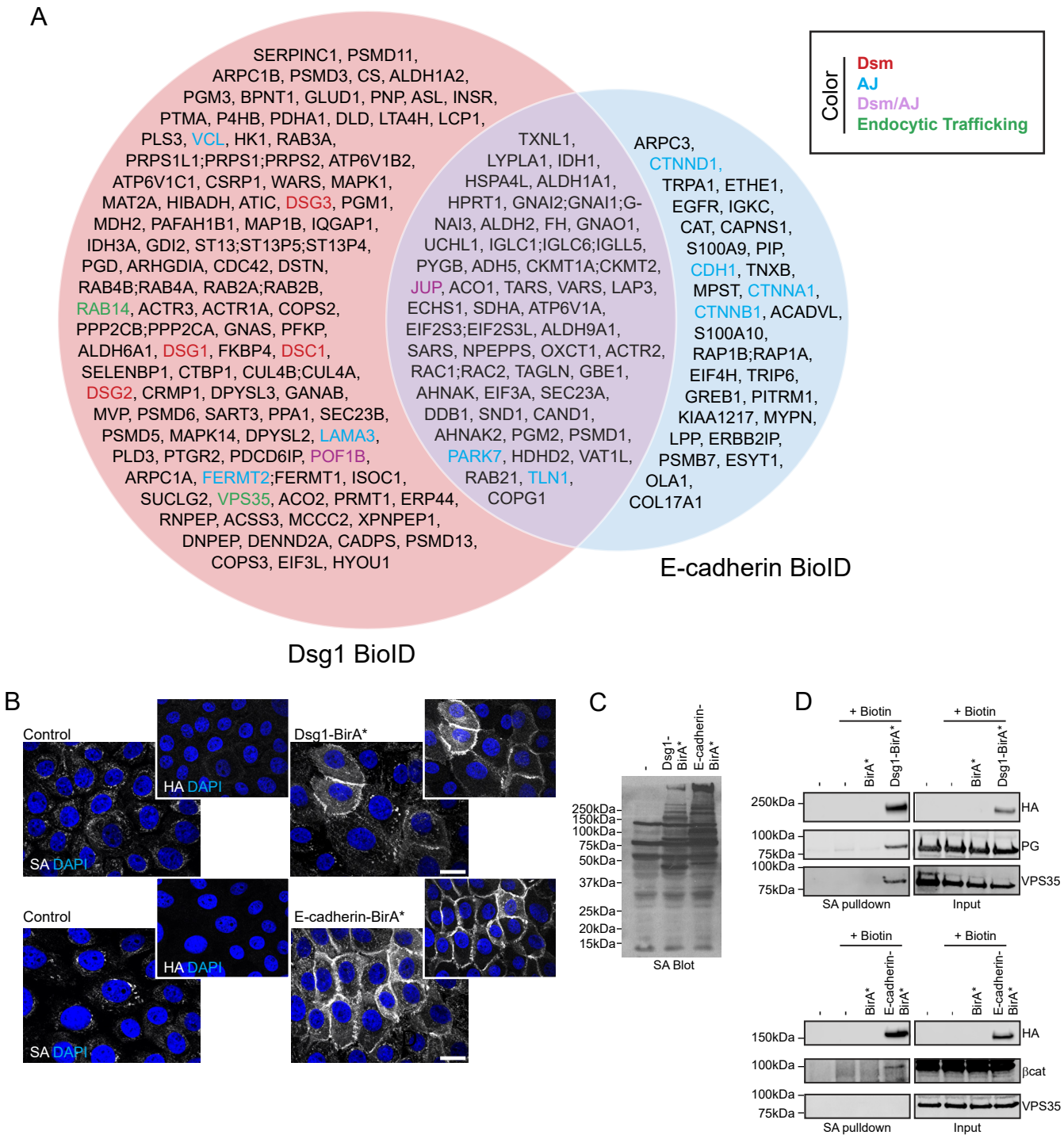

Figure S1. Dsg1 and E-cadherin BioID comparison and validation; Related to Figure 1.
(A) Venn diagram for comparison of hits found in Dsg1 and E-cadherin BioID screen. Color of text illustrates proteins contained in Gene Ontology (GO) gene sets of desmosome (Dsm, red, GO:0030057), adherens junction (AJ, blue, GO:0005912), shared in Dsm/AJ (purple), and endocytic recycling (green, GO:0032456).
(B) Keratinocytes +/- HA-tagged Dsg1-BirA* or E-cadherin-BirA* were differentiated for 24hrs before adding 50µM Biotin for another 24hrs in 1.2 mM $CaCl_2$ media. Cells were fixed and stained with an anti-HA tag antibody, Streptavidin-AF488 (SA), and DAPI. Scale bar, 20 µm.
