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Supplemental Information

The Dynamics of TGF-β Signaling Are Dictated by Receptor Trafficking via the ESCRT Machinery

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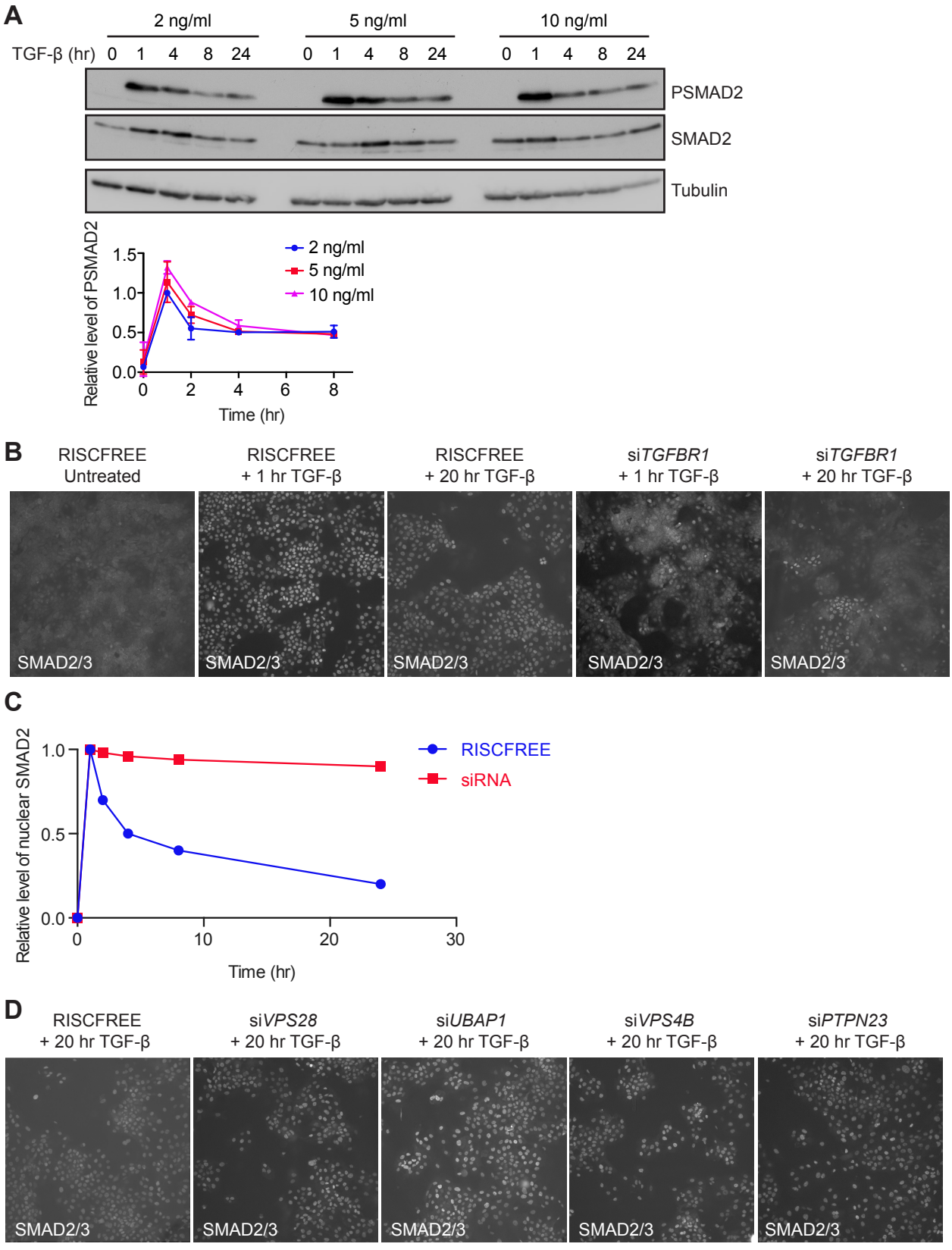

Figure S1, related to Figure 1. SMAD2/3 nuclear localization is increased after 20 hr of TGF-β stimulation when ESCRT components are knocked down.

(**A**) HaCaT cells were treated with 2, 5 or 10 ng/ml TGF-β for the times shown. Levels of PSMAD2, SMAD2 and Tubulin as a loading control were assayed by Western blotting. Quantifications are the average ± SD of two independent experiments.

(**B**) Shown are images from the 1536-well whole genome siRNA screen. HaCaTs were transfected with a whole genome siRNA library on a well-by-well basis, or with RISCFREE control siRNAs, and were either untreated or stimulated with TGF-β for 1 hr or 20 hr, fixed, stained for SMAD2/3 and imaged. Knockdown of TGFBR1 abrogates SMAD2/3 nuclear accumulation at both time points.

(**C**) Schematic of expected screen results. For control siRNAs, SMAD2/3 accumulate in the nucleus after 1 hr of TGF-β stimulation, then attenuate down to a lower level at 20 hr. For screen hits, SMAD2/3 accumulate normally in the nucleus after 1 hr of signalling, but then fail to attenuate.

(**D**) Shown are images from the 1536-well whole genome siRNA screen. Knockdown of ESCRT components increases SMAD2/3 nuclear localization after 20 hr of TGF-β stimulation.

