Animal injections and tissue processing

In general, 8- to 10-week-old adult females were ovariectomized and rested for 10 days before they received any injections. Mice were injected with sesame seed oil (0.1ml/mouse), E2 (1, 5, 15, 25, 100 ng/mouse), G-1 (0.5, 2, 10, 25, 50, 100 μg/mouse) or the same doses of G-1 given 30 min prior to E2 injection. They were sacrificed at the indicated times after the last injection. All test agents were dissolved in sesame oil (0.1 ml/mouse) and injected subcutaneously (sc). G-1 was dissolved in 50 μl DMSO/mg and then diluted in sesame oil. For studies with ^{3}H-thymidine or BrdU incorporation, mice were injected with [methyl-^{3}H]-thymidine (25 μCi/mouse, sc) or BrdU (50 mg/kg, ip) 2 h before sacrificing. Tissues were flash frozen and kept at –70 °C, or fixed in 10% neutral buffered formalin before paraffin embedding for subsequent studies.

Antibodies and other reagents

The affinity-purified polyclonal antibodies for actin (Cat#.sc-1615) and ERα (Cat# sc-542) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ERα [(pERα(Ser118)] (Cat# 05-973) was purchased from Millipore Corp. (Billerica, MA). Rabbit anti-human GPR30 (IMG-71861) was purchased from Imgenex Inc. (San Diego, CA). Goat anti-human GPR30 (Cat# AF5534) was purchased from R&D Systems, Inc. (Minneapolis, MN). The affinity-purified antibodies for ERK1/2 (Cat# 9102); phospho-ERK1/2 [pERK1/2(Thr202/Tyr204)] (Cat# 9101); and mouse anti-pERK1/2(Thr202/Tyr204) (E10) (Cat# 9106), were purchased from Cell Signaling (Beverly, MA). Secondary antibodies for Cy2-conjugated donkey anti-goat (Cat# 705-225-147), donkey anti-rabbit (Cat# 711-225-152), Cy3-conjugated donkey anti-rabbit (Cat# 711-165-152), donkey anti-mouse (Cat# 715-165-150), and peroxidase-conjugated goat anti-rabbit (Cat# 705-035-147) and donkey anti-goat (Cat# 705-035-147) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Peroxidase-conjugated goat anti-rabbit secondary antibody (ready-to-use) (Cat# 50-235) for immnohistochemistry and biotinylated antibody for BrdU (Cat# 93-3944) were obtained from

Zymed Laboratories Inc. (San Francisco, CA). G-1 and E2 were purchased from Caymen Chemicals, Inc., and Sigma Chemical Company, respectively.

Immunohistochemistry

Uterine sections were incubated with primary antibodies at 4°C overnight. The sections were washed in PBS and incubated with fluorescence- or peroxidase-conjugated secondary antibodies for 1 h at room temperature. Nuclear counter staining was followed with DAPI (for immunofluorescence) or hematoxylin (for DAB color substrates). The fluorescence signals were visualized by an inverted microscope (NIKON TE 2000U) with opti-grid structured light confocal system (Phylum) that has an all-motorized Z-focus device to capture high quality confocal images. For studies with BrdU incorporation, formaldehyde-fixed, paraffin-embedded tissue sections were stained for BrdU, using the manufacturer's instruction (Zymed Laboratories Inc., San Francisco, CA). Sections were lightly counterstained with hematoxylin. BrdU labeling index was determined for the luminal and glandular epithelial cells after counting at least 500 cells per animal on consecutive fields.

Probes for Northern blot hybridization

The full-length cDNA coding region of mouse GPR30 was obtained by RT-PCR using ovary RNA. The RT-PCR-derived fragment was subcloned into a pCRII-TOPO vector, and the identity of the clone was confirmed by nucleotide sequencing. The *Bip*, *c-Fos*, *c-Myc*, *cdkn1a*, *Ccnd1*, *ERα*, *Gpr30, Hbegf*, *Ltf, Pgr*, *Sik-SP*, and *Sfrp2* cDNA clones and ribosomal protein L7 (*Rpl7*) have been previously described (7, 9).