(C) Streptavidin-HRP Blot of whole cell lysate of the sample send to mass spectrometry for preliminary BioID screen with the conditions: Biotin-only control (-), Dsg1-BirA*, and E-cadherin-BirA*.
(D) Benchtop BioID of untransfected control (-) +/- Biotin, BirA*-only, and Dsg1-BirA* or E-cadherin-BirA*. Following streptavidin pulldown, the input and elution was run on a gel and immunoblotted for the HA-tagged Dsg1-BirA* or HA-tagged E-cadherin-BirA*, VPS35, and plakoglobin (PG) or β-catenin (βcat) as positive controls.

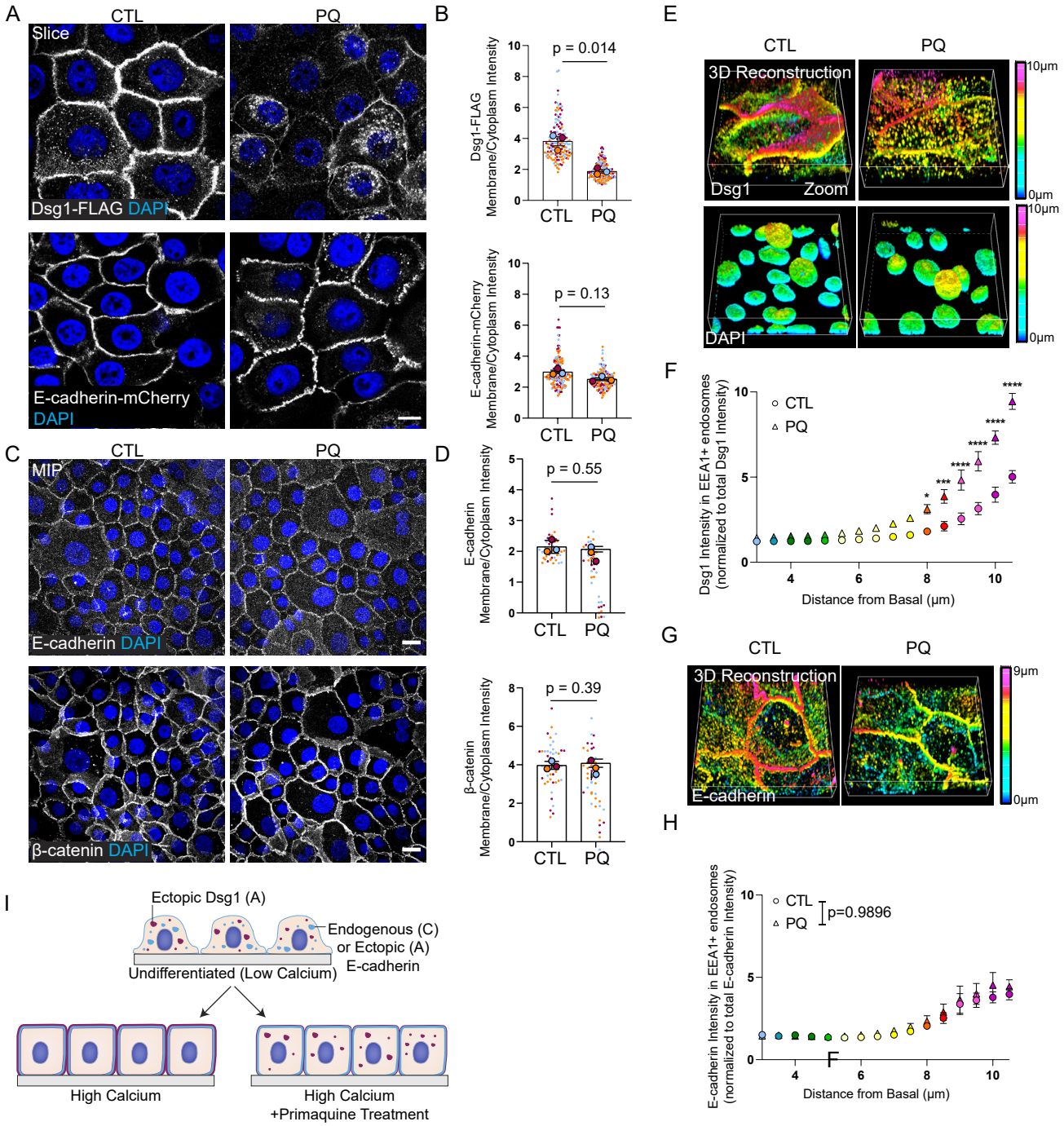

Figure S2. Primaquine treatment disrupts ectopic Dsg1, but not AJ proteins; Related to Figure 1.

