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Supplemental Information

Suppression of Membranous LRP5 Recycling, Wnt/β-Catenin Signaling, and Colon Carcinogenesis by 15-LOX-1 Peroxidation of Linoleic Acid in PI3P

Fuyao Liu, Xiangsheng Zuo, Yi Liu, Yasunori Deguchi, Micheline J. Moussalli, Weidong Chen, Peiying Yang, Bo Wei, Lin Tan, Philip L. Lorenzi, Shen Gao, Jonathan C. Jaoude, Amir Mehdizadeh, Lovie Ann Valentin, Daoyan Wei, and Imad Shureiqi

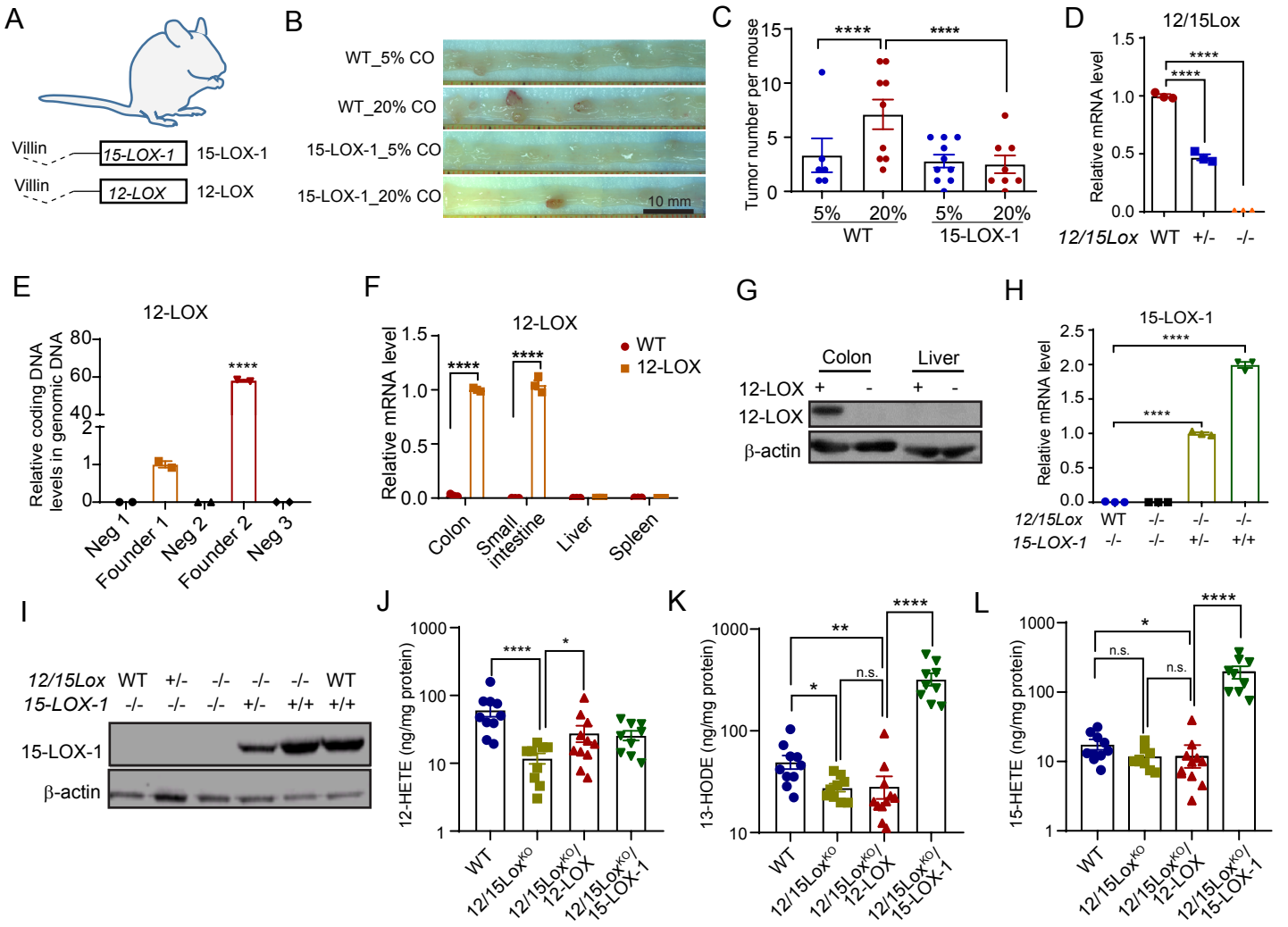

Figure S1. 15-LOX-1 suppresses linoleic acid (LA) promotion of azoxymethane (AOM)-induced colorectal carcinogenesis (CRC) in mice, related to Figure 1.

(A) Schematic of villin-*15-LOX-1* (15-LOX-1) and villin-*12-S-LOX* (12-LOX) mice. Dashed lines indicate the promoter region, followed by the indicated gene-coding region.

(B, C) *15-LOX-1* homozygotes and sex- and age-matched wild-type (WT) littermates were fed either 5% or 20% corn-oil (CO) diets and then treated with 7.5 mg/kg AOM via intraperitoneal injection once per week for 6 consecutive weeks. Mice were sacrificed 20 weeks after the last AOM injection and examined for tumor formation (n=8-10 mice per group). Representative colonic images **(B)** and the number of colonic tumors per mouse **(C)** are shown for the indicated mice groups. Values in C are mean ± SEM. **** *P < .0001* (2-sided Poisson).

(D) 12/15Lox relative mRNA expression in intestinal epithelial cells (IECs) of WT, *12/15Lox* $^{(+/-)}$, and *12/15Lox* $^{(-/-)}$ mice, measured by reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR). Values are mean ± SEM. **** *P < .0001* (one-way ANOVA with Bonferroni multiple-comparisons).

