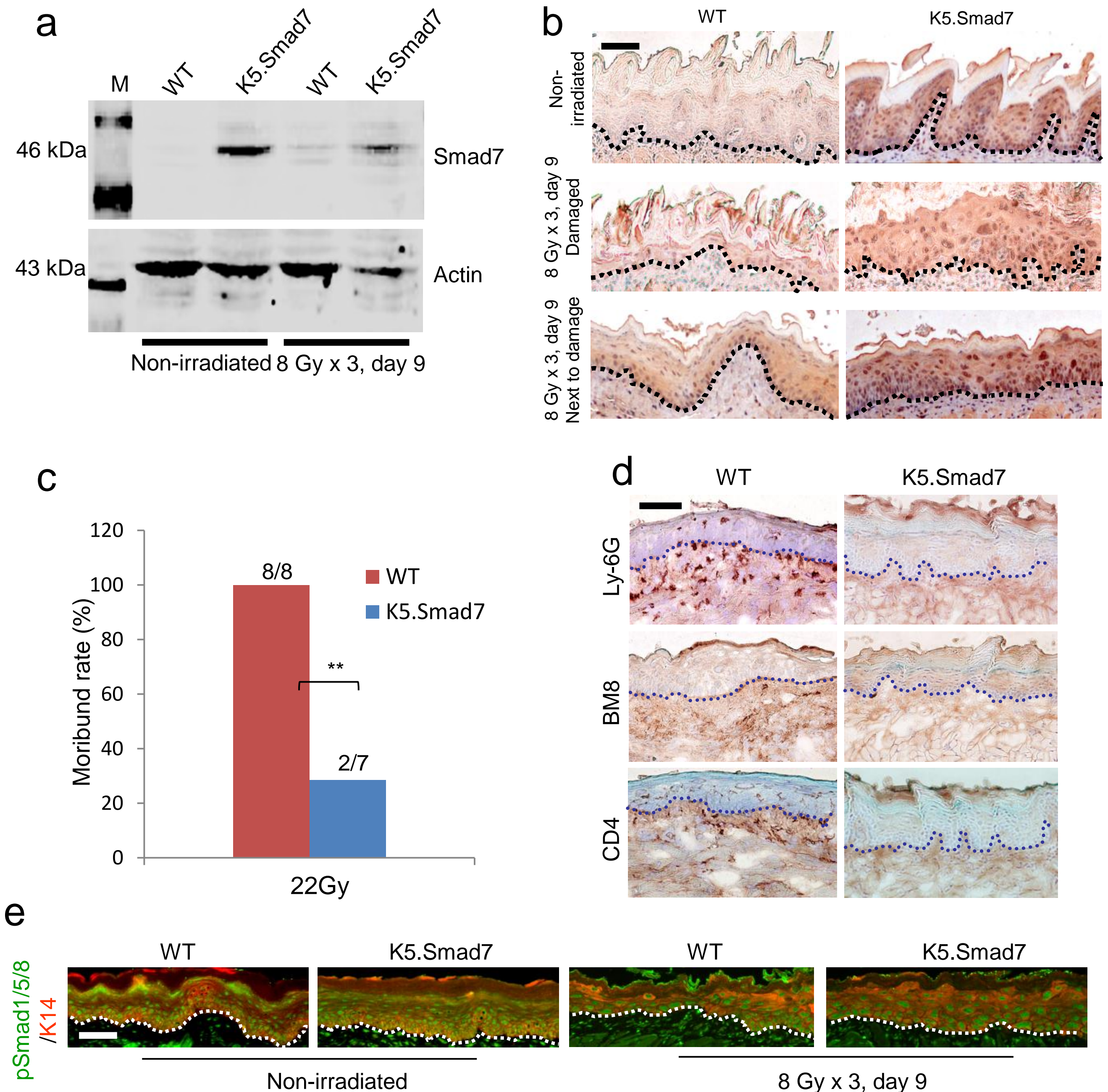
Preventive and therapeutic effects of Smad7 on radiation-induced oral mucositis

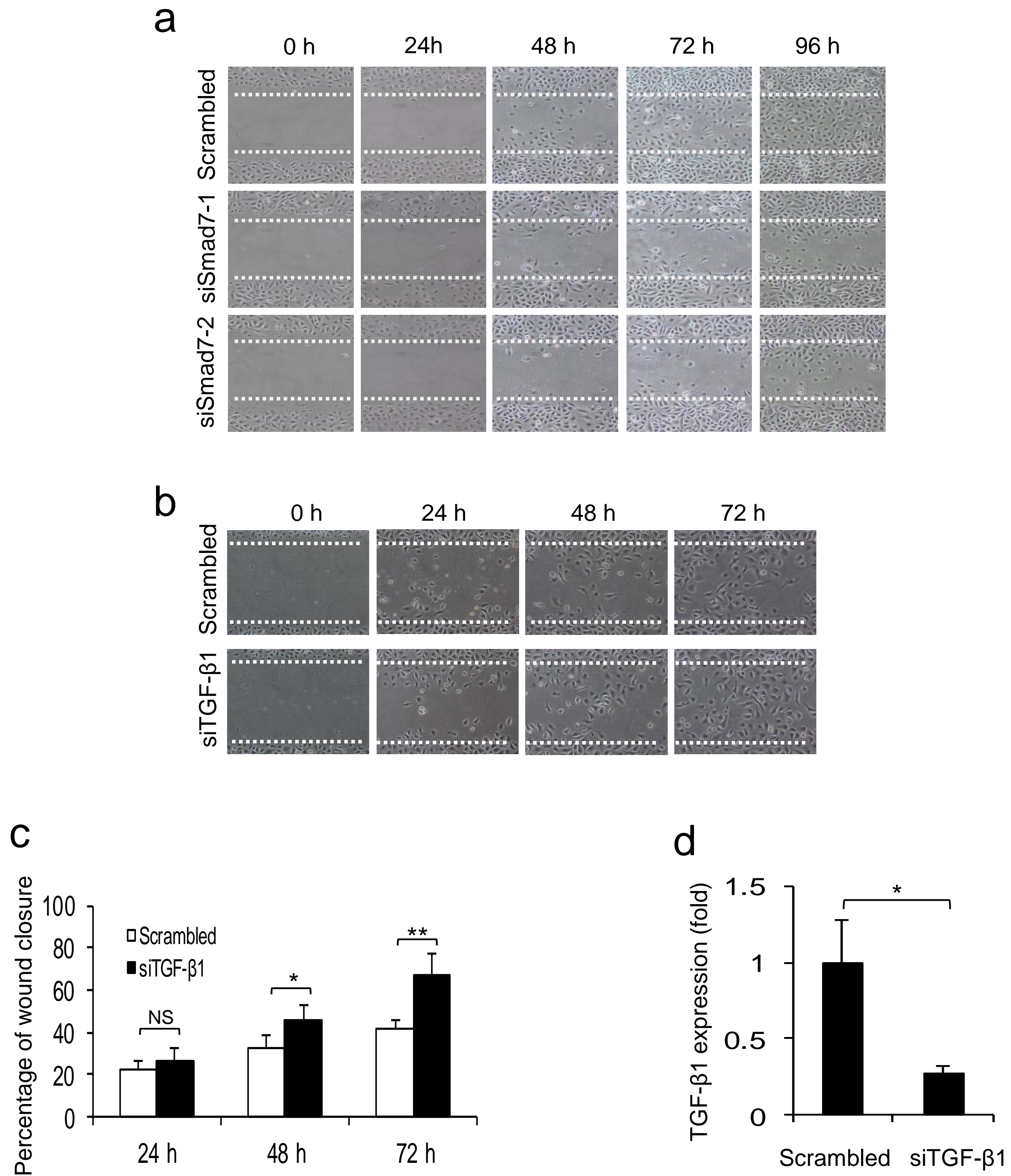
Gangwen Han*, Li Bian*, Fulun Li, Ana Cotrim, Donna Wang, Jian Bo Lu, Yu Deng, Gregory Bird, Anastasia Sowers, James B. Mitchell, J. Silvio Gutkind, Rui Zhao, David Raben, Peter ten Dijke, Yosef Refaeli, Qinghong Zhang, and Xiao-Jing Wang

*The authors contributed equally to this work.

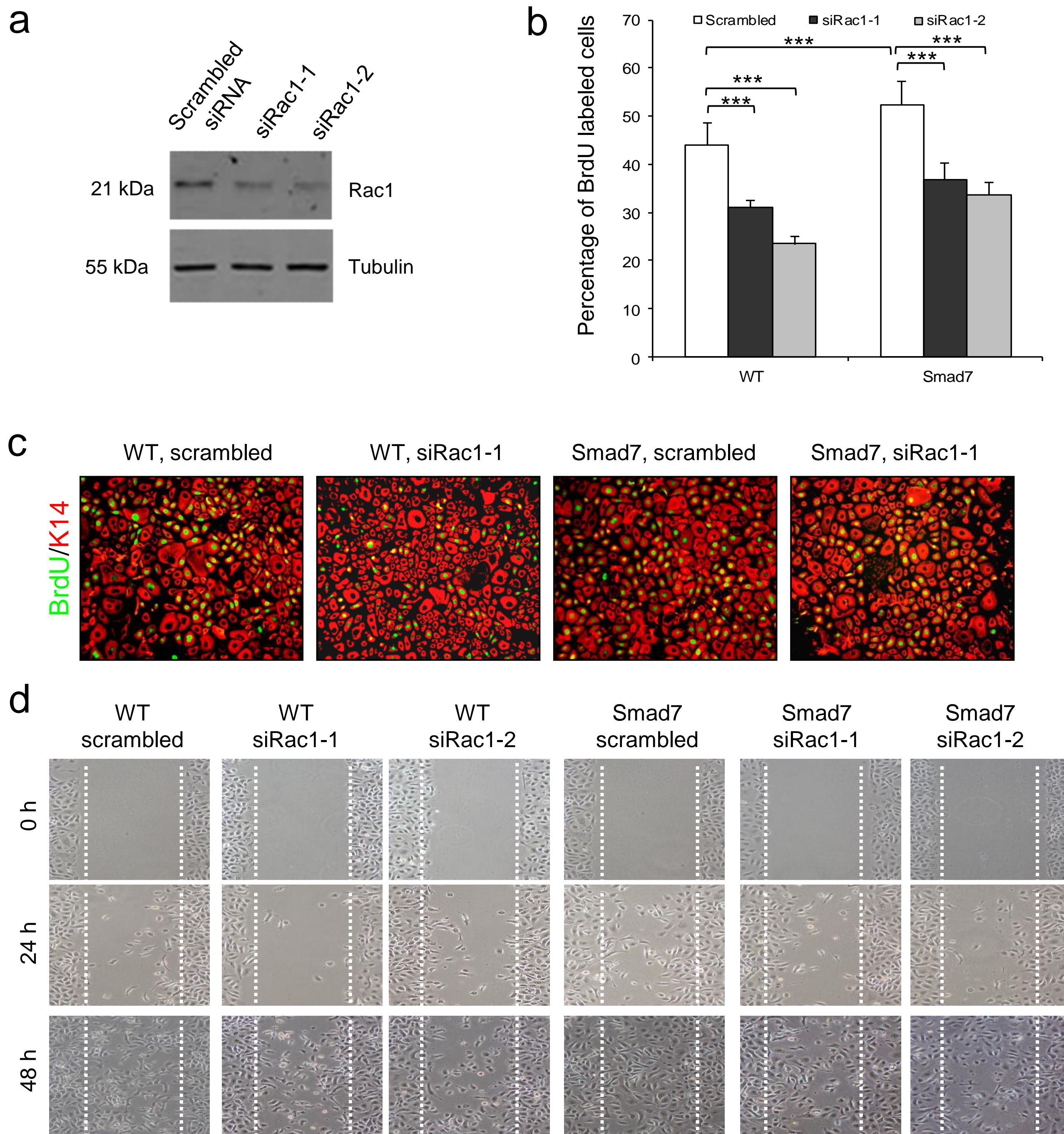
Supplementary Information



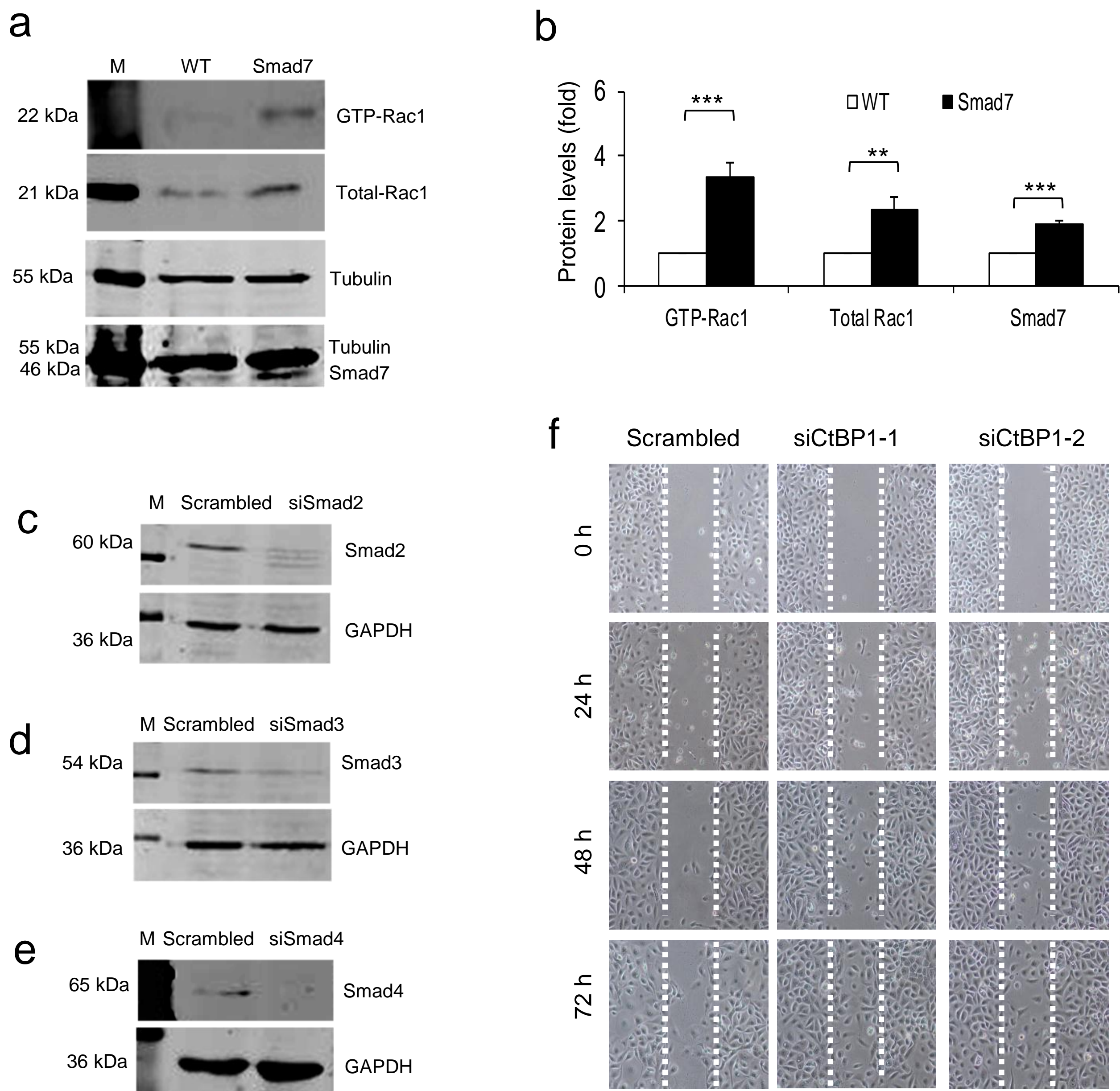
Supplementary Figure 1. Smad7 transgene expression in oral mucosa caused resistance to radiation-induced oral mucositis but