(A) Dsg1-FLAG and E-cadherin-mCherry immunofluorescence following overnight 200μM primaquine (PQ) treatment of undifferentiated keratinocytes. Scale bar, 10 μm.

(B) Quantification of Dsg1-FLAG and E-cadherin-mCherry membrane/cytoplasmic ratio following PQ treatment.

(C) Maximum intensity projection (MIP) of E-cadherin and β-catenin immunofluorescence following overnight 200μM PQ treatment of undifferentiated keratinocytes. Scale bar, 20 μm.

(D) Quantification of E-cadherin and β-catenin membrane/cytoplasmic ratio following PQ treatment.

(E) 3D reconstruction of Dsg1 and DAPI with a z-depth pseudocoloring illustrating Dsg1 localization in the stratified layer corresponding to Figure 1C.

(F) Quantification of Dsg1 intensity in EEA1+ vesicles with respect to the distance from the basal layer. Colors correspond to z-depth look up table in E.

(G) 3D reconstruction of E-cadherin staining (corresponding to Figure 1C) with a z-depth pseudocoloring illustration.

(H) Quantification of E-cadherin intensity in EEA1+ vesicles with respect to the distance from the basal layer. Colors correspond with the z-depth scale shown in G.

(I) Schematic demonstrating the effect of PQ treatment on endogenous E-cadherin (see panel C) and ectopic E-cadherin or Dsg1 (see panel A).

Statistics from three biological repeats using paired t-test (B, D) or two-way ANOVA with Sidak post-hoc test (F, H). Error bars are all SEM.