Construction of recombinant adenoviral plasmids

The full-length coding region of mouse GPR30 cDNA was generated by RT-PCR using primers carrying the linkers for *Xho*I at the 5'-ends as follows: 5'-

GGCTCGAGATGTACAGCAGCGTCTTCTT-3' (sense) and 5'-GGCTCGAGAGTAGCAGAGGCCAATGAT-3' (antisense). The amplified DNA fragment was cloned into the *Xho*I site of a shuttle vector pAdTrack cytomegalovirus (CMV), in the sense or antisense orientation with respect to a CMV promoter. The selected clones were sequenced to confirm the identity. All of the GPR30(S), GPR30(AS) and empty shuttle plasmids possess an additional CMV promoter which drives GFP expression independently. Plasmid DNAs were linearized with *Pme*I and subsequently cotransfected with pAdEasy-1 for recombination into *E. coli* BJ5183. The recombinant plasmid clones, harboring either GPR30(S) or GPR30(AS), were confirmed by restriction cutting using *Pac*I and by sequencing.

Virus injection procedures in mice

Adenoviral particles were inoculated directly into the uterine lumen of both horns (20 μl solutions in saline containing 1×10^{11} virus particles per horn) from the oviduct-uterus junction just before ovariectomy. Mice were given rest for 10 days before receiving injections of oil or E2 for 6 and 24 h treatments. Uterine tissues were appropriately collected for subsequent analysis.

SUPPLEMENTAL DATA

Supplement Fig. 1. Analysis of uterine wet weights for estrogen induced early response. Adult ovariectomized mice were given a single injection of oil (vehicle control), G-1 (100 μg/mouse), E2 (25 ng/mouse) or G-1+E2 and killed at 6h after the last injection. Uterine wet weights from 5-10 mice for each group were analyzed. The error bars represent standard errors. *, Values are statistically different compared with control ($P < 0.05$, ANOVA followed by Newman-Keul's multiple range test).

Supplement Fig. 2. Analysis of uterine epithelial cell proliferation by BrdU, pHH3 and Ki67 immunostaining. (A) Adult ovariectomized mice were given a single injection of oil (vehicle control), G-1 (100 μg/mouse), E2 (25 ng/mouse) or G-1+E2 and killed 24h after the last injection. Formaldehyde-fixed paraffin-embedded tissue sections were stained for BrdU, pHH3 and Ki67 as described in *Experimental Procedures*. Representative tissue sections are shown and reddish-brown nuclear deposits indicate the sites of positive immunostaining. (B) Quantitation of BrdU, pHH3 and Ki67 positive cells was presented here are after the analysis of at least ten sections from 3-5 different mice of each group. The error bars represent standard errors. *, Values are statistically different ($P < 0.05$) between compared groups based on ANOVA followed by Newman-Keul's multiple range test.