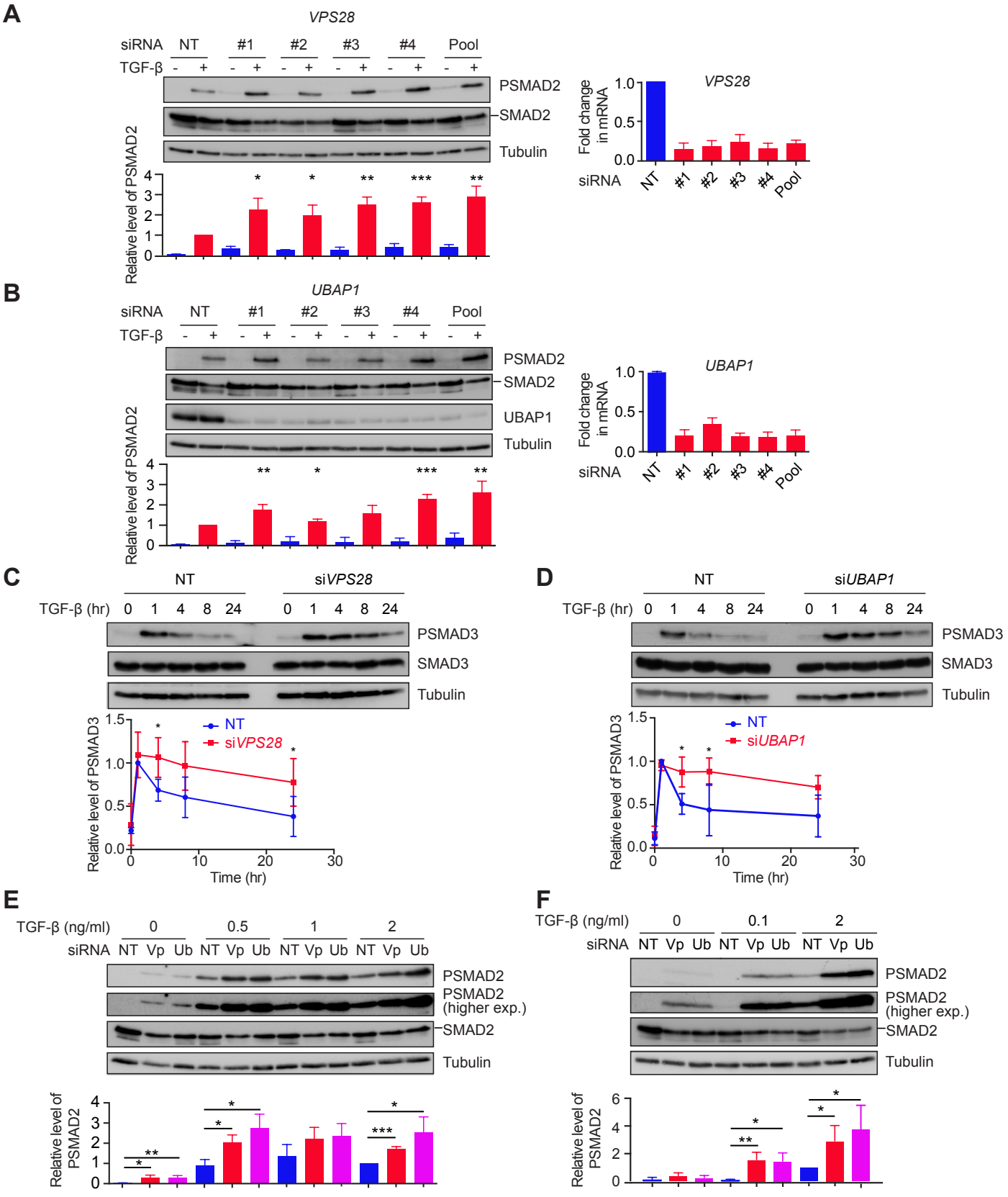

Figure S2, related to Figure 2. Characterization of *VPS28* and *UBAP1* siRNAs.

(**A** and **B**) HaCaT cells were transfected with non-targeting (NT) control siRNAs, 4 individual siRNAs targeting *VPS28* or a pool of the 4 together (A), or 4 individual siRNAs targeting *UBAP1* or a pool of the 4 together (B) and stimulated with TGF-β for 24 hr. Levels of PSMAD2, SMAD2/3, UBAP1 and Tubulin as a loading control were assayed by Western blot. Quantifications are the average ± SD of three independent experiments, normalized to NT samples treated for 24 hr. The extent of knockdown was assessed by qPCR, and the normalized average ± SD from the same three independent experiments is shown to the right.

(**C** and **D**) HaCaT cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *VPS28* or *UBAP1*, and stimulated with 2 ng/ml TGF-β for the times indicated. Levels of PSMAD3, SMAD3 and Tubulin as a loading control were assayed by Western blot. Quantifications are the normalized average ± SD of three independent experiments.

(**E** and **F**) HaCaT cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *VPS28* (Vp) or *UBAP1* (Ub), and stimulated with the doses of TGF-β indicated for 24 hr. Levels of PSMAD2, SMAD2/3 and Tubulin as a loading control were assayed by Western blot. A higher exposure of the PSMAD2 blot is also shown. Quantifications are the average ± SD of three independent experiments, normalized to NT samples treated with 2 ng/ml TGF-β.

