Published in final edited form as:

Gastroenterology. 2017 April ; 152(5): 1114–1125.e5. doi:10.1053/j.gastro.2016.12.017.

Heterotrimeric G Stimulatory Protein α Subunit Is Required for Intestinal Smooth Muscle Contraction in Mice

Xiaoteng Qin[1], **Shangming Liu**[1,2], **Qiulun Lu**[3], **Meng Zhang**[1], **Xiuxin Jiang**[4], **Sanyuan Hu**[4], **Jingxin Li**[5], **Cheng Zhang**[1], **Jiangang Gao**[6], **Min-Sheng Zhu**[7], **Robert Feil**[8], **Huashun Li**[9], **Min Chen**[10], **Lee S. Weinstein**[10], **Yun Zhang**[1], **Wencheng Zhang**[1]

[1]The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, The State and Shandong Province Joint Key Laboratory of Translational Cardiovascular Medicine, Qilu Hospital of Shandong University, Jinan, China [2]Key Laboratory of the Ministry of Education for Experimental Teratology, Department of Histology and Embryology, Shandong University School of Medicine, Jinan, China [3]Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology and Center for Human Genome Research, Huazhong University of Science and Technology, Wuhan, China [4]Department of General Surgery, Qilu Hospital of Shandong University, Jinan, China [5]Department of Physiology, School of Medicine, Shandong University, Jinan, China [6]School of Life Science and Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong University, Jinan, China [7]State Key Laboratory of Pharmaceutical Biotechnology and Model Animal Research Center and MOE Key Laboratory of Model Animal for Disease Study, Nanjing University, Nanjing, China [8]Interfakultäres Institut für Biochemie (IFIB), Signaltransduktion - Transgene Modelle, Universität Tübingen, Tübingen, Germany [9]ATCG Cancer Center, ATCG Corporation Ltd, Suzhou, China [10]Metabolic Diseases Branch, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Abstract

BACKGROUND & AIMS: The α subunit of the heterotrimeric G stimulatory protein (Gsα), encoded by the guanine nucleotide binding protein, α-stimulating gene (*Gnas*, in mice), is expressed ubiquitously and mediates receptor-stimulated production of cyclic adenosine monophosphate and activation of the protein kinase A signaling pathway. We investigated the roles of Gsα in vivo in smooth muscle cells of mice.

METHODS: We performed studies of mice with Cre recombinase-mediated disruption of *Gnas* in smooth muscle cells ($Gs\alpha^{SMKO}$ and SM22-$CreER^{T2}$, induced in adult mice by tamoxifen).

Reprint requests Address requests for reprints to: Wencheng Zhang, PhD or Yun Zhang, MD, PhD, Qilu Hospital of Shandong University, No. 107, Wen Hua Xi Rd, Jinan, Shandong, China 250012. zhangyun@sdu.edu.cn or zhangwencheng@sdu.edu.cn; fax: 86-531-82169257.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.12.017.

Conflicts of interest

The authors disclose no conflicts.

Intestinal tissues were collected for histologic, biochemical, molecular, cell biology, and physiology analyses. Intestinal function was assessed in mice using the whole-gut transit time test. We compared gene expression patterns of intestinal smooth muscle from mice with vs without disruption of *Gnas.* Biopsy specimens from ileum of patients with chronic intestinal pseudo-obstruction and age-matched control biopsies were analyzed by immunohistochemistry.

RESULTS: Disruption of *Gnas* in smooth muscle of mice reduced intestinal motility and led to death within 4 weeks. Tamoxifen-induced disruption of *Gnas* in adult mice impaired contraction of intestinal smooth muscle and peristalsis. More than 80% of these died within 3 months of tamoxifen exposure, with features of intestinal pseudo-obstruction characterized by chronic intestinal dilation and dysmotility. Gsα deficiency reduced intestinal levels of cyclic adenosine monophosphate and transcriptional activity of the cyclic adenosine monophosphate response element binding protein 1 (CREB1); this resulted in decreased expression of the forkhead box F1 gene (*Foxf1*) and protein, and contractile proteins, such as myosin heavy chain 11; actin, α2, smooth muscle, aorta; calponin 1; and myosin light chain kinase. We found decreased levels of Gsα, FOXF1, CREB1, and phosphorylated CREB1 proteins in intestinal muscle layers of patients with chronic intestinal pseudo-obstruction, compared with tissues from controls.

CONCLUSIONS: Gsα is required for intestinal smooth muscle contraction in mice, and its levels are reduced in ileum biopsies of patients with chronic intestinal pseudo-obstruction. Mice with disruption of *Gnas* might be used to study human chronic intestinal pseudo-obstruction.

Keywords

Mouse Model; CIP; Intestine; Digestion

Smooth muscle cells (SMCs) are the primary contractile components of digestive, cardiovascular, respiratory, and genitourinary systems. Contractile dysfunction of SMCs is associated with various diseases, including chronic intestinal pseudo-obstruction (CIP), atherosclerosis, hypertension, and asthma. The main feature of these diseases is decreased expression of proteins required for normal SMC contractile function.[1] Therefore, we need to understand the mechanisms that control the expression of contractile and regulatory proteins in SMCs, to determine how these processes are altered in pathologic conditions.

Many studies have identified that transcription factors, such as serum response factor (SRF) and the Foxo family of forkhead (Fox) proteins control the expression of contractile proteins in SMCs.[2] Knockout of SRF in intestinal SMCs of adult mice attenuated the expression of smooth muscle–specific genes and resulted in a phenotype of CIP.[3,4] Foxf1 binds to SRF and Myocd to regulate the expression of contractile proteins, and Foxf1 deletion in SMCs results in reduced contractile protein expression and impaired colonic contractility.[5]

The heterotrimeric G-protein α subunit (Gsα) is encoded by guanine nucleotide binding protein, α-stimulating (GNAS) (Gnas in mice), which is ubiquitously expressed in many cell types and responsible for receptor-stimulated cyclic adenosine monophosphate (cAMP) generation and activation of the protein kinase A pathway.[6] Protein kinase A can phosphorylate various downstream targets that are involved in a number of pathways. For example, protein kinase A phosphorylates cAMP response element binding protein 1

(CREB1) at Ser 133, to induce the expression of CREB1 target genes.[7] Accumulating evidence has demonstrated that aberrant expression of Gsα leads to various dysfunctions in cell growth, proliferation, apoptosis, differentiation, and metabolism.[8] Adipose tissue-specific disruption of Gsα results in a severely lean phenotype and early mortality.[9] Chondrocyte-specific ablation of Gsα leads to growth-plate defects and hypertrophic differentiation of growth-plate cartilage.[10] Recent studies have shown mutations in GNAS related to many human diseases: heterozygous mutation for loss of function in GNAS leads to Albright hereditary osteodystrophy,[11] whereas mutation activating GNAS results in McCune–Albright syndrome.[12] However, the roles of Gsα in SMCs have not been investigated.

In the present study, we examined the potential function of Gsα in SMCs by using mice with Acta2-Cre– or SM22-CreER^{T2}–mediated Gsα knockout (KO). Acta2-Cre–mediated Gsα KO (Gsα^{SMKO}) in smooth muscle reduced intestinal motility and led to early death, before 4 weeks. SM22-CreER^{T2}–mediated Gsα KO induced by tamoxifen in adult mice (adult Gsα^{SMKO}) impaired the contraction of intestinal smooth muscle and resulted in a phenotype similar to CIP. CIP is characterized by chronic intestinal dilation and dysmotility in the absence of mechanical obstruction.[13] These results demonstrate that Gsα plays a critical function in intestinal motility in mice.