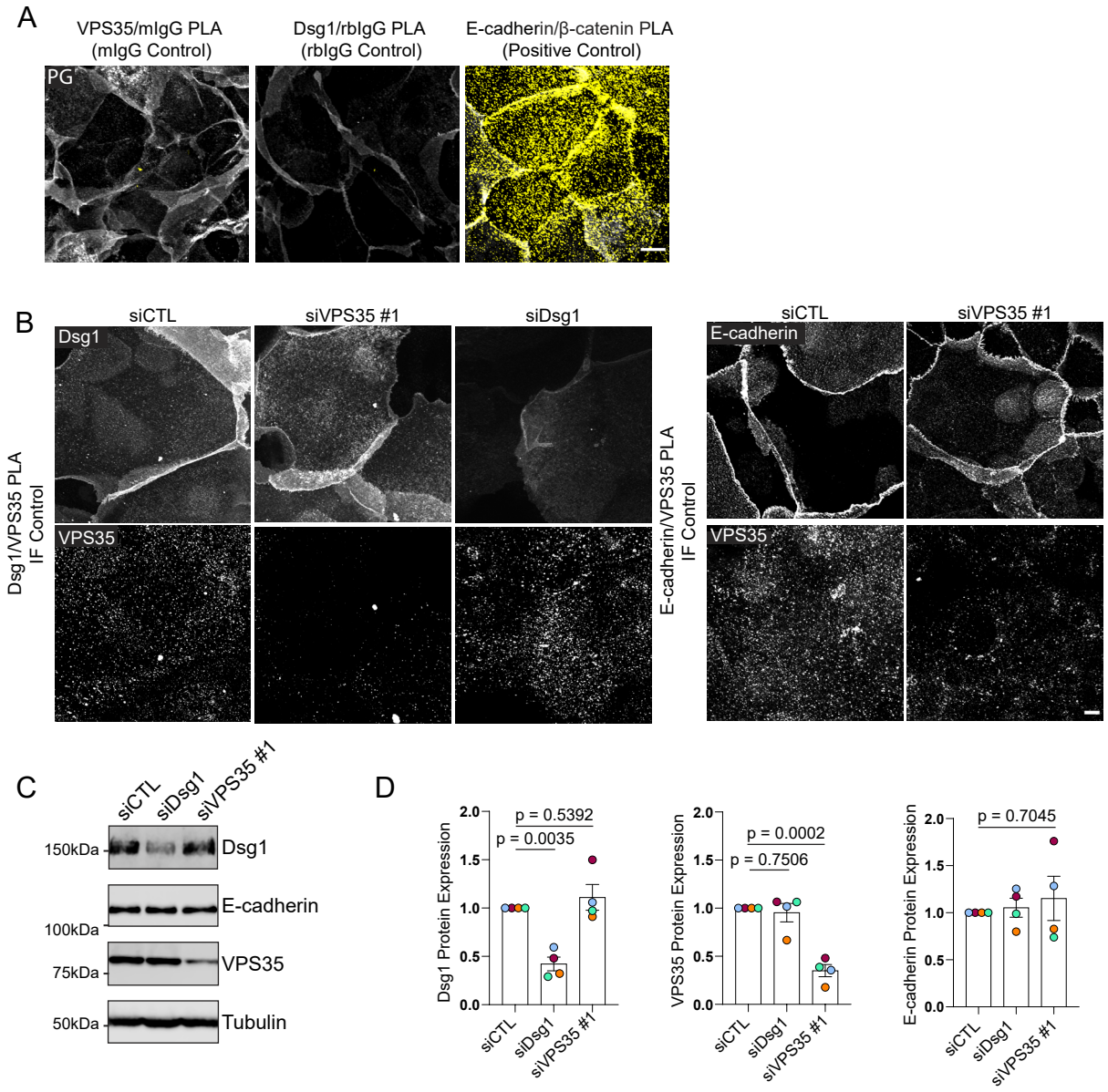

Figure S3. Controls for the PLA used to assess the proximity of VPS35 to Dsg1 or E-cadherin; Related to Figure 1.
(A) Non-specific interactions were tested by pairing anti-Dsg1 or anti-VPS35 antibodies with the species-specific IgG corresponding to its partner's species: mouse IgG (mIgG Control), rabbit IgG (rbIgG Control). An E-cadherin/β-catenin antibody pairing was used as a positive control for the PLA signal. Scale bar, 20 μm.
(B) Immunofluorescence (IF) was done in tandem with the PLA shown in Figure 1H using the same antibody pairings at the same dilutions to confirm positive protein recognition by the antibodies. Scale bar, 10 μm.
(C) Immunoblotting of lysates collected from cells grown in tandem with the PLA/IF coverslips to confirm efficient siRNA-mediated depletion of Dsg1 or VPS35. E-cadherin total levels do not change upon Dsg1 or VPS35 KD. Tubulin is included as a loading control.
(D) Quantification of the densitometry of the immunoblot normalized to control levels.
Statistics from three biological repeats using one-way ANOVA with Dunnett post-hoc test. Error bars are all SEM.