* indicates $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

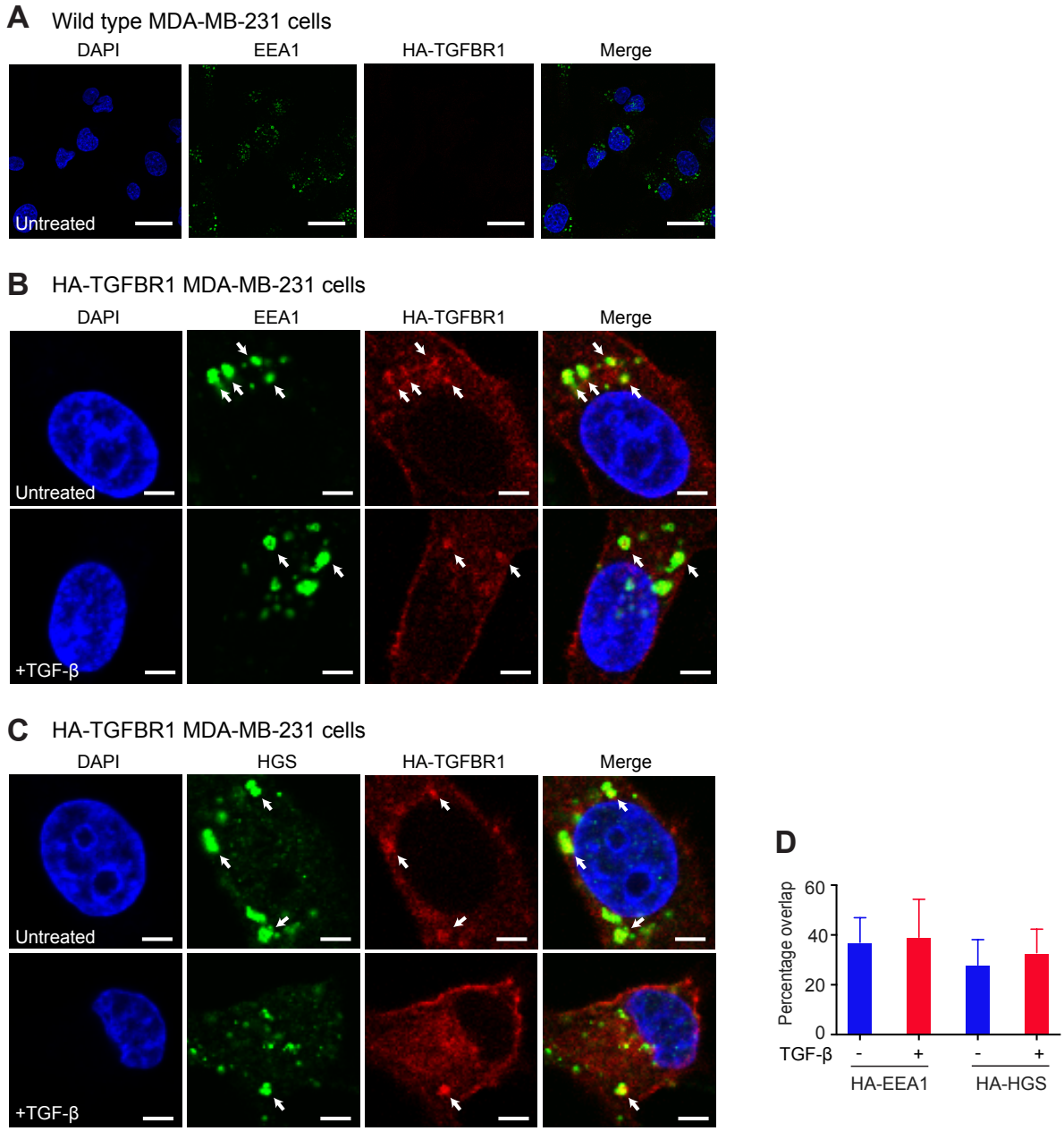

Figure S3, related to Figure 3. HA-TGFBR1 co-localizes with EEA1 and HGS.
(**A–C**) Wild type MDA-MB-231 cells (A) or MDA-MB-231 cells stably transfected with HA-TGFBR1 (B and C) were stimulated with TGF-β for 1 hr or left untreated as indicated. Cells were fixed and stained with antibodies against HA and EEA1 (A and B) or HA and HGS (C) and with DAPI to mark nuclei before confocal microscopy was performed to analyze protein localization. Shown are single slices from representative images of three independent experiments. White arrows indicate co-localization of HA with EEA1 or HGS. In (A) a field of cells is shown, whilst in (B) and (C) single cells are shown for clarity. In (A), the scale bar represents 30 µm, whilst in (B) and (C) it represents 5 µm.
(**D**) Quantifications were performed by first thresholding images to remove background, then determining the percentage overlap between signal in each channel, and are the average of 20 cells in each condition, with error bars corresponding to SDs. HA-EEA1 and HA-HGS indicate the percentage overlap between thresholded HA-TGFBR1 (red) and EEA1 (green) signals, or HA-TGFBR1 (red) and HGS (green) respectively.

