Supplementary Experimental Procedures

Immunoprecipitations (IPs)

For immunoprecipitations of Flag-Smad3, EpRas cells and its derivatives were either uninduced or induced with 2 ng/ml of TGF-β for 45 min and whole cell extracts were prepared using immunoprecipitation buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% NP-40, 1 mM DTT, 25 mM NaF, 25 mM Na β-glycerophosphate and protease inhibitors). Lysates were precleared with protein G beads and then incubated with anti-Flag beads (Sigma). Immunoprecipitates were fractionated on 15% SDS-polyacrylamide gels and analyzed by Western blotting with an anti-phosphorylated Smad1/3 antibody (# 9514, Cell Signaling Technology).

Luciferase assays

All Luciferase and β-Galactosidase assays were performed as previously described (1). EpRas cells and EpRas derivatives were transfected in 6-well plates with $CAGA_{12}$-Luciferase and pEF LacZ as an internal control (2). After 24 hrs, confluent cultures were seeded into 12-well plates and following overnight incubation, cells were induced with TGF-β for 8 hrs and assayed for luciferase activity using the Luciferase Reporter System (Promega). All experiments were performed in triplicate. Luciferase activity was normalized to β-Gal activity from the control EF-LacZ plasmid.

siRNA transfections

Small interfering RNA (siRNA) transfections in EpH4 cells were performed as described (3) using a Thermo Scientific Dharmacon siGENOME *SMART*pool against Smad3 (M-040706-01) or a non-targeting siRNA (D-001810-01-05). Cells were seeded the day prior to transfection in six-well plates. Each well was transfected with a 75 nM final concentration of siRNA using Dharmafect reagent 2. After 24 hours incubation, fresh media was added.

References

1. Pierreux, C. E., Nicolás, F. J., and Hill, C. S. (2000) *Mol. Cell. Biol.* **20**, 9041-9054
2. Bardwell, V. J., and Treisman, R. (1994) *Genes Dev* **8**, 1664-1677
3. Daly, A. C., Randall, R. A., and Hill, C. S. (2008) *Mol Cell Biol* **28**, 6889-6902

Supplementary Figure legends

Supplementary Figure 1. Smad3 levels increase in serum-starved EpH4 cells.
EpH4 cells were incubated in media containing 0.1% serum, maintaining them subconfluent, for 72 hours. They were then released by addition of media containing 10% serum ± 2 ng/ml TGF-β for 20 hours. Cells were harvested after the serum starvation and post release ± TGF-β and analyzed by FACS, to determine the number of cells in G1, S and G2/M, and by Western blotting, using antibodies against Smad3 and MCM7 as a loading control.

Supplementary Figure 2. Induction of Smad3 target genes, PAI-1 and JunB, in response to TGF-β in EpH4 cells with or without Smad3 knockdown and in EpRas cells.
EpH4, EpH4 cells transfected with an siRNA *SMART*pool against Smad3 and EpRas cells were induced with TGF-β (2 ng/ml) for 1, 6 and 9 hours. Lysates from cells harvested at the different time points were fractionated on a 15% SDS-polyacrylamide gel and Western blotted using antibodies against Smad3, PAI-1 and JunB. Grb2 serves as a loading control. Note that these are cycling cells so the difference in Smad3 levels between EpH4 and EpRas is not as dramatic as in quiescent cells.

Supplementary Figure 3. Knockdown of Smad3 in EpH4 cells results in an impaired response to TGF-β-induced growth inhibition.
A. EpH4 and EpH4 cells transfected with siRNA *SMART*pool against Smad3 were synchronized by contact inhibition and released into fresh medium in the presence or absence of TGF-β (2 ng/ml). Cells were harvested after the contact inhibition and after 20 hours ± TGF-β and analyzed by FACS to determine the number of cells in G1, S and G2/M. Percentages of cells in each phase of the cell cycle are given. **B.** Cells were also collected at the 20-hour time point for analysis by Western blotting with an antibody against Smad2/3 and MCM7 which serves as a loading control. **C.** The fold difference in the percentage of cells in G1 is plotted for both EpH4 cells and Smad3-depleted EpH4 cells, setting the percentage of cells in G1 in the absence of TGF-β 20 hours after release to 1. This normalizes for the observed difference in

proliferation rate of the EpH4 cells with and without the knockdown of Smad3.

