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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

MYPT1 Downregulation by Lipopolysaccharide-SIAH1/2 E3 Ligase-Ubiquitin-Proteasomal Degradation Contributes to Colonic Obstruction of Hirschsprung Disease

Smooth muscle contraction is evoked through multiple signaling pathways converging on myosin light chain phosphorylation.[1,2] Contraction-associated proteins such as MLCK, MYPT1, RLC, RhoA, ROCK, and CPI-17 are differentially expressed in different types of smooth muscle, leading to contractile diversity.[3–5] Herein we demonstrate that lipopolysaccharide (LPS) causes MYPT1 protein degradation via SIAH1/2 E3 ligases-ubiquitin-proteasomal pathway, which alters colonic contractile properties and results in obstructive phenotype.

Isolated colonic smooth muscle was treated with increasing doses of LPS ex vivo, causing significant reduction of MYPT1 protein (Supplementary Figure 1*A*). Compared with the proximal colon, MYPT1 protein in the distal colon was also reduced by LPS local treatment (Supplementary Figure 1*B*). Meanwhile, a single intraperitoneal injection of LPS (5 mg/kg) induced a significant reduction of MYPT1 within 24 hours, and the effect lasted for 5 days (Figure 1*A*). After injection of LPS (0.5 mg/kg) sequentially with a 24-hour interval, MYPT1 protein was maintained at a low level and recovered within 3 days after stopping the injection (Supplementary Figure 1*C*). Summarily, MYPT1 protein can be reduced by LPS and reversed by removing LPS.

We found MYPT1 mRNA level in colonic smooth muscle comparable with or without LPS (Supplementary Figure 1*D*). On treatment with MG132, MYPT1 protein was not affected by LPS (Supplementary Figure 1*E*). In pull-down by anti-

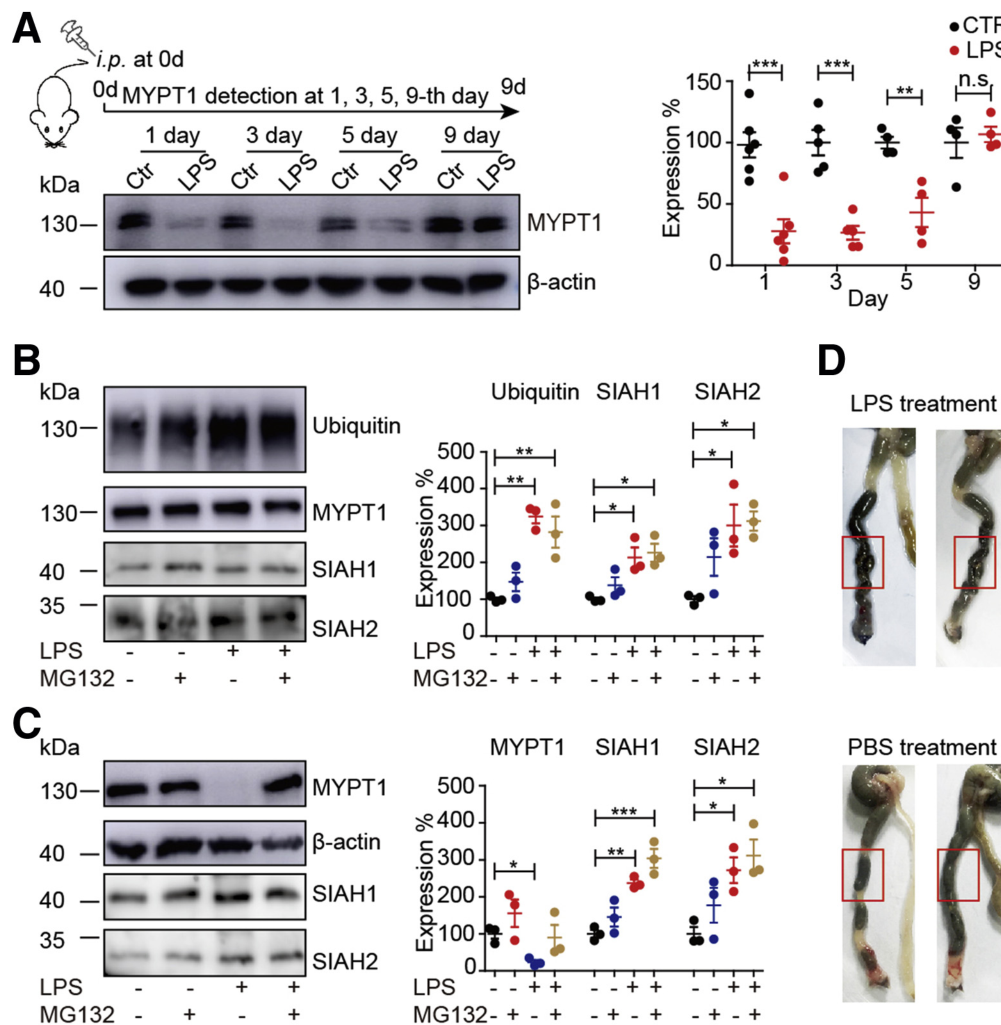

Figure 1. MYPT1 was downregulated by LPS-SIAH1/2 E3 ligase-ubiquitin-proteasomal degradation, leading to colonic obstruction. (*A*) Western blot showed MYPT1 expression after LPS intraperitoneal injection (n = 4–6) (*t* test). (*B* and *C*) Colonic smooth muscle from C57BL/6 mice was treated with or without LPS (0.1 mg/mL) and MG132 (50 μmol/L) for 24 hours and harvested for detection of constitutive expression of MYPT1, SIAH1, and SIAH2 by immunoblotting analysis (*C*); lysate was collected for immunoprecipitation with MYPT1, MYPT1-ubiquitin, SIAH2, and SIAH2 (*B*) (n = 3) (one-way analysis of variance [ANOVA]). (*D*) Position of feces in the colon of C57BL/6 mice after local treatment with LPS or phosphate-buffered saline. *Bars* represent the mean values ± standard error of the mean. *$P < .05$; **$P < .01$; ***$P < .001$.