did not affect pSmad1/5/8. (**a**) Smad7 western blots: undetectable in non-irradiated wildtype (WT) tongue and barely detectable after radiation. K5.Smad7 tongues have comparable Smad7 protein levels before and after radiation. M: molecular marker. (**b**) Smad7 immunostaining. Note that nuclei in some irradiated epithelial cells are hypertrophic. Dotted lines delineate epithelial-stromal boundary. (**c**) Reduced incidence of oral mucositis-induced morbidity in K5.Smad7 mice. Fisher's exact test is used to calculate the *P* value. **: *P* = 0.007. (**d**) K5.Smad7 tongue shows reduced infiltration of neutrophils (Ly-6G), macrophages (BM8) and activated T cells (CD4) compared to WT oral mucositis**.** Dotted lines delineate epithelial-stromal boundary. (**e**) No significant difference in pSmad1/5/8-nuclear positive cells (green) between WT and K5.Smad7 oral mucosa before or after radiation. Keratin (K14) immunostaining (red) highlights the epithelial compartment. Note that nuclei of irradiated epithelial cells are hypertrophic. The scale bar: 50 μm for all panels.



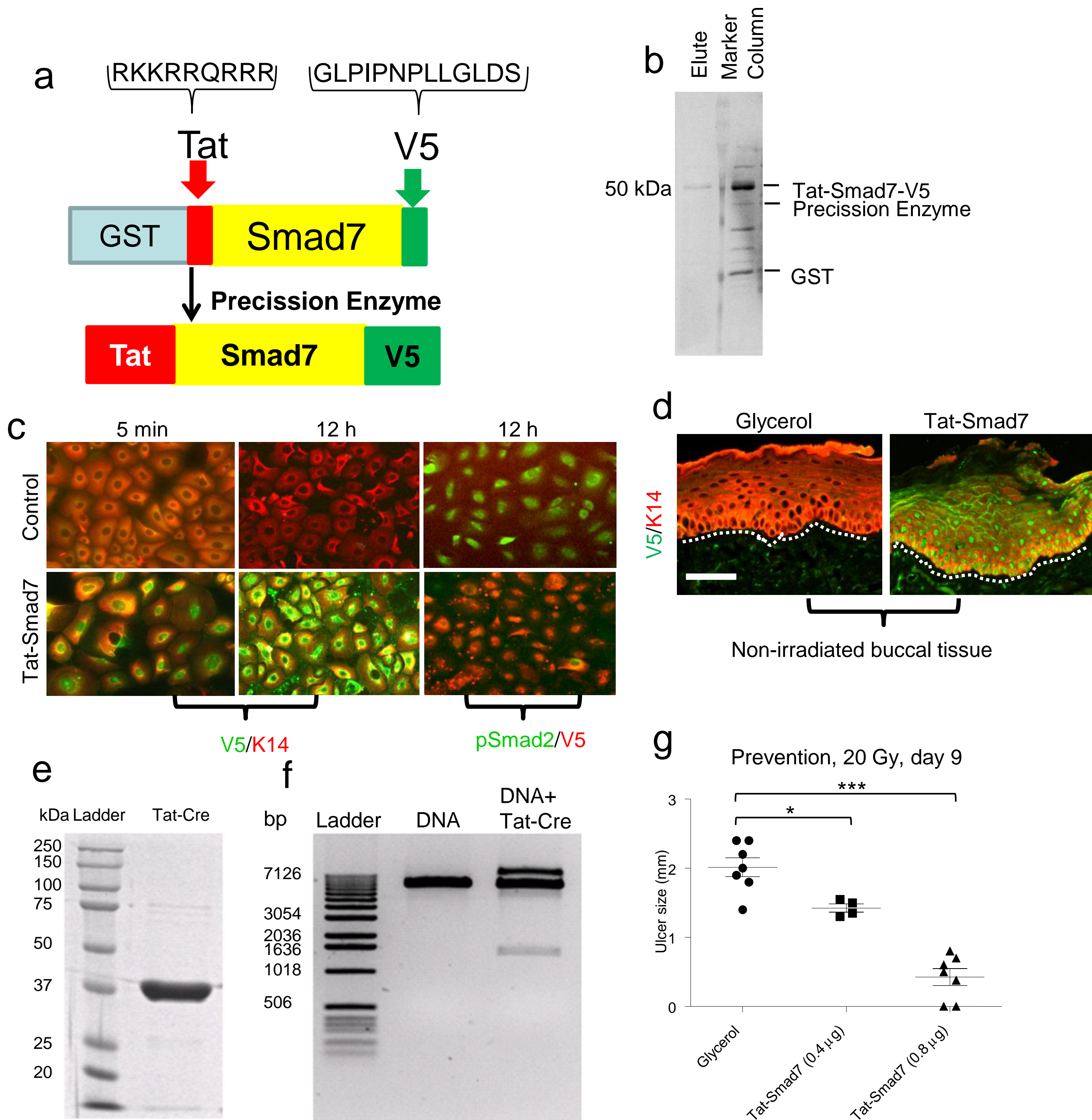
Supplementary Figure 2. Migration in spontaneously immortalized human oral epithelial cells (NOK-SI) was delayed by knocking down Smad7 but accelerated by knocking down TGF-β1. (**a, b**) Representative images of cell migration. Pairs of dotted lines