Supplementary Figure 4. Stably expressed Flag-Smad3 is responsive to TGF-β in clonal EpRas cell lines (EpRas S3 C1 and C2)

A. Flag-Smad3 is phosphorylated upon TGF-β stimulation in EpRas derivatives. EpRas cells and Flag-Smad3 expressing EpRas cell lines were either uninduced or induced with TGF-β1 for 45 min and lysates were incubated with anti-Flag beads. Immunoprecipitates were fractionated on a 15% SDS-polyacrylamide gel and Western blotted with an antibody against phosphorylated Smad3, which can also detect phosphorylated Smad1 in the inputs. **B.** Luciferase reporter assays in EpRas cells and EpRas Flag-Smad3 clones. Cells were transfected with the $CAGA_{12}$-Luc reporter and EF-LacZ and induced with TGF-β for 8 hrs where indicated. Luciferase activity was assayed and normalized. The data are the means and standard deviations of three independent experiments.

Supplementary Figure 5. Expression of Flag-Smad3 in EpRas S3 clone 2 restores the TGF-β growth inhibitory response in EpRas cells, but does not interfere with progression of EMT upon TGF-β stimulation.

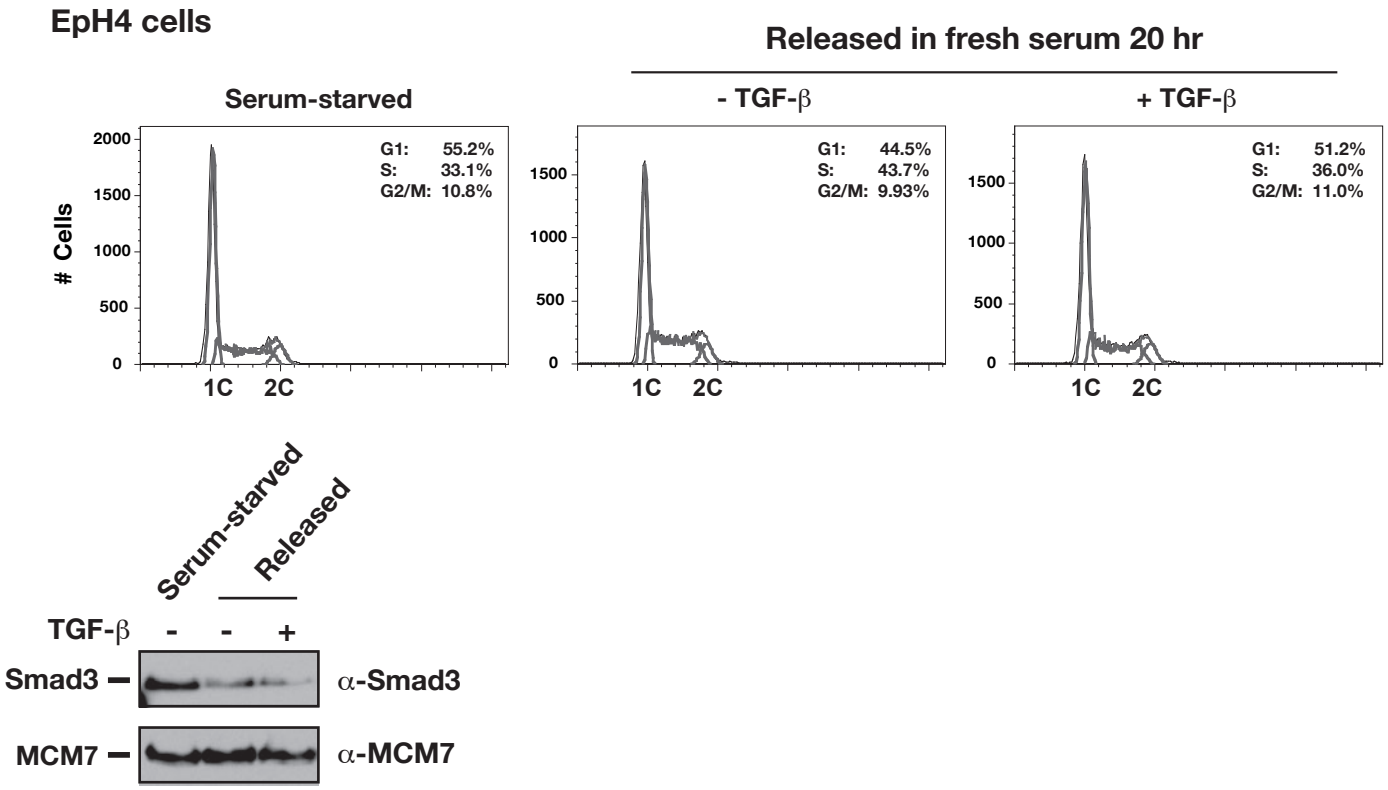
A. FACS-DNA profiles of EpRas S3 clone 2. EpRas S3 C2 cells were synchronized by contact inhibition and released into fresh medium in the presence or absence of TGF-β (2 ng/ml). Cells were collected at different time points and analyzed by FACS to determine the number of cells in G_1, S and G_2/M. The percentage of cells in each phase of the cell cycle are given. Cells were also collected for analysis by Western blotting (C). **B.** EpRas Smad3 C2 cells were plated out at low density and grown in the presence or absence of TGF-β1 (2 ng/ml). The medium was changed 1 day after seeding and then every other day. TGF-β1 was added to the cells upon medium change. Three days after plating, the cells were trypsinized and re-plated at equivalent density to day 1. Cells were grown for a total of 10 days and then were processed for immunofluorescence using an anti-E-cadherin antibody to analyze adherens junctions or an anti-Zona Occludens 1 (ZO-1) antibody to analyze tight junctions (left panel). Smad2/3 localization and actin reorganization was visualized with an anti-Smad2/3 antibody and Texas red-conjugated phalloidin respectively (right panel). The E-cadherin and ZO-1 staining was performed on one sample of cells, and the Smad2/3