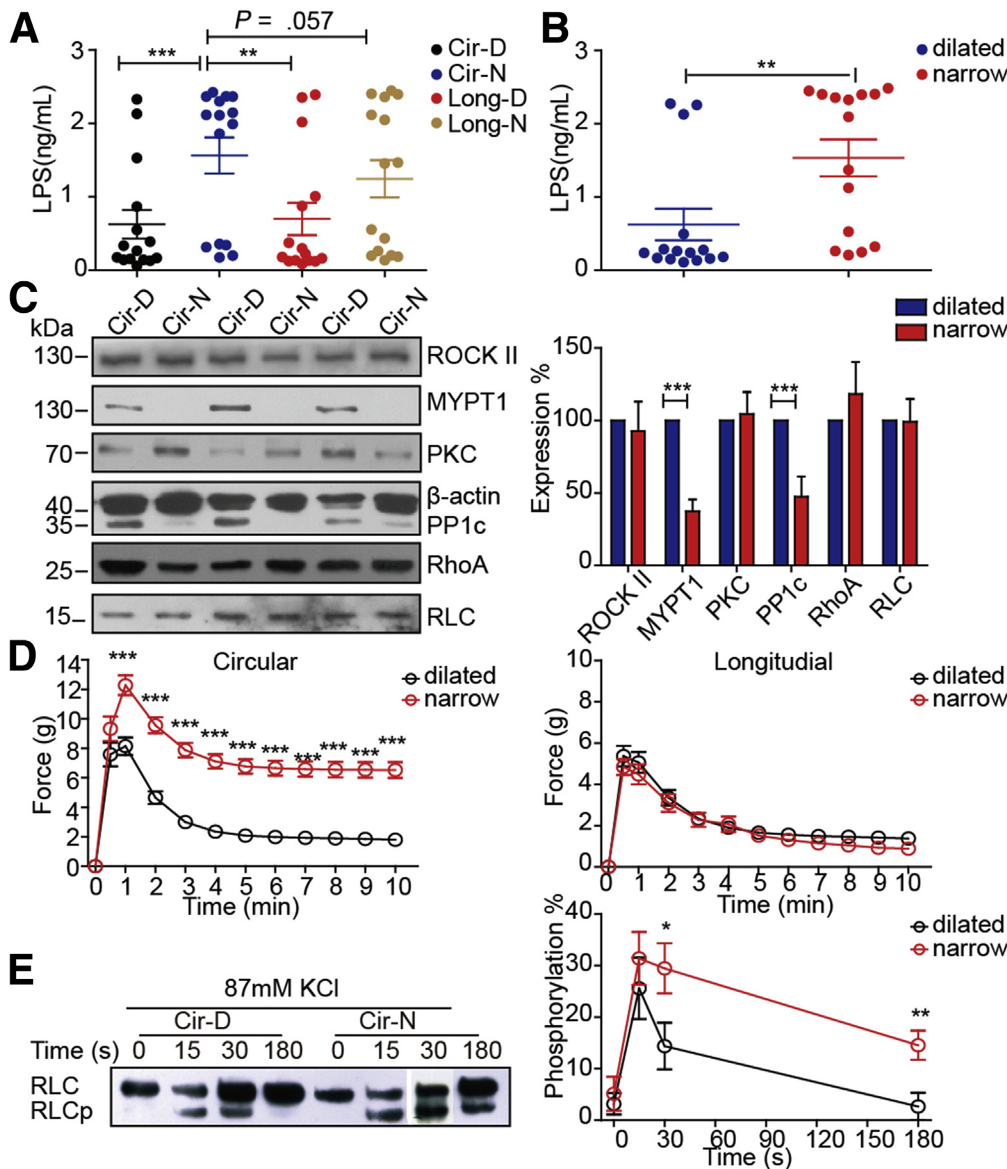

Figure 2. Cir-N showed high LPS concentration, MYPT1 deficiency, tonic-like contraction, and high RLC phosphorylation. (*A* and *B*) LPS was measured by limulus test in Cir-D, Cir-N, Long-D, and Long-N, respectively, (*A*) and in the whole layers of distal and longitudinal smooth muscle (*B*). (*C*) Western blots showed the protein levels in Cir-N (*left panel*). Quantification of proteins in the narrow segment shown as the percentage of those in the dilated segment (*right panel*) (pair *t* test). MYPT1, n = 51; PP1c, n = 34; ROCK2, n = 29; PKC, n = 31; RLC, n = 19; RhoA, n = 15. (*D*) Contraction of circular (*left panel*) and longitudinal (*right panel*) smooth muscle evoked by KCl. Cir-, n = 37; Long-, n = 20 (*t* test). (*E*) The pRLC level was expressed as percentage of total RLC stimulated by KCl (n = 8) (*t* test). *Bars* represent the mean values ± standard error of the mean. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

MYPT1 antibody, the ubiquitinated MYPT1 level was increased by LPS (Figure 1*B*). Pulled-down MYPT1 protein complex contained E3 ligases (SIAH1 and SIAH2) that have a binding motif with MYPT1.[6] SIAH1 and SIAH2 levels were elevated by LPS or LPS plus MG132 (Figure 1*B*). Furthermore, C3H/HeJ mice with a mutant TLR4 showed no apparent reduction of MYPT1 by LPS (Supplementary Figure 1*F*). Therefore, LPS/TLR4 enhances MYPT1 degradation through the SIAH1/2 E3 ligases-ubiquitin-proteasomal pathway.