(E-G) Generation and characterization of the novel villin-12-LOX (12-LOX) mouse line. Identification of villin-12-LOX founders: qPCR was used to measure levels of genomic human platelet 12-LOX cDNA in littermates after pronuclear injection of purified villin-12-LOX construct into fertilized oocytes. The genomic levels of 12-LOX coding DNA were calculated relative to the level of a calibrator sample (Founder 1). Neg indicates no detectable genomic 12-LOX coding DNA. Genotyping results of transgene villin-12-LOX mice are shown **(E)**. 12-LOX relative mRNA and protein expression levels in the indicated organs of 6-week-old 12-LOX mice and their WT littermates were measured by RT-qPCR **(F)** and Western blot analysis **(G)**, respectively. Values in E and F are mean ± SEM. **** *P<.0001* (one-way ANOVA for E and two-way ANOVA for F with Bonferroni multiple-comparisons)

(H, I) Characterization of 12/15Lox^{KO}/15-LOX-1 mouse line. 12/15Lox^{KO} mice were bred with villin-15-LOX-1 (15-LOX-1) mice to generate mice with 12/15Lox^{KO} with targeted human 15-LOX-1 expression in intestines (12/15Lox^{KO}/15-LOX-1). Shown are 15-LOX-1 relative mRNA and protein expression levels in IECs of the indicated mouse groups, measured by RT-qPCR **(H)** and Western blot analysis **(I)**, respectively. Values in H are mean ± SEM. **** *P<.0001 (*one-way ANOVA with Bonferroni multiple-comparisons).

(J-L) FVB mice (WT, 12/15Lox^{KO}, 12/15Lox^{KO}/12-LOX, or 12/15Lox^{KO}/15-LOX-1) were fed a 20% CO diet and treated with AOM as described in Figures 1A and B. Shown are eicosanoid metabolite profiles [12-HETE **(J)**, 13-HODE **(K)**, and 15-HETE **(L)**] in IECs measured by liquid chromatography–tandem mass spectrometry (n=9-11 mice per group). Values are mean ± SEM. **P<.05, **P<.01 and **** P<.0001* (one-way ANOVA with Bonferroni multiple-comparisons). n.s.: no significant difference.

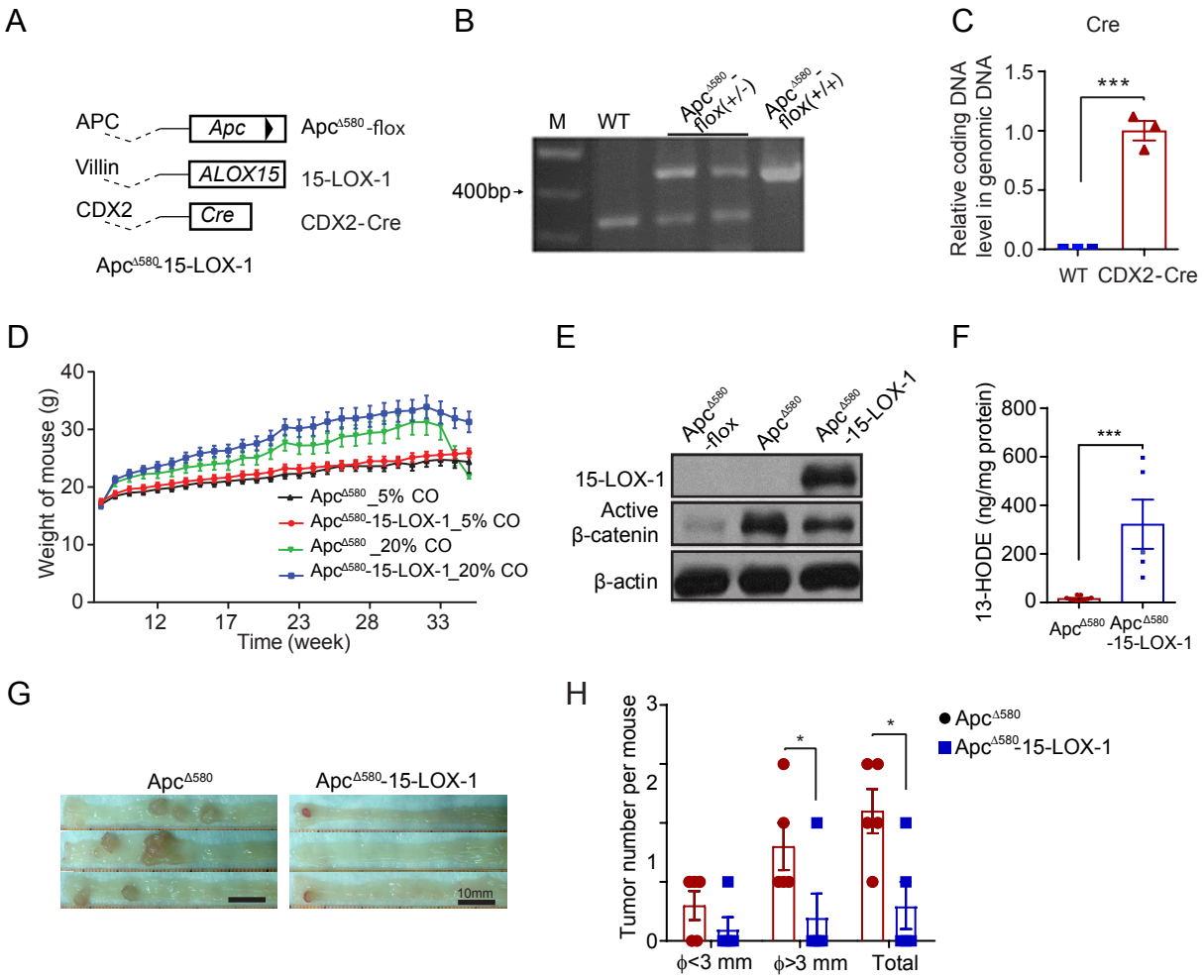

Figure S2. 15-LOX-1 suppresses linoleic acid (LA) promotion of *Apc* mutation-induced colorectal carcinogenesis (CRC) in mice, related to Figure 1.