and phalloidin staining on another. **C.** Expression of Flag-Smad3 in EpRas S3 C2 in quiescent cells and after release from quiescence (0-20 hrs). Lysates of EpRas S3 C2 at different timepoints were fractionated on a 15% SDS-polyacrylamide gel and blotted with an antibody against Smad3. Flag-Smad is detected as a band running with slightly lower mobility compared to endogenous Smad3 (see arrows). Grb2 serves as a loading control.

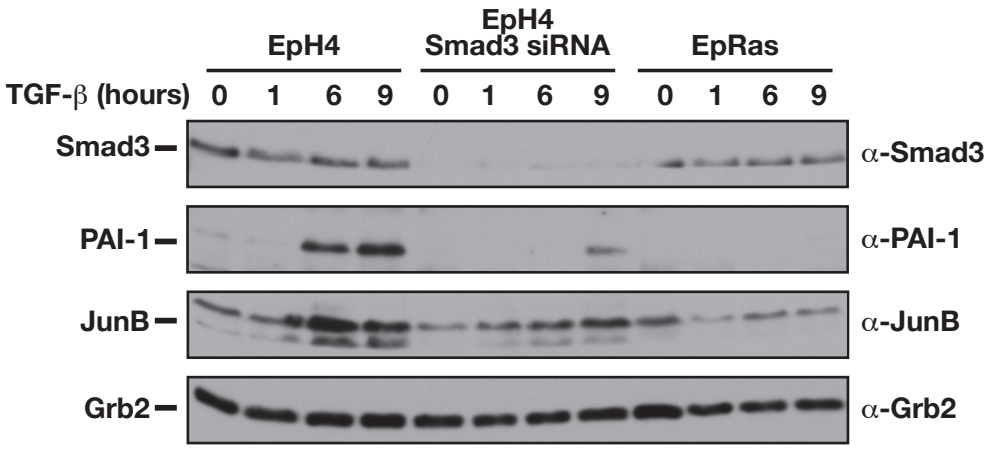
Supplementary Figure 6. Knockdown of Smad3 in EpH4 cells in not sufficient to induce EMT in response to TGF-β.