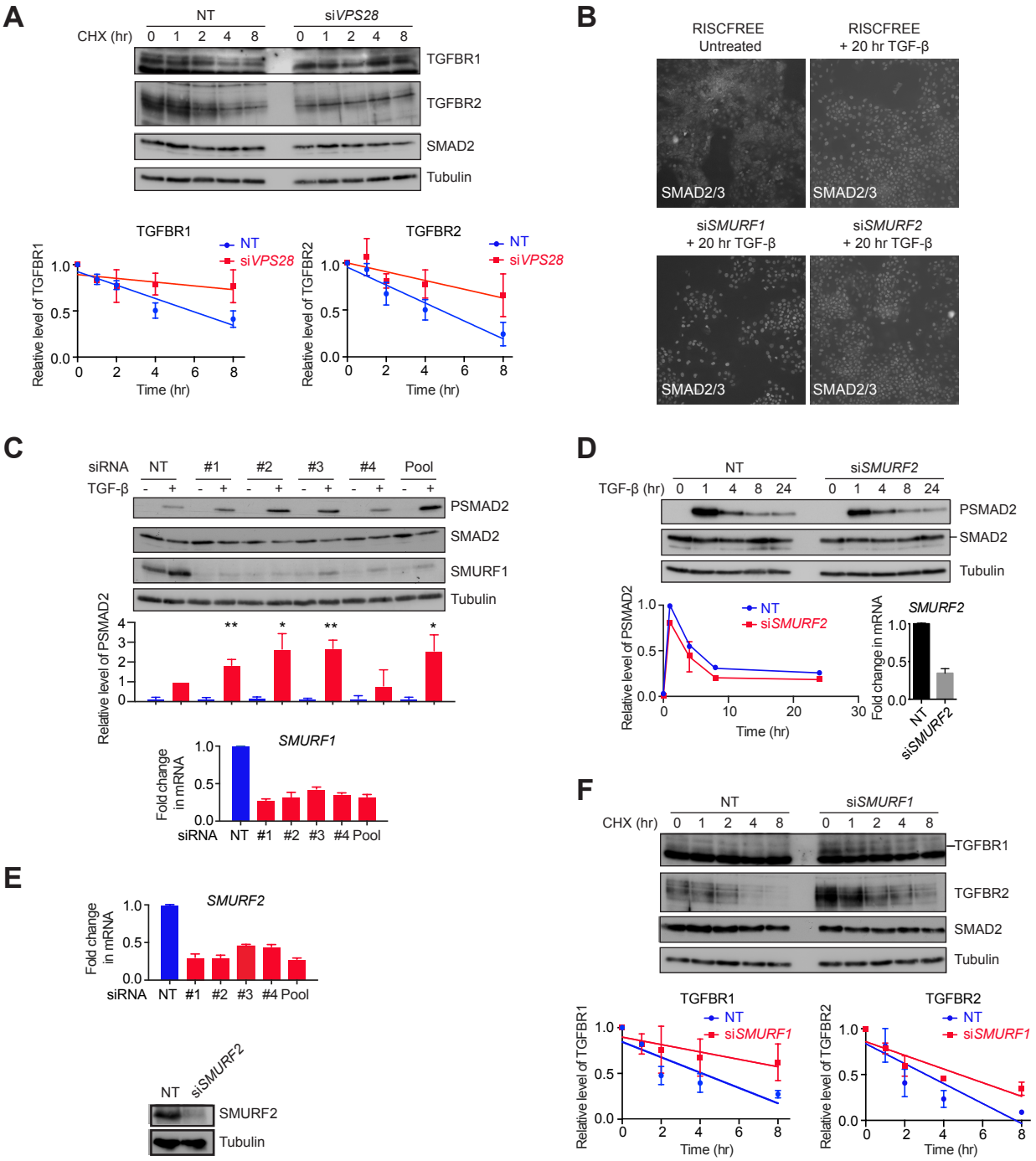

Figure S4, related to Figure 4. Knockdown of SMURF1 leads to a persistence in TGF-β-induced SMAD2 nuclear localization.
(**A**) HaCaT cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *VPS28*, and treated with cycloheximide (CHX) for the times indicated. Levels of TGFBR1, TGFBR2, SMAD2 and Tubulin as a loading control were assayed by Western blot. Quantifications are the average ± SD of three independent experiments, normalized to NT untreated samples.
(**B**) Shown are images from the 1536-well whole genome siRNA screen. HaCaTs were transfected with a whole genome siRNA library on a well-by-well basis, or with RISCFREE control siRNAs, stimulated for 20 hr with TGF-β, fixed, stained for SMAD2/3 and imaged. The effect of knockdown of SMURF1 and SMURF2 is shown.
(**C**) HaCaT cells were transfected with non-targeting (NT) control siRNAs, 4 individual siRNAs targeting *SMURF1* or a pool of the 4 together and stimulated with TGF-β for 24 hr. Levels of SMURF1, PSMAD2, SMAD2/3 and Tubulin as a loading control were assayed by Western blot. Quantifications are the average ± SD of three independent experiments, normalized to NT samples treated with TGF-β for 24 hr. * indicates $p<0.05$, ** $p<0.005$. The extent of knockdown was assessed by qPCR, and the normalized average ± SD from the same three independent experiments is shown below.
(**D**) HaCaT cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *SMURF2* and stimulated with TGF-β for the times indicated. Levels of PSMAD2, SMAD2/3 and Tubulin as a loading control were assayed by Western blot. Quantifications are the normalized average ± SD of three independent experiments. The extent of knockdown was assessed by qPCR, and the normalized average ± SD from the same three independent experiments is shown bottom right.
(**E**) HaCaT cells were transfected with non-targeting (NT) control siRNAs, 4 individual siRNAs targeting *SMURF2* or a pool of the 4 together. The extent of knockdown was assessed by qPCR, and the normalized average ± SD from three independent experiments is shown. Levels of SMURF2 and Tubulin as a loading control were also assayed by Western blot after transfection with NT or the siRNA pool.
(**F**) HaCaT cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *SMURF1* and treated with CHX for the times indicated. Levels of TGFBR1, TGFBR2, SMAD2/3 and Tubulin as a loading control were assayed by Western blot. Quantifications are the average ± SD of three independent experiments, normalized to NT untreated samples.
In (A) and (F) linear regression was used to determine a line of best fit.