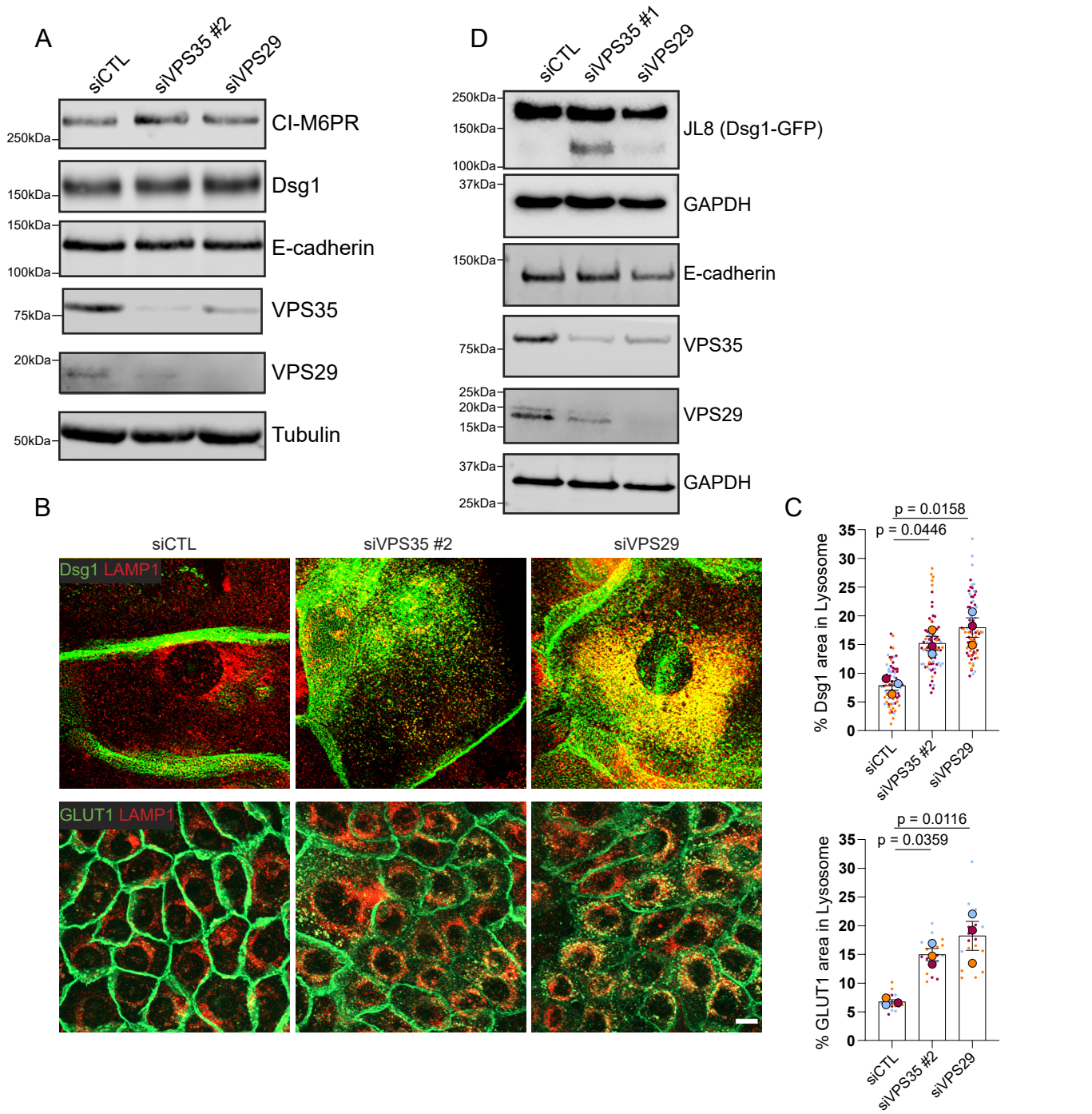

Figure S4. Verification of retromer component depletion and endogenous Dsg1 colocalization with the endo-lysosomal marker LAMP1; Related to Figure 2.

(A) Immunoblotting following siRNA-mediated depletion of retromer components shows efficient knockdown of VPS35 and VPS29 following 2 days in 1.2mM $CaCl_2$ media . Total levels of cation-independent mannose-6 phosphate receptor (CI-M6PR), E-cadherin and Dsg1 do not significantly change upon VPS35 or VPS29 knockdown. Tubulin is included as a loading control.

(B) Immunofluorescence of endogenous Dsg1 in stratified cells and GLUT1, a known retromer cargo, in basal cells co-stained with the endo-lysosomal marker, LAMP1, following retromer component depletion. Scale bar, 10 µm.

(C) Quantification of the % Dsg1 area and % GLUT1 area in LAMP1 stained endo-lysosomal vesicles.

(D) Immunoblotting of lysates collected from keratinocytes expressing Dsg1-GFP cultured in 1.2mM $CaCl_2$ media overnight showing efficient VPS35 and VPS29 knockdown. E-cadherin levels do not significantly change upon VPS35 or VPS29 knockdown. GAPDH is included as a loading control.

Statistics from three biological repeats using one-way ANOVA with Dunnett post-hoc test. Error bars are all SEM.