EpH4 cells, EpH4 cells transfected with siRNA a *SMART*pool against Smad3 (S3) or a non-targeting siRNA (NT) and EpRas cells were plated out at low density and either grown in the presence or absence of TGF-β (2 ng/ml). Cells were maintained subconfluent for 10 days as descibed above. siRNA transfection was performed in the EpH4 cells at day 1 and 4. Cells were processed for immunofluorescence using an anti-E-cadherin antibody to analyze adherens junctions or an anti-Zona Occludens 1 (ZO-1) antibody to analyze tight junctions. Smad2/3 localization and actin reorganization was visualized with an anti-Smad2/3 antibody and Texas red-conjugated phalloidin, respectively. Vimentin staining was also performed to visualize intermediate filaments, but it is shown just in EpRas cells due to its complete absence in EpH4 cells either in uninduced or induced conditions. The E-cadherin and ZO-1 staining was performed on one sample of cells, the Smad2/3 and phalloidin staining on another and Vimentin on a third sample. Smad3 levels are also shown by Western blotting, using Grb2 as a loading control, to confirm efficient siRNA knockdown of Smad3 in the EpH4 cells.

Supplementary Figure 7. Quantification of Smad3 levels relative to Smad2 levels in a panel of colorectal cancer cell lines. Cell lysates from EpH4, EpRas, HaCaT and a panel of colorectal cell lines were fractionated on a 15% SDS-polyacrylamide gel and Western blotted with an antibody against Smad2/3. LI-COR's Odyssey™ Infrared Imaging System using IRDye™ 800 (Rockland Immuno-chemicals) as the secondary antibody was used to quantify the levels of Smad3 and Smad2, and the ratio of Smad3 to Smad2 is shown.



Daly_Supplementary Figure 1



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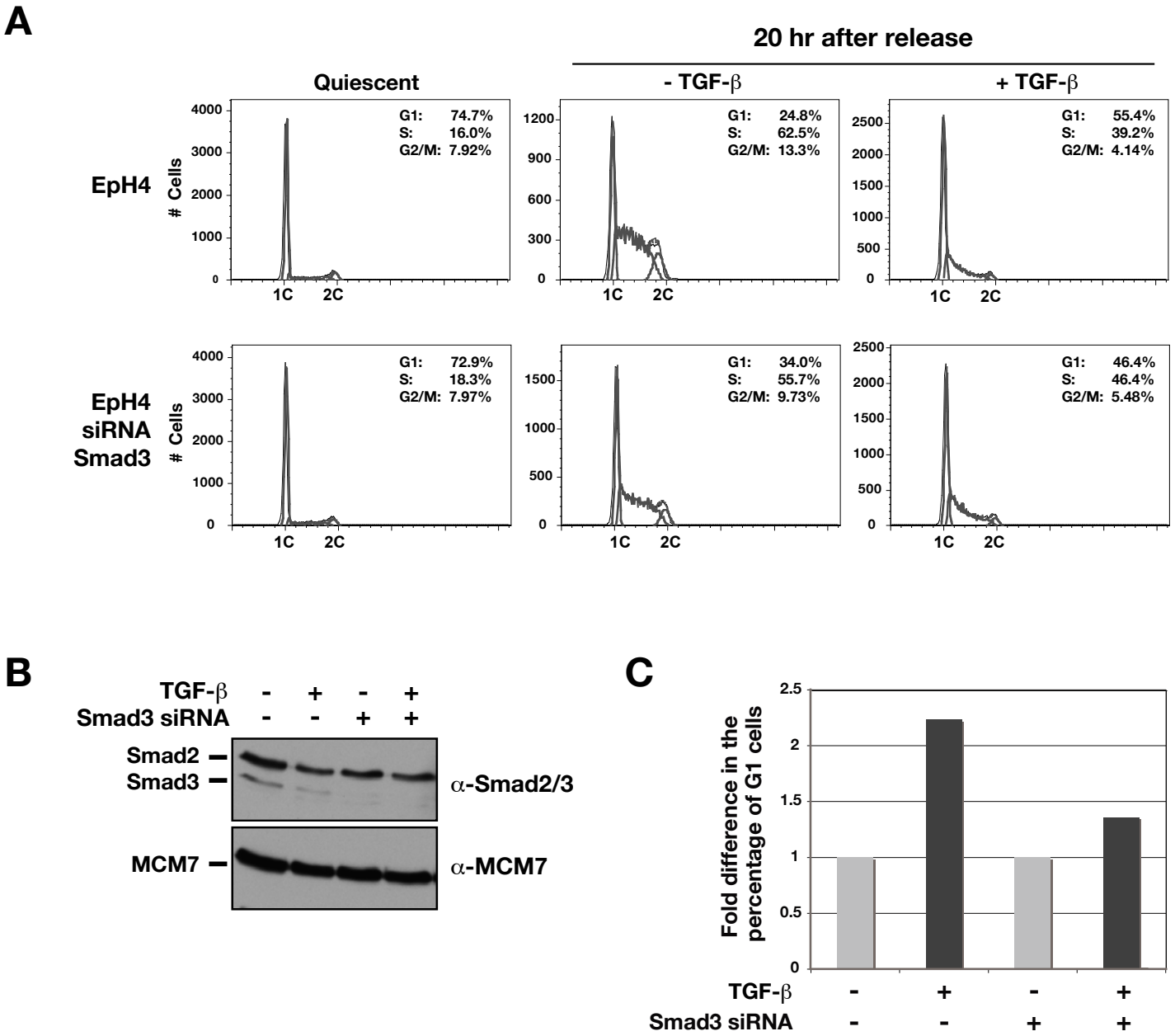