Materials and Methods

Generation of Smooth Muscle-Specific Gsα Knockout Mice

To ablate Gsα specifically in smooth muscle (Gsα^{SMKO}), Gsα$^{fl/fl}$ mice[14] were crossed with Acta2-Cre mice that express Cre recombinase under the control of the Acta2 promoter.[15] To generate adult smooth muscle–specific Gsα KO mice (adult Gsα^{SMKO}), Gsα$^{fl/fl}$ mice were crossed with SM22-CreER^{T2} mice expressing a tamoxifen-inducible Cre recombinase under control of the SM22 promoter.[16] Tamoxifen (1 mg/d) was injected intraperitoneally for 5 consecutive days. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Shandong University.

Analysis of Smooth Muscle Contractility

Force measurements were performed as described.[17] The mice were killed by cervical dislocation. Segments (6 mm long) of the jejunum and ileum were mounted in a 37°C organ bath and tied with surgical silk to the hooks of a force transducer (MLT0202; AD Instruments, Sydney, Australia) and lengthadjusting micrometer. After equilibrating in H-T buffer (137.0 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 5.6 mmol/L glucose, pH 7.4) for 15 minutes, the contractile effect of KCl (87 mM), acetylcholine (Ach) (1 mM) and electrical field stimulation (EFS) (20 Hz, 30 V, pulse train, 20 seconds) was investigated.

Whole-Gut Transit Time Test

To determine intestinal function, a whole-gut transit time test was performed with minor modification.[18] We injected 100 μL charcoal test meal (5% [w/v] charcoal in 0.9% NaCl) by orogastric gavage and monitored feces for the first appearance of black dye.

Human Cases

Full-thickness biopsy specimens were obtained from 5 female patients with a mean age of 51.2 ± 8.3 years who met the criteria for CIP. Age-matched female control specimens were obtained from patients (mean age, 50.3 ± 9.2 years) with diseases unrelated to gastrointestinal motility disorders. The use of human tissues was approved by the Medical Institutional Ethics Committee of Qilu Hospital, Shandong University, China.

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by using GraphPad Prism 3 (GraphPad Software Inc., San Diego, CA). Statistical comparisons between 2 groups involved Student *t* test and otherwise by 2-way analysis of variance and Bonferroni post-tests. $P < .05$ was considered statistically significant.

Results

Gsα Deficiency in Smooth Muscle Results in Postnatal Lethality

To elucidate the biological significance of Gsα expression in smooth muscle in vivo, *Gsα*$^{flox/flox}$ mice were crossbred with Acta2-Cre mice to generate *Gsα*$^{flox/+}$*/Acta2-Cre* mice, which were further intercrossed to obtain *Gsα*$^{flox/flox}$*/Acta2-Cre* mice (Gsα^{SMKO} mice) (Figure 1A). The littermate *Gsα*$^{flox/flox}$/Cre^{-} mice were used as controls. Immunohistochemistry showed nuclear localization of Cre in the smooth muscle layer of jejunum and ileum from Acta2-Cre mice (Figure 1B), suggesting Acta2-Cre–mediated recombination happening in the smooth muscle of jejunum and ileum. Western blots confirmed significantly reduced Gsα expression in the muscle of the jejunum, ileum, and aorta of Gsα^{SMKO} mice (Figure 1C). Immunohistochemistry further demonstrated Gsα deletion in the smooth muscle cells of aorta, jejunum, and ileum from the Gsα^{SMKO} mice (Supplementary Figure 1A-C). However, Gsα levels were not changed in the non–smooth muscle tissues, such as brain, skeletal muscle, and skin from control and Gsα^{SMKO} mice (Figure 1D).

Gsα^{SMKO} pups were born at the expected Mendelian ratio. At birth, the size and weight of Gsα^{SMKO} mice were comparable with those of their control littermates, but postnatal growth was retarded. From day 10 after birth, all Gsα^{SMKO} mice showed significantly reduced body weight relative to their controls (Figure 1E). At day 21, male Gsα^{SMKO} mice weighed 34.1% less than their controls (7.16 ± 1.04 g vs 10.86 ± 0.92 g, n = 6; $P < .01$). Importantly, Gsα^{SMKO} mice began to die at day 14 after birth. None of the Gsα^{SMKO} mice survived beyond postnatal day 28, whereas all control pups survived (Figure 1F). These data suggest that smooth muscle Gsα is essential for growth, and deficiency of Gsα can lead to serious growth retardation and early mortality. However, the detailed reason for the death of Gsα^{SMKO} mice is not clear.

Contraction Was Impaired in Gsα^{SMKO} Smooth Muscles

To evaluate the effects of Gsα deletion on intestinal motility, we measured rhythmic contractions of the jejunum and ileum from control and Gsα^{SMKO} mice at 3 weeks of age. Peristaltic contractile tension of jejunum and ileum from Gsα^{SMKO} mice was significantly

decreased compared with controls (Figure 2A). At necropsy, the whole digestive tract from 3-week-old $Gs\alpha^{SMKO}$ mice appeared normal, including the small intestine. Histologic examination showed comparable thickness of the Gsα-deficient and control ileal or jejunal muscle layers (Figure 2B).

To determine the contractile properties of Gsα-deficient smooth muscle, we measured the tension in isolated strips in response to KCl or the muscarinic agonist, ACh. Jejunal strips from $Gs\alpha^{SMKO}$ mice at 3 weeks of age showed only 38.2% of the tension of control tissue in response to KCl (Figure 2C). Similar results were obtained with ileal tissues (Figure 2D). In addition, intestinal smooth muscles for both jejunum and ileum from $Gs\alpha^{SMKO}$ mice showed only 35.7% to 43.0% of the tension of control tissue in response to ACh (Figure 2C and D). We also investigated the responses to EFS used to release ACh from parasympathetic nerves intrinsic in isolated tissues. The contraction forces obtained with EFS in jejunal or ileal smooth muscle were significantly reduced after Gsα knockout (Figure 2E). More importantly, intestinal contraction was impaired in both jejunum and ileum from $Gs\alpha^{SMKO}$ mice at 13 days of age (Supplementary Figure 2A and B). Therefore, Gsα plays a important role in normal intestinal smooth muscle contractions.

Smooth Muscle-Specific Gsα Deletion in Adult Mice Confers Severe Intestinal Obstruction

To explore the roles of smooth muscle Gsα in adult mice in vivo, $Gs\alpha^{lox/flox}$ mice were cross-bred with SM22-$CreER^{T2}$ mice expressing a tamoxifen-activated Cre recombinase to ablate Gsα expression in adult smooth muscle (adult $Gs\alpha^{SMKO}$; Figure 3A). We used $Gs\alpha^{flox/flox}/Cre^{-}$ mice with tamoxifen injection as controls. Immunohistochemistry demonstrated Gsα deletion in the smooth muscle cells of jejunum and ileum from the adult $Gs\alpha^{SMKO}$ mice (Supplementary Figure 1D and E).