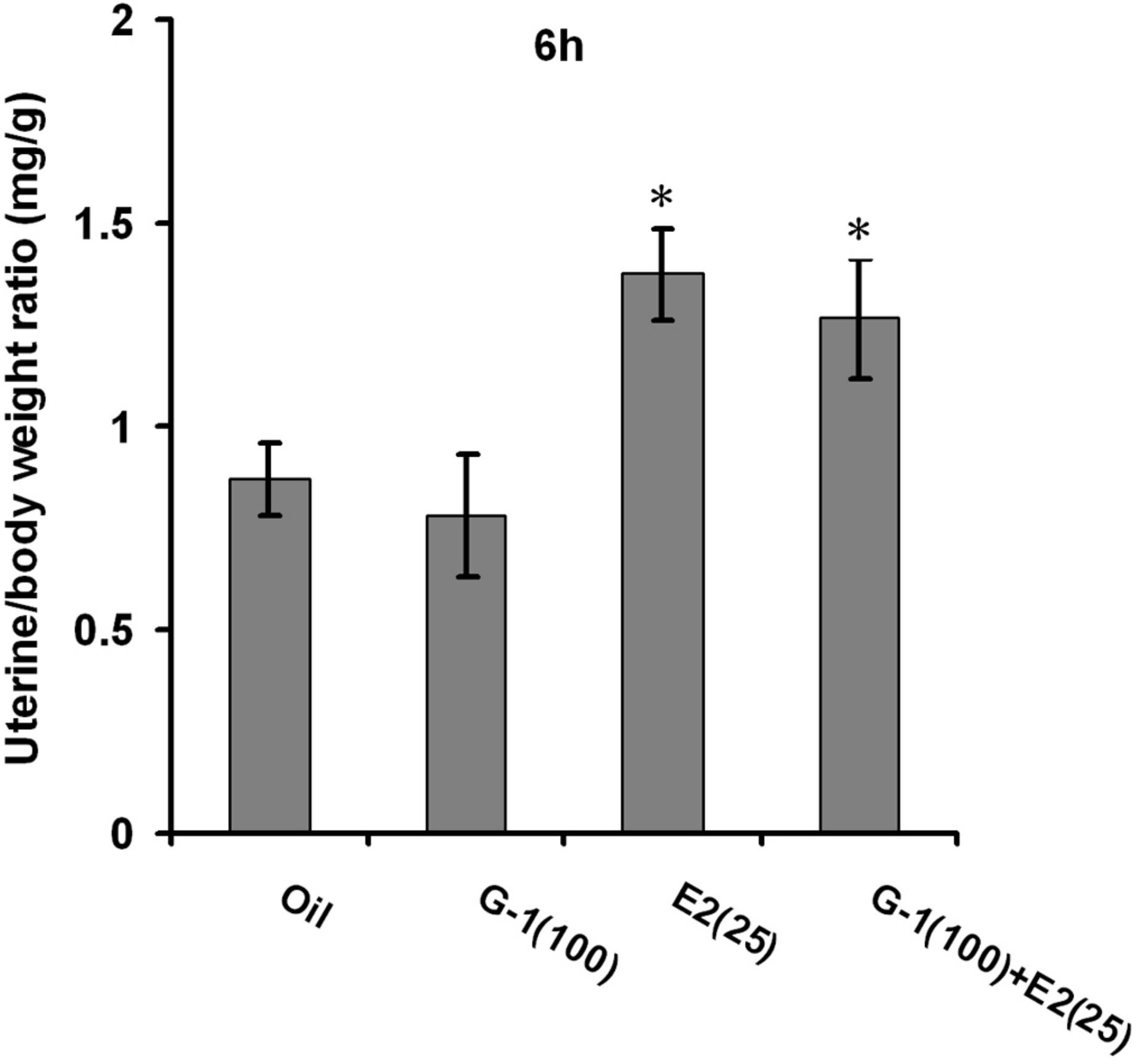
Supplement Fig. 3. **Direct visualization of GFP fluorescence in uterine tissue sections.** Ovariectomized mice were administrated with intraluminal injections (20 μl) of rAdGPR30(S) or saline (no virus control) and given rest for ten days. Uterine tissues were collected after injections of E2 (25 ng/mouse) for 6h. **Frozen uterine tissue sections (10 μm) were covered with mounting solution**

and then subjected to capture photomicrographs under fluorescence microscope for GFP (right panels) or under the phase for bright-fields (left panels). le, luminal epithelium and s, stroma.

Supplement Fig. 4. Effects of G15 by itself or in combination with E2 on uterine growth responses**.** Adult ovariectomized mice were given subcutaneous (*sc*) injections of oil (vehicle control), G15 (100 μg/mouse), E2 (25 ng/mouse) or the same doses of G15 30 min prior to E2. Mice were sacrificed 24 h after the last injection. BrdU was injected 2 h before sacrificing. A. Uterine wet weights from 5-6 mice for each group were analyzed. The error bars represent standard errors. *, Values are statistically different compared to the vehicle control ($P < 0.05$, ANOVA followed by Newman-Keul's multiple range test). B. BrdU incorporation: Formaldehyde-fixed paraffin-embedded tissue sections were stained for BrdU incorporation as described in Materials and Methods. Representative tissue sections are shown and reddish-brown nuclear deposits indicate the sites of positive immunostaining. Pictures were taken at 200X. le, luminal epithelium; ge, glandular epithelium; s, stroma cells. C Quantitation of BrdU-positive cells. The percentage of BrdU labeling index was determined for the luminal and glandular epithelial cells after counting at least 500 cells per animal on consecutive fields. The data presented after the analysis of at least 5-6 mice for each group. The error bars represent standard errors. *, Values are statistically different compared with control ($P < 0.05$, ANOVA followed by Newman-Keul's multiple range test).