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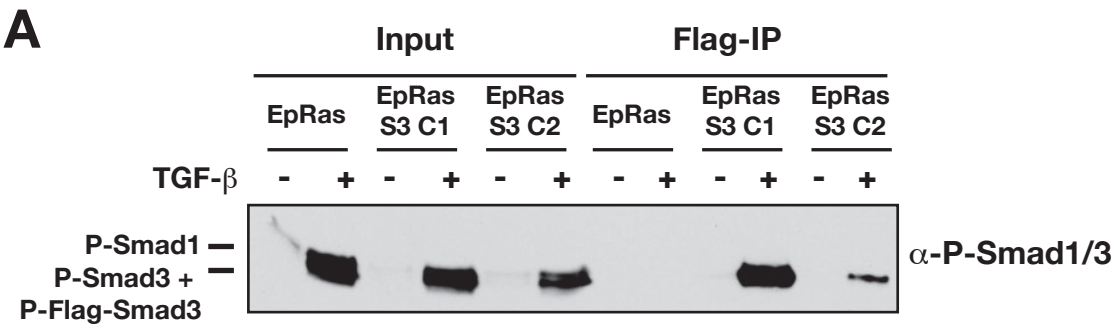

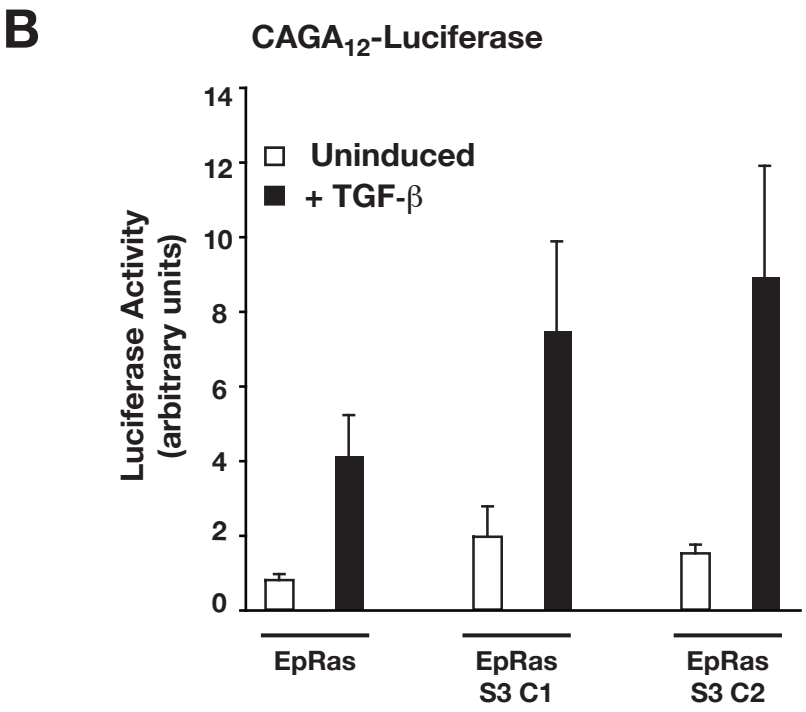

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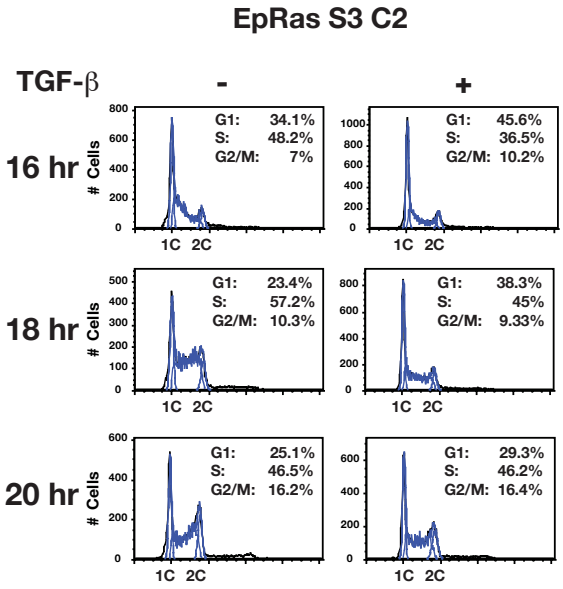
