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The RNA-Binding Protein RBPMS2 Regulates Development of Gastrointestinal Smooth Muscle

CÉCILE NOTARNICOLA,* CAROLINE ROULEAU,*,‡ LUDOVIC LE GUEN,* ANNE VIRSOLVY,* SYLVAIN RICHARD,*
SANDRINE FAURE,* and PASCAL DE SANTA BARBARA*

*INSERM U1046, Université Montpellier 1, Université Montpellier 2, Montpellier; and ‡CHRU Montpellier, Service d'Anatomie Pathologique, Montpellier, France

BACKGROUND & AIMS: Gastrointestinal development requires regulated differentiation of visceral smooth muscle cells (SMCs) and their contractile activities; alterations in these processes might lead to gastrointestinal neuromuscular disorders. Gastrointestinal SMC development and remodeling involves post-transcriptional modification of messenger RNA. We investigated the function of the RNA-binding protein for multiple splicing 2 (RBPMS2) during normal development of visceral smooth muscle in chicken and expression of its transcript in human pathophysiological conditions. **METHODS:** We used avian replication-competent retroviral misexpression approaches to analyze the function of RBPMS2 in vivo and in primary cultures of chicken SMCs. We analyzed levels of *RBPMS2* transcripts in colon samples from pediatric patients with Hirschsprung's disease and patients with chronic pseudo obstruction syndrome (CIPO) with megacystis. **RESULTS:** *RBPMS2* was expressed strongly during the early stage of visceral SMC development and quickly down-regulated in differentiated and mature SMCs. Misexpression of *RBPMS2* in differentiated visceral SMCs induced their dedifferentiation and reduced their contractility by up-regulating expression of *Noggin*, which reduced activity of bone morphogenetic protein. Visceral smooth muscles from pediatric patients with CIPO expressed high levels of *RBPMS2* transcripts, compared with smooth muscle from patients without this disorder. **CONCLUSIONS: Expression of *RBPMS2* is present in visceral SMC precursors. Sustained expression of *RBPMS2* inhibits the expression of markers of SMC differentiation by inhibiting bone morphogenetic protein activity, and stimulates SMC proliferation. *RBPMS2* transcripts are up-regulated in patients with CIPO; alterations in RBPMS2 function might be involved in digestive motility disorders, particularly those characterized by the presence of muscular lesions (visceral myopathies).**

Keywords: Developmental Biology; RNA-Binding Protein; Gastrointestinal Tract; Smooth Muscle Contraction.

Motility of the digestive tract is ensured by the contraction of visceral smooth muscles under the control of the autonomous enteric nervous system (ENS) and the interstitial cells of Cajal (ICC).[1–3] Dysfunction of just one of these cell types can lead to the development of gastrointestinal neuromuscular disorders in infants and adults.[4,5] Many studies have investigated the role of ENS damage in these diseases and shown that ENS absence caused by precocious differentiation or blockage of its migration triggers Hirschsprung disease (HSCR).[2–4] Alteration of ICC number, which perturbs the ICC network, also has been observed in different digestive motility disorders.[4,6] Until now, dysfunction of the downstream effector (visceral smooth muscle) rarely was investigated in gastrointestinal neuromuscular disorders.[4,6,7] Specifically, muscular lesions, classified as visceral myopathies, have been observed mainly in patients with chronic intestinal pseudo-obstruction (CIPO). However, the systematic analysis of muscular alterations has been overlooked mainly because of the poor knowledge of the molecular mechanisms involved in visceral smooth muscle cell (SMC) homeostasis. The limited number of valuable SMC markers is also an obstacle to the clinical investigation of visceral smooth muscle integrity in these motility disorders.

More is known about the molecular control of visceral SMCs during embryonic development. Visceral SMCs originate from the splanchnopleural mesoderm that forms the primitive visceral mesenchyme via activation of the hedgehog/bone morphogenetic protein (BMP) pathway.[2] Differentiation of visceral mesenchymal cells into visceral SMCs can be visualized first through their elongation and clustering and later by the expression of SMC-specific lineage markers, such as α-smooth muscle actin (αSMA), smooth muscle protein-22 (SM22), calponin, smoothelin, and smooth muscle myosin heavy chain (SM-MHC), which precedes contractile function.[8] Only a few studies have investigated the molecular mechanisms controlling visceral mesenchyme differentiation into SMCs.

Abbreviations used in this paper: αSMA, α-smooth muscle actin; BMP, bone morphogenetic protein; CIPO, chronic intestinal pseudo-obstruction; DMEM, Dulbecco's modified Eagle medium; E, embryonic day; ENS, enteric nervous system; FHL2, four and a half LIM domains 2; GFP, green fluorescent protein; HSCR, Hirschsprung disease; ICC, interstitial cells of Cajal; mRNA, messenger RNA; P-AKT, phosphorylated AKT; PH3, phospho-histone H3-Ser10; PSMAD, phosphorylated SMAD; QPCR, quantitative polymerase chain reaction; RBPMS2, RNA-binding protein for multiple splicing 2; RBP, RNA-binding protein; RCAS, Replication-Competent Avian leucosis Sarcoma virus; RRM, RNA recognition motif; SHH, sonic hedgehog; SMC, smooth muscle cell; SM-MHC, smooth muscle myosin heavy chain; SM22, smooth muscle protein-22; SRF, serum response factor.

Endodermal hedgehog signaling activation promotes visceral mesenchyme growth through expression of the *Patched* receptor and of *Bmp4* that drives its differentiation into SMCs.[9] Aberrant modulation of BMP activity induces visceral smooth muscle disorganization.[10] The fibroblast growth factor signaling pathway also is activated in visceral mesenchymal cells and is specifically down-regulated during visceral SMC differentiation, which is inhibited by sustained activation of the fibroblast growth factor pathway in vivo.[11] Although a substantial body of works has investigated visceral SMC development, the specific molecular mechanisms controlling its differentiation remain to be clarified.

Besides transcription, which represents the first step of gene expression, many post-transcriptional events regulate the final fate of messenger RNAs (mRNAs) in eukaryotic cells and determine their spatiotemporal pattern of expression. RNA-protein complexes control multiple steps of this process, including mRNA cellular localization, splicing, translational regulation, or mRNA degradation.[12] These complexes contain specific RNA-binding proteins (RBPs) that play important roles during development. The RNA recognition motif (RRM) proteins, a large RBP family involved in regulating RNA metabolism, are expressed in a tissue-specific manner, suggesting that they may participate in distinct developmental processes.[13] Moreover, postregulatory RNA events are involved in the control of differentiation and remodeling of smooth muscle tissues, such as heart and vessels,[14] suggesting that visceral smooth muscle development and plasticity could be regulated similarly. *RNA-binding protein for multiple splicing 2* (*RBPMS2*), a member of the RRM family, is expressed in vertebrate heart and gastrointestinal tract.[13,15] By using a microarray approach to identify modulators of visceral SMC development, we found that *RBPMS2* is expressed during the early stages of chick SMC development (unpublished data).[11]

Here, we investigated RBPMS2 function and mechanism of action during visceral SMC development and assessed its transcript expression in colon from patients with digestive motility disorders. We show that *RBPMS2* is an early marker of visceral SMC precursors and that *RBPMS2* misexpression in differentiated SMCs increases proliferation and hinders their contractile function. The RBPMS2-dependent contractile differentiation defect is mediated by inhibition of BMP signaling through up-regulation of *Noggin*, its major inhibitor. These results indicate that ectopic *RBPMS2* expression in vivo and in primary cultured SMCs phenocopies the alteration of the contractile function observed in patients with visceral myopathy.