Supplement Fig. 5. G15 mediated activity of GPR30 is not regulatory to E2-induced uterine ERK and ERα phosphorylation signals. Adult ovariectomized mice were given subcutaneous (*sc*) injections of oil (vehicle control), G15 (100 μg/mouse), E2 (25 ng/mouse) or the same doses of G15 30 min prior

to E2. Mice were sacrificed 2 and 24 h after the last injection. Mice injected with oil and sacrificed after 2 h served as vehicle controls. Western blot analysis of whole uterine tissue extracts for pERK1/2 (A) and pERα(Ser118) (B). These experiments were repeated at least four times with independent samples and a representative blot is presented. Fold changes in the levels of pERK1/2 and pERα(Ser118) were compared against the control, and the values were determined after normalization with ERK1/2 and Actin levels, respectively. The error bars represent standard errors. *, Values are statistically different compared with control ($P < 0.05$, ANOVA followed by Newman-Keul's multiple range test).



Supplement Fig. 1
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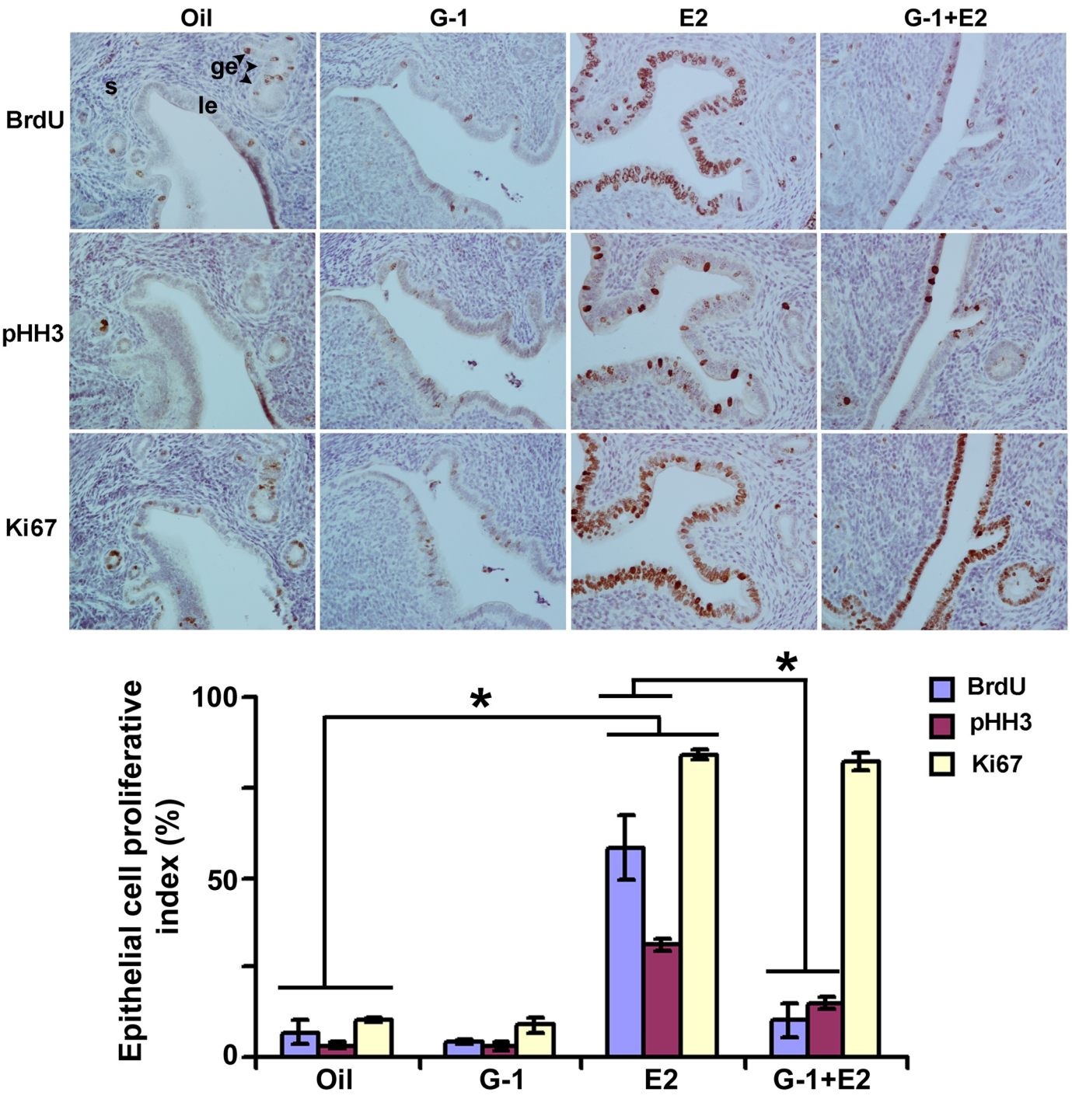