A



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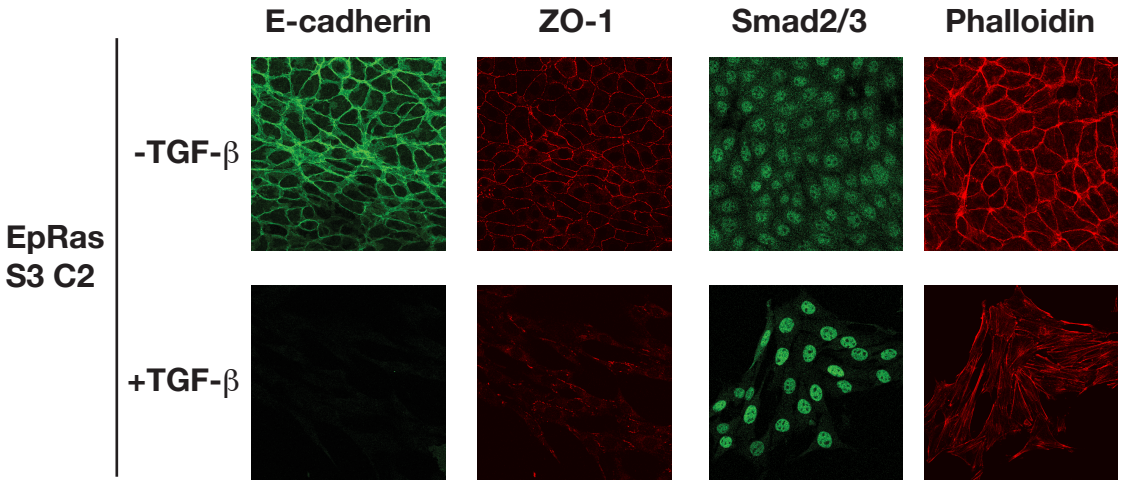

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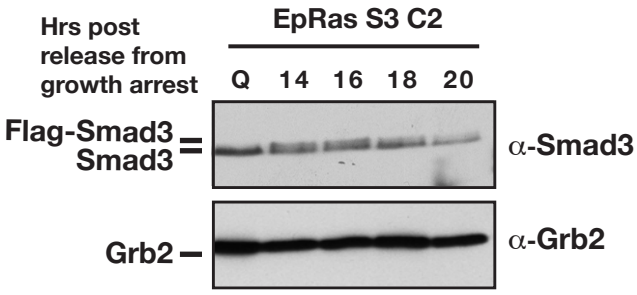