LPS local treatment in the distal colon caused 45% of mice to have packed feces in the colon lumen (Figure 1*D*), and circular smooth muscles showed an enhanced sustained phase of KCl-evoked contraction (Supplementary Figure 1*G*). These phenotypes did not appear in MYPT1^{SMKO} mice with the same treatment (Supplementary Figure 1*H*). Therefore, MYPT1 reduction by LPS causes an obstruction-like phenotype by altering colonic contraction.

To investigate the role of MYTP1 in Hirschsprung disease (HD), we collected fresh colons from HD patients: circular (Cir-) and longitudinal (Long-) muscle strips from dilated (D) and narrow (N) segments. LPS concentration was significantly higher in Cir-N than in Cir-D and Long-D and slightly higher than in Long-N (Figure 2*A*). The LPS average concentration in narrowed segments was

higher than in the dilated (Figure 2*B*). Meanwhile, compared with Cir-D, MYPT1 protein in Cir-N was decreased (Figure 2*C*), and PP1cδ was accordingly down-regulated (Figure 2*C*). In Long-N and Long-D, the contractile proteins were comparable (Supplementary Figure 2*A*). Moreover, Cir-N muscles produced a strong contractile response with a large, robust, and sustained tension by KCl, whereas Cir-D, Long-N, and Long-D displayed a phasic contraction (Figure 2*D*). H1152 and GF109203X inhibitors could not relax the KCl-contracted muscle, whereas sodium nitroprusside (SNP) and nifedipine could in Cir-N (Supplementary Figure 2*B*–*E*). On KCl stimulation, Cir-N smooth muscle from HD patients exhibited significantly higher regulatory light chain (RLC) phosphorylation than Cir-D at the sustained phase (Figure 2*E*), in accordance with its maintained force tension.

We generated MYPT1 smooth muscle–specific knockout mice, *Mypt1*$^{f/f}$; SMA Cre-, *Mypt1*$^{f/+}$; SMA-Cre^{+} and *Mypt1*$^{f/f}$; and SMA-Cre^{+}[7]. On KCl stimulation, the colonic circular muscle from homozygotes showed enhanced initial and sustained tension, whereas the vehicle from heterozygotes displayed a modest enhancement (Supplementary Figure 3*A*). Meanwhile, H1152 and GF109203 could not relax the mutant muscle, but SNP and nifedipine could (Supplementary Figure 2*B*–*E*). The number of ganglia and ganglionic cells in the mutant colon was significantly reduced (Supplementary Figure 3*F*). Collectively, MYPT1-deficient colon showed similar phenotypes including altered contractile properties and ganglia to Cir-N from HD patients, although the MYPT1^{SMKO} mice showed no colonic obstruction, comparable bowel motility, transit velocity, and eating/defecation activity.[7] Because circular smooth muscle layer constricts the bowel lumen and longitudinal muscle dilates the lumen and propels feces,[8,9] the disruption of this coordination might cause colonic obstruction.

We also established an *Ednrb*$^{-/-}$ line (Supplementary Figure 4*A*–*D*), an HD model with colonic obstruction and few ganglions. The high concentration of LPS, MYPT1 deficiency, and altered contractile property were also confirmed (Supplementary Figure 4*E*–*G*).

In summary, normal intestinal function relies on multiple factors such as smooth muscle and enteric nervous system. MYPT1 is a primary regulator of smooth muscle contraction.[7] LPS can degrade MYPT1 in colonic circular smooth muscle, thereby altering the contractile property and leading to colonic obstruction. Removing local LPS or targeting the SIAH/E3-mediated protein degradation pathway might be a useful strategy to treat colonic obstruction in HD.

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Abbreviations used in this paper: **ANOVA, analysis of variance; Cir, circular; D, dilated; HAEC, Hirschsprung-associated enterocolitis; HD, Hirschsprung disease; Long, longitudinal; LPS, lipopolysaccharide; N, narrow; RLC, regulatory light chain; SNP, sodium nitroprusside**

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2352-345X
https://doi.org/10.1016/j.jcmgh.2019.11.003

Received January 31, 2019. Accepted November 12, 2019.

Author contributions
W.Z., P.W., Y.B., W-B.T., and M-S.Z. designed the research; W.Z. and P.W. conducted all vitro and in vivo assays; H-X.L., W-W. J., and X-L.G. collected samples of HD; W-Q.H generated MYPT1 knockout mice and revised the manuscript; T.T. and J.S. helped with histologic assay; X.C. and Y.W. helped with protein assay; Y-Q.L. helped with mRNA assay; Y-Y.Z., L-S.W., and C-P.C. contributed new regents/analytic tools; C-J.L. and H-Q.C contributed data calibration; Z.W., W.P., Y.B., W-B.T., and M-S.Z. analyzed the data; and W.Z., P.W., W-B.T, and M-S.Z. wrote the manuscript.

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by the National Natural Science Funding of China (31330034 to M.-S.Z, 31371356/30971540 to H.-Q.C., and 81370473/81570467 to W.-B.T.).