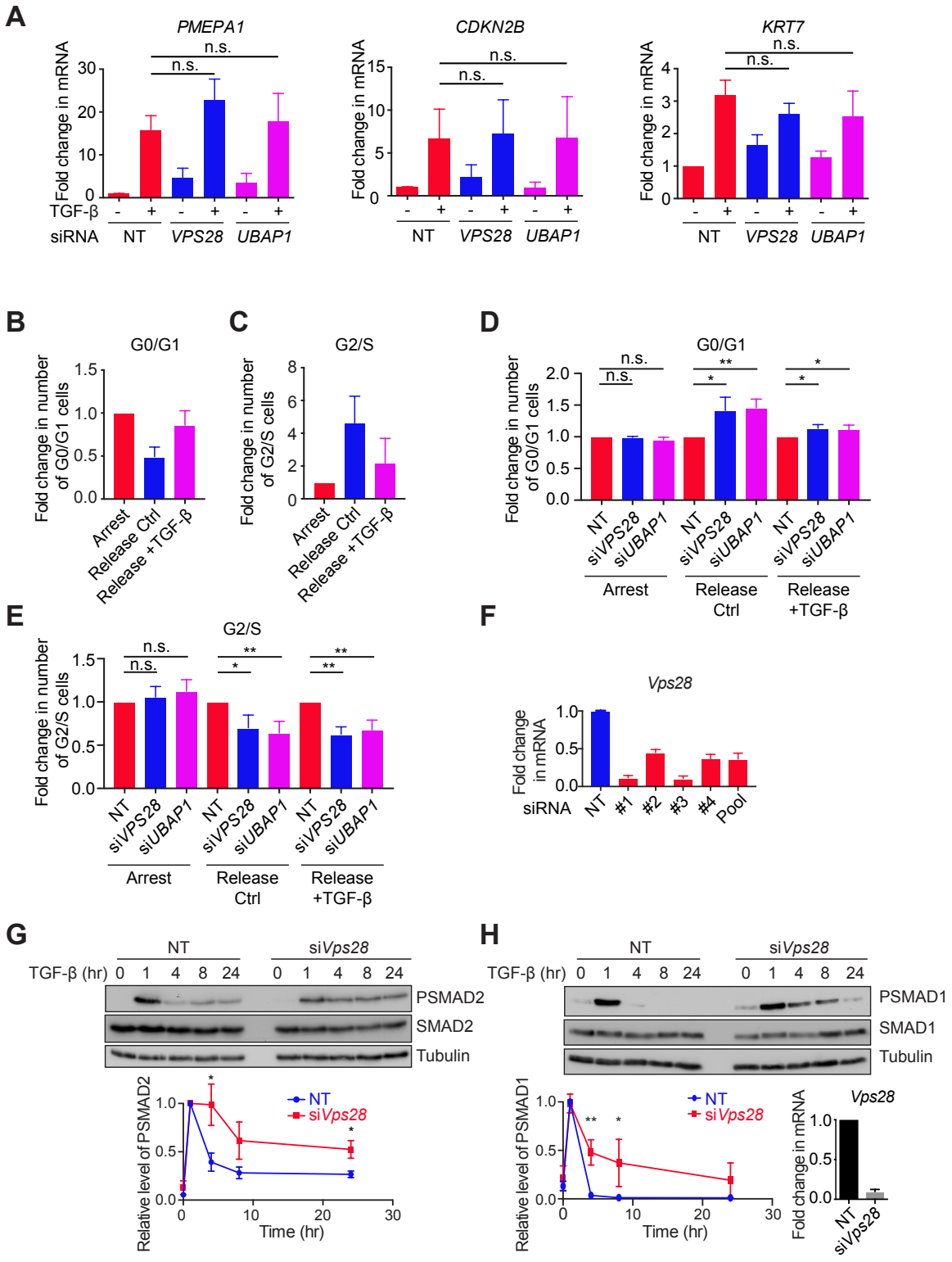

Figure S5, related to Figure 5. Downstream consequences of ESCRT knockdown on TGF-β responses.

(**A**) HaCaT cells were transfected with non-targeting (NT) control siRNAs, siRNAs targeting *VPS28* or siRNAs targeting *UBAP1* and stimulated with TGF-β for 24 hr. Gene expression were assayed by qPCR and is presented as the average ± SD of three independent experiments, normalized to levels in the NT untreated sample.

(**B–C**) HaCaT cells were serum starved for 24 hr to arrest the cell cycle (Arrest), then transferred into full serum media for 20 hr in the absence (Release Ctrl) or presence (Release +TGF-β) of 0.5 ng/ml TGF-β. Cells were fixed and cell cycle analysis performed, and cells assigned to a phase of the cell cycle. Shown is the average fold change ± SD in the proportion of cells in G0 or G1 (B) or G2/S (C), relative to arrested cells, from three independent experiments.

(**D–E**) HaCaT cells were transfected with NT siRNAs or siRNAs against *VPS28* or *UBAP1*, then treated as in (B). Shown is the average fold change ± SD in the proportion of cells in G0 or G1 (D) or G2 or S (E), relative to the NT sample in each condition, from three independent experiments. Examples of representative FACS profiles from these experiments are shown in Fig. 5B.

(**F**) NMuMG cells were transfected with non-targeting (NT) control siRNAs, 4 individual siRNAs targeting *Vps28* or a pool of the 4 together. The extent of knockdown was assessed by qPCR, and the normalized average ± SD from three independent experiments is shown.

(**G–H**) NMuMG cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *Vps28* and stimulated with TGF-β for the times indicated. Levels of PSMAD2, SMAD2 and Tubulin as a loading control (G) or PSMAD1, SMAD1 and Tubulin (H) were assayed by Western blot. Quantifications are the normalized average ± SD of three independent experiments. The extent of knockdown was assessed by qPCR, and the normalized average ± SD from the same three independent experiments is shown in the bottom right of panel H.

* indicates $p<0.05$, ** indicates $p<0.005$, n.s. indicates not significant.