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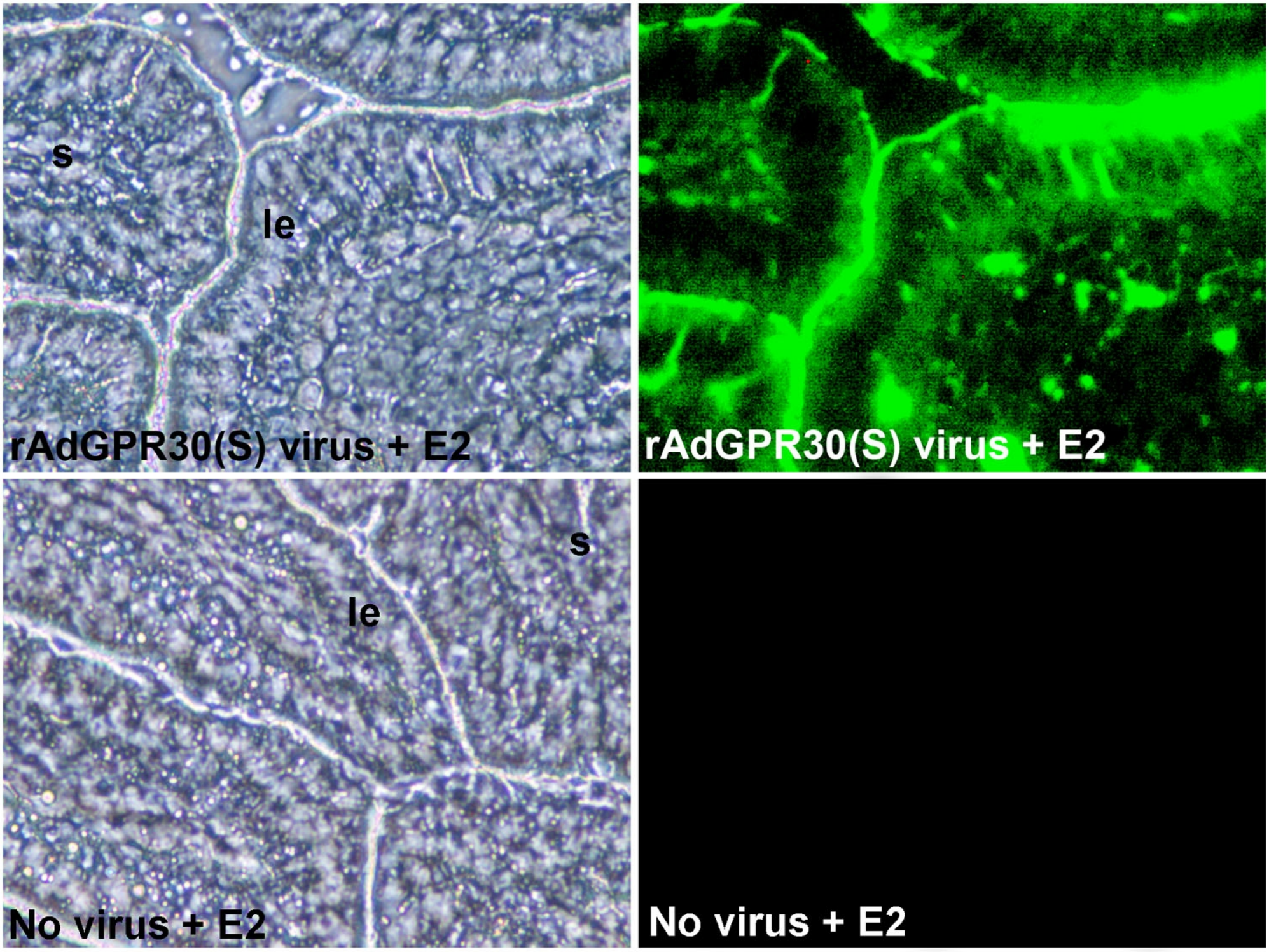