Supplementary Materials and Methods

Immunoprecipitation and Western blotting

Immunoprecipitation was performed according to the method as previously described.[1] Colonic smooth muscles were lysed by lysis buffer (50 mmol/L Tris.HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Roche, Basel, Switzerland]), and the debris was removed by centrifugation. The resultant lysates were pre-cleared with protein G Sepharose beads (lysates: protein G, 10:1) for 1 hour, followed by incubation with anti–MYPT-1 antibody overnight at 4°C. After adding protein G Sepharose beads and incubating for 3 hours at 4°C, Sepharose beads were collected and washed twice by centrifugation. Then we added 1 × loading buffer (10 mmol/L Tris-HCl, pH 8.0, 1% sodium dodecyl sulfate, 50 mmol/L dithiothreitol, 10% glycerol) to the beads, followed by incubation at 100°C for 10 minutes, and the protein sample was prepared by centrifugation at 12,000*g* for 1 minute.

To determine the expression of MYPT-1, ROCK-2, PKC, PP1cδ, and their related proteins, we isolated and homogenized circular and longitudinal smooth muscle layers. This procedure was performed as previously described.[2] The following primary antibodies were used for immunoprecipitation and immunoblot: anti–β-actin antibody (Sigma-Aldrich, St Louis, MO), anti–MYPT-1 antibody (Millipore, Burlington, MA), anti–ROCK-2 antibody (Santa Cruz Biotechnology, Dallas, TX), anti-PP1cδ antibody (Millipore), anti-PKC antibody (Millipore), anti-RhoA antibody (Santa Cruz Biotechnology), anti-ubiquitin antibody (Abcam, Cambridge, UK), anti–SIAH-1 antibody (Signalway Antibody Co, College Park, MD), and anti–SIAH-2 antibody (Santa Cruz Biotechnology).

Preparation of Mice Models

To produce smooth muscle–specific knockout mice, $Mypt1^{flox/flox}$ mice and SMA-Cre transgenic mice were crossed.[3] The resultant mice were $Mypt1^{+/+}$ ($Mypt1^{flox/flox}$), $Mypt1^{+/-}$ ($Mypt1^{+/flox}$; SMA-Cre) and $Mypt1^{-/-}$ ($Mypt1^{flox/flox}$; SMA-Cre). The strategy of genotyping was described in our previous report.[3]

Endothelin B receptor (*Ednrb*) knockout mice were generated by CRISPR/Cas9 technology. In brief, the Cas9 protein containing a C-terminal SV40 nuclear localization signal was synthesized and inserted into pUC57-sgRNA expression vector. Two gRNAs (gRNA1 and gRNA2) targeting sites of exon1 of *Ednrb* were designed, and sgRNA scaffold containing T7 promoter was amplified from pUC57-sgRNA. The gRNAs together with Cas9 mRNA were injected into C57BL/6 mouse zygotes. Primer pairs for genotyping were forward, 5′-CCA GTT GGT CTC CAG ACT GAA-3′; reverse, 5′-AAG GAT CTT GGC GGG ACT CCA GC-3′.

All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Model Animal Research Center of Nanjing University (Nanjing, China).

Force Measurement of Colonic Smooth Muscles From Mice

For measuring the force produced by mouse colonic muscles, the circular (3–5 mm in width) muscle strips from the distal colon (10 mm proximal to the anus)[4,5] were prepared and then mounted on a force transducer. Force measurements were performed according to our previously described methods.[3] The resting tension was set to approximately 0.5 g before force measurement. The muscles were stimulated by using a KCl-depolarization buffer containing 87 mmol/L KCl. The chemicals used for the contraction measurement, such as H1152 (Calbiochem, San Diego), GF109203X (Tocris, Bristol, UK), SNP (Sigma-Aldrich), and nifedipine (Sigma-Aldrich), were diluted with HEPES-Tyrode (H-T) buffer to achieve the indicated concentrations.

Force Measurement of the Colonic Smooth Muscles From Hirschsprung Disease

The dilated and narrow colon biopsies were collected from 87 HD patients who received transanal endorectal pull-through or laparoscope-assisted transanal endorectal pull-through operations[6,7] at Nanjing Children Hospital. The ages of patients were 4 ± 0.3 months, and the gender ratio (male: female) was 3:1. The percentage of patients with preoperative Hirschsprung-associated enterocolitis (HAEC) was 21.79%, and the percentage of patients with postoperative HAEC was 11.53%. Patients with preoperative HAEC were treated with antibiotic treatment before surgery, whereas the others were treated through enema with 0.9% NaCl. The percentage of patients with more than 15 cm length of narrow segments was 24.36%, and percentage of those with segment less than 15 cm length was 75.64%. All biopsies were immediately stored in pre-cold and pre-oxygenated H-T buffer[3] and then subjected to force measurement within 2 hours.

After removing the mucosa from colon, strips of circular and longitudinal muscle (1.5 × 10.0 mm)[8] from the dilated colon and the narrow colon were cut along the direction of the muscle fibers and were mounted on a force transducer (MLT0202; AD Instruments, Spain) that was connected to a PowerLab recording device (AD Instruments, Australia). The strips were mounted in circular or longitudinal orientation and were equilibrated in H-T buffer for 30 minutes at 37°C before force measurement. The resting tension was set to approximately 1.0 g.[9]

Measurement of Regulatory Light Chain Phosphorylation

Urea/glycerol–polyacrylamide gel electrophoresis was performed to separate the non-phosphorylated RLC from the phosphorylated RLC.[10] This procedure was performed as previously described.[3] Western blotting using an RLC-specific antibody was performed to visualize the RLC-containing bands.[10] The amount of monophosphorylated RLC relative to the total amount of RLC protein was determined by using a Jieda 801 Image Analysis System 3.3.2 (JEDA

Science-Technology Development Co, Ltd, Nanjing, China) and was expressed as a percentage.