Materials and Methods

Chick Embryonic Gastrointestinal Tissues

Fertilized White Leghorn eggs from Haas Farm (Kaltenhouse, France) were incubated at 38°C in humidified incubators. Gastrointestinal tissues from chick embryos were dissected as described.[16]

Human Colon Samples

Tissue samples from pediatric patients (1 month to 1 year) were from the collection of the Lapeyronie Hospital (Montpellier, France) as previously described.[17] Control samples were right colon specimens from 3 neonates who underwent ileocolic resection for congenital cystic duplication of the terminal ileum. Full-thickness, large-bowel specimens were obtained after rectosigmoidectomy from 3 patients diagnosed with HSCR or 3 patients diagnosed with slow-transit constipation without HSCR and with megacystis (CIPO patients).

Avian Retroviral Misexpression System

Myc-tagged chick full-length *RBPMS2* was cloned into the Replication-Competent Avian Leucosis Sarcoma virus strain A (RCAS[A]) vector to produce replication-competent retroviruses that express Myc-*RBPMS2*. The RCAS(A)-*Noggin* and RCAS stain B with green fluorescence protein coding region (RCAS[B]-GFP) retroviral constructs were described previously.[11,16] Retroviral constructs were transfected into the DF-1 chicken fibroblast cell line (ATCC-LGC) to produce retroviruses. Retroviruses were injected into the splanchnopleural mesoderm of stage-10 chicken embryos to target the stomach mesenchyme.[10,16] Eggs then were placed at 38°C until harvested.

Primary Cultured SMCs and Analysis

Primary cultures from embryonic day 15 (E15) gizzard muscle were prepared as described.[18] Briefly, the tunica muscularis carefully was separated from the serosa and tunica mucosa before collagenase dissociation. Isolated cells were cultured in Dulbecco's modified Eagle medium (DMEM) in the presence of 0.2% bovine serum albumin and 5 μg/mL insulin in Matrigel-coated plates (VWR, Fontenay-sous-Bois cedex, France) to maintain the cell differentiation status (>95% of isolated cells were Desmin- and αSMA-positive, data not shown). Differentiated SMCs then were infected with different retroviruses and maintained in culture for 1–7 days. In our conditions, avian retroviruses have a high tropism to infect SMCs and low tropism for enteric neurons (Supplementary Figure 1). SMC contractility was monitored with a Nikon inverted microscope and cells were imaged before and after treatment with 10^{-3} mol/L carbachol (Sigma, France), a muscarinergic agonist, as previously published.[18] In rescue experiments, differentiated SMCs infected with retroviruses during 3 days were treated with 20 ng/mL of purified BMP4 (Humanzyme, Chicago, IL) for 4 days.

Isometric Tension Measurement During Contractile Activity

E15 gizzards were cut at the level of the ventral and dorsal tendons to avoid damaging the muscular bundles.[11] The right part of the organ connected to the duodenum was used for the measurements (control, n = 23; *RBPMS2* misexpression, n = 21). Organs were mounted between 2 stainless steel hooks and placed in an organ bath filled with Tyrode-HEPES solution with 2.5 mmol/L $CaCl_2$ continuously bubbled with 95% O_2/5% CO_2 and maintained at 37°C. Changes in isometric tension were recorded using an IT1-25 force transducer and an IOX computerized system (EMKA Technologies, Paris, France). Gizzards initially were stretched at a resting tension of 0.5 g and, after a 60-minute equilibration period, contraction was induced with cumulative doses of carbachol (10^{-6} to 10^{-3} mol/L). Effects were evaluated by measuring the maximum tension, and data were expressed as changes relative to the basal tension (contraction in g) for 10^{-4} mol/L carbachol concentration.

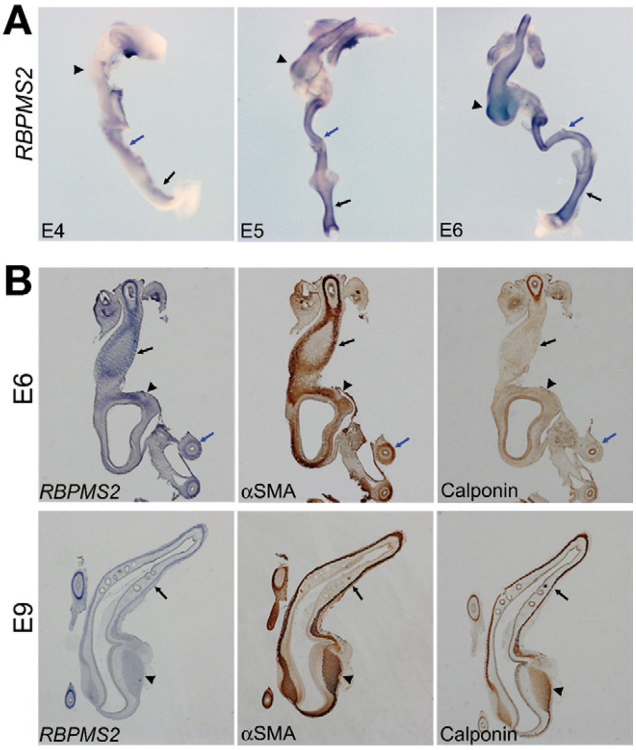

Figure 1. Dynamic expression of *RBPMS2* in chick gastrointestinal mesenchyme. (*A*) Whole-mount in situ hybridization of E4, E5, and E6 gastrointestinal tracts using the *RBPMS2* riboprobe. *Arrows*, *arrowheads*, and *blue arrows* indicate the colon, developing stomach, and small intestine, respectively. (*B*) Serial longitudinal sections of E6 (*upper panels*) and E9 (*lower panels*) stomachs analyzed by in situ hybridization with the *RBPMS2* riboprobe and by immunohistochemistry with anti-αSMA and anticalponin antibodies. *Arrows*, *arrowheads*, and *blue arrows* indicate the anterior glandular stomach, posterior muscular stomach, and duodenum, respectively.

Western Blotting, In Situ Hybridization, Immunodetection, Microarray Experiments, Quantitative Polymerase Chain Reaction

See the Supplementary Materials and Methods section for more detail.

Results

Dynamic Expression of RBPMS2 *in Chick Gastrointestinal Mesenchyme*

To determine the role of RBPMS2 in the digestive visceral smooth muscle, first we analyzed its expression during chick gastrointestinal tract development. *RBPMS2* started to be expressed at embryonic day 4 (E4), an early stage of gastrointestinal tract development, in the regions of the future stomach, midgut, and colon (Figure 1*A*). At E5, *RBPMS2* was expressed strongly in the developing stomach, small intestine, and colon, with the exception of the cecum (Figure 1*A*). Additional expression was observed in the developing lungs. At E6, *RBPMS2* transcripts accumulated in the stomach, small intestine, and colon (Figure 1*A*). *RBPMS2* expression was temporally and spatially comparable with that of *αSMA*, the earliest known SMC marker, which is expressed in the developing and differentiated visceral smooth musculature (Supplementary Figure 2). By using paraffin sections we showed that, at E6, *RBPMS2* and αSMA expression overlapped in the undifferentiated visceral mesenchyme (Figure 1*B*, *upper panels*). However, after visceral smooth muscle differentiation (E9), *RBPMS2* expression rapidly decreased in the smooth muscle layer, whereas calponin (which is a marker of differentiated SMCs) expression increased (Figure 1*B*, *lower panels*). In summary, *RBPMS2* expression was highest in undifferentiated visceral mesenchymal cells and progressively decreased with muscle differentiation, thus identifying it as an early marker of visceral smooth muscle precursor cells.