Control and adult $Gs\alpha^{SMKO}$ mice were monitored at various times after tamoxifen injection to determine the pathologic phenotype. From 28 days after the first tamoxifen injection, the body weight was lower for adult $Gs\alpha^{SMKO}$ than control mice (Figure 3B). From 3 weeks after tamoxifen injection, adult $Gs\alpha^{SMKO}$ mice showed reduced physical body motility (data not shown). The lower abdomen started to bloat and increase in volume, and adult $Gs\alpha^{SMKO}$ mice began to die (Figure 3C). During this period, intake of food and excretion of feces were markedly reduced (Figure 3D). We found >80% lethality within 3 months after the first application of tamoxifen (Figure 3C). In contrast, tamoxifen-treated $Gs\alpha^{flox/flox}/Cre^{-}$ control mice appeared normal, with no deaths. In all adult $Gs\alpha^{SMKO}$ mice, gross necropsy showed severe intestinal situs with extremely dilated and food-filled ileum (Figure 3E). Motility of the intestine was determined by functional assays. Charcoal, a black dye, was injected into the stomach and stool was monitored. Despite a comparable length of the intestinal tract, adult $Gs\alpha^{SMKO}$ mice showed about 3-fold longer whole-gut transit time than control mice (Figure 3F). Adult $Gs\alpha^{SMKO}$ mice might develop a severe motility disorder similar to some symptoms of human patients with CIP, leading to cachexia and death.

Gsα Deficiency in Adult Smooth Muscle Impairs Contractile Activity

Histologic analyses showed marked changes in the intestinal tract of adult $Gs\alpha^{SMKO}$ mice (Figure 4A and B). At 3 weeks after tamoxifen injection, the small intestine was dilated,

with the mean diameter significantly increased (1.6- ± 0.3-fold) for the ileum compared with controls (3566 ± 1011 μm vs 2254 ± 562 μm; $P < .05$). The mean diameter of the jejunum did not change significantly (1.1- ± 0.2-fold; 2890 ± 539 μm vs 2540 ± 149 μm; $P = .156$). However, the cross-sectional thickness of the ileal muscle layer was increased (2.4- ± 0.5-fold for the longitudinal muscle layer and 2.1- ± 0.4-fold for the circular muscle layer; $P < .05$), whereas the thickness of jejunal muscle was not altered (1.2- ± 0.2-fold for longitudinal muscle and 1.3- ± 0.3-fold for circular muscle; $P > .05$). Inspection of intestinal cross sections showed noticeably enlarged muscle cells. The number of nuclei in an arc of the ileal wall equal to one-tenth the circumference did not differ between adult $G\alpha_{s}^{SMKO}$ and control mice (longitudinal muscle: 120 ± 20 vs 116 ± 15; $P = .69$; circular muscle: 114 ± 9 vs 109 ± 8; $P = .77$). The thicker muscle layers were due largely to SMC hypertrophy.

Gsα knockout in intestinal smooth muscle did not affect the levels of β-adrenergic receptors (ADRB1 and ADRB2) (Supplementary Figure 3A and B). Contractile functions of the intestinal tunica muscularis were tested by tension recordings of smooth muscle tissues (jejunum and ileum). The tension responses to KCl in adult $G\alpha_{s}^{SMKO}$ muscle was only about 25% of that observed in control muscle (jejunum, 34.3% and ileum, 15.4%). Similar results were obtained with ACh (Figure 4C and D) and EFS (Figure 4E). These data clearly indicate that Gsα regulates depolarization- and agonist-mediated intestinal smooth muscle contractions.

Gsα Deletion Reduced the Expression of Contractile Proteins and cAMP/CREB1 Signaling

We performed a genome-wide microarray assay of control and Gsα-deficient intestinal smooth muscle to profile differentially expressed genes that might be involved in Gsα regulation. As shown in Figure 5A and Supplementary Table 1, we found 1165 genes with >2.0-fold up-regulation and 904 genes with >2-fold down-regulation in 3 independent experiments. To understand some important biological processes affected by Gsα in SMCs, a gene ontology analysis was performed and revealed Gsα roles in the regulation of transcription, phosphorylation, cell proliferation, cell cycle, cell differentiation, cell adhesion, cell migration, and immune response (Figure 5B). Pathway analysis with the Kyoto Encyclopedia of Genes and Genomes database further classified the functional annotations of genes and revealed up-regulated and down-regulated genes significantly enriched in top 10 pathways (Figure 5C). To verify the microarray analysis data, we examined differentially expressed genes that are involved in smooth muscle contraction from control and adult $G\alpha_{s}^{SMKO}$ mice at 3 weeks after tamoxifen injection. The messenger RNA (mRNA) levels of contractile proteins, including myosin heavy chain 11 (MYH11); actin, α2, smooth muscle, aorta (ACTA2); calponin 1 (CNN1); and myosin light chain kinase, were significantly reduced in the Gsα-deficient jejunum (Figure 5D), which were further confirmed by Western blot analysis (Supplementary Figure 3A and B). In analyzing transcription factors that control the expression of contractile proteins in SMCs, serum response factor (SRF) mRNA levels were not changed in the Gsα-deficient jejunum. However, Myocd mRNA levels were slightly decreased and Foxf1 mRNA levels were significantly reduced with Gsα deletion (Figure 5D), which was further confirmed by Western blot analysis (Figure 5F).

Because Gsα is responsible for receptor-stimulated cAMP generation and subsequent CREB1 activation, we measured cAMP levels and CREB1 activity with Gsα KO. As expected, Gsα KO reduced cAMP levels in basal or response to isoproterenol (Figure 5E). Both phospho-CREB1 at Ser 133 and total CREB1 levels were significantly reduced with Gsα KO (Figure 5F), isoproterenol-induced CREB1 phosphorylation was also decreased in Gsα-deficient smooth muscle (Figure 5E). Our data suggest that Gsα KO results in reduced cAMP/CREB1 signaling and expression of contractile proteins, thereby impairing smooth muscle contraction.

CREB1 Binds With Foxf1 Promoter and Regulates its Expression

We next dissected how Gsα/CREB1 regulates Foxf1 expression. We analyzed the promoter of Foxf1 by using the Transcription Factor Database (http://jaspar.genereg.net), an Internet-based transcription-factor binding-site program. One CRE site was found in the Foxf1 promoter (Figure 6A), so CREB1 may bind to the Foxf1 promoter and regulate its expression. The cAMP activator forskolin, used to induce CREB1 activity, could increase Foxf1 expression in intestinal SMCs (Figure 6B).

To confirm that CREB1 can bind to the Foxf1 promoter, we used chromatin immunoprecipitation assay. Forskolin induced the binding of CREB1 to the CRE site in the Foxf1 promoter (Figure 6C). To further analyze the role of the CREB1 binding site on Foxf1 promoter activity, we mutated the core CREB1 binding site in CRE and then inserted it into a luciferase plasmid. Forskolin significantly increased luciferase activity from the WT but not CRE-mutant Foxf1 promoter (Figure 6D). Therefore, CREB1 binds with the Foxf1 promoter and regulates its expression.

Levels of Gsα, Foxf1, and p-CREB1/CREB1 Were Reduced in the Intestinal Muscle Layer of Patients With Chronic Intestinal Pseudo-Obstruction

A genome-wide microarray assay of intestinal smooth muscle from patients with CIP and age-matched control was conducted to profile differentially expressed genes. As shown in Supplementary Figure 4A and Supplementary Table 2, we found 125 genes with >2.0-fold up-regulation and 111 genes with >2-fold down-regulation in CIP group compared with control group. Pathway analysis with the Kyoto Encyclopedia of Genes and Genomes database classified the functional annotations of genes and revealed up-regulated and down-regulated genes significantly enriched in top 9 pathways (Supplementary Figure 4B). There are 2069 differentially expressed genes from Gsα knockout group. The human CIP group and Gsα knockout group shared 48 common differentially expressed genes (Supplementary Figure 4C), of which 21 genes were up-regulated and 27 genes including Gsα, CREB1, Foxf1, and contractile proteins were down-regulated (Supplementary Figure 4D and E). The phenotype of the adult $Gs\alpha^{SMKO}$ mice shares some aspects of CIP pathology in humans.