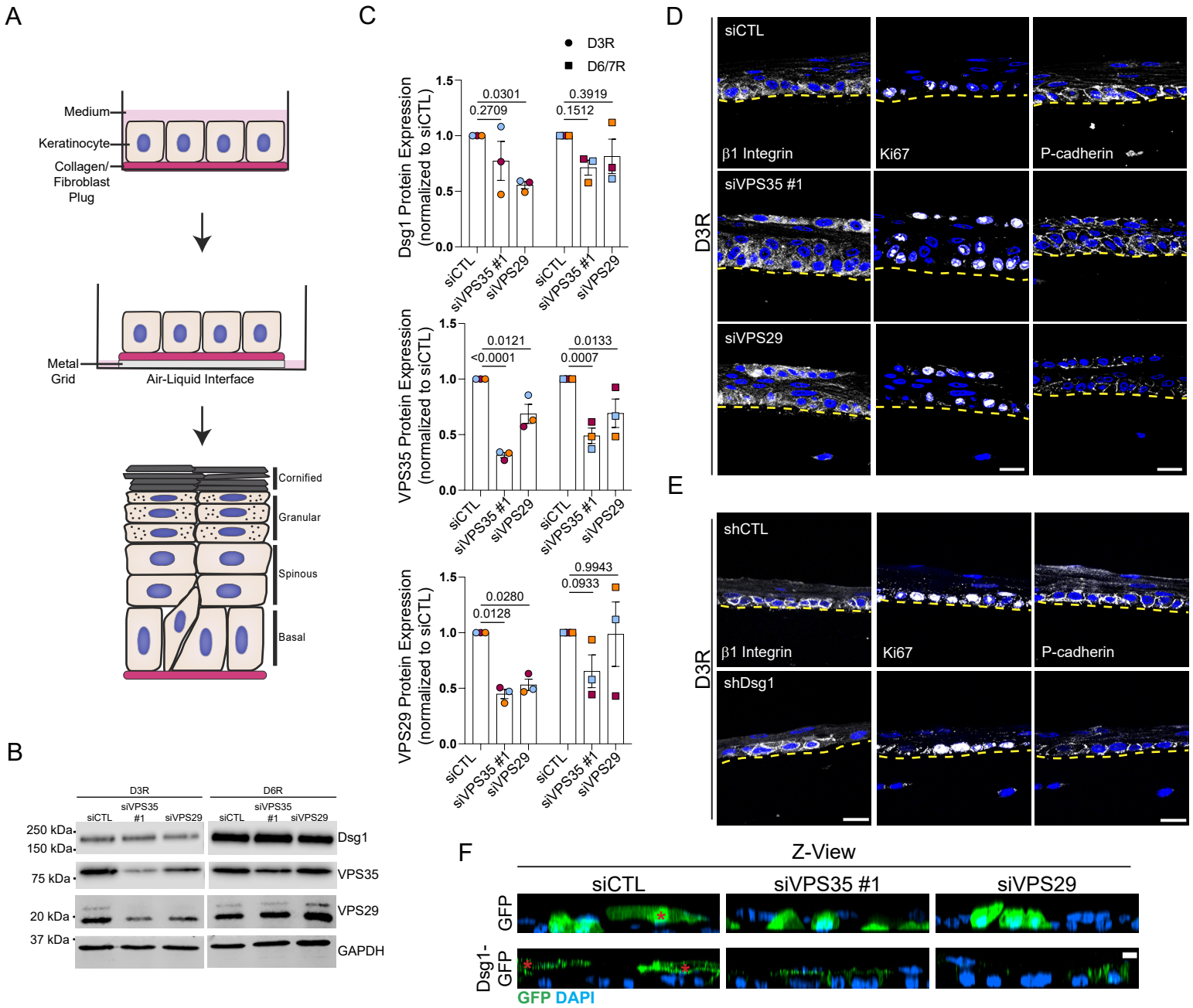

Figure S5. 3D organotypic epidermal culture experimental design, immunoblots of day 3 and day 6-7 3D organotypic cultures, and altered expression of basal cell markers in retromer knockdown Day 3 rafts; Related to Figure 4.

(A) Keratinocytes were seeded on collagen plugs containing fibroblasts, which were subsequently lifted to an air-liquid interface by placing them onto a metal grid and feeding the culture from the bottom to induce stratification. Cultures were harvested 3 days or 6-7 days after lifting to the air liquid interface.

(B) Efficient depletion of VPS35 and VPS29 was observed in day 3 organotypic cultures, but protein levels begin to recover in day 6-7 cultures. GAPDH is included as a loading control.

(C) Quantification of immunoblots in (B).

(D) Tissue sections from 3D control and retromer-depleted rafts stratified for 3 days (D3R) were stained for basal markers, β1 integrin, Ki67, and P-cadherin. Scale bar, 20 μm.

(E) Tissue sections from 3D control and Dsg1-depleted rafts stratified for 3-4 days (representative images are D3R) were stained for basal markers, β1 integrin, Ki67, and P-cadherin. Scale bar, 20 μm.

(F) Representative z-view for Figure 4E. Nuclei are stained with DAPI. Red asterisks mark GFP positive stratified cells. Scale bar, 10 μm.

Statistics from three biological repeats using one-way ANOVA with Dunnett post-hoc test. Error bars are all SEM.