(A) Schematic of Apc$^{\Delta580}$–15-LOX-1 mice. Apc$^{\Delta580}$ mice were bred with 15-LOX-1 mice to produce Apc$^{\Delta580}$;15-LOX-1, designated as Apc$^{\Delta580}$–15-LOX-1 mice. Dashed lines indicate the promoter region, followed by the indicated gene-coding region. Arrow represents *Apc* exon 14 flanked with *loxP* sites.

(B) Genotyping of mice carrying *Apc$^{\Delta580}$-flox* alleles, in which *Apc* exon 14 is flanked with *loxP* sites by regular PCR. *Apc* WT band: 320 bp. *Apc$^{\Delta580}$* flox band: 430 bp. M represents DNA ladder marker.

(C) *Cre* genotyping for CDX2-*Cre* mice. Genomic *Cre*-recombinase coding DNA levels were measured by quantitative PCR. WT had no detectable genomic *Cre*-recombinase coding DNA.

(D) Apc$^{\Delta580}$ and Apc$^{\Delta580}$ –15-LOX-1 mice at 4 weeks were fed 5% or 20% corn oil (CO) diets, and the body weights of the mice were assessed every week until age 35 weeks. Body weight curves of the four mouse groups are shown (n=14-20 per group).

(E, F) Normal colonic epithelial cells of *Apc$^{\Delta580}$*-flox, Apc$^{\Delta580}$, and Apc$^{\Delta580}$–15-LOX-1 mouse groups were scraped and harvested at age 20 weeks for measuring 15-LOX-1 and active β-catenin levels by Western blot analysis **(E)** and 13-HODE levels by liquid chromatography–tandem mass spectrometry **(F)**. Values are mean ± SEM. *** *P<.001* (unpaired *t* test).

(G, H) 15-LOX-1 expression inhibited CRC in Apc$^{\Delta580}$ mice. Apc$^{\Delta580}$ mice and Apc$^{\Delta580}$ –15-LOX-1 mice fed a standardized diet with fixed LA content (7% CO) were followed for 25 weeks and evaluated for tumor formation (n=5 per group). Representative colon photographs **(G)** and the colonic tumor numbers per mouse with tumor size distributions **(H)** of Apc$^{\Delta580}$ and Apc$^{\Delta580}$–15-LOX-1 mice are shown. Values are mean ± SEM. * *P* < *.05* (2-sided Poisson).