To further confirm the expression of Gsα/CREB1/Foxf1 signaling in intestines of patients with CIP, immunohistologic staining was performed. Compared with control intestinal muscle layers, layers of patients with CIP showed significantly reduced levels of Gsα, Foxf1, and p-CREB1/CREB1 (Figure 7A and B), which indicates the important role of Gsα/CREB1/Foxf1 signaling in the development of human chronic intestinal pseudo-obstruction.

Discussion

To determine the role of Gsα in SMCs in vivo, we generated 2 kinds of smooth muscle-specific Gsα-KO mice in different Cre recombinase mice. Acta2-Cre–mediated Gsα KO resulted in reduced intestinal contraction and early death before 4 weeks for unknown reasons. Adult Gsα-KO mice were induced with time-controlled recombination by tamoxifen. Gsα KO in adult SMCs led to dilation and impaired contraction of the intestine, which resulted in defective peristalsis in vivo and most death within 3 months; both toxicity and malnutrition were assumed to be the probable cause of death. In the molecular mechanism, Gsα regulates the expression of contractile proteins through cAMP/CREB1-mediated Foxf1 and plays an important role in normal intestinal smooth muscle contraction (Figure 7C). Our study revealed the roles of smooth muscle Gsα at the animal level, which will increase our understanding of Gsα multifunctions.

The phenotype of Gsα-KO mice is caused by decreased Foxf1 expression. Foxf1 has been identified as an important transcription factor for the SMC genes encoding the main effectors of contraction.[5] Foxf1 binds to SRF and Myocd to regulate the expression of smooth muscle contractile proteins. Smooth muscle–specific Foxf1 deletion results in neonatal lethality and impaired contractility.[5] Other transcription factors, such as SRF, GATA6, and MEF2 also play a role in regulating SMC gene expression.[19] Smooth muscle–specific SRF mice showed a similar phenotype as our adult $Gs\alpha^{SMKO}$ mice.[3,4] Recent studies also showed down-regulated SMC-specific markers in various motility disorders in humans. For example, the jejunal circular smooth muscle layer showed no ACTA2 expression in idiopathic forms of CIP.[20] MYH11 was down-regulated in colon biopsy specimens of patients with idiopathic megacolon and slow-transit constipation.[21] Similarly, $Gs\alpha^{SMKO}$ mice showed intestinal motility dysfunction accompanied by down-regulation of SMC-specific markers, such as ACTA2 and MYH11.

Gsα deletion in intestinal smooth muscle resulted in decreased contractile responses. Gsα-deficient muscle strips displayed much lower contractile force than control strips, independent of the agonist applied. The main cause is presumably changes in the contractile machinery of intestinal smooth muscle because key downstream regulators, such as myosin light chain kinase and effectors of contraction, such as ACTA2 and MYH11 are down-regulated in Gsα-deficient SMCs. However, Gsα-dependent cAMP signaling contributes to smooth muscle relaxation.[22] The different conclusions are explained by different research methods. The previous study used chemical regents to elevate cAMP levels to explore its function, which were transient and sometimes not specific. In our mouse KO model, long-term deletion of Gsα caused changes in downstream regulators, including phospho-CREB1 (Ser 133) and total CREB1, which down-regulated smooth muscle contractile proteins and reduced contractility.

Reduced contractility with Gsα deficiency has significant consequences for the intestinal performance. The retarded passage of charcoal showed that whole-gut transit time was approximately 3-fold increased as compared with controls. Furthermore, the dilated intestine in adult $Gs\alpha^{SMKO}$ mice may result from chronic pseudo-obstruction associated with

hypertrophy of the smooth muscle in the intestinal wall, which is likely an adaptive response to increased workload of smooth muscle with the diminished contractility.

CIP may be congenital or acquired secondary to other disorders, such as postoperative ileus and autoimmune or infectious diseases.[4] A proper diagnosis and treatment of this rare and disabling disorder is difficult because the underlying pathology is poorly understood. CIP can be classified as neuropathic or myopathic forms based on histopathology data and patterns of motility disorder.[23] Smooth muscle myopathies may be responsible for the disease in approximately one-third of cases of congenital CIP.[24] In this study, we describe a mouse model of adult smooth muscle–specific Gsα KO that displays clinical manifestations similar to CIP. Moreover, we report on the reduced levels of Gsα and Foxf1 in the intestine of CIP patients, so smooth muscle dysfunction resulting from Gsα deficiency might contribute to the development of this disease in humans.

In conclusion, we have shown that Gsα deficiency greatly decreases the contractility of intestinal smooth muscle. Gsα regulates the levels of smooth-muscle contractile proteins through CREB1-mediated Foxf1 expression, so it is crucial for normal intestinal smooth muscle contractility and functions. Mutations in the GNAS gene or altered Gsα levels may play an important role in the development of smooth muscle myopathies, such as CIP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Prof Xiao Yang from the State Key Laboratory of Proteomics, Collaborative Innovation Center for Cardiovascular Disorders, Genetic Laboratory of Development and Diseases, Institute of Biotechnology, Beijing, PR China, for providing the Acta2-Cre mouse for this study.

Funding

This study was supported by grants from the National Natural Science Foundation of China (81570393 to Wencheng Zhang, 81425004, 91339109 and 81270350 to Cheng Zhang, 31400771 to Meng Zhang), the Taishan Scholar Project of Shandong Province of China (tsqn20161066 to Wencheng Zhang), the Natural Science Foundation of Shandong Province (ZR2015HM002 to Wencheng Zhang), the Program of Introducing Talents of Discipline to Universities (B07035 to Yun Zhang), the State Program of National Natural Science Foundation of China for Innovative Research Group (81321061 to Yun Zhang), the State Key Program of National Natural Science of China (61331001 and 81530014 to Yun Zhang) and the International Collaboration and Exchange Program of China (81320108004 to Yun Zhang).

Abbreviations used in this paper:

ACh	acetylcholine
ACTA2	actin, alpha 2, smooth muscle, aorta
cAMP	cyclic adenosine monophosphate
CIP	chronic intestinal pseudo-obstruction
CREB1	cAMP response element binding protein 1

EFS	electrical field stimulation
Fox	Foxo family of forkhead
GNAS	guanine nucleotide binding protein, α-stimulating
Gsα	heterotrimeric G stimulatory protein α subunit
KO	knockout
mRNA	messenger RNA
MYH11	myosin heavy chain 11
SMC	smooth muscle cell
SRF	serum response factor