delineate the scratch wound. Quantification of cell migration and efficiency of Smad7 knockdown are presented in Fig. 2d and Fig. 2e. Scrambled: scrambled siRNA. (**c**) Quantification of cell migration after TGF-β1 knockdown from 3 separate experiments. (**d**) qRT-PCR showing TGF-β1 knockdown efficiency. Data are presented as mean ± s.d. and Two-tail Student T-test was used to calculate *P* values. *: $P < 0.05$, **: $P < 0.01$. NS: no significance.



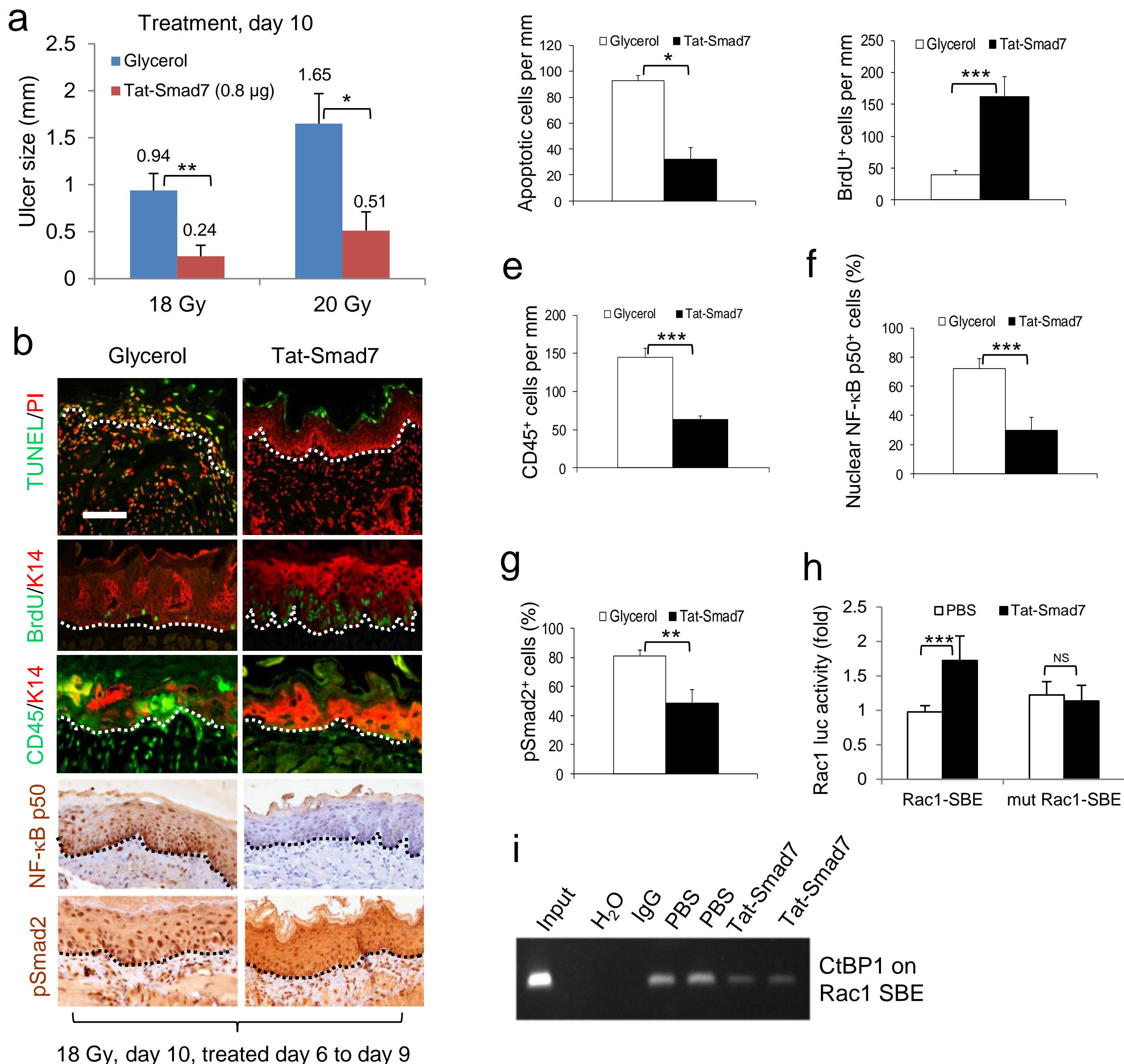
Supplementary Figure 3. Knocking down Rac1 reduced proliferation and migration of wildtype (WT) and Smad7 transgenic keratinocytes. (**a**) Western blot for Rac1 48 hours after Rac1 siRNA (siRac1-1, siRac1-2) transfection. Control: Scrambled siRNA. (**b**) Percentage of BrdU labeled cells in WT and Smad7 transgenic keratinocytes with or without Rac1 knockdown. Data from 3 separate experiments were presented as mean ± s.d. and Two-tail Student T-test was used to calculate *P* values. ***: $P < 0.001$. (**c**) Representative immunofluorescence of BrdU positive cells presented in (**b**). An antibody against keratin 14 (K14, red) was used for counterstain. (**d**) *In vitro* cell migration assay for WT and Smad7 transgenic keratinocytes after Rac1 knockdown. Pairs of dotted lines delineate the scratch wound. Quantification of cell migration is presented in Fig. 2g.