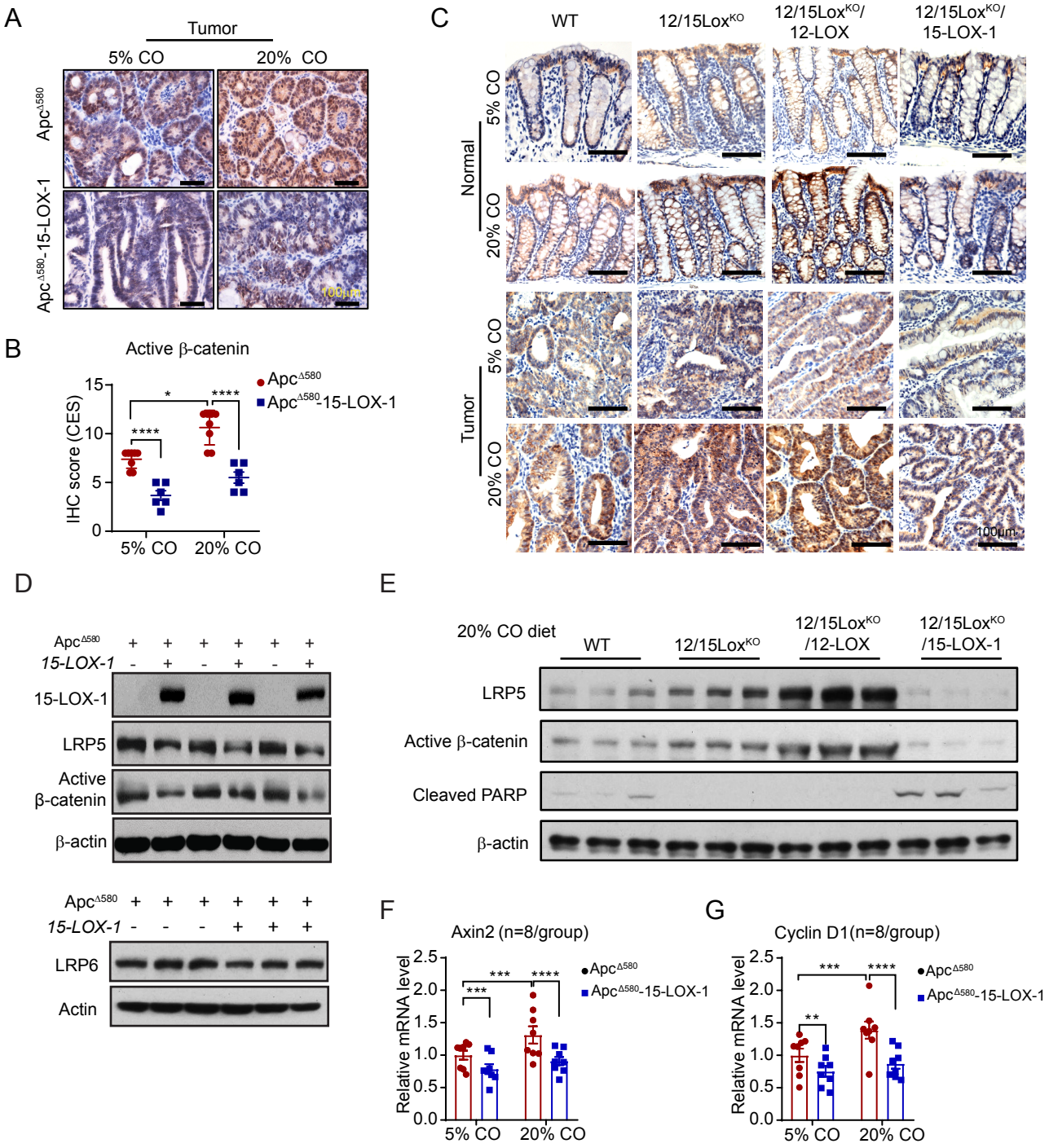

Figure S3. 15-LOX-1 suppresses active β-catenin and LRP5 expression in normal and tumor colonic epithelial cells, related to Figures 1 and 2.

(A, B) Representative immunohistochemistry (IHC) staining images (**A**) and quantitative active β-catenin IHC composite expression scores (CES) (**B**) in tumor colonic tissues from the indicated mouse groups, as described in **Figures 1D-F** (n=6-8 mice per group). Values are mean ± SEM. * *P < .05* and **** *P < .0001* (two-way ANOVA with Bonferroni multiple-comparisons).

(C) Representative IHC staining images of active β-catenin in normal and tumor colonic tissues from the indicated mouse groups as described in **Figures S1J-L**.

(D) LRP5 and active β-catenin (**top**) and LRP6 (**bottom**) protein levels in normal IECs of $Apc^{\Delta 580}$ and $Apc^{\Delta 580}$-15-LOX-1 littermates at age 4 weeks were measured by Western blot analysis (n=3 pairs).

(E) LRP5, active β-catenin, and cleaved PARP protein levels for the indicated mice fed with 20% corn oil (CO) as described in **Figure 1A** were measured by Western blot analysis (n=3 per group).

(F, G) $Apc^{\Delta 580}$ and $Apc^{\Delta 580}$–15-LOX-1 mice at 4 weeks were fed 5% or 20% CO diets for 16 weeks. The mRNA expression levels of Axin2 **(F)** and cyclin D1 **(G)** in the IECs of the mice were measured by reverse transcription real-time quantitative polymerase chain reaction. Values are mean ± SEM. ***P<.01, ***P<.001 and **** P<.0001* (two-way ANOVA with Bonferroni multiple-comparisons).