References

1. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004;84: 767–801. [PubMed: 15269336]
2. Owens GK. Molecular control of vascular smooth muscle cell differentiation and phenotypic plasticity. Novartis Found Symp 2007;283:174–191. [PubMed: 18300422]
3. Angstenberger M, Wegener J, Pichler B, et al. Severe intestinal obstruction on induced smooth muscle–specific ablation of the transcription factor SRF in adult mice. Gastroenterology 2007;133:1948–1959. [PubMed: 18054566]
4. Mericskay M, Blanc J, Tritsch E, et al. Inducible mouse model of chronic intestinal pseudo-obstruction by smooth muscle-specific inactivation of the SRF gene. Gastroenterology 2007;133:1960–1970. [PubMed: 18054567]
5. Hoggatt AM, Kim JR, Ustiyan V, et al. The transcription factor Foxf1 binds to serum response factor and myocardin to regulate gene transcription invisceral smooth muscle cells. J Biol Chem 2013;288:28477–28487. [PubMed: 23946491]
6. Lei R, Zhang K, Wei Y, et al. G-protein α-subunit Gsα is required for craniofacial morphogenesis. PLoS One 2016;11:e0147535. [PubMed: 26859889]
7. Tasken K Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev 2004; 84:137–167. [PubMed: 14715913]
8. Lu C, Xia J, Zhou Y, et al. Loss of Gsα impairs liver regeneration through a defect in the crosstalk between cAMP and growth factor signaling. J Hepatol 2016; 64:342–351. [PubMed: 26386161]
9. Chen M, Chen H, Nguyen A, et al. Gsα deficiency in adipose tissue leads to a lean phenotype with divergent effects on cold tolerance and diet-induced thermogenesis. Cell Metab 2010;11:320–330. [PubMed: 20374964]
10. Sakamoto A, Chen M, Kobayashi T, et al. Chondrocyte-specific knockout of the G protein G(s)alpha leads to epiphyseal and growth plate abnormalities and ectopic chondrocyte formation. J Bone Miner Res 2005;20: 663–671. [PubMed: 15765186]
11. Patten J, Johns DR, Valle D, et al. Mutation in the gene encoding the stimulatory G protein of adenylate cyclase in Albright's hereditary osteodystrophy. N Engl J Med 1990;322:1412–1419. [PubMed: 2109828]
12. Shenker A, Weinstein LS, Moran A, et al. Severe endocrine and nonendocrine manifestations of the McCune-Albright syndrome associated with activating mutations of stimulatory G protein GS. J Pediatr 1993; 123:509–518. [PubMed: 8410501]
13. Connor FL, Di Lorenzo C. Chronic intestinal pseudo-obstruction: assessment and management. Gastroenterology 2006;130:S29–S36. [PubMed: 16473068]

14. Chen M, Gavrilova O, Zhao WQ, et al. Increased glucose tolerance and reduced adiposity in the absence of fasting hypoglycemia in mice with liver-specific Gs alpha deficiency. J Clin Invest 2005;115:3217–3227. [PubMed: 16239968]
15. Wu Z, Yang L, Cai L, et al. Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse. Respir Res 2007;8:1. [PubMed: 17207287]
16. Kühbandner S, Brummer S, Metzger D, et al. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15–22. [PubMed: 11020712]
17. He WQ, Peng YJ, Zhang WC, et al. Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice. Gastroenterology 2008;135:610–620. [PubMed: 18586037]
18. van der Zee R, Welling GW. A simple method to determine the transit time in mice. Z Versuchstierkd 1983; 25:233–237. [PubMed: 6320554]
19. Miano J Serum response factor: toggling between disparate programs of gene expression. J Mol Cell Cardiol 2003;35:577–593. [PubMed: 12788374]
20. Knowles C, Silk DB, Darzi A, et al. Deranged smooth muscle α-actin as a biomarker of intestinal pseudo-obstruction: a controlled multinational case series. Gut 2004;53:1583–1589. [PubMed: 15479676]
21. Wedel T, van Eys GJ, Waltregny D, et al. Novel smooth muscle markers reveal abnormalities of the intestinal musculature in severe colorectal motility disorders. Neurogastroenterol Motil 2006;18:526–538. [PubMed: 16771768]
22. Murthy K Signaling for contraction and relaxation in smooth muscle of the gut. Annu Rev Physiol 2006; 68:345–374. [PubMed: 16460276]
23. Connor F, Lorenzo CD. Chronic intestinal pseudo-obstruction: assessment and management. Gastroenterology 2006;130(Suppl 1):S29–S36. [PubMed: 16473068]
24. Hyman P, Bursch B, Sood M, et al. Visceral pain-associated disability syndrome: a descriptive analysis. J Pediatr Gastroenterol Nutr 2002;35:663–668. [PubMed: 12454583]

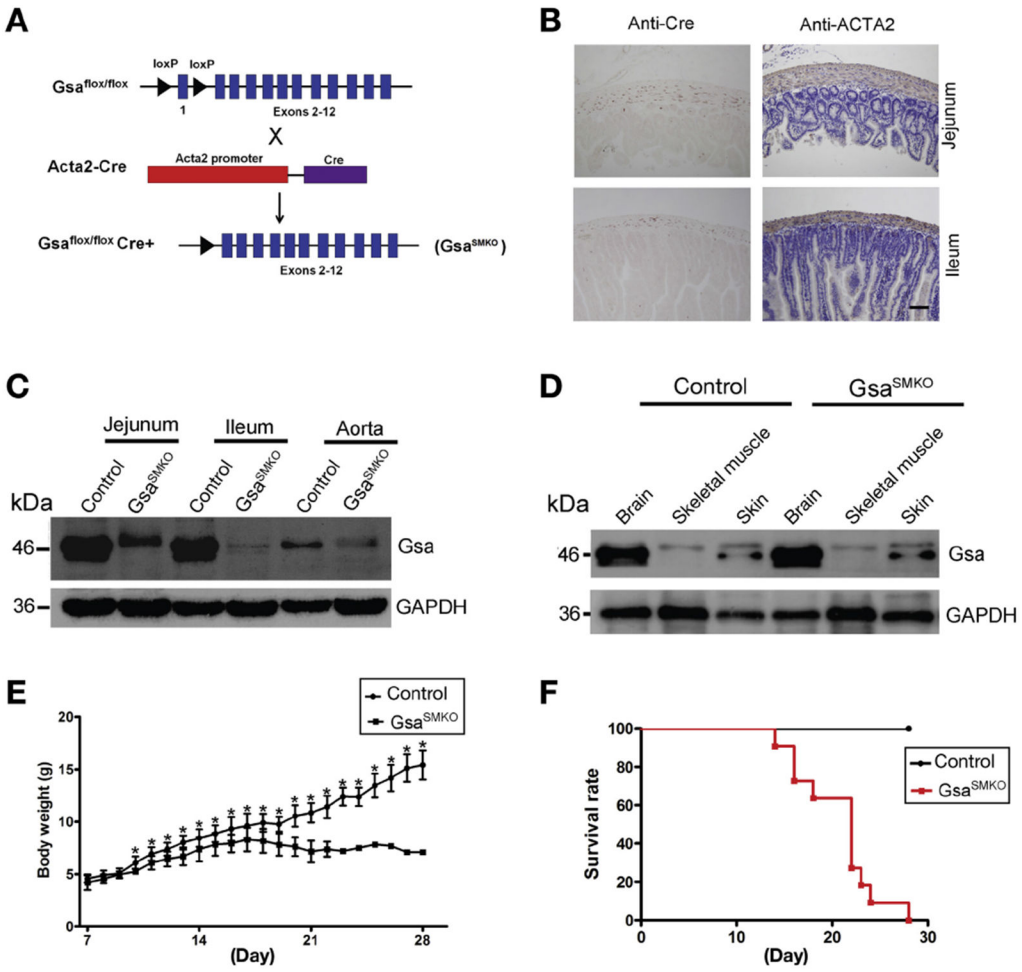

Figure 1.