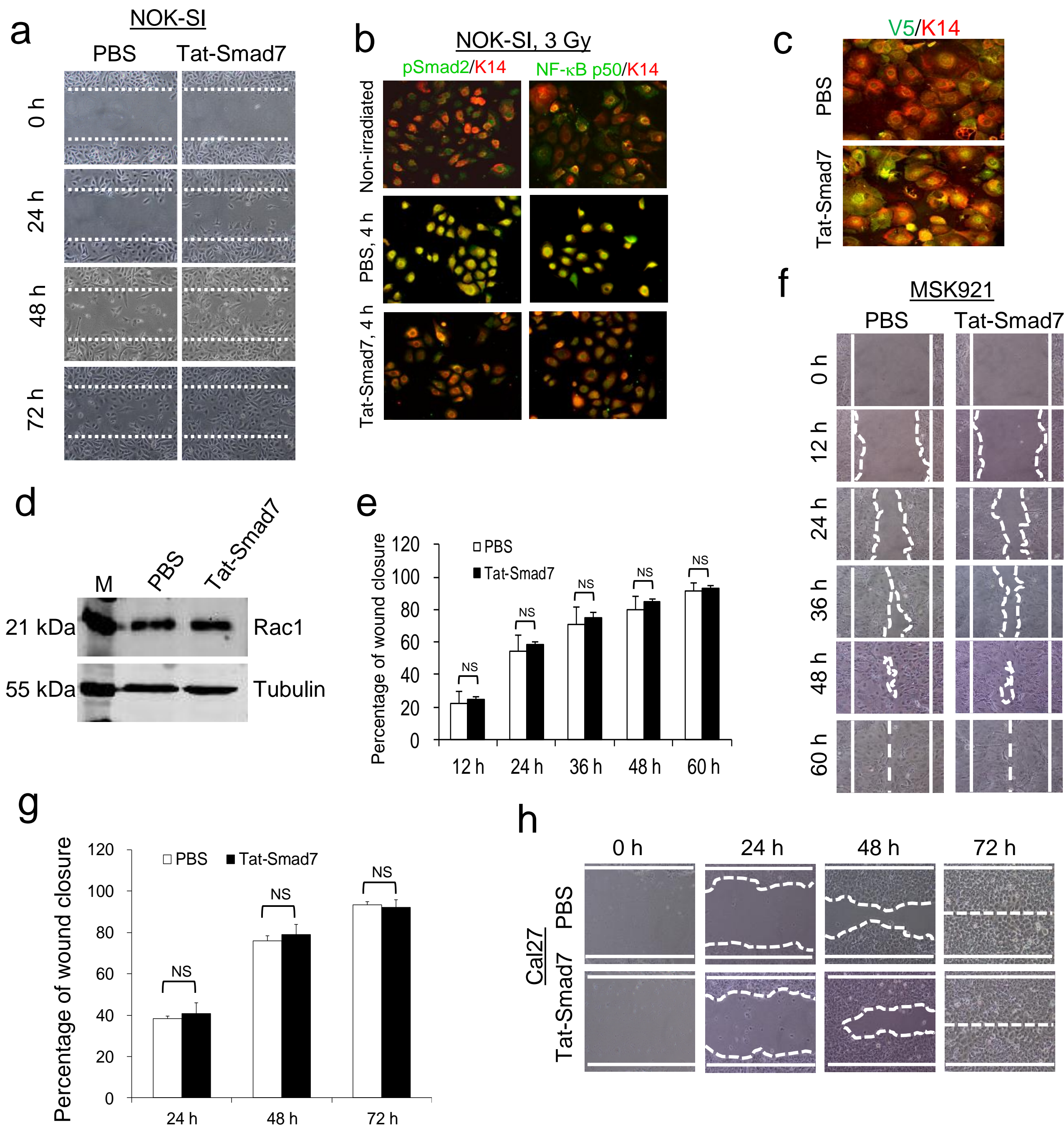
Supplementary Figure 4. Increased Rac1 expression by Smad7, knockdown efficiencies of individual Smads, and increased keratinocyte migration by knocking CtBP1. (a) Western blot for GTP-Rac1 and total Rac1 in wildtype (WT) and Smad7 transgenic keratinocytes. Additional samples are shown in Fig. 3b. M: molecular marker. (**b**) Quantification of GTP-Rac1, total Rac1 and Smad7 in WT and Smad7 transgenic keratinocytes shown in (**a**) and in Fig. 3b. The protein level in WT keratinocytes of each blot was normalized as "1". Data is presented as mean ± s.d. and Two-tail Student T-test was used to calculate *P* values. **: $P < 0.01$, ***: $P < 0.001$. (**c-d**) Western blots for Smad2, Smad3 and Smad4 knockdown in human keratinocytes. Their effects on Rac1 expression are shown in Fig. 3c. M: molecular marker. GAPDH: internal protein control by reprobing the same blot. (**f**) NOK-SI cell migration. Pairs of dotted lines delineate the scratch wound. Quantification of cell migration and efficiency of CtBP1 knockdown are shown in Fig. 4a and Fig. 4c.