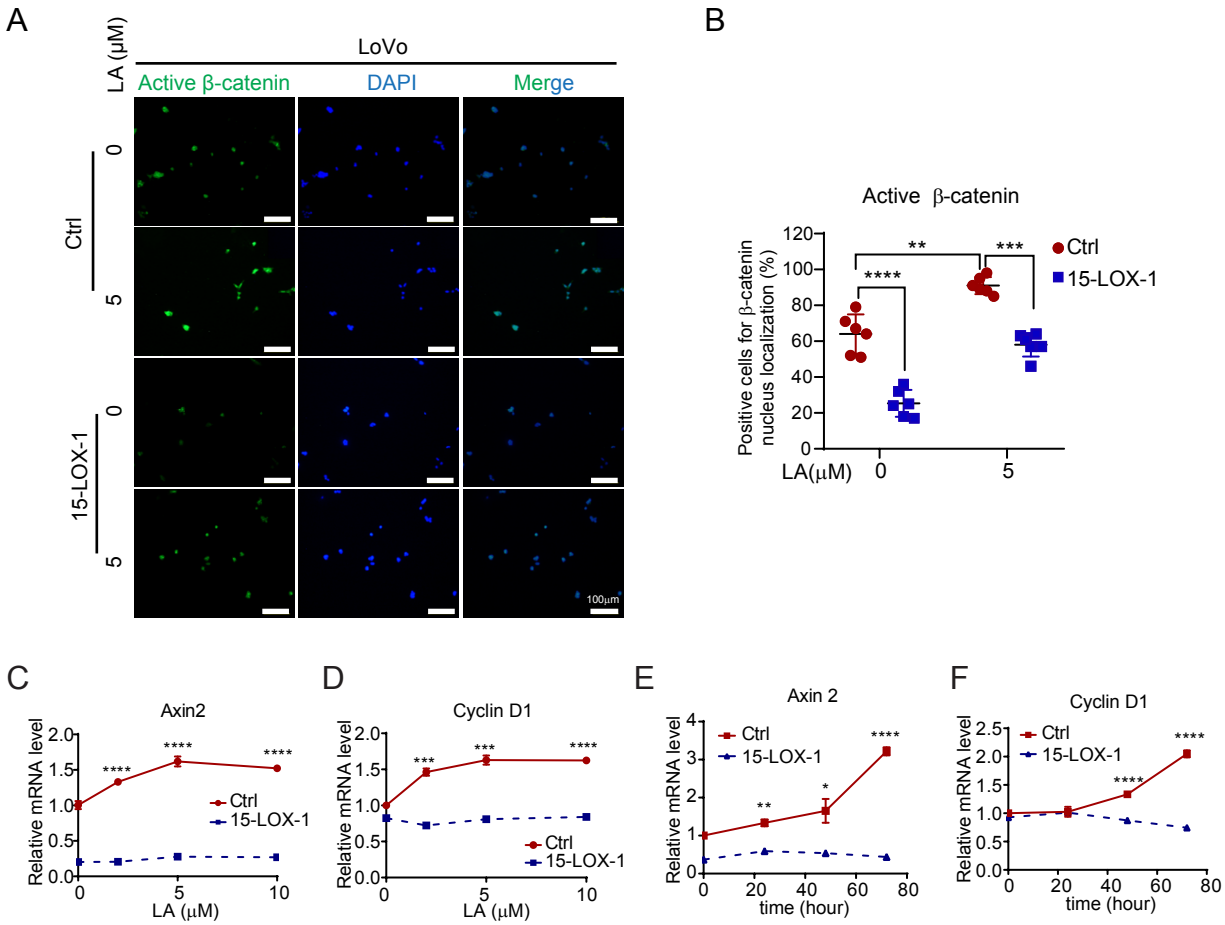

Figure S4. 15-LOX-1 suppresses active β-catenin and LRP5 expression in human colorectal cancer cells, related to Figure 2.
(A, B) Immunofluorescence staining of active β-catenin in LoVo cells stably transduced with either control (Ctrl) or 15-LOX-1 lentivirus and treated with 5μM LA for 48 hours. The cells with more fluorescence in the nucleus than cytoplasm were scored as positive cells for active β-catenin nuclear localization; positive and total number of cells were counted in six random fields by fluorescence microscopy. Representative immunofluorescence images (**A**) and quantitation of active β-catenin nuclear localizations (**B**) are shown. Values are mean ± SD. *** P<.01, *** P<.001 and **** P<.0001* (two-way ANOVA with Bonferroni multiple-comparisons). Three repeated experiments showed similar results.
(C-F) Axin2 and cyclin D1 mRNA expression in LoVo cells stably transduced with either control (Ctrl) or 15-LOX-1 lentivirus and treated with LA at different concentrations in culture media supplemented with 5% dialyzed fetal bovine serum for 48 hours (**C, D**) or treated with 5μM LA at different time points **(E, F),** measured by RT-qPCR. Values are mean ± SD. **P<.05, **P<.01, *** P<.001 and **** P<.0001* (two-way ANOVA with Bonferroni multiple-comparisons).

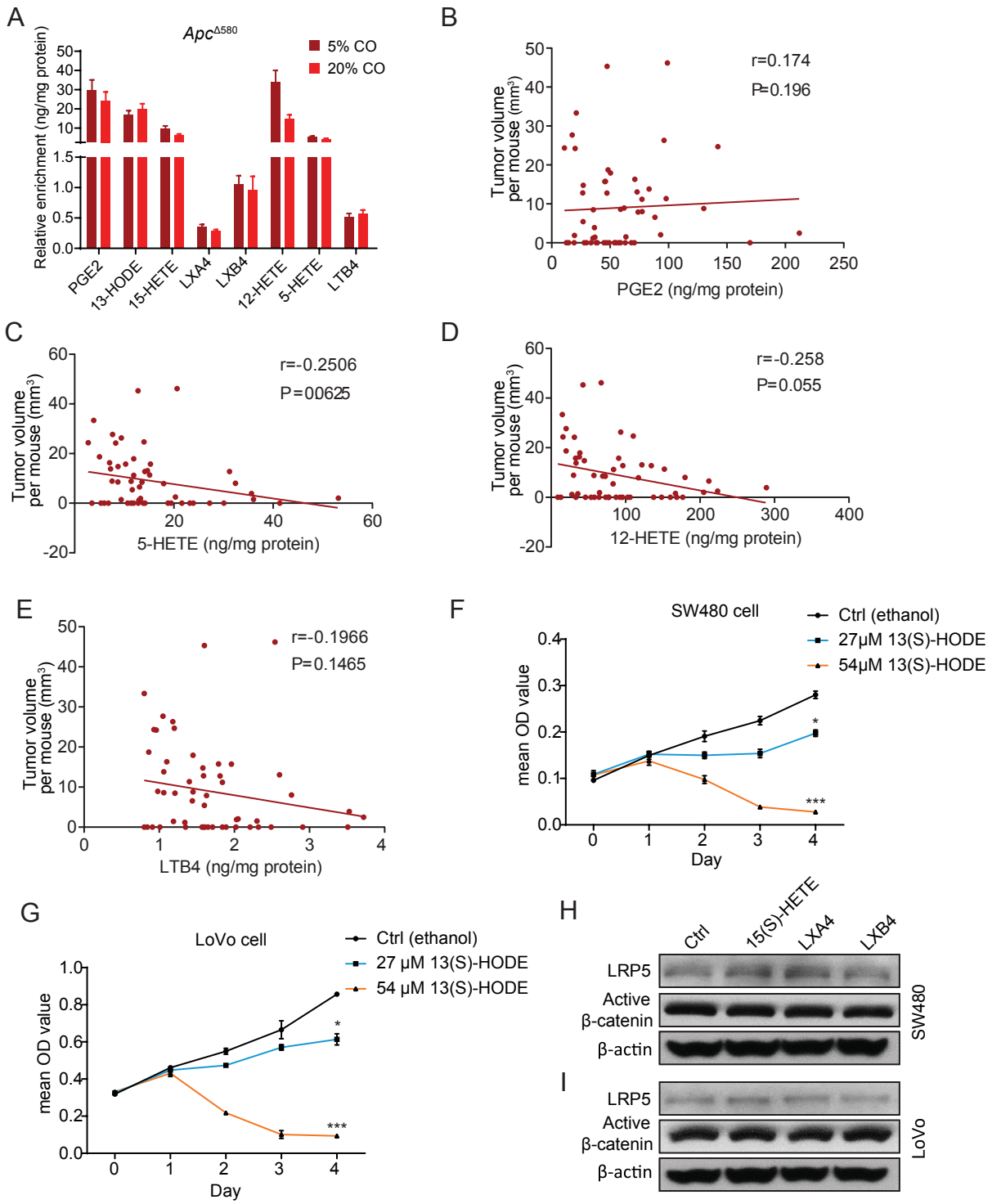

Figure S5. Profile analysis of 15-LOX-1–mediated eicosanoid metabolites in Apc$^{\Delta 580}$ mice and 13(S)-HODE suppression of human colorectal cancer proliferation, related to Figure 4.