Smooth-muscle deficiency in the Gsα results in postnatal lethality. (*A*) Schematic diagram of transgenic mice used to generate $Gs\alpha^{SMKO}$ mice. (*B*) Immunohistochemistry of Cre and ACTA2 in cross sections of jejunum and ileum from Acta2-Cre mice. Scale bar = 50 μm. (*C*) Western blot analysis of Gsα protein level in the jejunum, ileum, and aorta from $Gs\alpha^{SMKO}$ mice. (*D*) Western blot analysis of Gsα in the brain, skeletal muscle, and skin from control and $Gs\alpha^{SMKO}$ mice. (*E*) A typical growth curve for male mice from postnatal days 7–28 (n = 6). **P* < .05 vs control. (*F*) Kaplan–Meier survival plot of control and $Gs\alpha^{SMKO}$ mice (n = 11). **P* < .05 vs control.

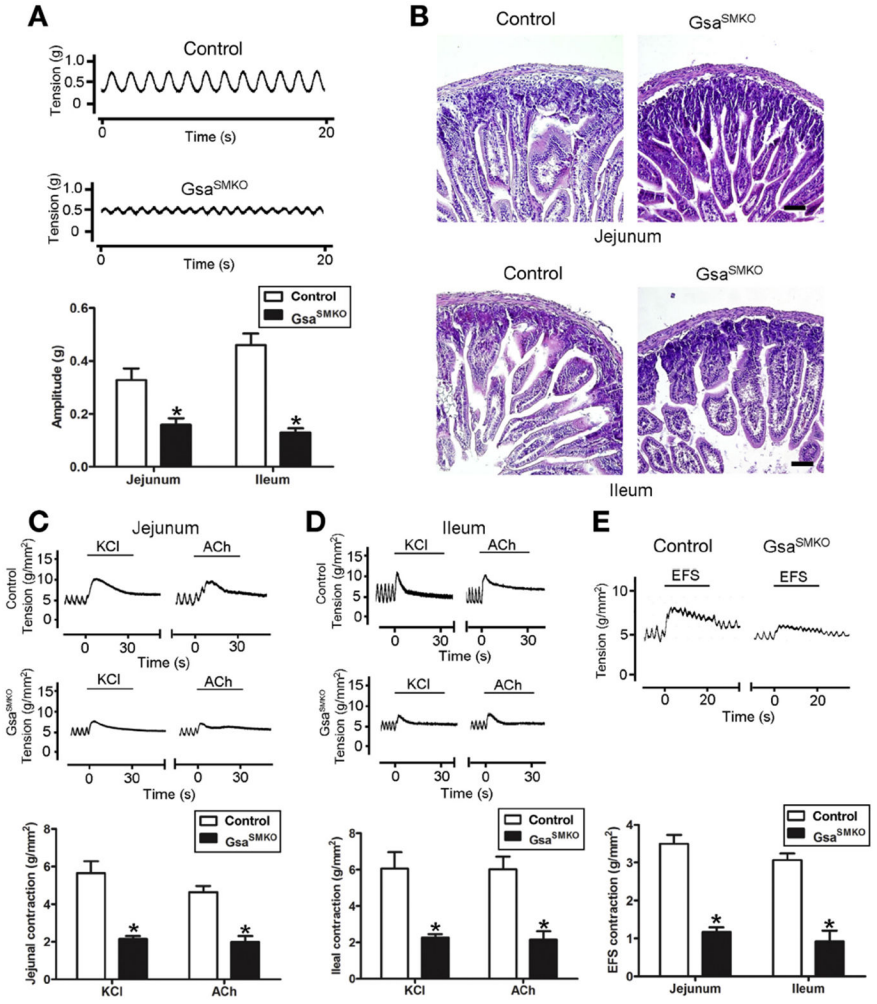

Figure 2.

Impaired contraction in Gsα^{SMKO} smooth muscles. (*A*) Representative spontaneous contractions in jejunal segments from control and Gsα^{SMKO} mice and contraction amplitudes (n = 5). **P* < .05 vs control. (*B*) Transverse sections of jejunum and ileum from control and Gsα^{SMKO} mice stained with H&E. Scale bar = 50 μm. (*C*, *D*) Representative recordings of contraction in jejunum (*C*) and ileum (*D*) from control and Gsα^{SMKO} mice with 87 mM KCl or 1 μM ACh. *Bars* show duration of stimulation. Shows quantification of contraction responses to KCl and ACh for jejunum and ileum (n = 5). **P* < .05 vs control. (*E*) Representative recordings of contraction in jejunum from control and Gsα^{SMKO} mice with EFS. Shows quantification of contraction responses to EFS (n = 5). **P* < .05 vs control.

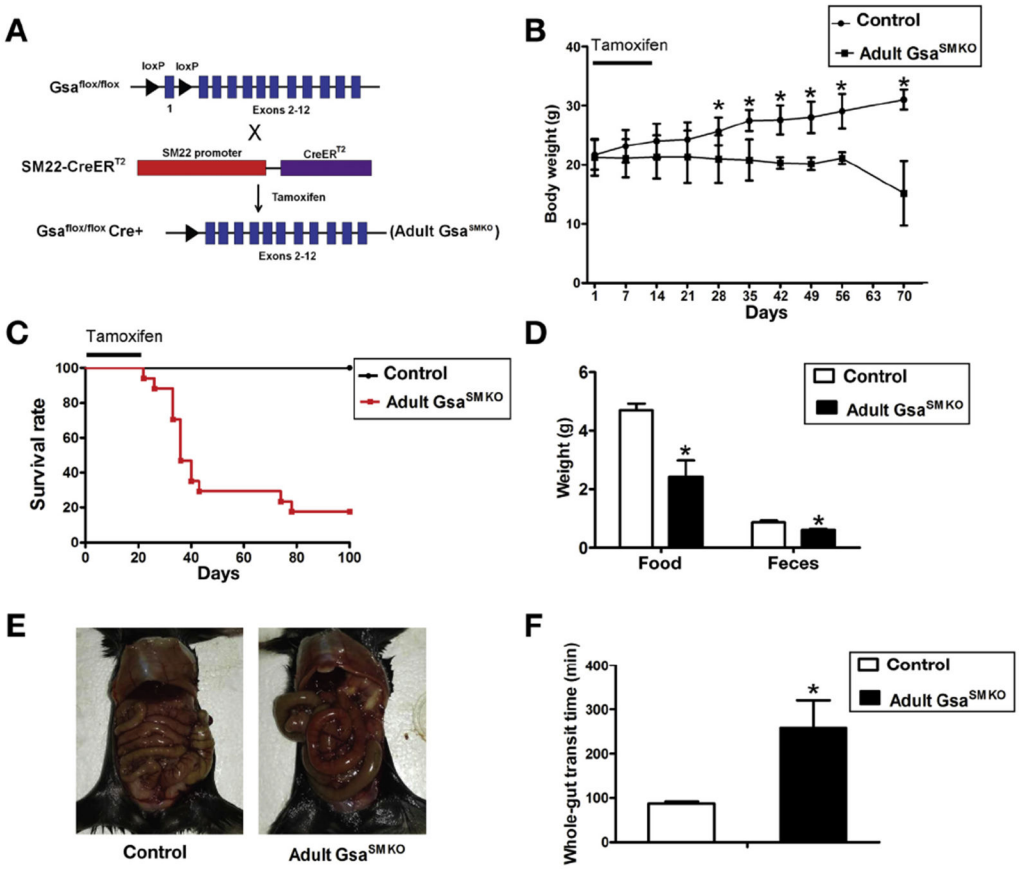

Figure 3.

