

# Supporting Information

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## SI Materials and Methods

**Cell Culture.** All cells were incubated at 37 °C maintained at 5% CO<sub>2</sub>. Littermate-derived Lkb1<sup>+/+</sup> and Lkb1<sup>-/-</sup> MEFs were isolated from day 14 postcoitum Lkb1<sup>+/+</sup> and Lkb1<sup>lox/lox</sup> embryos and grown in DMEM plus 10% FBS (HyClone), penicillin, and streptomycin. Both genotypes were infected with a Cre expressing adenovirus and subsequently immortalized with an INK4a shRNA expressing lentivirus. Ampk $\alpha$ 1/ $\alpha$ 2<sup>+/+</sup> and Ampk $\alpha$ 1/ $\alpha$ 2<sup>-/-</sup> SV40-immortalized MEFs were kindly provided by Dr. Keith Laderoute with permission from Dr. Benoit Viollet (1). Ampk $\alpha$ 1/ $\alpha$ 2<sup>+/+</sup> and Ampk $\alpha$ 1/ $\alpha$ 2<sup>-/-</sup> MEFs were plated at a density of 1 × 10<sup>5</sup> and Lkb1<sup>+/+</sup> and Lkb1<sup>-/-</sup> MEFs were plated 2.0 × 10<sup>5</sup> per well in 6-well dishes and grown in DMEM plus 10% FBS (HyClone), penicillin, and streptomycin. Twenty-four h after plating, MEFs were then left untreated, treated with 50 nM rapamycin (LC Laboratories) or 100 μM CoCl<sub>2</sub> (Sigma-Aldrich) for 24 h.

**Mouse Colony Maintenance, Treatment Regimen, and Polyp Measurement.** Lkb1<sup>+/+</sup> and Lkb1<sup>+/-</sup> mice, maintained on an FVB/N genetic background, were monitored for the development of gastrointestinal polyps as previously described (2). Mice with clinical signs of disease were euthanized and necropsied. The groups were vehicle treated or RAPA treated mice. Mice were treated with either vehicle (5% Tween 80, 5% PEG400 solution), or 10 mg/kg rapamycin by i.p. injection once a day for 5 days with 2 days rest for a period of either 1 or 2 months as indicated. The mean latency, distribution of polyps, and polyp phenotype were comparable to previous studies (2–4). Polyps were scored and total polyp burden was measured as described (5). Ki67-stained polyps were scored from 5 polyps for each group (Vehicle or Rapamycin treated). For each polyp, 3 fields of view at a 32× magnification were randomly selected and scored. A total of 200 nuclei from epithelial cells total per field of view was counted. Within the same field all Ki67 positively stained nuclei from epithelial cells were counted. The number of Ki67+ cells was divided by the total number of nuclei, and the percentages for each group were averaged, and a *P* value was determined. All experimental procedures in mice were approved by the Salk Institute and University of California at San Diego Institutional Animal Care and Use Committees.

**Tissue Isolation and Biochemistry.** Polyps and adjacent tissue were harvested immediately and either processed for histological analysis or snap frozen in liquid nitrogen for molecular studies. These samples were then placed frozen into Nunc tubes and homogenized in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphate, 50 mM NaF, 5 mM β-glycero-phosphate, 50 nM calyculin A, 1 mM Na3VO<sub>4</sub>, complete protease inhibitor mixture (Roche Diagnostics)] on ice for 30 s using a tissue homogenizer. MEFs were lysed in boiling SDS-lysis buffer [10 mM Tris (pH 7.5), 100 mM NaCl, and 1% SDS] after the indicated treatments. After trituration, lysates were equilibrated for protein levels using the BCA method (Pierce) and resolved on 6–12% SDS/PAGE gels, depending on the experiment. Gels were transferred to PVDF and western blotted according to the antibody manufacturer's suggestions (6, 7).

**Histology and Immunohistochemistry.** Mouse tissues were fixed in 10% formalin overnight and embedded in paraffin. For immunohistochemistry, slides were deparaffinized in xylene and eth-

anol and rehydrated in water. Antigen retrieval using sodium citrate pH 6.0 or Tris EDTA pH 10.0 buffer was performed according to the manufacturer's instructions. Slides were quenched in hydrogen peroxide (3%) to block endogenous peroxidase activity and then washed in TBST buffer. Slides were blocked in 5% normal serum for 1 h at room temperature. Slides were incubated with primary antibody diluted in blocking buffer, washed and a secondary biotinylated goat-anti-mouse IgG antibody was applied. The avidin-biotin peroxidase complex method (Vector) was used and staining was visualized using the DAB chromophore (DAB, ABC; Vector). Slides were counterstained with hematoxylin and mounted with Fluoromount (Southern Biotech). The antiphosphoribosomal protein S6 (S235/236) (Cell Signaling Technology), Ki67 (SP6) (Neomarkers), and GLUT1 (GT11-A Rabbit polyclonal antibody; Alpha Diagnostics Int.) and HIF-1α (rabbit polyclonal antibody; Novus Biologicals LLC) antibodies were diluted according to manufacturer's suggestions.