(A-E) Apc$^{\Delta 580}$ and Apc$^{\Delta 580}$-15-LOX-1 littermates at 4 weeks were fed either 5% or 20% corn oil (CO) diets, sacrificed at age 16 weeks, and examined for tumor formation (n=14 mice per group). **(A)** Eicosanoid metabolite profiles of the normal intestinal epithelial cells (IECs) of the indicated Apc$^{\Delta 580}$ mouse groups were measured by liquid chromatography–tandem mass spectrometry. **(B-E)** Spearman correlation analysis of colonic tumor volumes and levels of PGE2 **(B)**, 5-HETE **(C),** 12-HETE **(D)**, and LTB4 **(E)** in IECs of all examined Apc$^{\Delta 580}$ and Apc$^{\Delta 580}$-15-LOX-1 mice (n=56).

(F, G) 13(S)-HODE suppresses human CRC proliferation. SW480 **(F)** and LoVo **(G)** cells were treated with 0, 27, and 54μM 13(S)-HODE for 0, 1, 2, 3, and 4 days. Cell viability and proliferation were measured by MTT assay as presenting absorbance of optical density (OD) at 590 nm. Values are mean ± SD. **P<.05 and *** P<.001 compared with Ctrl* (two-way ANOVA with Bonferroni multiple-comparisons).

(H, I) SW480 **(H)** and LoVo **(I)** cells were treated with 20μM 15(S)-HETE, 100nM LXA4, and 200nM LXB4 for 48 hours, and the cells were harvested and examined for LRP5 and active β-catenin expression by Western blot analysis.