Smooth muscle-specific Gsα KO in adult mice confers severe intestinal obstruction. (*A*) Schematic diagram of transgenic mice used to generate adult Gsα^{SMKO} mice induced by tamoxifen injection. (*B*) A typical growth curve for male control and adult Gsα^{SMKO} mice after tamoxifen injection (n = 8). *P < .05 vs control. (*C*) Kaplan–Meier survival plot of control and adult Gsα^{SMKO} mice after tamoxifen injection (n = 17). *P < .05 vs control. (*D*) Food intake and feces excretion after 4 weeks of tamoxifen injection (n = 5 each group). (*E*) Gastrointestinal tract of control and adult Gsα^{SMKO} mice. Note the dilated ileum in the adult Gsα^{SMKO} mouse. (*F*) Whole-gut transit time for control and adult Gsα^{SMKO} mice (n = 5). *P < .05 vs control.

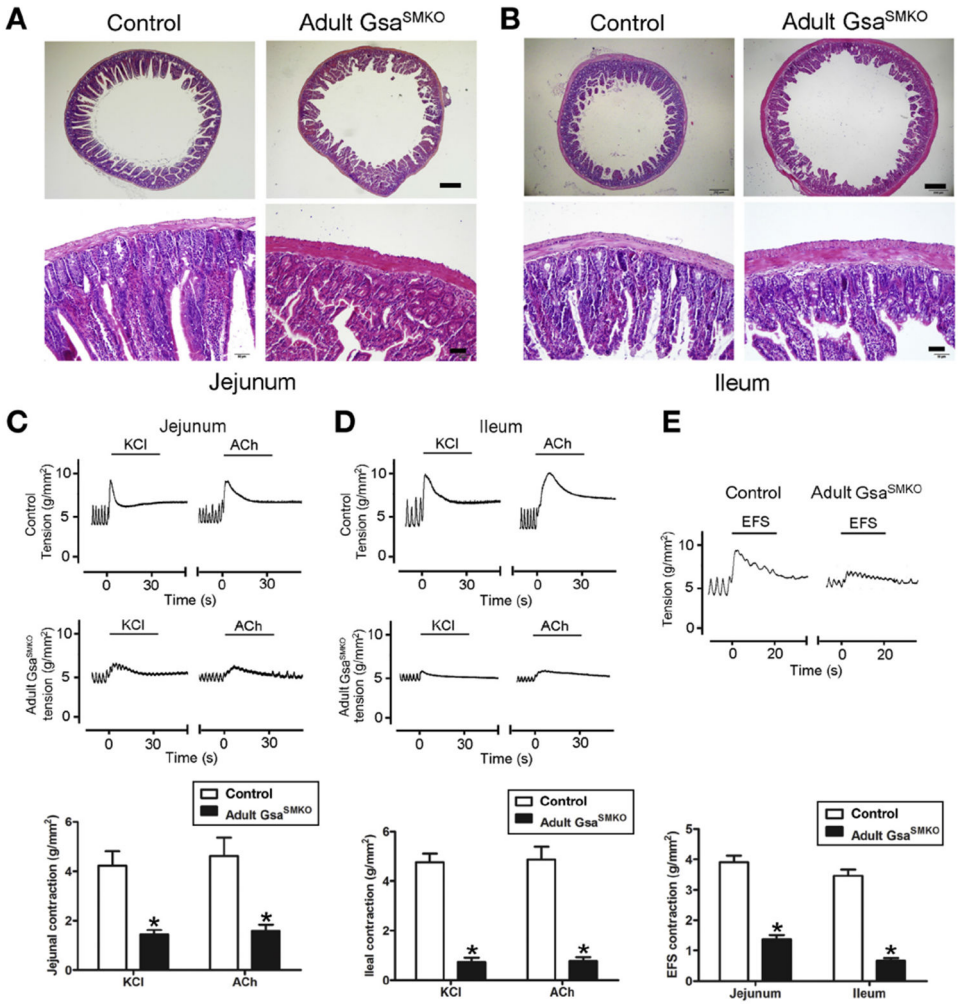

Figure 4.

Gsα deletion in adult smooth muscle impairs contractility. (*A*, *B*) Transverse sections of jejunum (*A*) and ileum (*B*) from control and adult Gsα^{SMKO} mice stained with H&E. The structure of jejunum and ileum from adult Gsα^{SMKO} mice shows the hypertrophic smooth muscle layer. Scale bars for (*A*, *B*): *upper row* = 200 μm; *lower row* = 50 μm. (*C*, *D*) Representative recordings of jejunum (*C*) and ileum (*D*) from control and adult Gsα^{SMKO} mice with 87 mM KCl or 1 μM ACh. Shows quantification of contraction responses to KCl and ACh for jejunum and ileum (n = 5). **P* < .05 vs control. (*E*) Representative recordings of contraction in jejunum from control and adult Gsα^{SMKO} mice with EFS. Shows quantification of contraction responses to EFS (n = 5). **P* < .05 vs control.

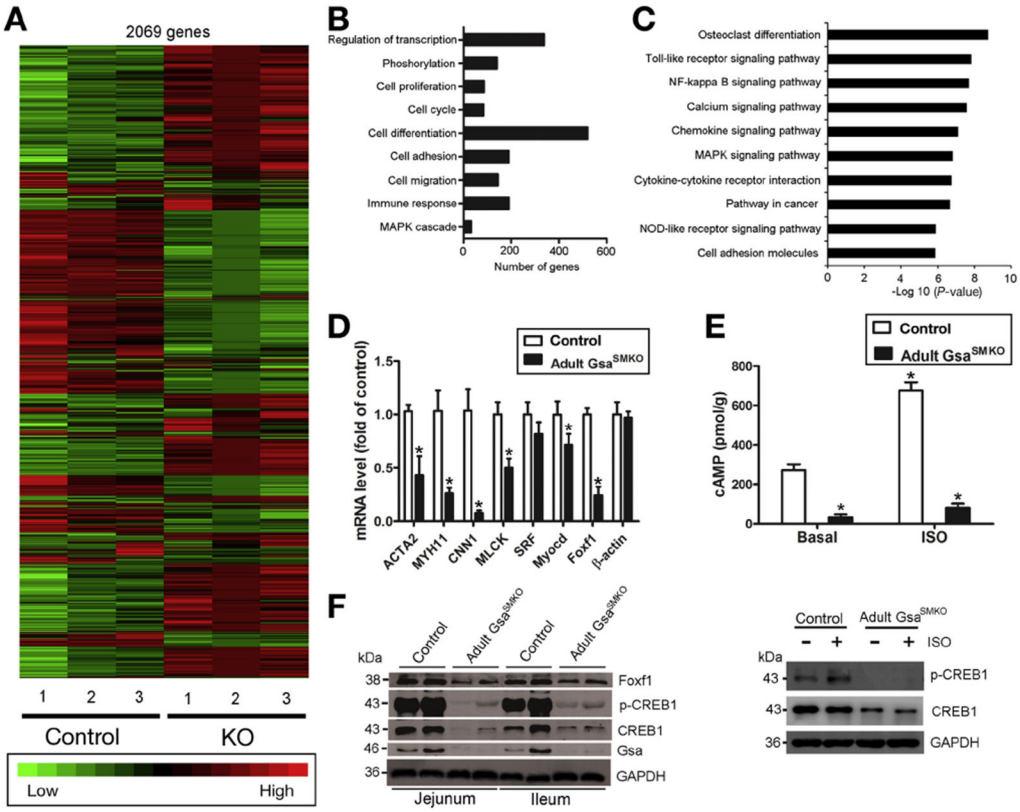

Figure 5.