Histologic Analysis

Eight- to twelve-week-old MYPT1^{SMKO} mice were killed by cervical dislocation. The entire colon (proximal colon, 10 mm to cecum; distal colon, 10 mm to anus) was fixed in 4% formaldehyde at 4°C for 2 hours, dehydrated in butyl alcohol at 4°C overnight, embedded in paraffin, and cut into 7-μm sections.[11] The sections were stained by hematoxylin/eosin, and the ganglionic cells in the entire colon were examined under microscopy images (Dotslide; Olympus, Tokyo, Japan).

Statistics

Data were presented as the mean ± standard error of the mean. The differences between 2 groups were evaluated by paired or unpaired *t* tests. Multiple group comparison was performed by using one-way ANOVA, followed by Tukey's test. $P \leq .05$ was considered statistically significant. All statistical analyses were performed by using GraphPad software (San Diego, CA).

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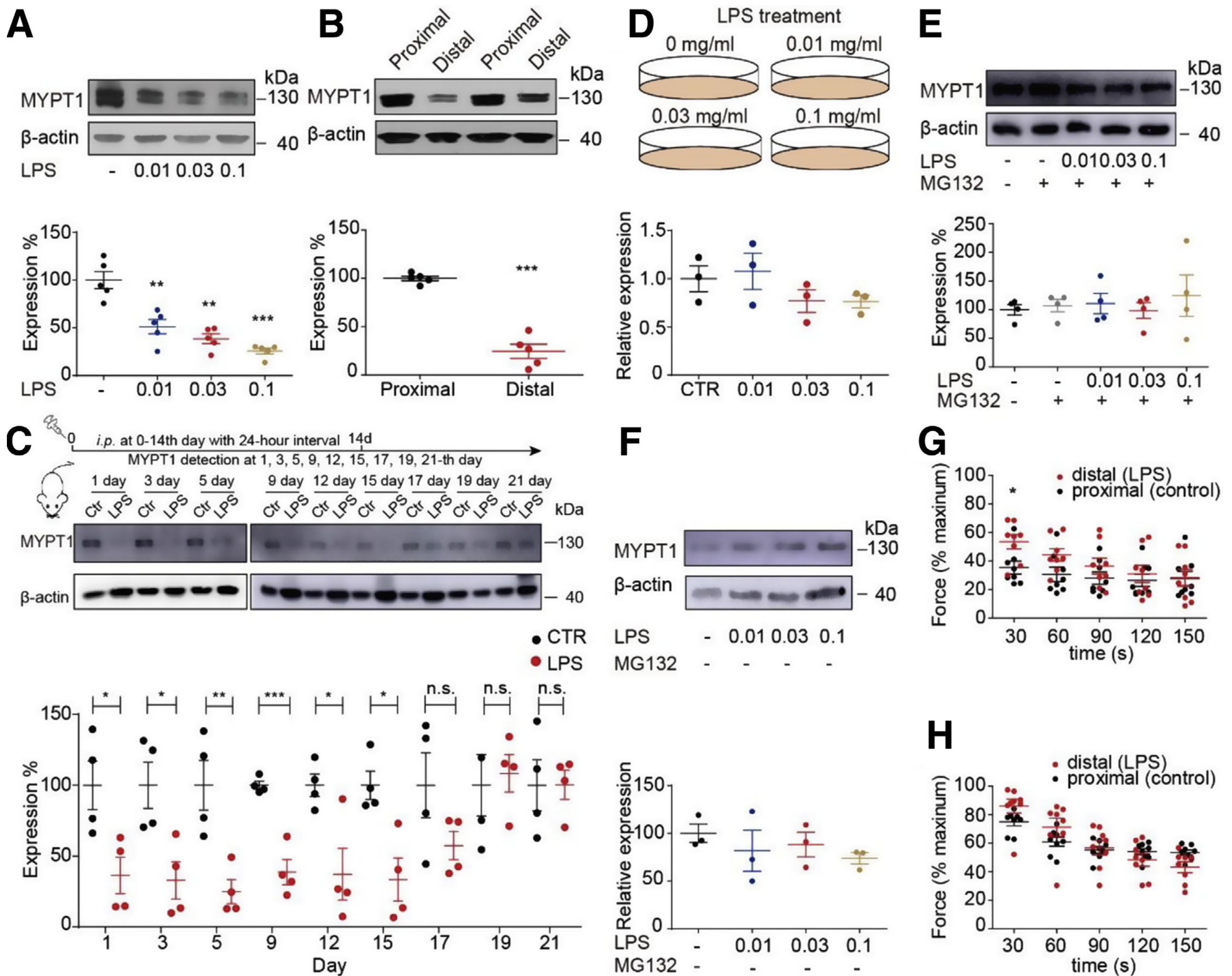
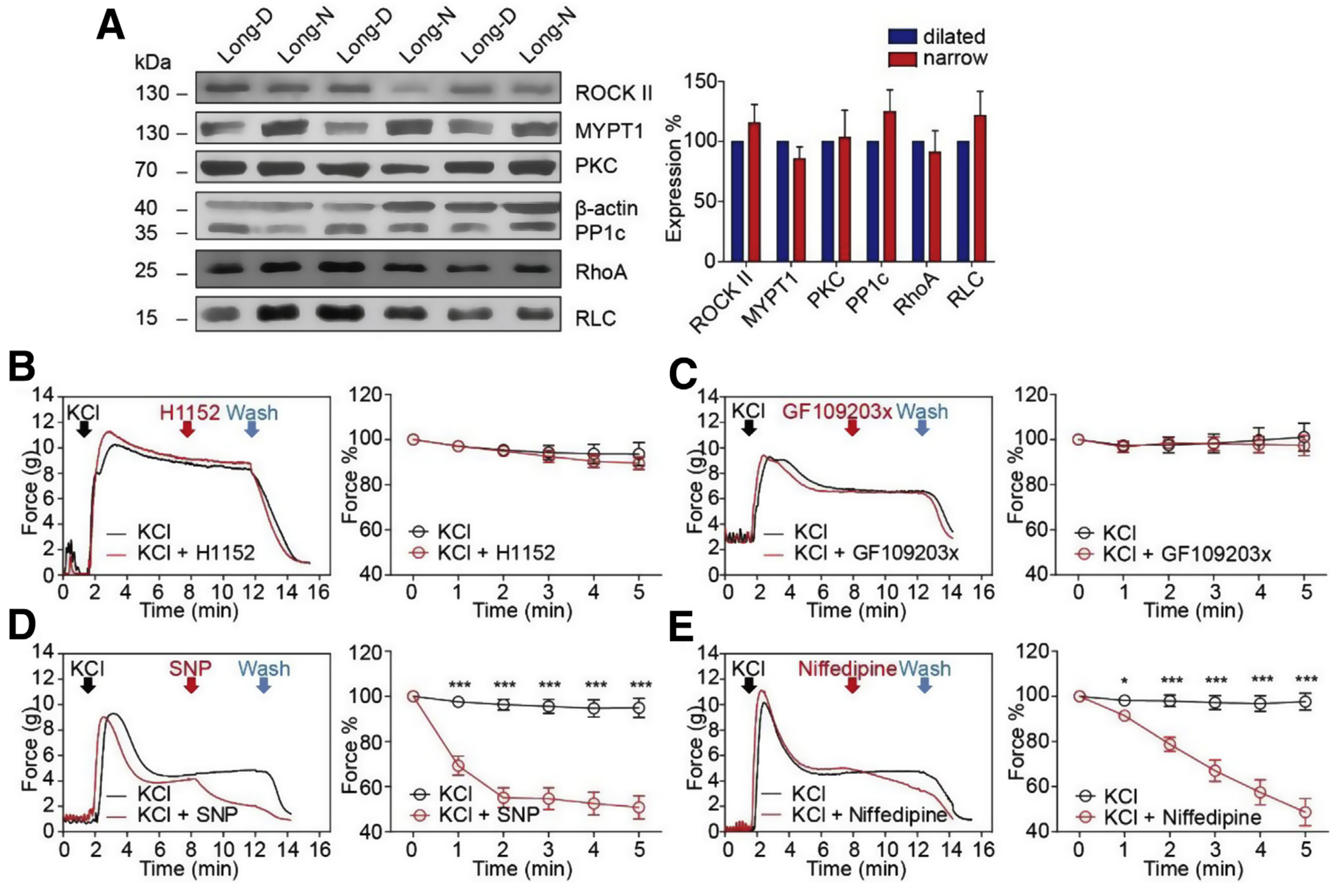
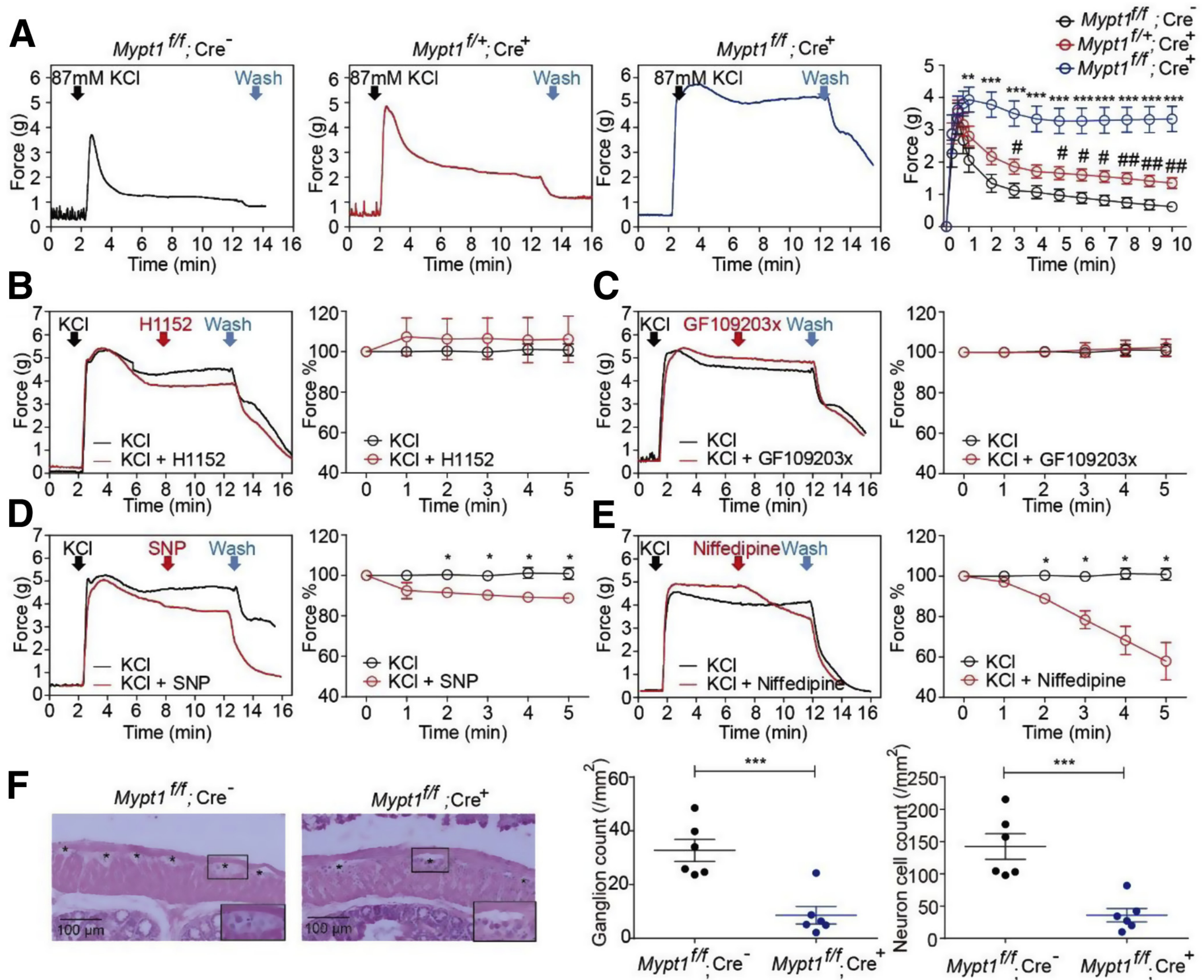