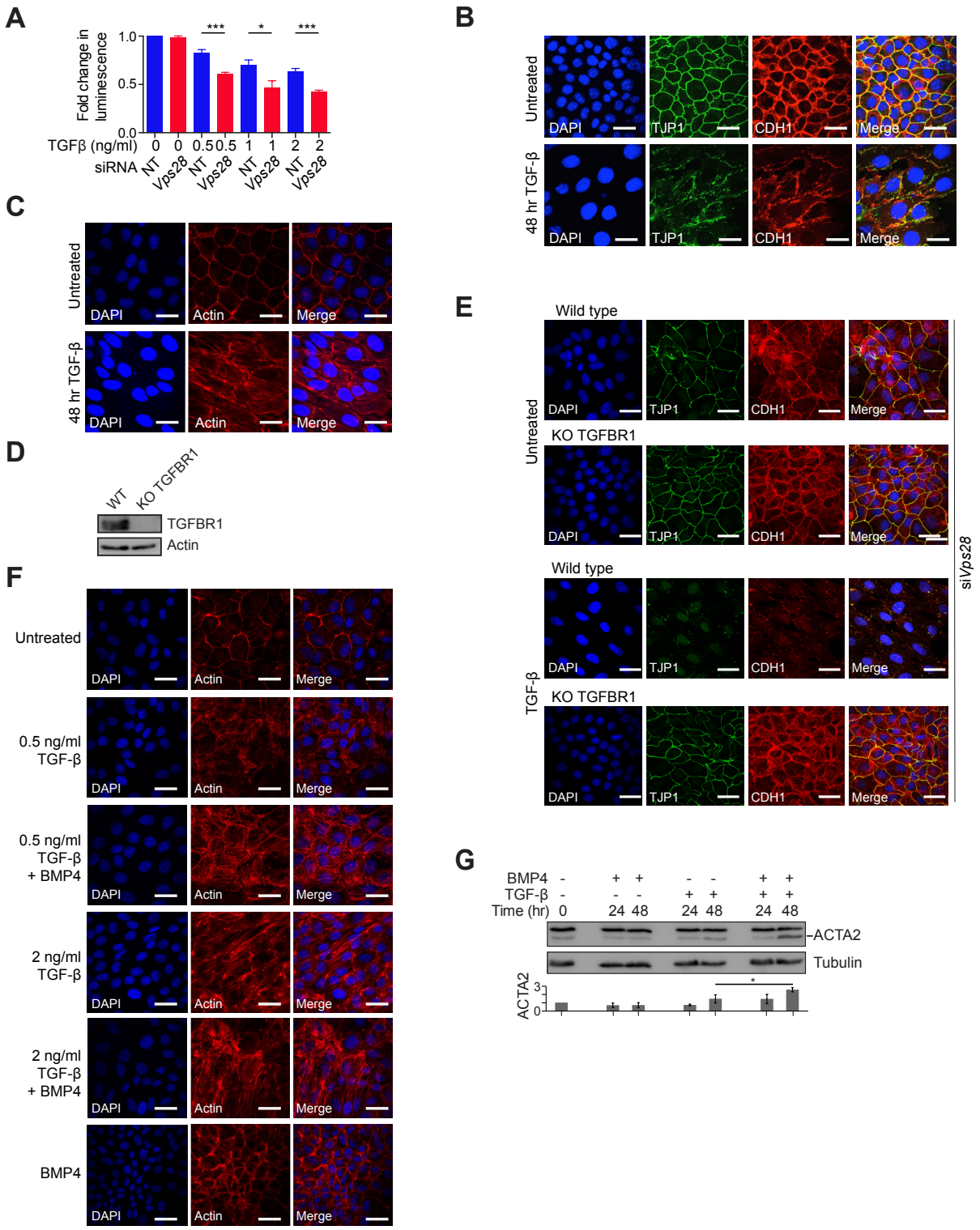

Figure S6, related to Figure 6. Knockdown of ESCRT components promotes signaling through SMAD1 in NMuMG cells and thus promotes EMT.

(**A**) NMuMG cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *Vps28*, and stimulated with 0.5, 1 or 2 ng/ml TGF-β as indicated. A CellTiter-Glo Luminescent Cell Viability Assay was performed to assess cell number. Shown is the normalized luminescence ± SDs from three independent experiments.

(**B**) NMuMG cells were treated or not with 2 ng/ml TGF-β for 48 hr. Cells were fixed and stained for TJP1/CDH1 and with DAPI to mark nuclei and imaged by confocal microscopy. Shown is a maximal Z-projection for each condition, alone and as a merge.

(**C**) NMuMG cells were treated or not with 2 ng/ml TGF-β for 48 hr. Cells were fixed and stained for Actin with phalloidin and with DAPI to mark nuclei and imaged by confocal microscopy. Shown is a maximal Z-projection for each condition, alone and as a merge.

(**D**) Levels of TGFBR1 and Actin as a loading control were assayed by Western blotting of lysates from wild type (WT) NMuMGs cells or those with TGFBR1 knocked out (KO TGFBR1).

(**E**) NMuMG cells, either wild type, or with TGFBR1 knocked out (as in D), were transfected with siRNAs targeting *Vps28* and then either untreated or treated with TGF-β for 48 hr. Cells were fixed and stained for TJP1/CDH1 and DAPI to mark the nuclei.

(**F**) NMuMG cells were treated with 0.5 or 2 ng/ml TGF-β with or without 10 ng/ml BMP4, or with 10 ng/ml BMP4 alone for 48 hr. Cells were fixed and stained for Actin with phalloidin and with DAPI to mark nuclei and imaged by confocal microscopy. Shown is a maximal Z-projection for each condition, alone and as a merge.

(**G**) NMuMG cells were treated with 10 ng/ml BMP4, 0.5 ng/ml TGF-β or the combination of the two for 24 or 48 hr. Levels of ACTA2 and Tubulin as a loading control were assayed by Western blot. Quantifications are the average ± SD of three independent experiments, normalized to untreated samples.

In all cases, scale bar indicates 30 μm. * indicates $p<0.05$, *** indicates $p<0.0005$.