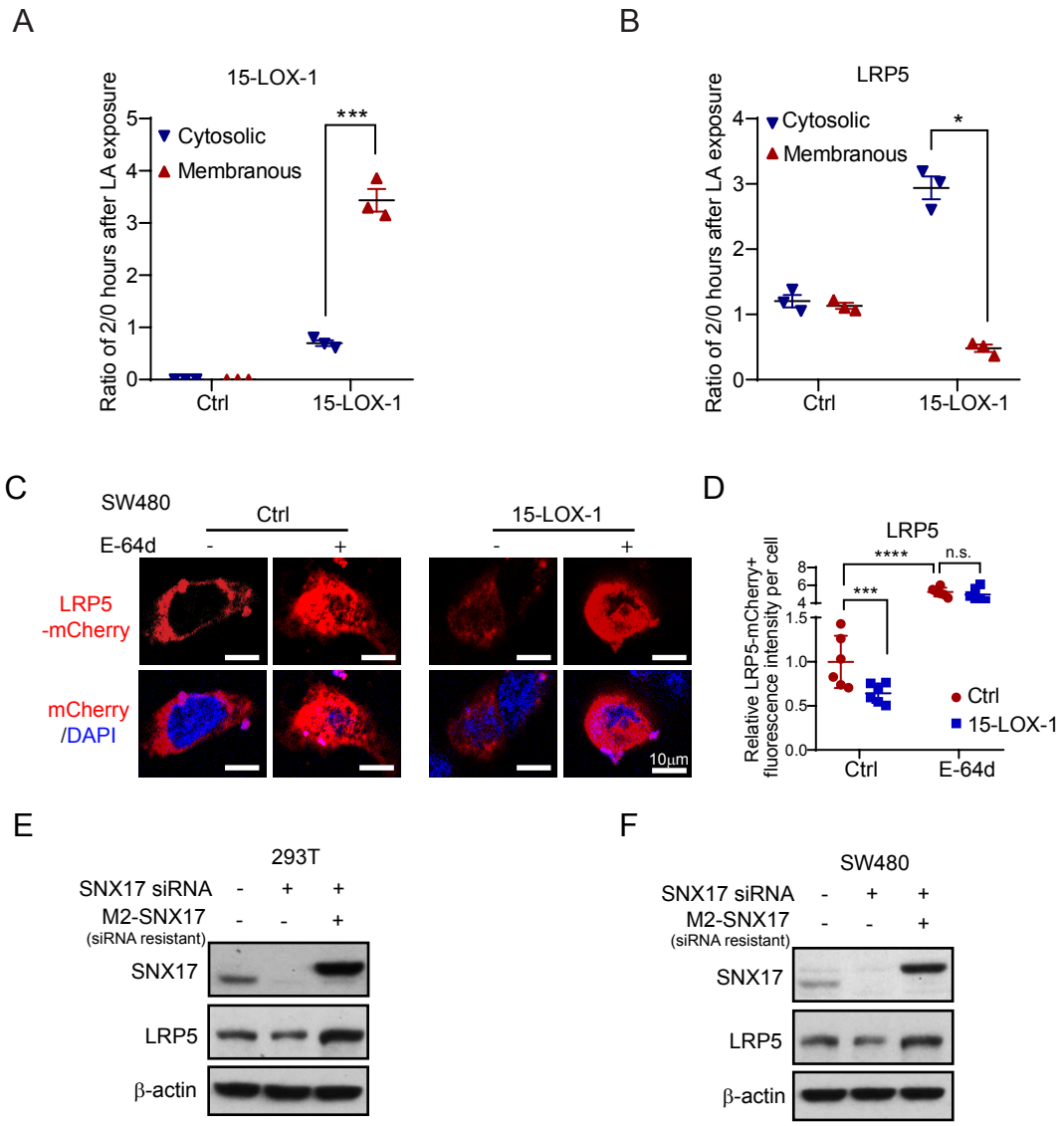

Figure S6. 15-LOX-1 reduces cell membranous LRP5 levels, related to Figures 5 and 6.
(A, B) LoVo cells stably transduced with control (Ctrl) or 15-LOX-1 lentivirus treated with 10μM linoleic acid (LA) for 2 hours were processed for cytoplasmic (C) and membranous (M) protein fractions and then analyzed for LRP5 and 15-LOX-1 protein expression by Western blot analysis. The 2-hour to 0-hour ratios of cytoplasmic protein band densities normalized to β-actin and membranous protein band densities normalized to Na^+-K^+-ATPase for 15-LOX-1 **(A)** and LRP5 **(B)** in LoVo cells transduced with Ctrl or 15-LOX-1 lentivirus, corresponding to **Figure 5D**, are shown**.** Values are mean ± SEM. **P<.05 and *** P<.001* (two-way ANOVA with Bonferroni multiple-comparisons).
(C, D) SW480 cells were co-transfected by mCherry-tagged LRP5 expression vector with control vector or 15-LOX-1 expression vector for 48 hours, followed by treatment with E-64d (10μM) for 1 hour. Representative microphotographs (**C**) and quantification (**D**) of LRP5 localization traced with mCherry fluorescence protein from six random fields under a confocal microscope are shown. Values are mean ± SD. **** P<.001 and **** P<.0001* (two-way ANOVA with Bonferroni multiple-comparisons). n.s.: no significant difference. Three repeated experiments showed similar results.
(E, F) 293T **(E)** and SW480 **(F)** cells were transfected with SNX17 siRNA or control siRNA for 24 hours and then transfected with an siRNA resistant M2-SNX17 expression vector or control vector for another 24 hours. Cells were harvested and then analyzed for SNX17 and LRP5 protein expression by Western blot.

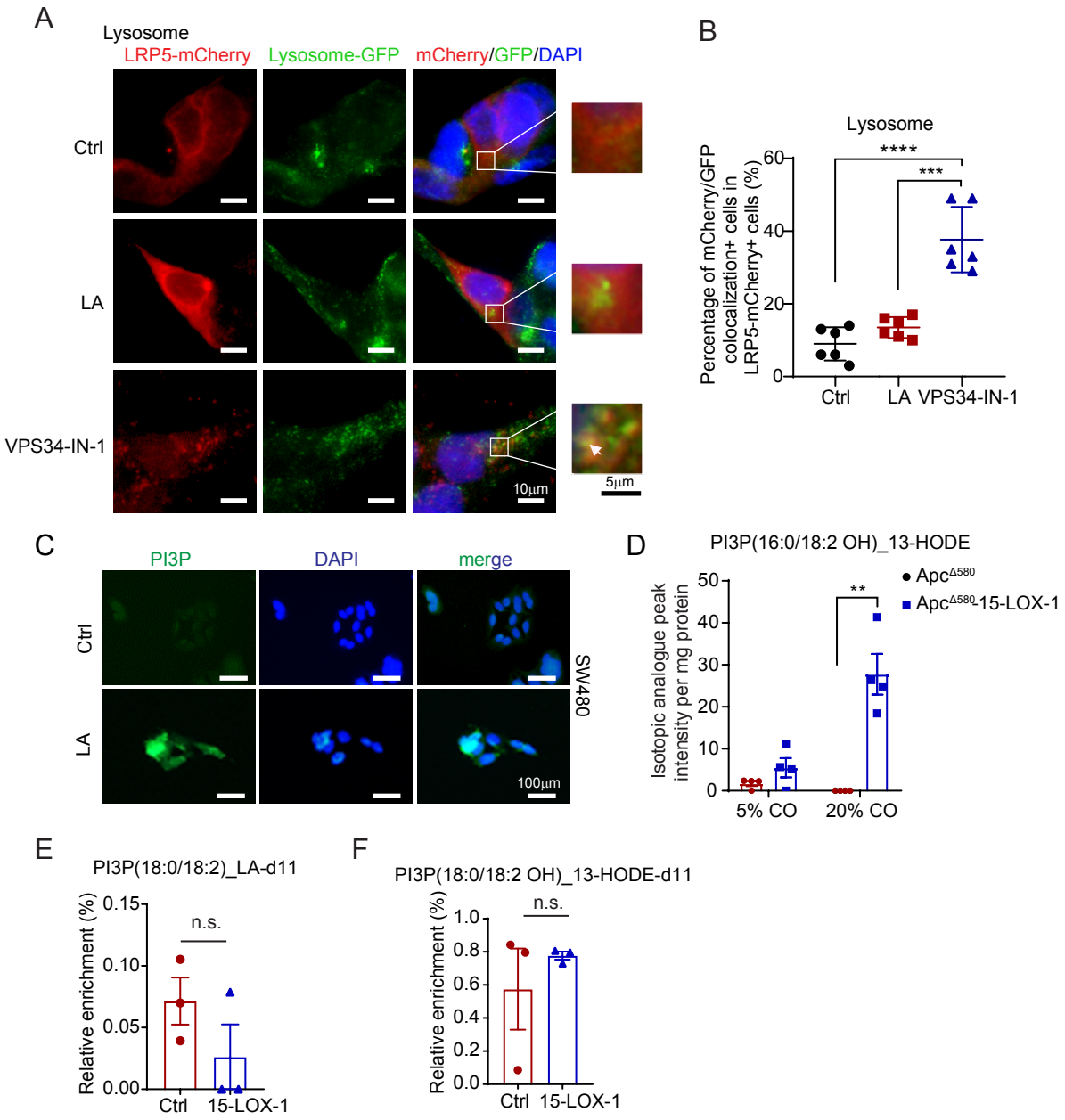

Figure S7. Linoleic acid (LA) increases phosphatidylinositol 3-phosphate (PI3P) production, and 15-LOX-1 increases PI3P_13-S-HODE production, related to Figure 7.