Sustained RBPMS2 *Expression Alters Gastrointestinal Development, SMC Differentiation, and Contractile Function*

To investigate RBPMS2 function, we maintained *RBPMS2* expression throughout visceral muscle development and differentiation by using an avian replication-competent retroviral misexpression system that allows in vivo targeting of specific genes in the stomach mesenchyme.[11,16] Sustained *RBPMS2* expression resulted in a dramatic alteration of the stomach morphology (Figure 2*A*). Specifically, the proventriculus, which is the glandular part of the chick stomach (Figure 2*A*, *white arrowheads*), was hypertrophied, whereas the gizzard (the muscular part, Figure 2*A*, *white arrows*) was denser and malformed in comparison with controls that overexpressed GFP alone (Figure 2*A*). However, sustained *RBPMS2* expression did not affect stomach development and patterning, as revealed by the normal expression of the mesenchymal marker *BARX1* and of the endodermal marker *sonic hedgehog* (*SHH*) in *RBPMS2* misexpressing stomachs (Figure 2*B*). Similarly, the determination of visceral SMCs was not affected because positive αSMA cells still were observed in the smooth muscle layer (Figure 2*C*); however, SMC differentiation was hindered as indicated by the reduction of calponin expression in *RBPMS2* misexpressing stomachs in comparison with GFP controls (Figure 2*C*, Supplementary Figure 3). HuC/D-positive neurons were organized into well-defined plexuses, suggesting that *RBPMS2* misexpression did not induce detectable changes in ENS migration and differentiation (Figure 2*C*). Finally, the SMC proliferation rate was 1.55-fold higher in *RBPMS2* misexpressing stomachs than in GFP controls (Figure 2*C*, Supplementary Figure 4), as indicated by the expression of phosphorylated histone 3-Ser10 (PH3), a standard marker of G2/M transition. Similarly, sustained *RBPMS2* expression in the developing colon mesenchyme did not affect SMC determination, whereas it inhibited calponin expression (Supplementary Figure 5). Altogether, these results indicate that a tight regulation of *RBPMS2* expression is important for the normal development and differentiation of gastrointestinal smooth muscle. In addition, we investigated the contractile function at the organ level in control and *RBPMS2*-overexpressing gizzards (E15) by recording the smooth muscle contraction in ex vivo organ experiments (Figure 2*D*). Addition of carbachol triggered

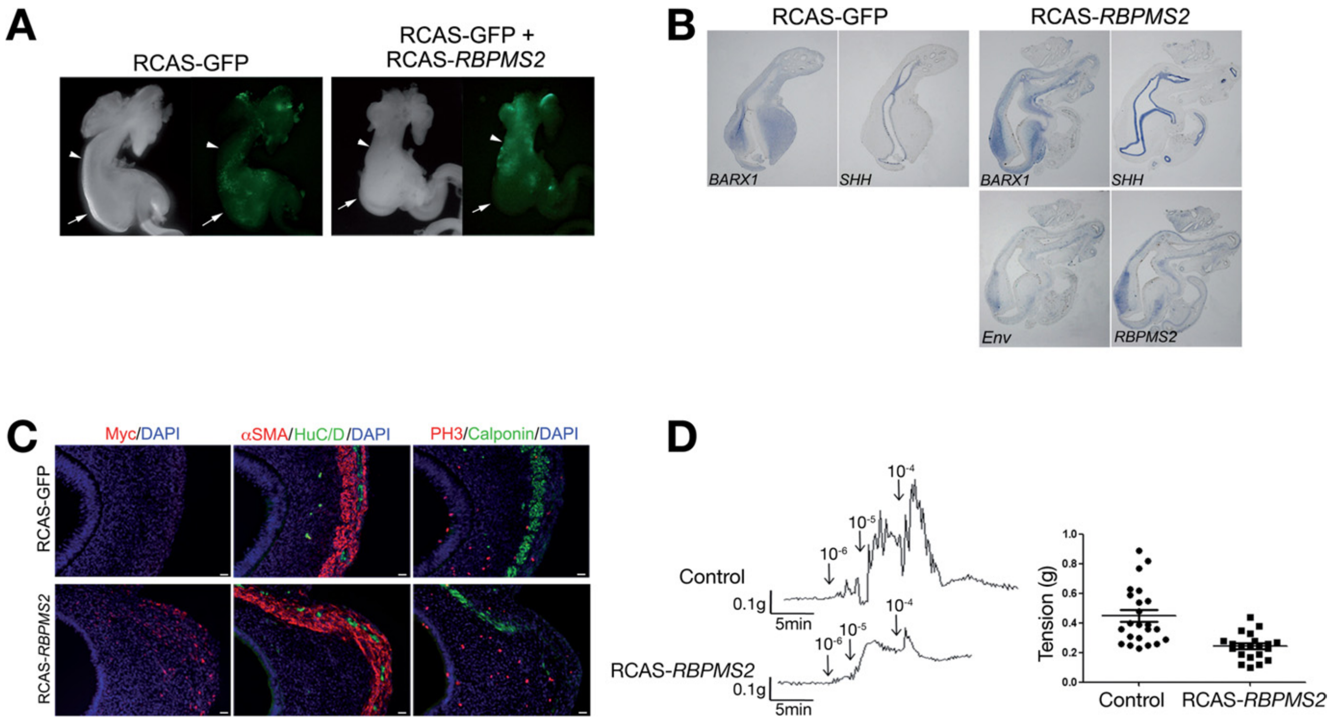

Figure 2. Sustained *RBPMS2* expression alters chick gastrointestinal development, SMC differentiation, and contractile function. (*A*) E9 stomachs after retroviral misexpression of RCAS-GFP (as control) or both RCAS-*RBPMS2* and RCAS-GFP. The presence of retroviruses is confirmed by direct observation of GFP expression. *White arrows* and *arrowheads* indicate the gizzard and the proventriculus, respectively. (*B*) Serial longitudinal sections of E9 stomach analyzed by in situ hybridization after RCAS-*RBPMS2* misexpression using retroviral *Envelop* (*Env*), *RBPMS2*, *BARX1*, and *SHH* riboprobes. Control E9 stomachs were analyzed with *BARX1* and *SHH* riboprobes. (*C*) Serial transversal sections of E9 stomachs infected with RCAS-GFP or RCAS-*RBPMS2* retroviruses and analyzed by immunofluorescence. Nuclei visualized with Hoechst. Antibodies against SMC (αSMA and calponin), neuronal (HuC/D), and mitotic (PH3) markers were used. Anti-Myc antibodies were used to identify cells infected by RCAS-*RBPMS2* retroviruses. *Scale bar*, 20 μm. (*D*) Measurement of contractile activity in isolated E15 gizzards. Typical recording showing stimulation with 10^{-6} to 10^{-4} mol/L carbachol in normal and *RBPMS2*-overexpressing organs (*left panels*). Maximum tension after 2 minutes of 10^{-4} mol/L carbachol stimulation in normal (n = 23) and *RBPMS2*-overexpressing (n = 21) gizzards (*right panel*). Values are the mean ± standard error of the mean.

the contraction of gizzard muscles in a dose-dependent manner with a maximal tension reached at 10^{-4} mol/L. The contraction was weaker in gizzards overexpressing *RBPMS2* (0.24 ± 0.02 g; n = 21) than in control gizzards (0.45 ± 0.04 g; n = 23; $P < .001$).

To determine how RBPMS2 regulates visceral SMC differentiation, we established primary cultures on Matrigel of visceral differentiated SMCs from E15 gizzard muscles in serum-free medium supplemented with insulin. In this condition, cultured differentiated SMCs can display phenotypic modulation upon exogenous stimulation.[18,19] Control primary cultured SMCs were spindle-shaped and homogenously expressed αSMA and calponin, 2 SMC contractile markers, in highly organized filament bundles (Figure 3*A*). SMCs then were infected with replication-competent retroviruses (RCAS-*RBPMS2* construct or RCAS-empty [control]) for 3 days. Although in control cells the expression of αSMA and calponin remained unchanged (Figure 3*A*, Supplementary Figure 6), in SMCs infected with Myc-tagged *RBPMS2* their expression was lost (Figure 3*A*, Supplementary Figure 7). However, Myc-positive cells still expressed the mesenchymal marker Desmin (Figure 3*A*), confirming that they were mesenchymal-derived cells. Quantitative reverse-transcription polymerase chain reaction (QPCR) using RNA from cells infected with RCAS-*RBPMS2* or RCAS-empty for 3 days confirmed the down-regulation of *calponin*, *αSMA*, and *SM22* expression (Figure 3*B*). Analysis of the effect of *RBPMS2* overexpression on *serum response factor* (*SRF*) and its co-activator *Myocardin*, which control numerous steps of SMC differentiation,[20] indicated that *Myocardin* mRNA was up-regulated (2-fold), whereas *SRF* expression was not significantly affected (Figure 3*B*). Similarly, *four and a half LIM domains 2* (*FHL2*) mRNA, which inhibits the induction of smooth muscle contractile genes regulated by SRF,[21] was up-regulated (Figure 3*B*, Supplementary Figure 8). To analyze the impact of ectopic *RBPMS2* expression on Myocardin protein level, we co-transfected primary cultured SMCs with a plasmid-encoding myocardin and either *RBPMS2* or empty (control) retroviral constructs. In SMCs overexpressing *RBPMS2*, the myocardin protein level was increased in comparison with control (Supplementary Figure 9). Because myocardin accumulates in response to proteasome inhibition by MG132,[22] we transfected primary cultured SMCs with a plasmid encoding for myocardin with or without 5 μmol/L MG132. Ectopic myocardin expression increased calponin expression (Supplementary Figure 9). Moreover, in accordance with published work,[22] proteasome inhibition by MG132 induced accumulation of myocardin, but reduced