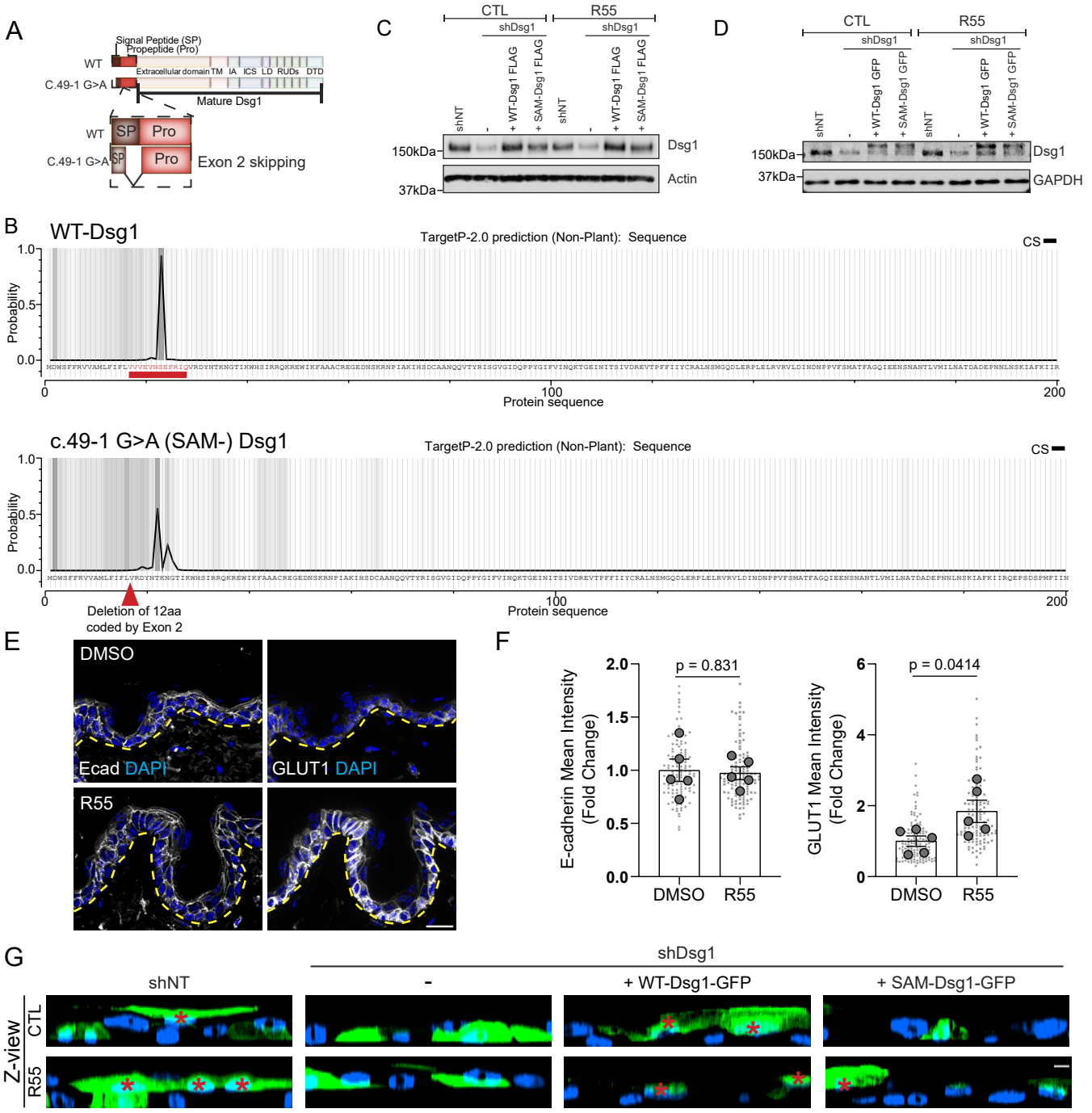

Figure S6. Signal peptide and cleavage site prediction of WT-Dsg1 and SAM-Dsg1. R55 treatment does not affect total protein levels of Dsg1, and upregulates GLUT1 expression, but not E-cadherin; Related to Figures 5 and 6.
(A) c.49-1 G>A has been reported to result in the skipping of exon 2 during transcription, which codes for amino acids located in the signal peptide sequence of Dsg1.
(B) Amino acid sequence of Dsg1 with and without the deletion of exon 2 were inputted into TargetP 2.0 (http://www.cbs.dtu.dk/services/TargetP/) to test the probability of signal peptide recognition. WT-Dsg1 has a 0.981 likelihood of containing a signal peptide and a 0.9370 probability of a cleavage site (CS) at position 23-24. SAM-associated Dsg1 mutation c.49-1 G>A results in a deletion of 12 amino acids encoded by Exon 2 resulting in a 0.5477 likelihood of containing a signal sequence and a 0.5527 probability that the cleavage site is between positions 22-23. The plot shows the probability and location of the cleavage site (CS), as well as the attention weight, which weights the amino acids that have higher importance to the model illustrating them with a darker shading.
(C) Immunoblot of lysates expressing shNT, shDsg1, shDsg1+WT-Dsg1-FLAG or shDsg1+SAM-Dsg1-FLAG +/-R55 and probed for Dsg1. Actin was used as a loading control. Whole cell lysates shown here were collected in tandem with experiments shown in Figure 5A-D.

(D) Immunoblot of lysates expressing shNT, shDsg1, shDsg1+WT-Dsg1-GFP or shDsg1+SAM-Dsg1-GFP +/-R55 and probed for Dsg1. GAPDH was used as a loading control. Whole cell lysates shown here) were collected in tandem with experiments shown in Figure 6A, B.
(E) Sections from DMSO or R55 treated dorsal mouse skin were stained with anti-E-cadherin, anti-GLUT1, and DAPI. Scale bar, 20 μm.
(F) Quantification of E-cadherin (left) and GLUT1 (right) mean fluorescence intensity in the mouse epidermis following R55 treatment.
(G) Representative z-view for Figure 6A. DAPI (blue) marks nuclei. Red asterisks mark GFP positive stratified cells. Scale bar, 10 μm.
Statistics from five biological repeats using unpaired t-test. Error bars are all SEM.