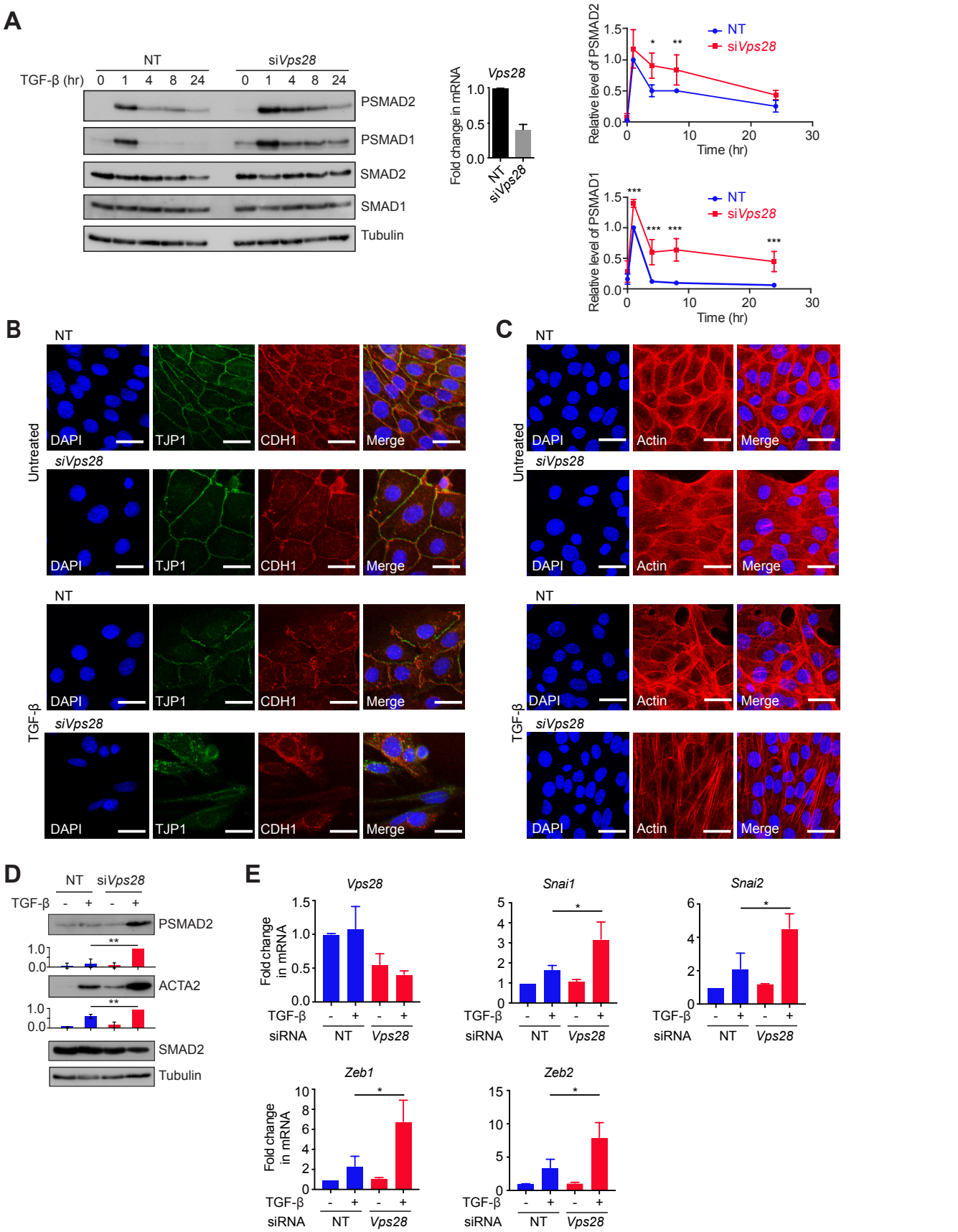

Figure S7, related to Figure 6. Knockdown of ESCRT components potentiates the ability of EpRas cells to undergo TGF-β-induced EMT.

(**A**) EpRas cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *Vps28*, and stimulated with 2 ng/ml TGF-β for the times indicated. Levels of PSMAD2, PSMAD1, SMAD2/3, SMAD1 and Tubulin as a loading control were assayed by Western blot. Quantifications (far right) are the normalized average ± SD of three independent experiments. The extent of knock-down (middle panel) was assessed by qPCR, and the normalized average ± SD from the same three independent experiments is shown.

(**B–E**) EpRas cells were reverse transfected with non-targeting (NT) control siRNAs or siRNAs targeting *Vps28*, and treated or not with 0.5 ng/ml TGF-β after 24 hr. After 72 hr, cells were re-plated and re-transfected with siRNAs as before, and treated or not with 0.5 ng/ml TGF-β for a further 96 hr. Cells were fixed and stained for TJP1 and CDH1 (B) or Actin using phalloidin (C) and DAPI to mark nuclei and imaged by confocal microscopy. Shown is a maximal Z-projection for each condition, alone and as a merge. Scale bar indicates 30 μm. Levels of PSMAD2, SMAD2/3, ACTA2 and Tubulin as a loading control were assayed by Western blot (D). Quantifications are the normalized average ± SD of three independent experiments. Expression levels of the genes indicated were assayed by qPCR (E) and are the average ± SD of three independent experiments, normalized to levels in the NT untreated sample. * indicates $p<0.05$, ** indicates $p<0.005$, *** indicates $p<0.0005$.

Table S2, related to STAR Methods. List of primers for qPCR experiments and siRNA target sequences.