For immunocytochemistry on human PJS and control samples, the avidin-biotin complex (ABC) method was performed. Briefly, slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature. For antigen retrieval, sections were heated in pressure cooker in 10 mM citrate buffer (pH 6.0) for pS6 (Ser-235/236, rabbit polyclonal antibody, 1:200 dilution; Cell Signaling Technology), and in Tris-EDTA buffer (pH 8.0) for GLUT1 (GT12-A 1:100 dilution; Alpha Diagnostics) before staining. The P-S6 and GLUT1 antibodies were applied and incubated overnight at 4 °C. Application of the monoclonal antibody for HIF-1α (clone H1α67, Cat#NB100-105; mouse monoclonal antibody, 1:10,000 dilution; Novus Biologicals) was followed by sequential 15 min incubations with biotinylated link antibody, streptavidin-biotin-peroxidase complex, amplification reagent, and streptavidin-peroxidase. Immunodetection was performed with an LSAB 2 system (DAKO). Hematoxylin was used as a counterstain. A known GLUT1 and P-S6 positive breast carcinoma or normal skin sample with known HIF-1α expression were used as positive controls, and the same samples were used as negative controls by replacing the primary antibody with PBS. For every sample, immunostaining was repeated at least twice to confirm the results. Staining was graded for intensity as negative or positive by 2 independent observers (C.-L.W. and S.W.), and there were no major discrepancies between the 2 observers.

**Immunocytochemistry and Imaging.** For immunocytochemistry, sections were deparaffinized in xylene and ethanol and rehydrated in water. Antigen retrieval was performed using sodium citrate pH 6.0. Sections were blocked in 5% normal serum for 1 h at room temperature and incubated with hydroxyprobe-1 mouse monoclonal antibody 1 (MAB1, Natural; Amersham Pharmacia International Inc.) at a 1:50 dilution for 40 min at RT. A secondary anti-rabbit Alexa488 secondary antibody (1:1,000; Molecular Probes) and DAPI stain were then used. Coverslips were mounted in FluoromountG (SouthernBiotech) and images were acquired on a Zeiss Axioplan2 epifluorescence microscope coupled to the Openlab software using the 20× objective.

**Detection of Hypoxic Regions in Polyps.** To allow assessment of the hypoxic regions within tumors, mice were injected i.p. with 60 mg/kg (in wt/vol PBS) pimonidazole (hydroxyprobe-1TM, Natural; Amersham Pharmacia International Inc.), 1.5 h before

being killed. Stomach and small intestine were dissected out, processed, and embedded into paraffin. For immunohistochemistry, 5- $\mu$ m sections were deparaffinized in xylene and ethanol and rehydrated in water. Antigen retrieval using sodium citrate pH 6.0 buffer was performed according to the manufacturer's instructions. Sections were quenched in hydrogen peroxide (3%) and then washed in TBST buffer. Sections were blocked in 5% normal serum for 1 h at room temperature and then incubated with hydroxyprobe-1 mouse monoclonal antibody 1 (MAb1, Natural; Amersham Pharmacia International Inc.) at a 1:50 dilution for 40 min at room temperature. A secondary biotinylated goat-anti-mouse IgG antibody was applied, and staining was visualized by using the DAB chromophore (ABC, DAB; Vector). Sections were counterstained with hematoxylin and mounted with Fluoromount (SouthernBiotech).