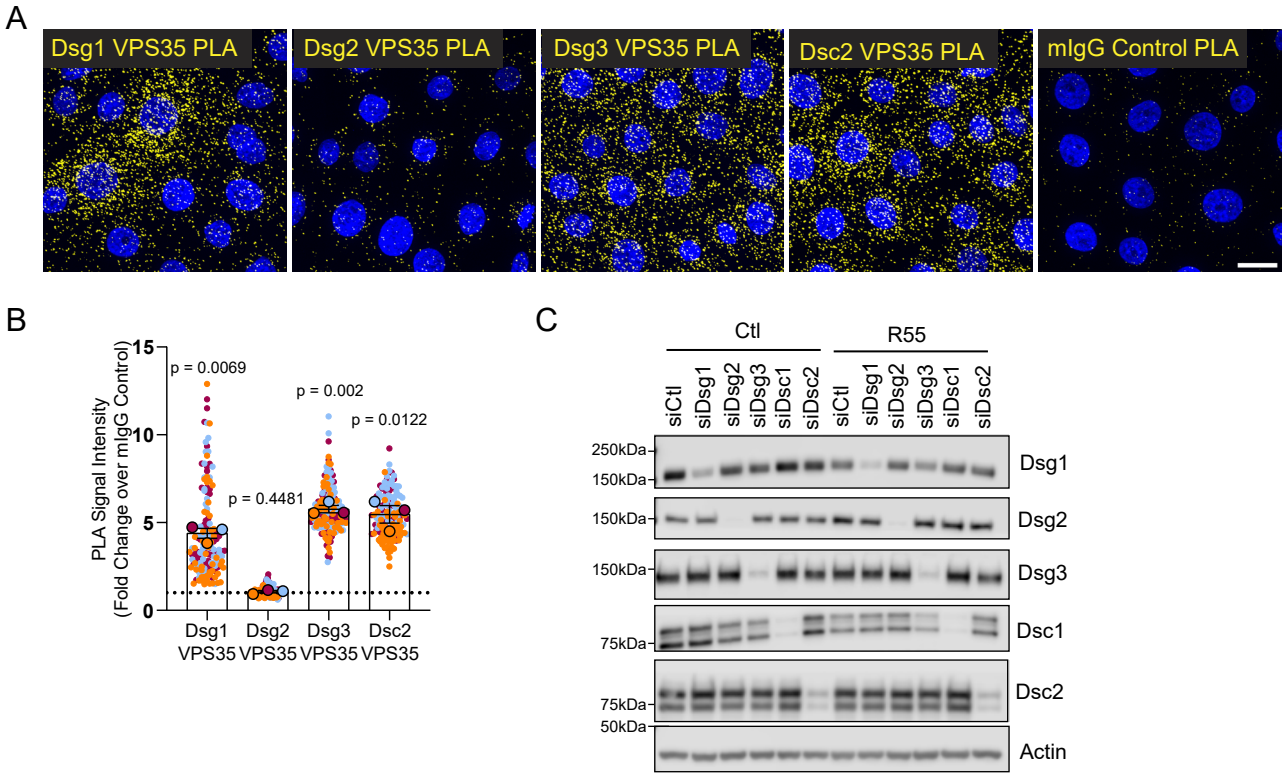

Figure S7. Dsg3 and Dsc2, but not Dsg2, are also in close proximity with VPS35; Related to Figure 6.
(A) PLA using an anti-VPS35 antibody paired with an anti-Dsg1, Dsg2, Dsg3, or Dsc2 antibody to test the proximity of VPS35 with endogenous desmosomal cadherins in situ (yellow) after 6hr in 1.2mM $CaCl_2$ media. DAPI marks nuclei (blue). As a control, mIgG and VPS35 antibodies were used as a negative control. Scale bar, 20 μm
(B) Quantification of VPS35/Dsg1, VPS35/Dsg2, VPS35/Dsg3, and VPS35/Dsc2 PLA signal normalized to mIgG control.
(C) Immunoblotting lysates from Figure 6C stratification assay confirms efficient knockdown of Dsg1, Dsg2, Dsg3, Dsc1, and Dsc2.
Statistics from three biological repeats using one sample t-test with theoretical mean of 1. Error bars are all SEM.