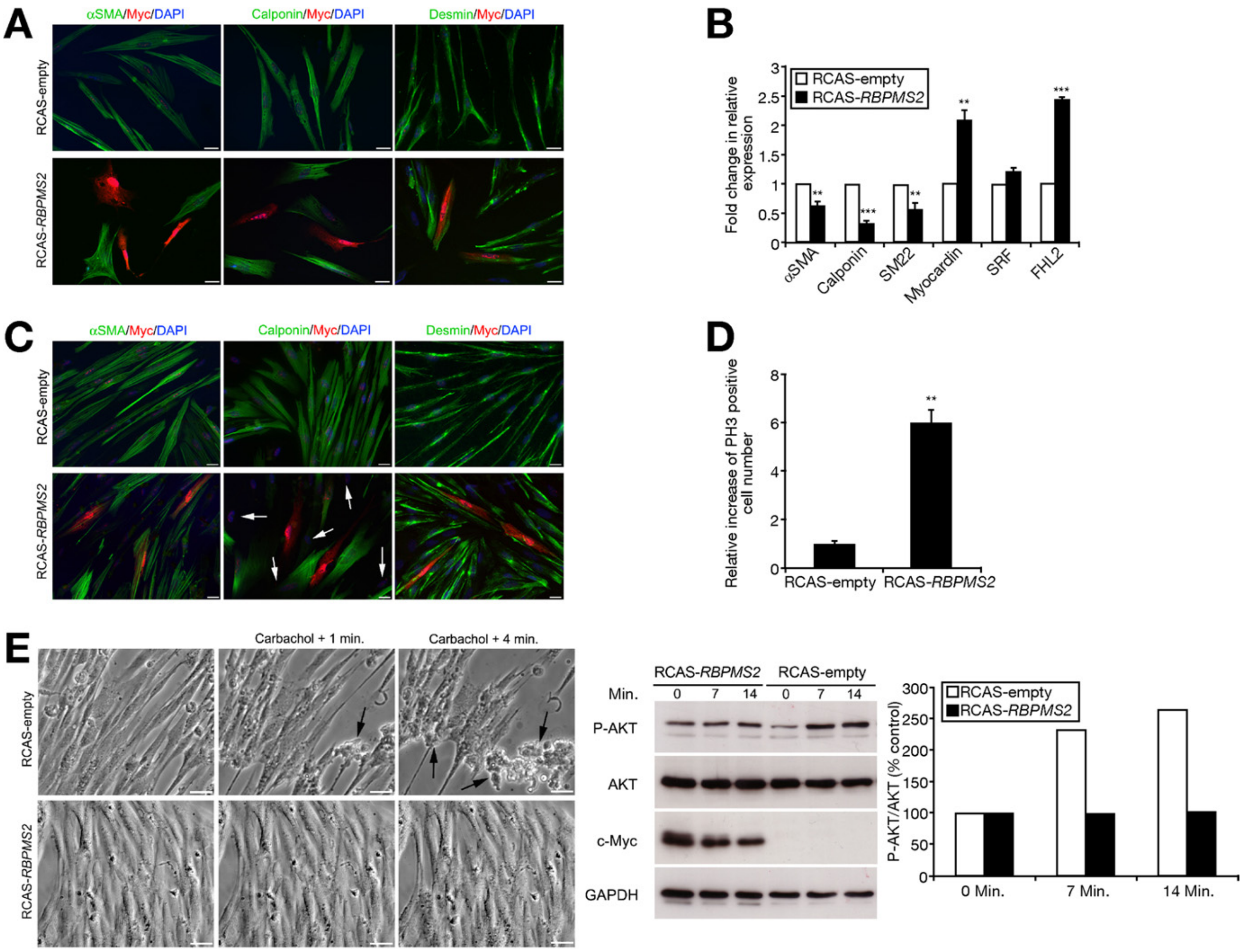

Figure 3. *RBPMS2* overexpression hinders SMC differentiation and ability to contract. Immunofluorescence analysis of primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for (*A*) 3 and (*C*) 7 days. Nuclei were visualized with Hoechst. Antibodies against SMC (αSMA and calponin) and mesenchymal cell (desmin) markers were used. Anti-Myc antibodies were used to identify cells infected by RCAS-*RBPMS2* retroviruses. *Scale bar*, 20 μm. *White arrows* indicate Myc-negative cells with loss of calponin expression. (*B*) Gene expression analysis by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 3 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-*RBPMS2* vs RCAS-empty). $^{**}P < .01$; $^{***}P < .001$. (*D*) Quantification of mitotic cells using anti-PH3 antibodies in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 7 days. Values are the mean ± standard error of the mean of 2 independent experiments. $^{**}P < .01$. (*E*) Contractility assays in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 7 days and then stimulated with 10^{-3} mol/L carbachol (*left panels*). Images were acquired before and at 1 minute and 4 minutes after carbachol addition. *Arrows* show contracted cells in control cultures. *Scale bar*, 40 μm. Immunoblot analysis of phosphorylated P-AKT (62 kilodaltons) and total AKT (62 kilodaltons) in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* for 7 days and treated with 10^{-4} mol/L carbachol for 7 and 14 minutes (*middle panel*). Anti-Myc antibodies detected the expression of Myc-tagged RBPMS2 (26 kilodaltons). Loading was verified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (37 kilodaltons). *Right panel* shows the quantification of the Western blot data.

calponin expression (Supplementary Figure 9). Altogether, these findings indicate that ectopic *RBPMS2* expression in visceral SMCs induces accumulation of myocardin, which is critical for its function. After 7 days, αSMA expression was recovered in *RBPMS2*-overexpressing SMCs, whereas loss of calponin expression spread to adjacent nontransfected (Myc-negative) cells (Figure 3*C*, *white arrows*). As before, *RBPMS2*-overexpressing cells were desmin-positive (Figure 3*C*). Moreover, the number of PH3-positive cells was 6-fold higher in cells that overexpressed *RBPMS2* than in controls (Figure 3*D*), suggesting a global change in the proliferative status of SMCs upon *RBPMS2* deregulated expression.