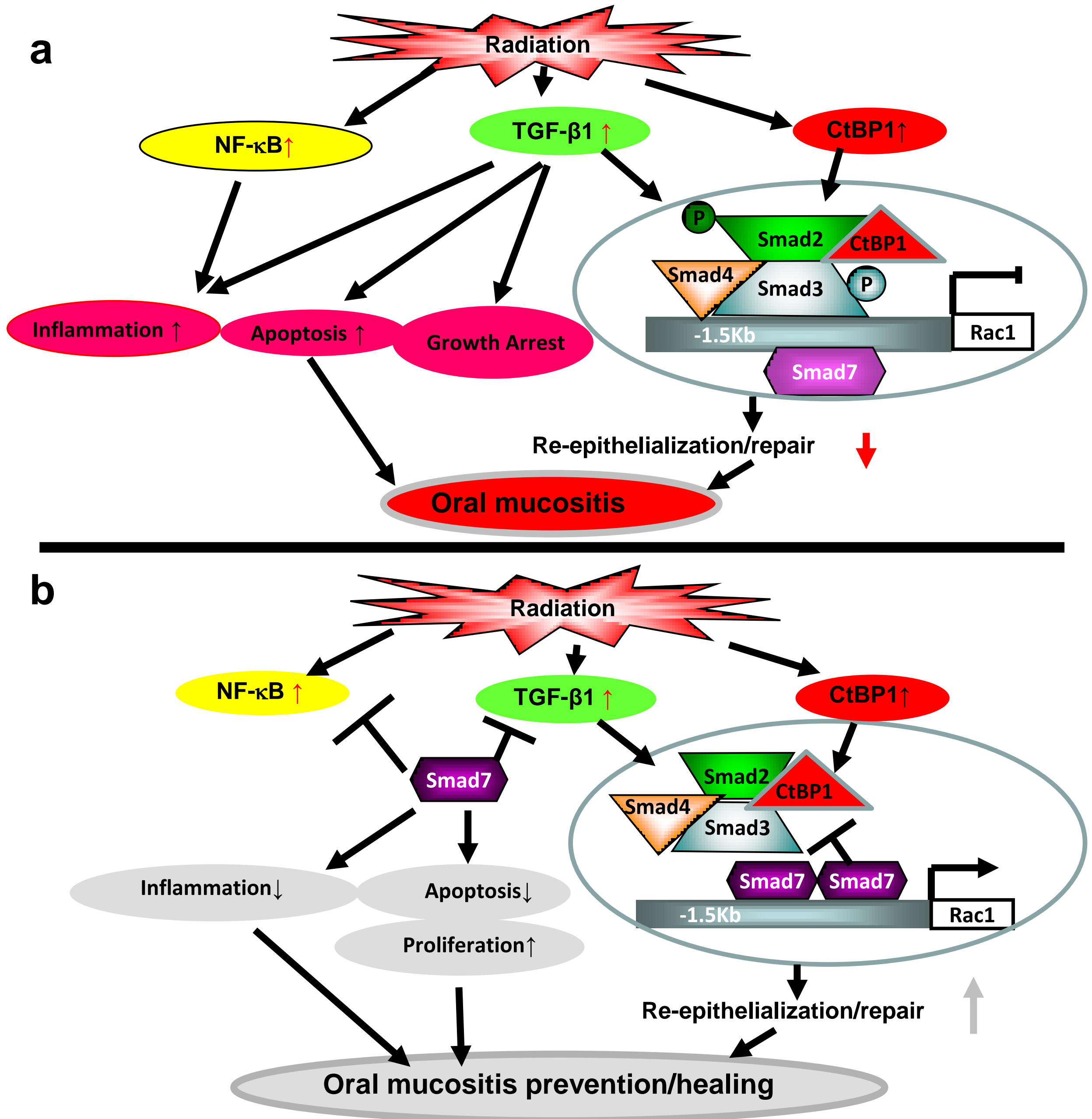
Supplementary Figure 5. Purification and characterization of Tat-Smad7 and Tat-Cre proteins. (**a**) Schematic of Tat-Smad7 protein. (**b**) Coomassie stained SDS-PAGE gel for purified Tat-Smad7 protein. (**c**) Tat-Smad7 protein transduction to keratinocytes. Left and middle panels: Tat-Smad7 staining (green) using a V5 antibody, counterstained with a K14 antibody (red). Cells showed Tat-Smad7 in the nucleus 5 min after transduction and in both nucleus and cytoplasm 12 hours after transduction. Right panels: Tat-Smad7 abrogated Smad2 phosphorylation (pSmad2, green). V5 (red) counterstain visualizes Tat-Smad7 transduced cells. (**d**) V5 antibody staining detects Tat-Smad7 transduction in buccal mucosa 12 hours after Tat-Smad7 (1 µg) topical application. A K14 antibody was used for counterstain. Scale bar: 50 µm for both panels. (**e**) Sliver stained SDS-PAGE gel for purified Tat-Cre protein with the same Tat and V5 tags in (**a**). (**f**) Agarose gel showing activity of Tat-Cre: Tat-Cre cuts out a 1,460 bp floxed fragment from the 7,650 bp vector pLL3.7 (Addgene). (**g**) Tat-Smad7 protein preventive treatment reduced 20 Gy radiation-induced oral ulcers. Data are expressed as mean ± s.e.m. and Two-tail Student T-test is used to calculate P values. *: $P < 0.05$, ***: $P < 0.001$.



Supplementary Figure 6. Effects of Tat-Smad7 treatment on oral mucositis. (**a**) Reduced ulcer size in Tat-Smad7 (0.8 µg daily, day 6 to day 9) treated oral mucosa. Samples were harvested on day 10. N = 8 per group, except N= 7 for 20 Gy Glycerol group. **(b)** Immunostaining of molecular markers for samples from (**a**). Scale bar: 50 µm for the top two panels and 25 µm for other panels. Propidium iodide (PI) and K14 were used as counterstain. (**c-g**) Quantifications of immunostaining shown in (**c**). 3-4 samples were used. **(h)** Luciferase assay. Tat-Smad7 treatment increased activity of the Rac1 promoter with SBE but not the mutant SBE in mouse keratinocytes. **(i)** ChIP assay for CtBP1 binding to the SBE of mouse Rac1 promoter in Tat-Smad7 treated mouse keratinocytes. Data are expressed as mean ± s.e.m (**a**) or mean ± s.d (**c-h**) and Two-tail Student T-test is used to calculate P values. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. NS: no significance.