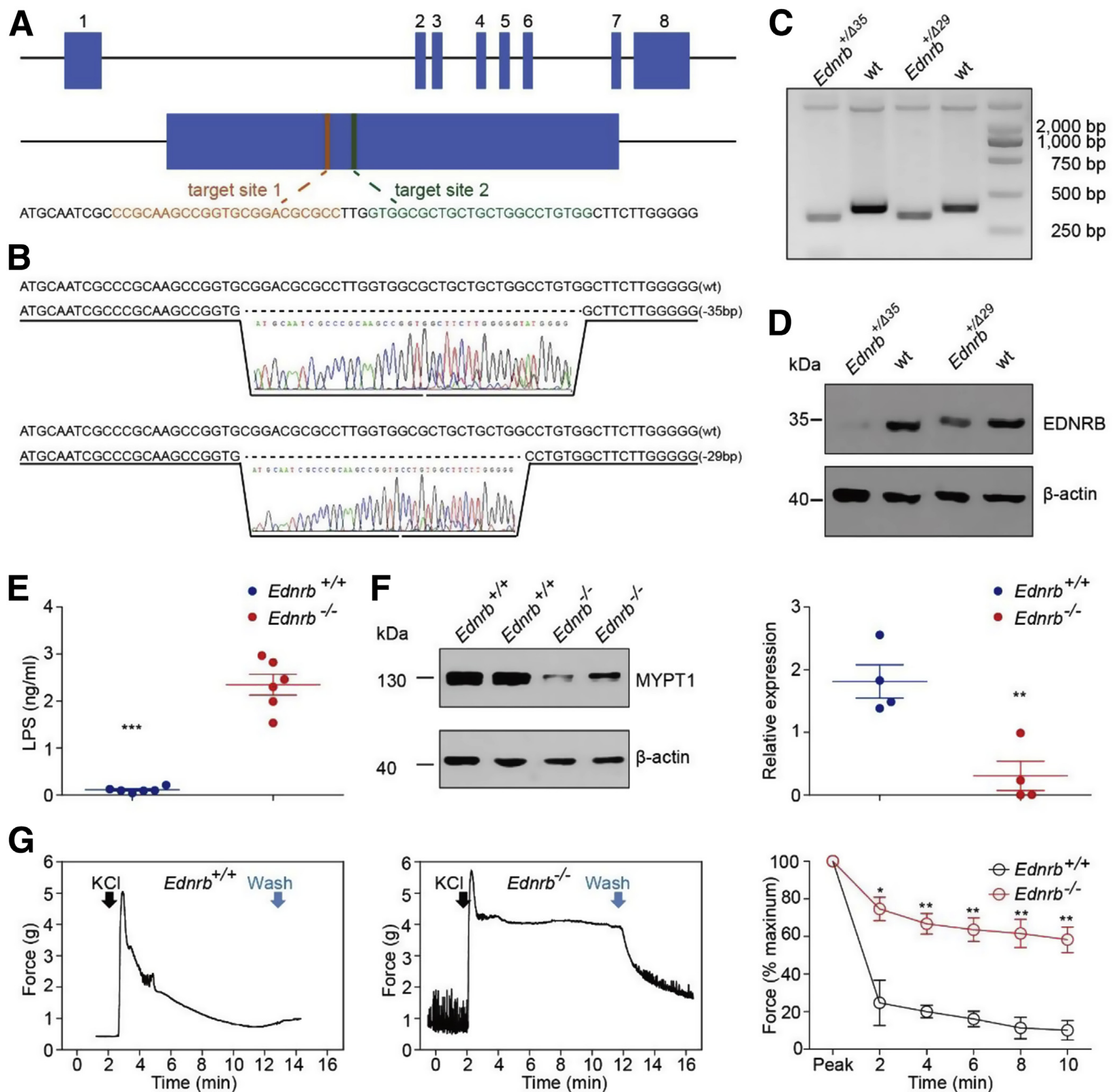
Supplementary Figure 1. LPS treatment induced altered contractile property by decreasing expression of MYPT1 through TLR4 both. (*A*) In ex vivo experiment, mouse (C57BL/6) colon segments were isolated, followed by treatment of increased dose of LPS. The protein of the treated muscles was sampled for Western blot assay (n = 5) (one-way ANOVA). (*B*) LPS-containing cotton balls (20 μg) were inserted into distal about 1 cm from the anus for 14 days. Then MYPT1 protein was measured in the distal and proximal sections of the colons (paired *t* test) (n = 5). (*C*) C57BL/6 mice were injected every other day with LPS (0.5 mg/kg body weight) (n = 4) for 14 days. Colonic smooth muscles were sampled at indicated time points and subjected to MYPT1 protein measurement with Western blot. Amount of loaded protein was normalized by using β-actin. (*D*) Quantitative polymerase chain reaction showing relative mRNA expression of MYPT1 in smooth muscle treated with LPS (n = 3) (one-way ANOVA). (*E*) Colonic smooth muscle from C57BL/6 mice was treated with MG132 (a proteasome inhibitor) (50 μm) and LPS by increased dose for 24 hours and harvested for Western blot analysis (n = 4) (one-way ANOVA). (*F*) Colonic smooth muscle from C3H/HeJ mice was treated with LPS by increased dose for 24 hours (n = 3) (one-way ANOVA). (*G* and *H*) After same treatment as (*B*), contractility of proximal and distal colonic smooth muscles was measured with KCl stimulation. Quantitation of percentage force with sustain to the maximum force in distal and proximal colon with C57/B6 (*G*) and *Mypt1*^{SMKO} (*H*) mice, respectively (n = 8) (*t* test). *Bars* represent mean values ± standard error of the mean. *$P < .05$; **$P < .01$; ***$P < .001$. CTR, control.