List of primers used for qPCR experiments

Gene	Primer Fwd	Primer Rev	Species
VPS28	5'-CGGGAGAGGGAGAAG TACGA-3'	5'-TTCTCCAGGGCTTGC ATTGT-3'	Human
Vps28	5'-CTACTCCTGGTGTTG GAGCC-3'	5'-GTCATACTTCTCCCG CTCCC-3'	Mouse
UBAP1	5'-CAGCGGCATTCAGGT TCTAA-3'	5'-GGCAAGGAGAAGCCA ATAGGTA-3'	Human
PTPN23	5'-GCCAGCTGTGAAGAA GTTTGT-3'	5'-ACAGCCCTCAAAGTC TCGTG-3'	Human
VPS4B	5'-AGAAGGGGAATGACA GTGATGG-3'	5'-GGCTCCTTCAAGTCC AGCAA-3'	Human
TGFBR1 Exon/Exon	5'-GAACTTCCAACTACTG GCCCT-3'	5'-AATGACAGCTGCCAG TTCCA-3'	Human
TGFBR1 Intron/Exon	5'-GCCACCTACAGTGTTTT TGTCG-3'	5'-AATGACAGCTGCCAG TTCCA-3'	Human
TGFBR2 Exon/Exon	5'-GCTGGGGGCTCGGTC TA-3'	5'-GCCTCCATTTCCACA TCCGA-3'	Human
TGFBR2 Intron/Exon	5'-GCTGGGGGCTCGGTC TA-3'	5'-GGGACCACTCACCCG ACTT-3'	Human
AKAP12	5'-TGAAGAGAAACCTGC TCCGT-3'	5'-CGTTTTCTGCTCTTC GGTTC-3'	Human
CDKN1A	5'-ACTCTCAGGGTCGAA AACGG-3'	5'-ATGTAGAGCGGGCCT TTGAG-3'	Human
JUNB	5'-ATACACAGCTACGGG ATACGG-3'	5'-GCTCGGTTTCAGGAG TTTGT-3'	Human
TPM1	5'-GCAAATGTGCCGAGC TTGAA-3'	5'-CTGCGAGTACTTCTC AGCCT-3'	Human
ATF3	5'-GAGCCTGGAGCAAAA TGATG-3'	5'-TTGACAAAGGGCGTC AGGTT-3'	Human
FN1	5'-ACAAACACTAATGTTA ATTGCCCA-3'	5'-CGGGAATCTTCTCTG TCAGCC-3'	Human
PLAU	5'-CGACTCCAAAGGCAG CAATG-3'	5'-TGCTGCCCTCCGAAT TTCTT-3'	Human
COL4A2	5'-GGATGGCTATCAAGG GCCTG-3'	5'-CTGGCACCTTTTGCT AGGGA-3'	Human
PMEPA1	5'-AACGCTCTTTGTTCCA GAGCATGG-3'	5'-TCACCACCACCATCA CCATCATCA-3'	Human
CDKN2B	5'-GGGACTAGTGGAGAA GGTGC-3'	5'-CATCATCATGACCTG GATCGC-3'	Human
KRT7	5'-GGAGCCGTGAATATC TCTGTG-3'	5'-GAGAAGCTCAGGGCA TTGCT-3'	Human
SNAI1	5'-GCTGCAGGACTCTAA TCCAGA-3'	5'-ATCTCCGGAGGTGGG ATG-3'	Human

SNAI2	5’-TCGGACCCACACATTACCTTG-3’	5’-AAAAGGCTTCTCCCCCGTGT-3’	Human
SMURF1	5’-TTGGCGGGAGATGTCGAAC-3’	5’-GGTCAGGGAGCCTGAAGAAG-3’	Human
SMURF2	5’-TCAAGCTGCGCCTGACAGTA-3’	5’-GCAAATGGATCAGGAAGTCGGAA-3’	Human
Snai1	5’-TTGCCGCAGGTGGCTGATGG-3’	5’- TCCCAGCCAGTGGGTTGGCT-3’	Mouse
Snai2	5’-CATTGCCTTGTGTCTGCAAG-3’	5’-AGAAAGGCTTTTCCCCAGTG-3’	Mouse
Zeb1	5’-TATCACAATACGGGCAGGTG-3’	5’-GCCAGCAGTCATGATGAAAA-3’	Mouse
Zeb2	5’-CAGATCAGCACCAAATGCTAAC-3’	5’-ACACTCCGTGCACTTGAACTT-3’	Mouse
GAPDH	5’-CTTCAACAGCGACACCCACT-3’	5’-GTGGTCCAGGGGTCTTACTC-3’	Human
Gapdh	5’-TCTTGTGCAGTCCCAGCCT-3’	5’-CAATATGGCCAAATCCGTTCA-3’	Mouse

List of siRNA target sequences used

Gene	**Target sequence**	**Species**
VPS28_1	GCUCAGAAAUCAGCUCUAU	Human
VPS28_2	GCAUGUCGGCGUCAGAUGA	Human
VPS28_3	GGAGAAGUACGACAACAUG	Human
VPS28_4	GACGAAUUCUGCCGCAAGU	Human
UBAP1_1	UAAAGUUGGUCUACCUAUU	Human
UBAP1_2	CAUUAUGGCUCAGUUAUUG	Human
UBAP1_3	GUACGAGUGUGUCCUCAGA	Human
UBAP1_4	GAACGGGCAACCCUAGAUU	Human
PTPN23_1	GUGCACAGGUGGUAGAUUA	Human
PTPN23_2	GCAAGUCUGUGGCCCAUGA	Human
PTPN23_3	GAGCAGGCCUGUAUUCUCU	Human
PTPN23_4	GCGCUUCGCUUCACUAUGG	Human
VPS4B_1	GAAGCCGCACGUAGAAUUA	Human
VPS4B_2	CGAUAGAUCUGGCUAGCAA	Human
VPS4B_3	UGAGGAAAUGAGCGAUAUA	Human
VPS4B_4	GGAAGCAGACUUUCGGGAA	Human
SMURF1_1	GCACUAUGAUCUAUAUGUU	Human
SMURF1_2	AAAGAGAUCUAGUCCAGAA	Human
SMURF1_3	GGAAGAAGGUUUGGAUUAC	Human
SMURF1_4	AGUAGGGUGUGGACGCAAA	Human
SMURF2_1	GAUGAGAACACUCCAAUUA	Human
SMURF2_2	GACCAUACCUUCUGUGUUG	Human
SMURF2_3	CAAAGUGGAAUCAGCAUUA	Human
SMURF2_4	GAACAACACAAUUUACAGA	Human
Vps28_1	GUACAAAGCUGCCUUCCGA	Mouse
Vps28_2	GGAACAAGCCGGAGCUGUA	Mouse

Vps28_3	GAAGUAAAGCUCUACAAGA	Mouse
Vps28_4	CAGCUCCAUUGAUGAAUUU	Mouse
NT_1	UAGCGACUAAACACAUCAA	Human
NT_2	UAAGGCUAUGAAGAGAUAC	Human
NT_3	AUGUAUUGGCCUGUAUUAG	Human
NT_4	AUGAACGUGAAUUGCUCAA	Human