Supplementary Figure 7. Effects of Tat-Smad7 treatment on migration of human keratinocytes and tumor cell lines. (**a**) Tat-Smad7 accelerates NOK-SI cell migration. Quantification from 4 separate experiments is shown in Fig. 6f. Pairs of dotted lines delineate initial wounds (**b**) Tat-Smad7 treatment in NOK-SI cells attenuated radiation-induced pSmad2 and NF-κB p50 nuclear localization. (**c**) V5 staining of MSK921 cells 2 hours after Tat-Smad7 treatment. K14 staining was used as counterstain. (**d**) Rac1 western analysis in MSK921 60 hours after Tat-Smad7 treatment. M: molecular marker. (**e**) Quantification of MSK921 cell migration from 3 separate experiments. (**f**) A representative MSK921 cell migration assay treated with Tat-Smad7 and PBS. Pairs of solid lines delineate initial wounds. Dotted lines highlight the forefront of migrated cells. (**g**) Quantification of Cal27 cell migration from 3 separate experiments. (**h**) Representative images for (**g**). Pairs of solid lines delineate initial wounds. Dotted lines highlight the forefront of migrated cells. Data are expressed as mean ± s.d. and the Two-tail Student T-test is used to calculate *P* values. NS: no significance.



Supplementary Figure 8. Potential mechanisms of Smad7-mediated protection and healing of oral mucositis. (**a**) Radiation activates NF-κB, increases TGF-β1 and CtBP1. NF-κB and TGF-β1 induce inflammation. TGF-β1 induces apoptosis, growth arrest and activates Smad-2, -3 and -4 which recruit CtBP1 to the Rac1 promoter to repress Rac1 transcription, leading to blunted re-epithelialization. The combination of almost no epithelial regeneration and excessive inflammation causes oral mucositis. (**b**) Smad7 blocks NF-κB and TGF-β1-induced inflammation and blocks TGF-β1-induced apoptosis and growth arrest. Smad7 relieves Rac1 transcriptional repression by either preventing TGF-β1-mediated Smad activation (phosphorylation) or competing with signaling Smads/CtBP1 transcriptional repression complex in binding to the Rac1 promoter. Increased Rac1 induced by Smad7 contributes to keratinocyte migration during re-epithelialization. Smad7-mediated acceleration of epithelial regeneration and reduction in inflammation lead to prevention or healing of oral mucositis.