Supplementary Figure 2. Relaxant effects of ROCK, PKC, L-type calcium channel inhibitors, and SNP on KCl-evoked contraction of Cir-N from HD and colon from MYPT1^{SMKO} mice. (*A*) Western blots showing relative amounts of proteins in Long- smooth muscles (*left panel*). Quantification of protein levels in the narrow segment as percentage of those in the dilated segment (*right panel*) (paired *t* test). MYPT1, n = 51; PP1c, n = 34; ROCK2, n = 29; PKC, n = 31; RLC, n = 19; RhoA, n = 15. (*B–E*) Representative tracings of Cir-N pre-contracted using 87 mmol/L KCl, followed by exposure to 0.3 μmol/L H1152 (*B*), 5 μmol/L GF109203X (*C*), 100 μmol/L SNP (*D*), or 3 μmol/L nifedipine (*E*). Relative ratios of relaxed force during the sustained phase, which were expressed as percent of force of contraction after addition of KCl at same time point as addition of the vehicle (force % = $(F_{vehicle} - F_{inhibitor})/F_{vehicle}$, F = force) in the *left panel*. *Bars* represent mean values ± standard error of the mean; n = 3. **P* < .05; ****P* < .001 (*t* test).



Supplementary Figure 3. Colon from MYPT1^{SMKO} mice presented a similar phenotype compared with Cir-N from HD. (*A*) Representative tracings of circular smooth muscle of distal colon treated with 87 mmol/L KCl from *Mypt*$^{f/f}$; SMA Cre-, *Mypt1*$^{f/+}$; SMA Cre+, and *Mypt1*$^{f/f}$; SMA Cre+ mice. Quantification of force responses of circular smooth muscle to treatment with KCl (one-way ANOVA) (n = 6). (*B–E*) Representative tracings of circular distal colon from MYPT1^{SMKO} mice pre-contracted using 87 mmol/L KCl, followed by exposure to 0.3 μmol/L H1152 (*B*), 5 μmol/L GF109203X (*C*), 100 μmol/L SNP (*D*), or 3 μmol/L nifedipine (*E*). Relative ratios of the relaxed force during the sustained phase, which were expressed as percent of force of contraction after addition of KCl at the same time point as addition of the vehicle (force % = $(F_{vehicle} - F_{inhibitor})/F_{vehicle}$, F = force) in *left panel*. (*F*) H&E staining of colons showed significant reduction in number of ganglionic aggregates and total ganglionic cells in *Mypt*$^{f/f}$; SMA Cre- and *Mypt1*$^{f/f}$; SMA Cre+ mice. Quantitation of the ganglion and the total neuron cells count normalized by colon area (*t* test) (n = 6). *Asterisk* indicates a ganglion. *Bars* represent mean values ± standard error of the mean. $^{**}P < .01$; $^{***}P < .001$; $^{\#}P < .05$; $^{\#\#}P < .01$.



Supplementary Figure 4. Colonic phenotype of *Ednrb*$^{-/-}$ mice. (*A*) Schematic representation of *Ednrb* knockout strategy by CRISP-Cas9 technology. (*B* and *C*) DNA sequencing isolated from chimeric (+/-) and wild-type (+/+) mice tail presented the deleted segment. (*D*) Western blot analysis of EDNRB protein expression in the colon from *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (*E*) Limulus test showing concentration of LPS in *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice (n = 6). (*F*) Western blots showing decreased expression of MYPT1 in *Ednrb*$^{-/-}$ mice (n = 4). (*G*) Representative tracings of responses of circular smooth muscle from colon of *Ednrb*$^{+/+}$ (*left panel*) and *Ednrb*$^{-/-}$ (*middle panel*) mice evoked by 87 mmol/L KCl. Quantification of force tension of circular smooth muscle from HD colons that were evoked by KCl (*right panel*) (n = 6). *Bars* represent mean values ± standard error of the mean. *$P < .05$; **$P < .01$; ***$P < .01$ (*t* test). bp, base pairs.