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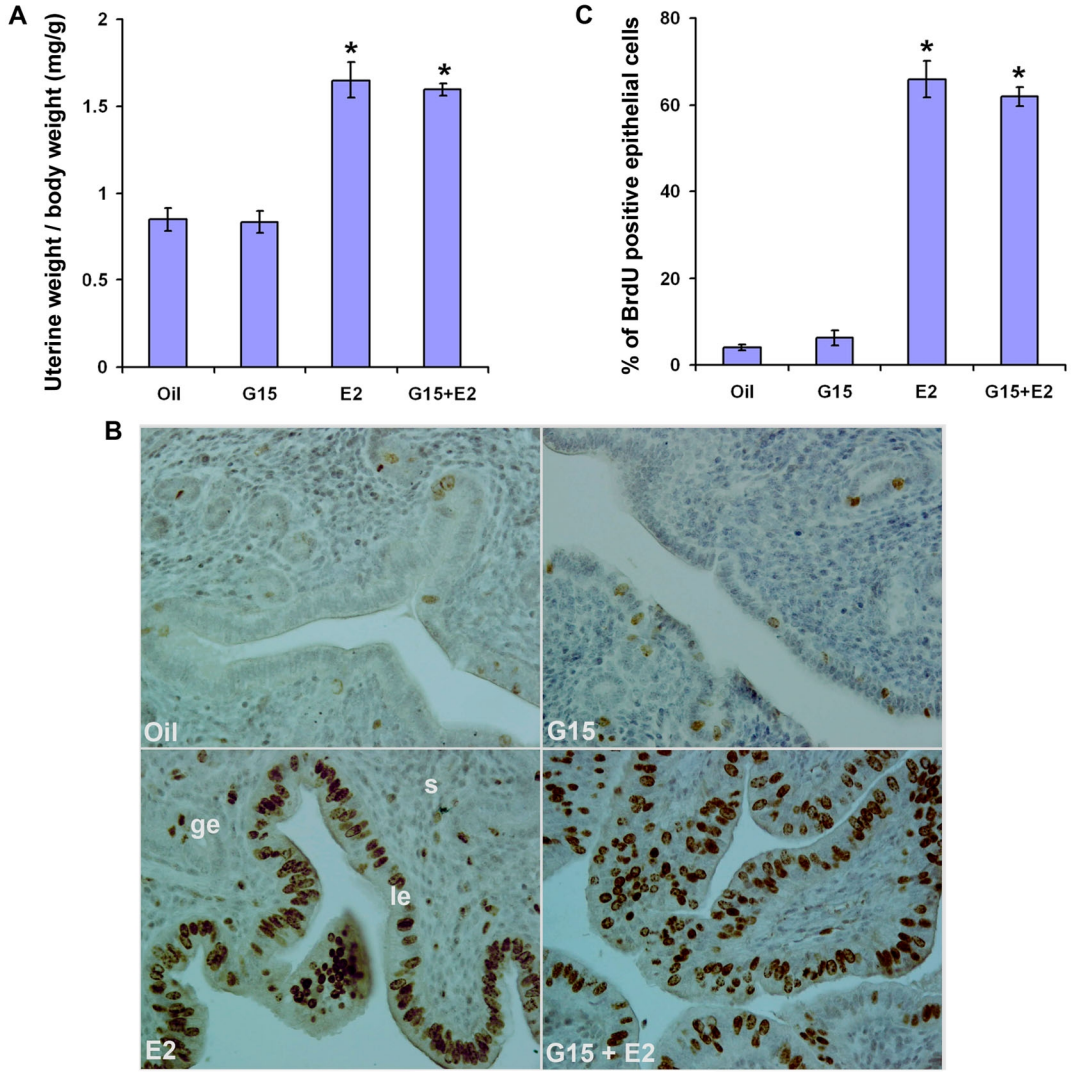

