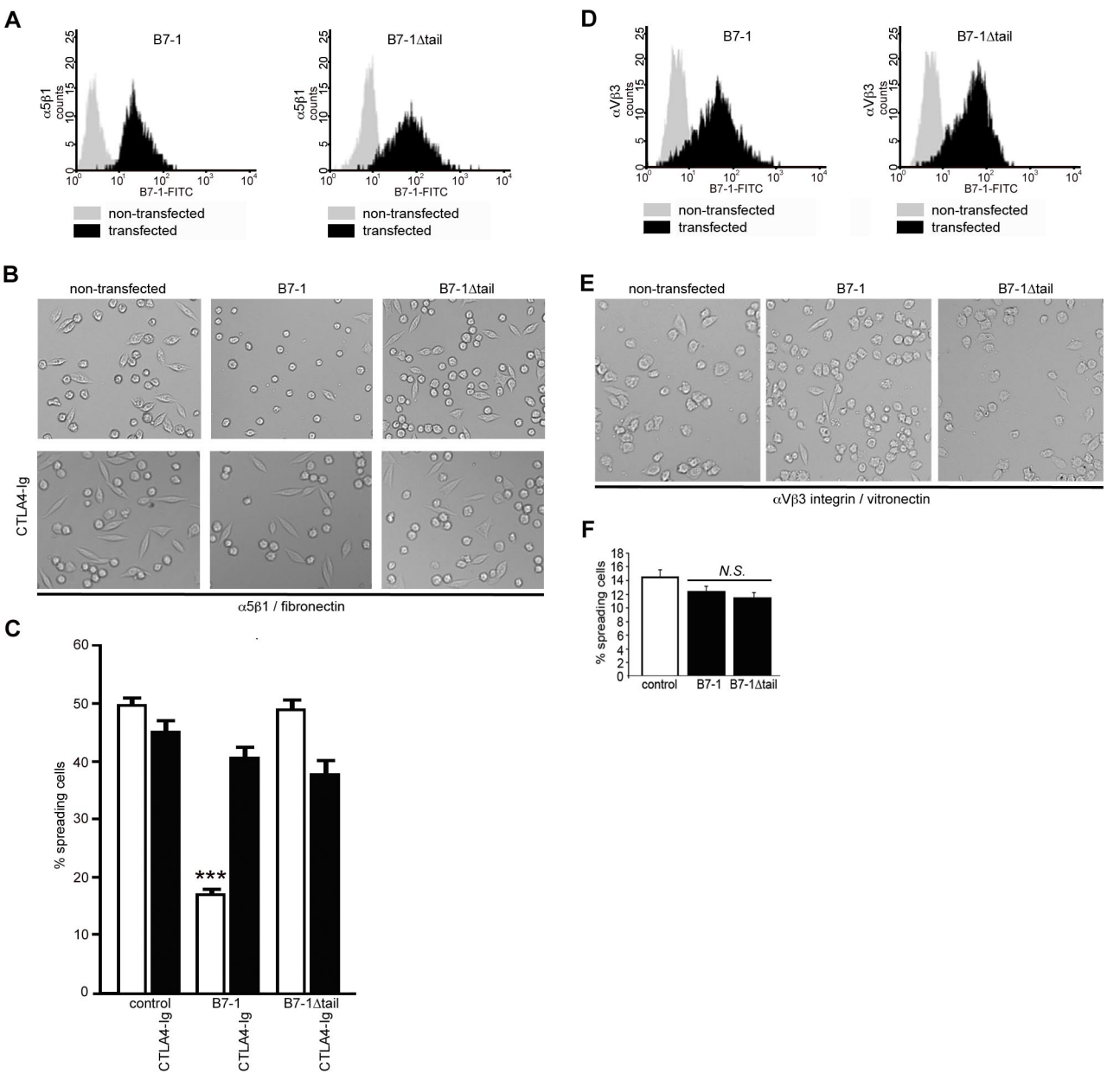

# Supplementary Figure 1



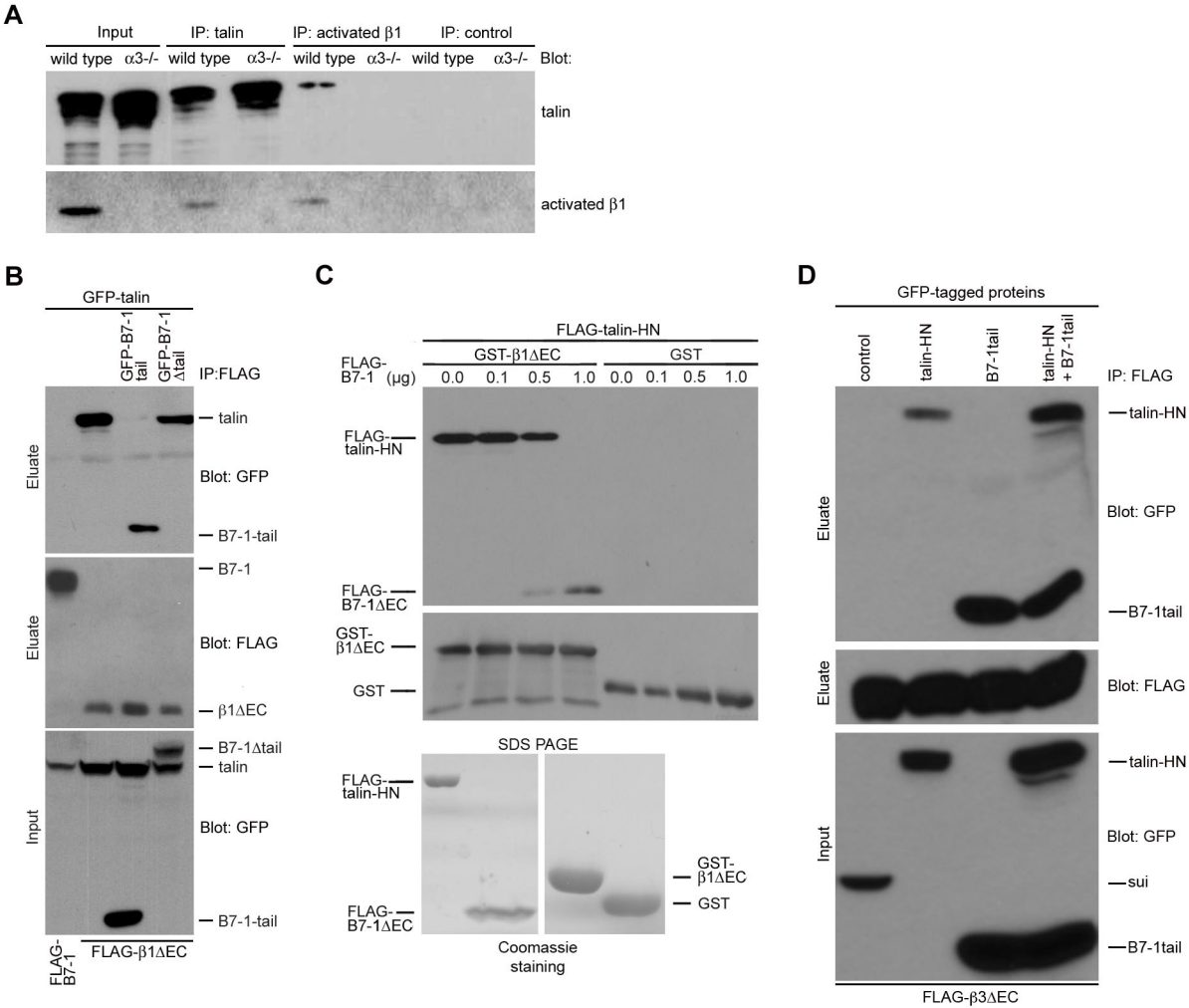

# Supplementary Figure 2




Supplementary Figure 3




# Supplementary Figure 4




# Supplementary Figure 5