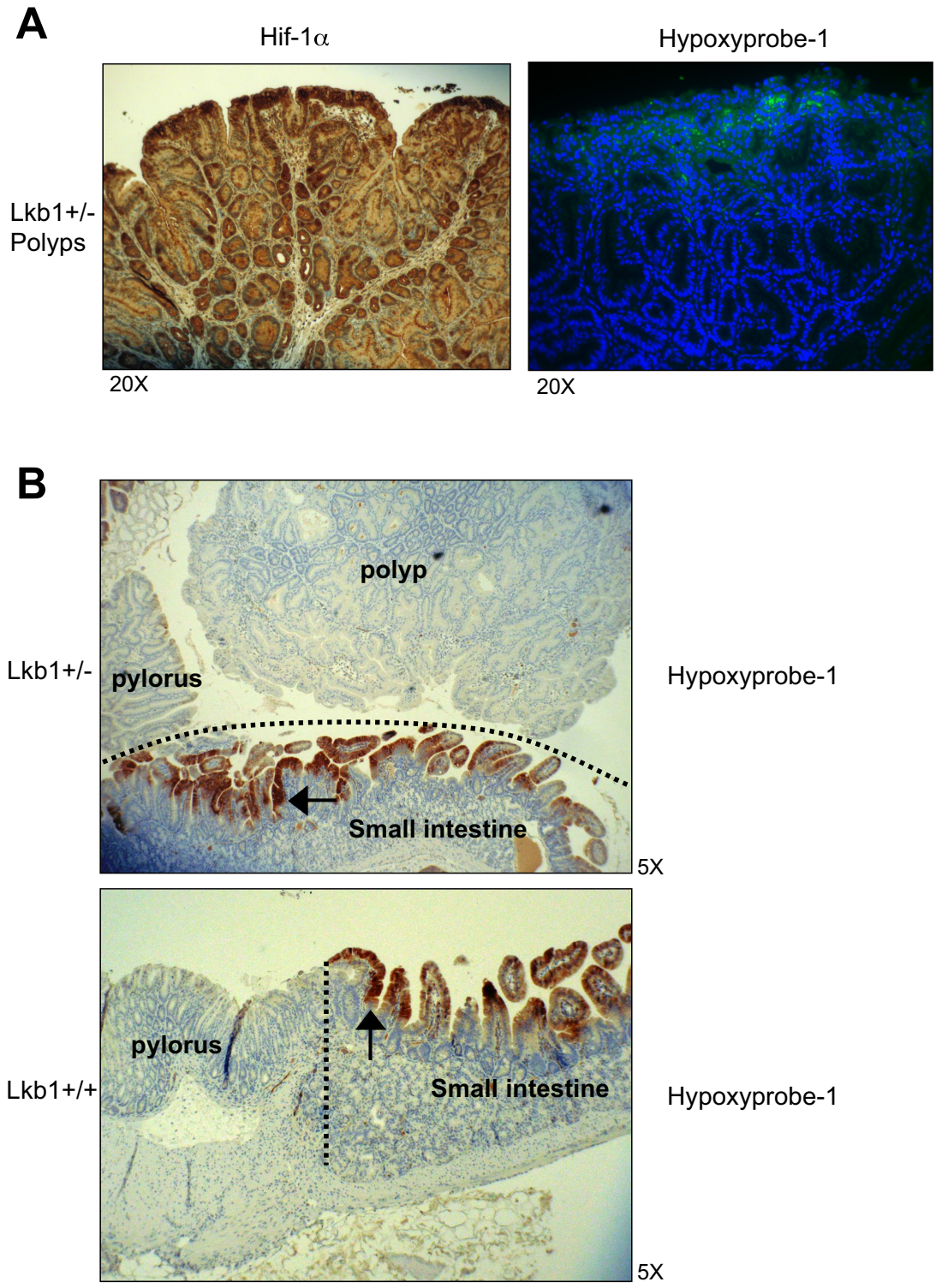
**Patient Samples.** Formalin-fixed, paraffin-embedded sections of 5 PJPs located in small bowel and 8 PJPs located in colon were obtained from 8 patients with PJS (5 from Massachusetts General Hospital, Boston, and 3 from Biomedicum, Helsinki, Finland) for immunohistochemical staining with the P-S6 and

GLUT1 antibodies. An additional 9 sections of formalin-fixed, paraffin-embedded PJPs (4 from colon, 4 from small intestine, 1 from stomach) were obtained from 7 patients with PJS from Massachusetts General Hospital for immunohistochemical staining with the HIF-1 $\alpha$  antibody. Normal small bowel mucosa controls and normal colonic mucosa controls were obtained at Massachusetts General Hospital. The studies were approved by the Human Study Committees in both hospitals. The histopathological diagnosis of PJPs was confirmed in all cases by 2 independent pathologists. Consecutive 5- $\mu$ m tissue sections were cut from each tissue block for immunohistochemical analysis.

**FDG PET Analysis.** Mice were warmed for 30 min in an isolator box on a recirculating water pad kept at  $\approx 30^\circ\text{C}$  to normalize their body temperature for injection (i.v. 250 uCi, 0.1 mL) of 18-fluoro-deoxyglucose (F<sup>18</sup>-FDG). Mice were anesthetized with 2% Isoflurane and kept under anesthesia until imaging (2 bed positions, 10 min per position) 1 h after injection by microPET (Vista DR; GE Healthcare). Individual animals were scanned once or twice in a 3-week period. Ten scans were performed on +/- mice and 5 scans on +/+ mice.

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**Fig. S2.** Polyps from Lkb1<sup>+/-</sup> mice are normoxic while retaining HIF-1 $\alpha$  expression. Immunohistochemical (IHC) and immunocytochemical (ICC) analysis of gastrointestinal sections from 13-month-old Lkb1<sup>+/-</sup> and Lkb1<sup>+/+</sup> mice probed with antibodies against Hif-1 $\alpha$  or hypoxyprobe-1. *A Left* represents IHC on polyps probed with an antibody against Hif-1 $\alpha$ . *Right* represents ICC on polyps probed with an antibody against hypoxyprobe-1 as indicated by positively stained cells in green. DAPI was used as a nuclear counter stain (blue). *(B)* IHC on gastrointestinal sections probed with an antibody against hypoxyprobe-1. Arrows indicate positively stained epithelial cells in the small intestine. Dashed lines represent the separation between pyloric region and small intestine. IHC and ICC results are representative of polyps from 3 mice of each genotype.