To investigate the effects of *RBPMS2* overexpression on visceral SMC contractility, primary cultured SMCs were stimulated with carbachol. Many control cells (RCAS-empty) became round and detached from the disc surface, showing their effective capacity to contract (Figure 3*E*). Conversely, *RBPMS2*-overexpressing SMCs were more broad-shaped than control cells and, after addition of carbachol, only few became round and detached from the disc surface (Figure 3*E*). Recently, intestinal SMC dedifferentiation and inability to contract upon carbachol stimulation were correlated with decreased expression of *cholinergic receptor muscarinic 3* mRNA and of carbachol-induced phosphorylated AKT (P-AKT).[23]

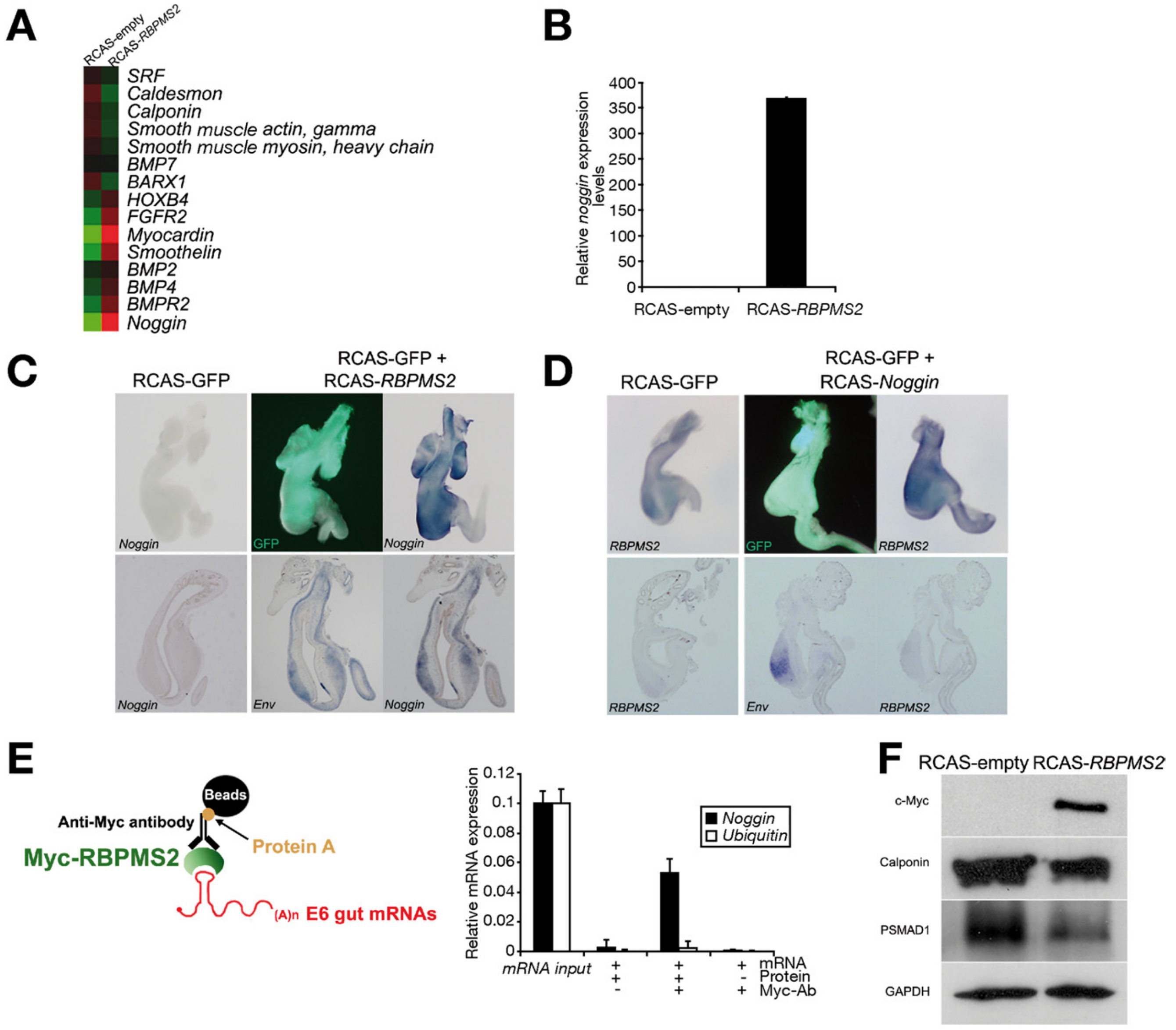

Figure 4. RBPMS2 inhibits BMP signaling through *Noggin* up-regulation. (*A*) Identification of RBPMS2 target genes by microarray using RNA from primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* for 3 days. Highest signals are in *red*, and lowest signals are in *green*. (*B*) Analysis of *Noggin* expression by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 3 days. Data were normalized to *ubiquitin* expression and shown as mean ± standard deviation. In situ hybridization analysis in whole-mount (*upper panels*) and serial longitudinal sections (*lower panels*) of E7 stomachs infected with RCAS-GFP (control), or (*C*) RCAS-GFP and RCAS-*RBPMS2* or (*D*) RCAS-*Noggin* retroviruses. Infection was confirmed by observation of GFP expression or detection of *Env* expression. (*E*) Immunoprecipitation assays to identify mRNAs that interact with RBPMS2. Schematic representation of the approach (*left panel*) and QPCR to detect RBPMS2-associated mRNAs using specific primers (*right panel*). The mean expression levels ± standard error of the mean of *Noggin* and *ubiquitin* were measured in each condition. (*F*) Immunoblot analysis of PSMAD1 (55 kilodaltons) and Calponin (34 kilodaltons) expression in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* for 3 days. Anti-Myc antibodies detected the expression of Myc-tagged RBPMS2 (26 kilodaltons). Loading was verified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (37 kilodaltons).

Similarly, we found that *cholinergic receptor muscarinic 3* mRNA expression was decreased by 56% in *RBPMS2*-overexpressing SMCs compared with controls (Supplementary Figure 10). Moreover, addition of 10^{-4} mol/L carbachol for 14 minutes resulted in an increase of P-AKT to 264% in control SMCs, although the total level of AKT was unchanged. Conversely, no detectable change in P-AKT level was observed in *RBPMS2*-overexpressing SMCs (Figure 3*E*). These experiments show that *RBPMS2* sustained expression in differentiated SMCs hinders their ability to contract and favor their proliferation, leading to their dedifferentiation.

RBPMS2 *Inhibits BMP Signaling Through Induction of Noggin Expression*

To identify the underlying molecular mechanism(s) of RBPMS2 action, we analyzed the gene expression profiles of primary cultured SMCs infected with RCAS-*RBPMS2* or RCAS-empty retroviruses for 3 days (Figure 4*A*) by microarray analysis.

RBPMS2 overexpression in differentiated SMCs induced the down-regulation of *calponin* and also of markers of SMC differentiation, such as *caldesmon* and *SM-MHC*. Conversely, *Noggin*, the BMP signaling pathway inhibitor, was 360-fold up-regulated (Figure 4*A*), whereas BMP transcriptional targets, including *PITX2*, *ID2*, and *ID4*, were down-regulated, suggesting a major inhibition of BMP activity (data not shown). *Noggin* up-regulation was confirmed by QPCR using primary SMCs harvested after 3 days of RCAS-*RBPMS2* infection (Figure 4*B*).

Because *Noggin* expression pattern has not been described in detail,[24] we performed in situ hybridization and showed that *Noggin* was expressed in gut mesenchymal derivatives as early as *RBPMS2* (Supplementary Figure 11). Moreover, because misexpression of *Noggin* in stomach induces a hypertrophic phenotype[16] that is highly reminiscent of the defects observed upon *RBPMS2* misexpression, we monitored *Noggin* expression by in situ hybridization after *RBPMS2* misexpression in the gastrointestinal mesenchyme as before. *Noggin* was strongly up-regulated in stomach and lung in comparison with controls (Figure 4*C*). Conversely, misexpression of *Noggin* had a moderate impact on the spatiotemporal expression of *RBPMS2* (Figure 4*D*). Altogether, these results show that RBPMS2 induces *Noggin* expression and accumulation in vivo and in primary cultured SMCs.