Gsα deletion reduced the expression of contractile proteins and cAMP/CREB1 signaling. (*A*) Microarray assay of genes with >2.0-fold up-regulation or >2.0-fold down-regulation in Gsα-deficient intestinal smooth muscle compared with control. (*B*) Gene ontology enrichment analysis of differentially expressed genes. The number of genes with a significantly changed expression is shown in parentheses. (*C*) Top 10 enrichment pathways based on the Kyoto Encyclopedia of Genes and Genomes database. (*D*) Quantitative reverse transcription polymerase chain reaction of genes related to smooth muscle contraction for validation of the microarray results (n = 5). **P* < .05 vs control. (*E*) The jejunum tissue from control and adult $Gs\alpha^{SMKO}$ mice was stimulated with 10 μM isoproterenol (ISO) for 5 minutes followed by cAMP measurement and Western blot to detect the levels of p-CREB1 and total CREB1. n = 5; **P* < .05 vs control. (*F*) Western blot analysis of protein levels of p-CREB1, total CREB1, and Foxf1 in muscle tissue from control and adult $Gs\alpha^{SMKO}$ mice.

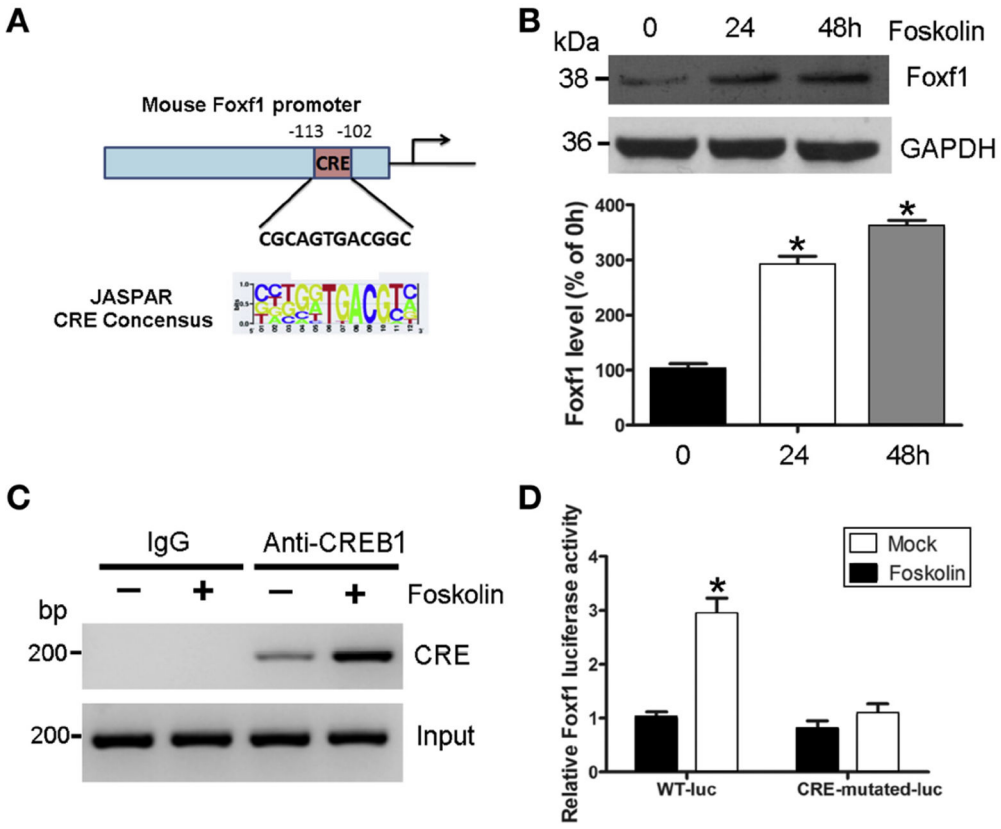

Figure 6.

CREB1 binds with Foxf1 promoter and regulates its expression. (*A*) Predicted CRE site in the mouse Foxf1 promoter. (*B*) Mouse intestinal SMCs were treated with 10 μM forskolin for 24 hours and Western blot was used to detect Foxf1 expression. The levels of Foxf1 were quantified (n = 4). **P* < .05 vs 0 hours. (*C*) Mouse intestinal SMCs were treated with 10 μM forskolin for 12 hours, chromatin immunoprecipitation was then used to assay the CREB1 binding site in the Foxf1 promoter. (*D*) Mouse intestinal SMCs were transfected with wild-type or mutant Foxf1 promoter luciferase constructs, then treated with forskolin for 24 hours and luciferase activity was measured. Results of the luciferase promoter assay are presented as fold change ± SEM of firefly/*Renilla* luciferase activity (n = 4). **P* < .05 vs WT-luc + Mock.

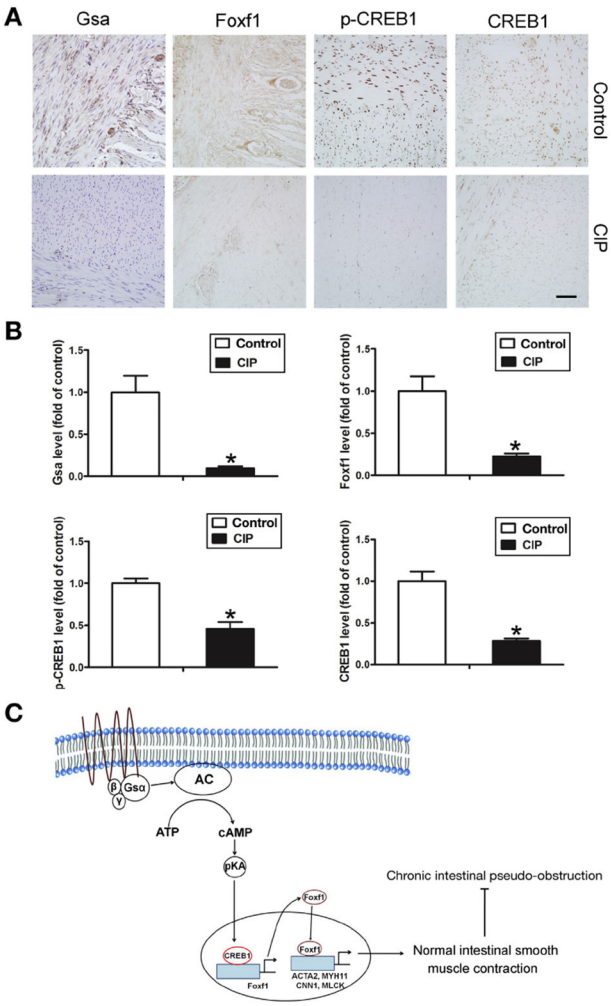

Figure 7.

Levels of Gsα, Foxf1, and p-CREB1/CREB1 were reduced in the intestinal muscle layer of CIP patients. (*A*) Paraffin-embedded control and CIP specimens from patients were stained with primary antibodies for Gsα, Foxf1, p-CREB1, and total CREB1, then secondary antibody horseradish peroxidase–3,3′-diaminobenzidine (DAB) detection reagents (*brown*). Scale bar = 50 μm. (*B*) Quantification of Gsα, Foxf1, p-CREB1, and total CREB1 levels by normalization to DAB intensity in control specimens (set to 1) (n = 5). **P* < .05 vs control. (*C*) Diagram for Gsα-regulated intestinal smooth-muscle function via CREB1/Foxf1. AC, adenylyl cyclase.