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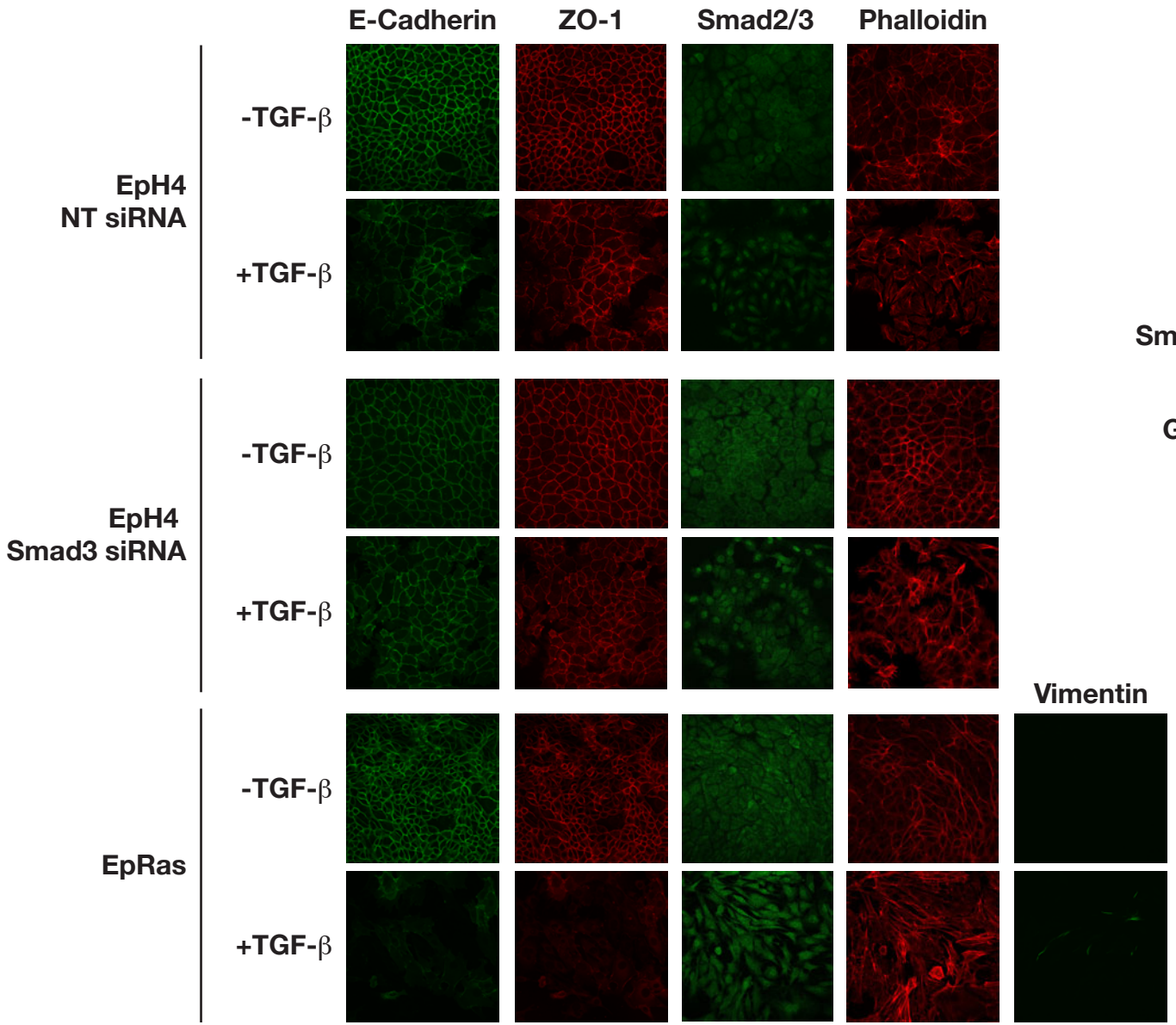