Because RBPMS2 can bind to RNAs via its RRM domain, we investigated whether *Noggin* up-regulation in primary SMCs and in vivo upon *RBPMS2* misexpression could be caused by interaction between RBPMS2 and *Noggin*. First, Myc-tagged RBPMS2 from infected DF-1 cells was immunoprecipitated with anti-Myc antibodies bound to protein A Sepharose beads in the presence of total RNA from E6 gastrointestinal mesenchyme. QPCR showed that *Noggin* was strongly amplified (6% of total *Noggin* mRNA) after immunoprecipitation of Myc-tagged RBPMS2 (Figure 4*E*, Supplementary Figure 12), indicating that *Noggin* mRNA and RBPMS2 are present in a common RNA-protein complex. Then, we investigated the impact of the RBPMS2/*Noggin* interaction on the activity of the BMP signaling pathway with antibodies against the activated and phosphorylated intracellular BMP effectors SMAD1, 5, and 8 (namely PSMAD1) that we previously characterized as tools to evaluate the activity of BMP pathway.[10,25] In control primary SMCs, high expression of PSMAD1 was associated with calponin expression. In cells infected with RCAS-*RBPMS2* for 3 days, calponin expression decreased concomitantly with a strong reduction of PSMAD1 expression (Figure 4*F*), showing an inhibitory effect of RBPMS2 on the BMP signaling pathway, but also indirectly a positive effect of RBPMS2 on *Noggin* mRNA transcription or stabilization.

These results show that RBPMS2 positively regulates *Noggin* expression, leading to inhibition of BMP activity.

Noggin Downstream of RBPMS2 *Alters SMC Differentiation*

To evaluate whether Noggin is an essential relay of RBPMS2, we focused on Noggin function during visceral SMC development and differentiation. We thus misexpressed *Noggin* in stomach throughout visceral muscle development and analyzed the differentiation of visceral SMCs. *Noggin* misexpression inhibited calponin expression in comparison with GFP controls, showing an alteration of visceral SMC differentiation without significant changes of the proliferative rate in vivo (Supplementary Figure 4). Moreover, immunofluorescence analysis of primary cultured SMCs after 3 days of infection with RCAS-*Noggin* retroviruses showed that αSMA was expressed uniformly, whereas calponin expression was strongly decreased (Figure 5*A*). QPCR analysis of these cells showed that *calponin* and *SM22* were down-regulated, whereas *αSMA*, *myocardin*, and *FHL2* were induced (Figure 5*B*, Supplementary Figure 8). *Noggin* overexpression also increased myocardin protein level (Supplementary Figure 9), as previously observed with *RBPMS2* overexpression. Conversely, *Noggin* overexpression did not affect the proliferation rate of primary SMCs, as indicated by the absence of significant variations in the number of PH3-positive RCAS-*Noggin* cells in comparison with control cells (RCAS-empty) (Figure 5*C*; 7 days of infection). These experiments show a common repressive action of RBPMS2 and Noggin on SMC differentiation, but divergent effects on the proliferative rate of primary cultured SMCs.

Noggin inhibits BMP signaling by interfering with homodimerization or heterodimerization of BMP ligands, thus blocking their interaction with receptors and preventing their activation.[25] Because our results suggest that the RBPMS2 effect on calponin expression in SMCs is mediated through inhibition of the BMP pathway via Noggin induction, we assessed the impact of *RBPMS2* misexpression on SMC differentiation in the presence or absence of BMP4, the most strongly expressed BMP ligand in the gastrointestinal musculature.[2,16,19] As previously observed in vascular SMC cultures, addition of BMP4 for 4 days increased differentiation of primary SMCs (data not shown).[19] Addition of 20 ng/mL BMP4 to RCAS-*RBPMS2*–infected SMCs restored calponin expression in the uninfected neighboring cells, but not in the infected cells (Figure 5*D*, *right panel*, and Figure 5*E* for quantification). Indeed, *RBPMS2* overexpression in SMCs decreases calponin expression in infected (Myc-positive) and uninfected (Myc-negative) neighboring cells (Figures 3*C* and 5*D*, *left panel*), suggesting that RBPMS2 acts both in an autocrine and paracrine manner. QPCR analysis confirmed that BMP4 addition to cells infected with RCAS-*RBPMS2* restored expression of both *calponin* and *SM22* (Figure 5*F*).

These experiments show that Noggin hinders SMC differentiation downstream of RBPMS2.

RBPMS2 *Transcripts Are Highly Expressed in Visceral Smooth Muscles of Patients With CIPO*

Intestinal motility disorders in infants comprise many heterogeneous diseases that are classified as gastrointestinal neuromuscular disorders and have clinical