Supplement Fig. 4

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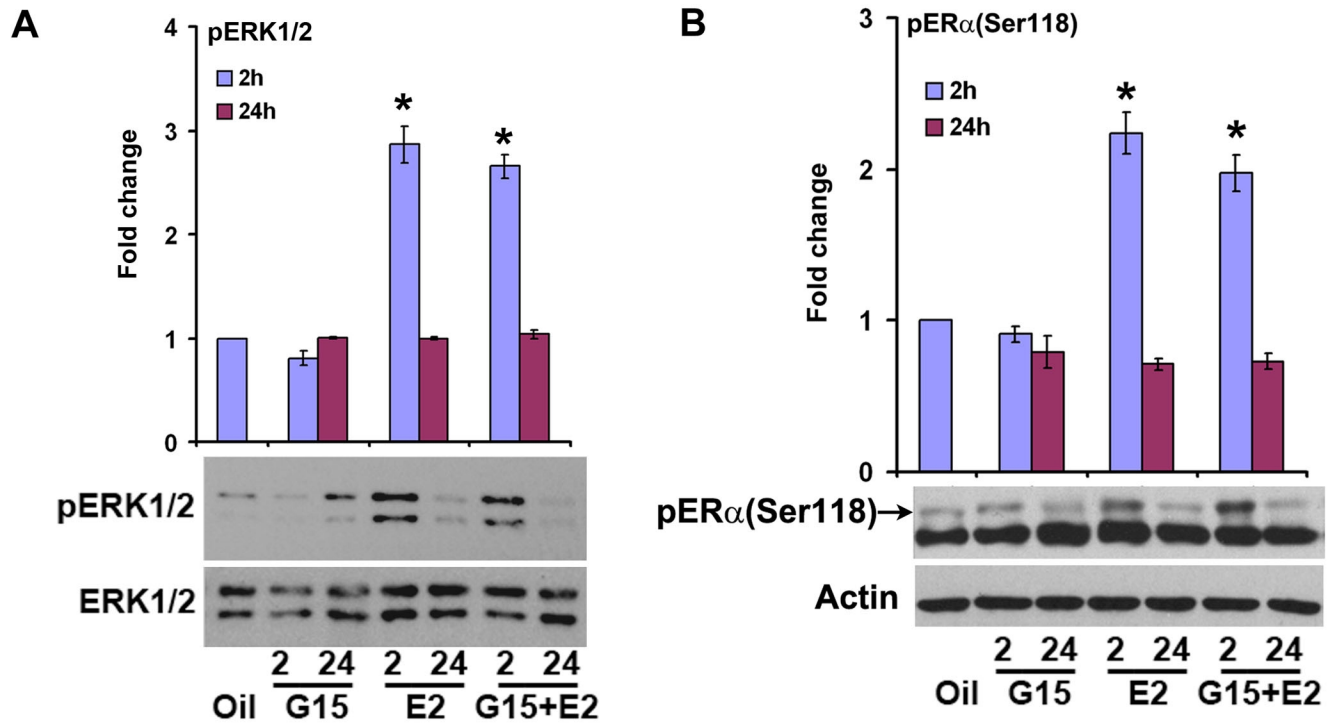

Supplement Fig. 5
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