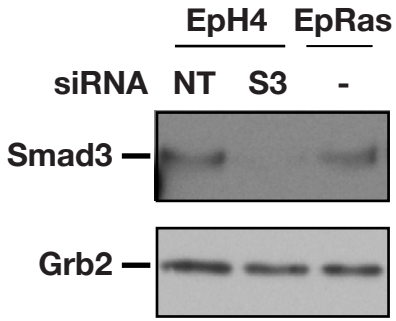

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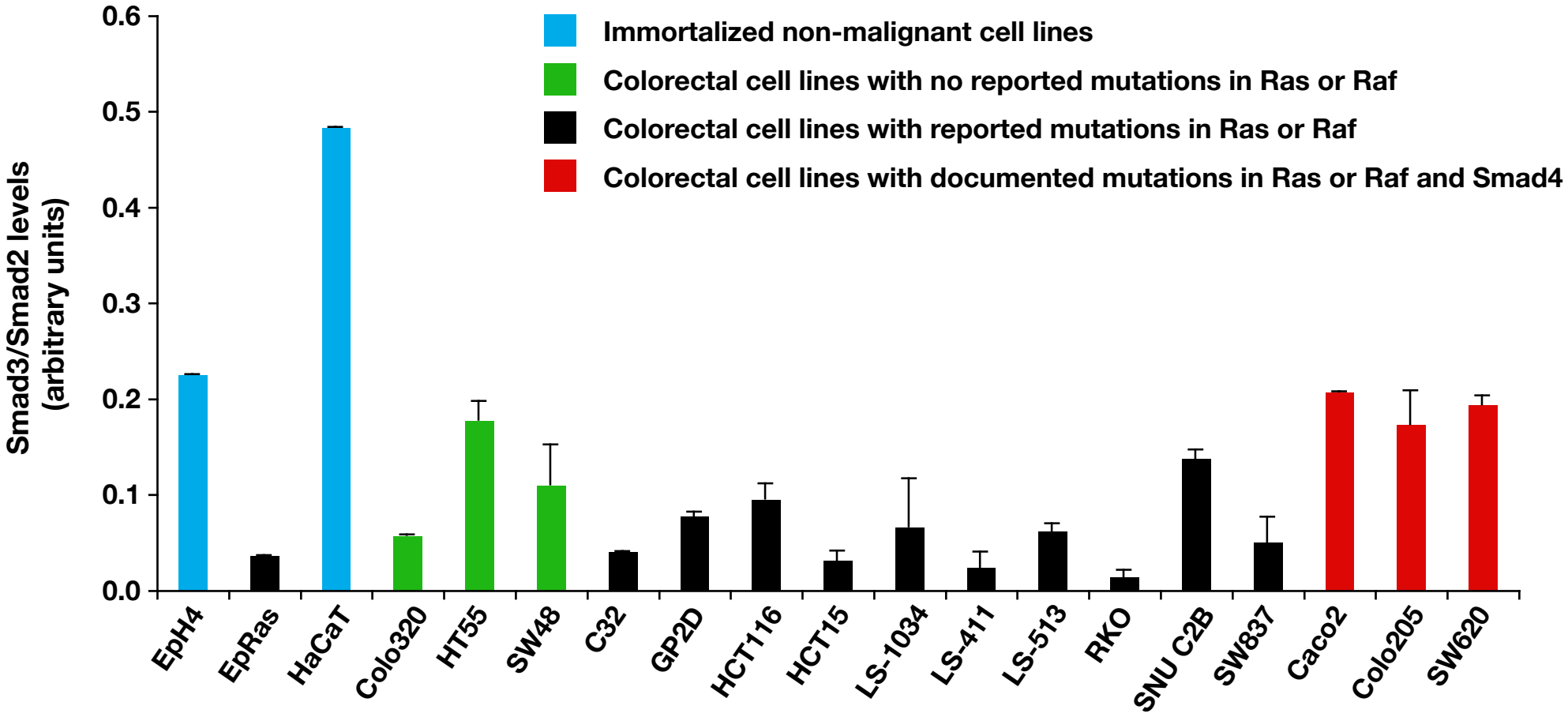

Daly_Supplementary Figure 7