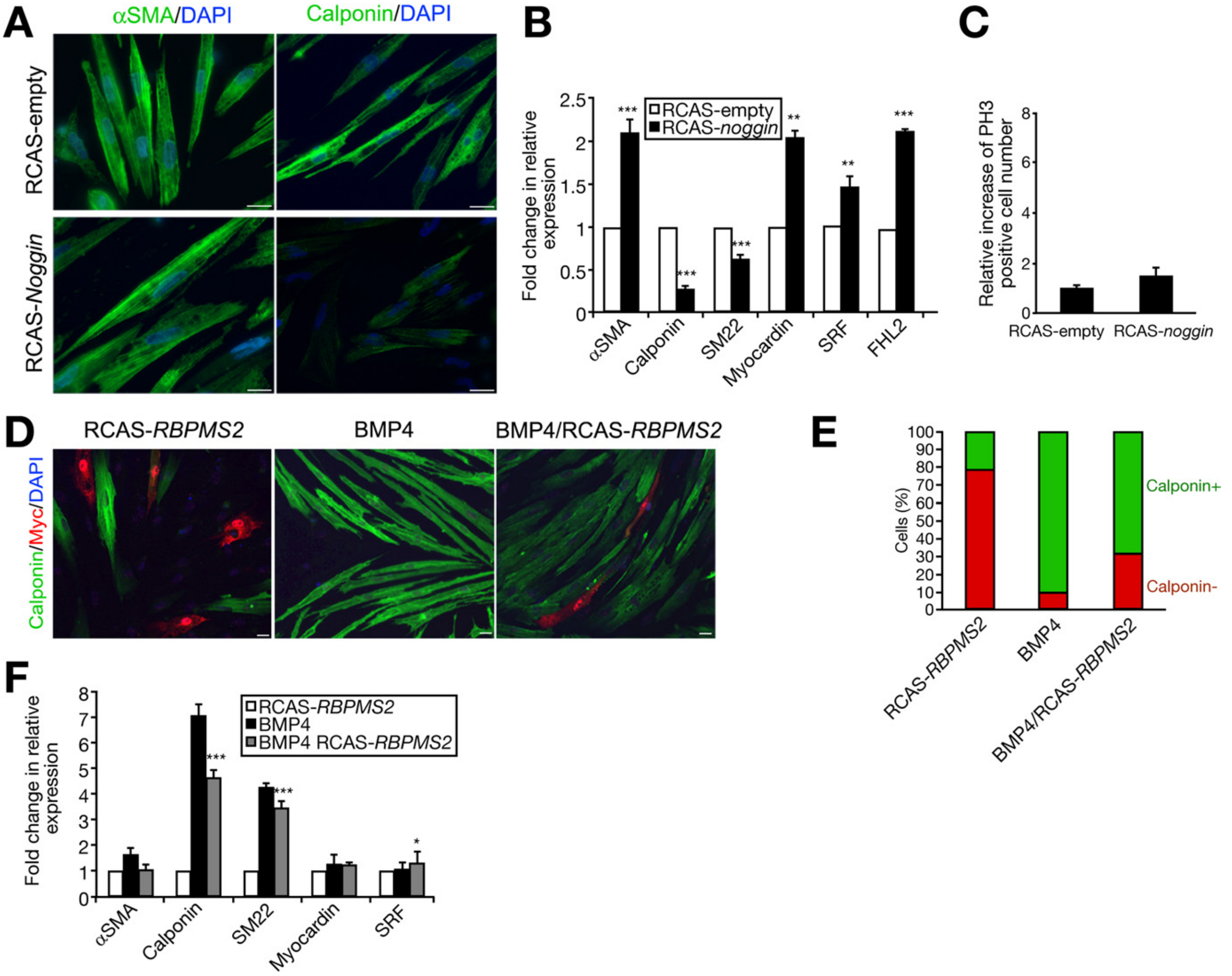

Figure 5. *Noggin* overexpression decreases the expression of SMC contractile proteins downstream of RBPMS2. (*A*) Immunofluorescence analysis of primary cultured SMCs infected with RCAS-empty or RCAS-*Noggin* retroviruses for 3 days. Nuclei were visualized with Hoechst. Antibodies directed against SMC markers (αSMA and calponin) were used. *Scale bar*, 20 μm. (*B*) Analysis of gene expression by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-*Noggin* retroviruses for 3 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-*Noggin* vs RCAS-empty). $^{**}P < .01$; $^{***}P < .001$. (*C*) Quantification of mitotic cells using anti-PH3 antibodies in primary cultured SMCs infected with RCAS-*Noggin* retroviruses for 7 days. Values are the mean ± standard error of the mean of 2 independent experiments. No statistical significance. (*D*) Immunofluorescence analysis of primary cultured SMCs infected with RCAS-*RBPMS2* retroviruses in the presence or not of BMP4 for 4 days. Nuclei were visualized with Hoechst. Antibodies against calponin and Myc were used. *Scale bar*, 20 μm. (*E*) Quantification of calponin-positive cells in the different conditions described in *panel D*. (*F*) QPCR analysis of gene expression in primary SMCs infected with RCAS-*RBPMS2* retroviruses for 7 days alone or in the presence of BMP4 in the different conditions described in *panel D*. Normalized expression levels then were converted to fold changes ± standard deviation (treatment vs RCAS-*RBPMS2*). $^{*}P < .05$ and $^{***}P < .001$ both compare with RCAS-*RBPMS2*.

symptoms ranging from simple constipation to intestinal occlusion.[4,5] Recently, specific smooth muscle defects were shown to be involved in the pathogenesis of pediatric digestive motility disorders.[7] Because our findings suggests that RBPMS2 might be involved in visceral SMC development and differentiation, we analyzed the expression of *RBPMS2* transcripts in colon biopsy specimens from pediatric patients with a history of chronic constipation associated with megacystis (CIPO), or aganglionosis (HSCR) and from neonates without digestive motility disorders (controls). Histologic analysis revealed the presence of regular circular and longitudinal smooth muscle layer in each case. Vacuolization of smooth muscles was observed in the circular smooth muscle of CIPO patients, a finding characteristic of visceral myopathy[4] (Figure 6, *upper panels*, *inset*, and *arrowheads*). In situ hybridization showed that *RBPMS2* transcripts were strongly expressed only in circular smooth muscle of colon biopsy specimens from patients with CIPO, whereas its expression was significantly lower or absent in controls and in patients with HSCR (Figure 6, *lower panels*). Conversely, *RBPMS2* transcripts were expressed in ENS of colon sections from control neonates and patients with CIPO,

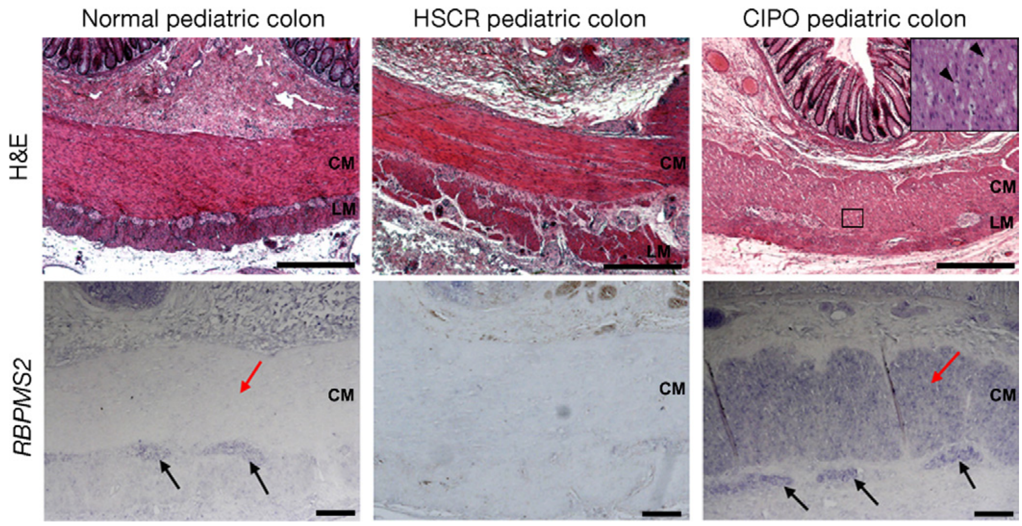

Figure 6. *RBPMS2* transcript expression is abnormally high in smooth muscles from colon specimens of patients with CIPO. H&E staining of colon sections from pediatric patients with CIPO, HSCR, and from neonates without digestive motility disorders (controls) (*upper panels*). The visceral musculature is composed of circular smooth muscles (CM) and longitudinal smooth muscles (LM). High-magnification inset of the CIPO circular muscle layer shows extensive vacuolization (*arrowheads*). In situ hybridization using an anti-human *RBPMS2* riboprobe of consecutive sections from the same specimens (*lower panels*). *Arrows* and *red arrows* indicate staining in ganglia cells and in the circular smooth muscle layer, respectively.

but not in patients with HSCR (owing to congenital absence of ganglia) (Figure 6).

These results suggest that visceral myopathies are associated with abnormal *RBPMS2* transcript expression in visceral smooth muscles.

Discussion

In this work, we investigated the expression and function of the RNA-binding protein RBPMS2 in the developing chick gastrointestinal tract. *RBPMS2* expression in the chick gastrointestinal mesenchymal layer is regulated temporally because it is high at E4–E6 and then progressively is reduced at a later stage. This dynamic expression pattern corresponds to the progression of visceral undifferentiated mesenchymal cells into differentiated SMCs. Only few genes have such a dynamic expression pattern in visceral SMC precursors. *αSMA* is expressed as early as *RBPMS2*, but then is maintained also in differentiating SMCs, when *calponin*, *SM-MHC*, and *myocardin* also are expressed. These data identify RBPMS2 as a marker of undifferentiated visceral SMCs.

We then show that sustained *RBPMS2* expression hinders visceral SMC differentiation in vivo and in SMC primary cultures through up-regulation of *Noggin* expression that leads to inhibition of the BMP signaling pathway. Previous studies showed that exogenous stimulation of BMP activity could prevent dedifferentiation of vascular SMCs in cell culture, suggesting that BMP activation is essential for modulating the vascular SMC phenotype.[19] Our findings confirm this observation in vivo and suggest that *Noggin* is a key regulator during visceral SMC development and also may be involved in the phenotypic regulation of SMCs in culture (Figure 7).

We also found differences between the action of RBPMS2 and Noggin mainly in SMC primary cultures. Indeed, *RBPMS2*, but not *Noggin*, overexpression induced SMC proliferation and transient repression of αSMA expression. Therefore, in addition to the Noggin-BMP axis, RBPMS2 might regulate other pathways that contribute to the dedifferentiation and increased proliferation of visceral SMCs, thus dissociating the effect on proliferation from the effect on differentiation.