(A, B) 293T cells were transfected with mCherry-tagged LRP5 expression vector for 24 hours, then incubated with CellLight Lysosome-GFP, BacMam 2.0. Twenty hours later, cells were treated with 100μM LA or 1μM VPS34-IN-1 for 4 hours. Representative microphotographs of mCherry-tagged LRP5 localization into GFP-tagged lysosomes **(A)** and quantitative results for the percentage of cells with mCherry and GFP fluorescence colocalization out of total mCherry positive cells that were counted in six random fields by confocal microscopy (**B**) are shown. Values are mean ± SD. ****P<.001 and ****P<.0001* (one-way ANOVA with Bonferroni multiple-comparisons)**.** Three repeated experiments showed similar results.

(C) Immunofluorescence staining of PI3P in SW480 WT cells treated with 5μM LA or solvent for 48 hours. Representative images are shown.

(D) $Apc^{\Delta 580}$ and $Apc^{\Delta 580}$-15-LOX-1 littermates at age 4 weeks fed 5% or 20% corn oil (CO) diets were sacrificed at age 14 weeks. The intestinal epithelial cells (IECs) of these mice were scraped and examined for patterns of PI3P incorporated with (16:0/18:2 OH)_13-HODE by liquid chromatography–high resolution mass spectrometry (n=3-4 mice per group). Values are mean ± SEM. ***P<.01* (two-way ANOVA with Bonferroni multiple-comparisons).

(E, F) SW480 cells stably transduced with control (Ctrl) or 15-LOX-1 lentivirus were treated with 100μM LA-d11 in culture medium containing 5% dialyzed fetal bovine serum for 36 hours. PI3P profiling was traced with d11 labeling by liquid chromatography–high resolution mass spectrometry (LC-HRMS). The percentage of d11-labeled PI3P incorporated with (18:0/18:2)_LA over total PI3P incorporated with (18:0/18:2)_ LA **(E)**, or d11-labeled PI3P incorporated with (18:0/18:2 OH)_13-HODE over total PI3P incorporated with (18:0/18:2 OH)_13-HODE **(F),** was calculated and presented. Values are mean ± SEM. n.s.: no significant difference (unpaired *t* test).

Table S1. 5%- and 20% corn oil diets, related to Figure 1.

Dietary component	5% corn oil	20% corn oil
Corn oil, %	5	20
DL-methionine, g/100 g	0.30	0.353
Dextrose, g/100 g	65	44.682
Cellulose, g/100 g	5	5.88
Casein, g/100 g	20	23.55
Choline bitartrate, g/100 g	0.2	0.235
AIN-76A mineral mixture, g/100 g	3.50	4.12
AIN-76A vitamin mixture, g/100 g	1.00	1.18
BHT, %	0.02	0.02
BHQ, %	0.02	0.02
Kcal/g	3.85	4.53

Table S2. Tumor incidence of CRC in the indicated mouse experiments, related to Figure 1.

Corresponding to Figures S1B and C

Mouse group	Diet (Corn oil, %)	Mice numbers at the end	Mice bearing colon tumors (incidence, %)
WT	5	6	100
WT	20	9	100
15-LOX-1	5	10	90
15-LOX-1	20	8	87.5

Corresponding to Figures 1A-C

Mouse group	Diet (Corn oil, %)	Mice numbers at the end	Mice bearing colon tumors (incidence, %)
WT	5	19	100
12/15Lox^{KO}	5	18	100
12/15Lox^{KO}/12-LOX	5	18	100
12/15Lox^{KO}/15-LOX-1	5	17	76.5
WT	20	18	100
12/15Lox^{KO}	20	17	100
12/15Lox^{KO}/12-LOX	20	19	100
12/15Lox^{KO}/15-LOX-1	20	19	84

Corresponding to Figures 1D-F

Mouse group	Diet (Corn oil, %)	Mice numbers at the end	Mice bearing colon tumors (incidence, %)
Apc$^{\Delta 580}$	5	14	71
Apc$^{\Delta 580}$-15-LOX-1	5	14	36
Apc$^{\Delta 580}$	20	14	86
Apc$^{\Delta 580}$-15-LOX-1	20	14	64

Corresponding to Figures S1S and T

Mouse group	Mice numbers at the end	Mice bearing colon tumors (incidence, %)
Apc$^{\Delta 580}$	5	100
Apc$^{\Delta 580}$-15-LOX-1	5	40

Table S3. The quantitative data of the band densities of LRP5 normalized to β-actin for Figures 5A and B, related to Figure 5.

Corresponding to Figure 5A for SW480 cells

Group	The change percentage at 2 hours: (2h-0h)/0h	The change percentage at 4 hours: (4h-0h)/0h
Control	-35%	-65%
15-LOX-1	- 50%	-85%

Corresponding to Figure 5B for LoVo cells,

Group	The change percentage at 2 hours: (2h-0h)/0h	The change percentage at 4 hours: (4h-0h)/0h
Control	+10%	-42%
15-LOX-1	- 3%	-62%

Table S4. Representative cell internalization distribution presenting as percentage of 50000 examined cells with R1>0 by imaging flow cytometry, corresponding to Figure 5H, related to Figure 5.

Group	LA-0 hour	LA-2 hour
Control	21%	19%
15-LOX-1	32%	45%