We show that *RBPMS2* and *Noggin* overexpression in differentiated SMCs hinders their differentiation associated with myocardin up-regulation. Indeed, recently, Yin et al[22] reported that myocardin accumulation inhibits its

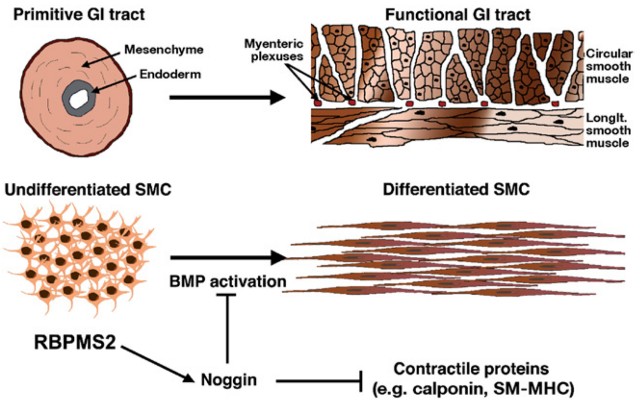

Figure 7. Model of the gastrointestinal SMC development regulated by the RBPMS2 and BMP pathways. RBPMS2 regulates the early stage of SMC differentiation through the control of the expression of the BMP inhibitor Noggin and the inhibition of contractile proteins.

own function and that proteosomal degradation of myocardin is required for its full transcriptional activity. Similarly, we show that myocardin accumulation results in a reduction of calponin expression (a marker of differentiated SMCs). In addition, *RBPMS2* and *Noggin* misexpression led to up-regulation of the SRF target gene *FHL2*, which interacts with SRF and inhibits induction of smooth muscle contractile genes by SRF.[21] Altogether, these results support the notion that activation of the RBPMS2/Noggin pathway inhibits SMC contractile genes through functional alteration of the myocardin/SRF pathway.

In conclusion, we show that, in chick embryos, *RBPMS2* is expressed during the early stages of visceral SMC development and that its expression is progressively lost during differentiation of visceral smooth muscles. Ectopic expression of *RBPMS2* in primary culture of differentiated SMCs triggers an increase of their proliferative rate and hinders their contractile function, which also was observed at the organ level. Our findings show that regulated expression of *RBPMS2* is important for the correct development and differentiation of visceral SMCs. We then found that *RBPMS2* transcript expression was significantly higher in circular smooth muscle cells from colon specimens of pediatric patients with CIPO (digestive dysmotility syndrome in the absence of physical obstruction of the bowel),[4–7,26] whereas its expression was very low or absent in specimens from patients without digestive motility disorders or with HSCR (a developmental ENS disorder). Some investigators have reported abnormal architecture of the tunica muscularis of colon specimens from patients with CIPO, suggesting a potential primary alteration of the visceral smooth muscles.[6,7] In summary, we identified *RBPMS2* as a new marker of visceral SMC remodeling that could be useful for the characterization of smooth muscle alteration in visceral myopathies. Future studies are needed to investigate the potential function of RBPMS2 in CIPO etiopathogenesis, particularly the alteration in the tunica muscularis.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of

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Reprint requests

Address requests for reprints to: Pascal De Santa Barbara, PhD, INSERM U1046, Université Montpellier 1, Université Montpellier 2, Montpellier, France. e-mail: pascal.de-santa-barbara@inserm.fr; fax: (33) 467-415-231.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Western Blotting

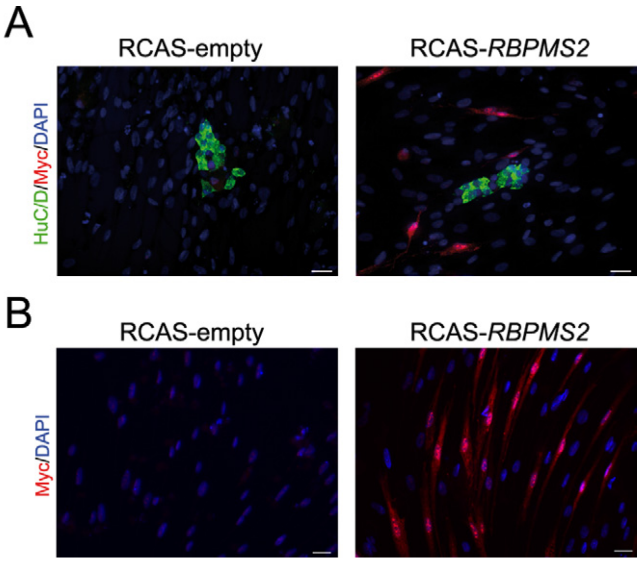
For protein immunoblotting, protein extracts (10 μg) were separated on 10% polyacrylamide gels (Bio-Rad Laboratories, France) and then blotted on nitrocellulose membranes. Membranes were incubated with primary antibodies (anti-Myc from Ozyme [Montigny-Le-Bretonneux, France], anti-avian calponin from Sigma-Aldrich, anti-phosphoSMAD1 from Cell Signaling [Danvers, MA], anti-myocardin from Santa Cruz Biotechnology [Santa Cruz, CA], anti–glyceraldehyde-3-phosphate dehydrogenase from Sigma-Aldrich, and anti-total AKT and anti–P-AKT from Cell Signaling) overnight and then with the relevant horseradish peroxidase–conjugated secondary antibodies. Detection was performed by chemiluminescence on Kodak films. Glyceraldehyde-3-phosphate dehydrogenase expression was used to confirm equal loading.

In Situ Hybridization and Immunodetection

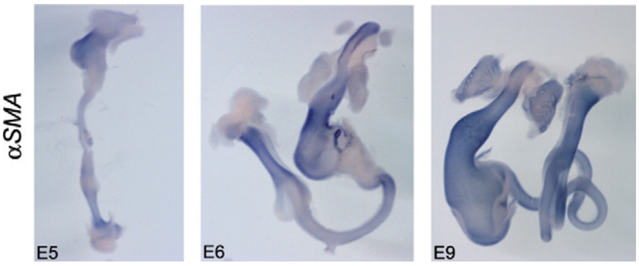
Immunofluorescence and immunohistochemistry experiments with chick and human paraffin-embedded sections were performed as described.[11,17] For immunodetection, anti-αSMA (Sigma-Aldrich), anti-Myc (Ozyme), anti-avian calponin (Sigma-Aldrich), anti-HuC/D (Invitrogen, France), anti-desmin (Euromedex, Mundolsheim Cedex, France), and anti-phospho-histone H3-Ser10 (Millipore, Molsheim, France) antibodies were used. Nuclei were stained with Hoechst (Invitrogen). H&E staining was performed using standard procedures. In situ hybridization experiments using dissected gut or paraffin sections were performed as described.[11,16] Anti-sense riboprobes were generated by PCR amplification using specific primer sets and subcloned. The following chick templates were used: *αSMA*, *Env*, *RBPMS2*, *BARX1*, *SHH*, and *Noggin*.[24] Human *RBPMS2* complementary DNA was isolated, sequenced, and used to prepare riboprobes for in situ hybridization. Images were acquired using a Nikon-AZ100 stereomicroscope and a Carl-Zeiss AxioImager microscope.

Microarray Experiments and QPCR

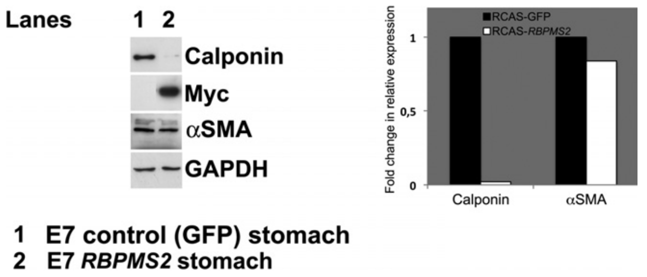
Total RNAs were extracted from SMC primary cultures with the HighPure RNA Isolation kit (Roche Diagnostic, France) and reverse transcription was performed as described.[11] For microarray experiments, resulting complementary DNAs were biotinylated and hybridized to Affymetrix GeneChip Chicken Genome Arrays (Santa Clara, CA) following the manufacturer's protocols (IRB, CHRU Montpellier, France).[11] For QPCR, gene expression levels were measured using LightCycler technology (Roche Diagnostics). PCR primers (Supplementary Table 1) were designed using the LightCycler Probe Design software 2.0. Each sample was assayed from 3 independent experiments performed in triplicate. Expression levels were determined with the LightCycler analysis software (version 3.5) relative to standard curves. Data were represented as the mean level of gene expression relative to the expression of the reference gene *ubiquitin*. Data were analyzed using the Student t test and results were considered significant when the P value was less than .05 (*), $P < .01$ (**), or $P < .001$ (***) in Figures 3 and 5 and Supplemental Figures 4, 8, and 10.



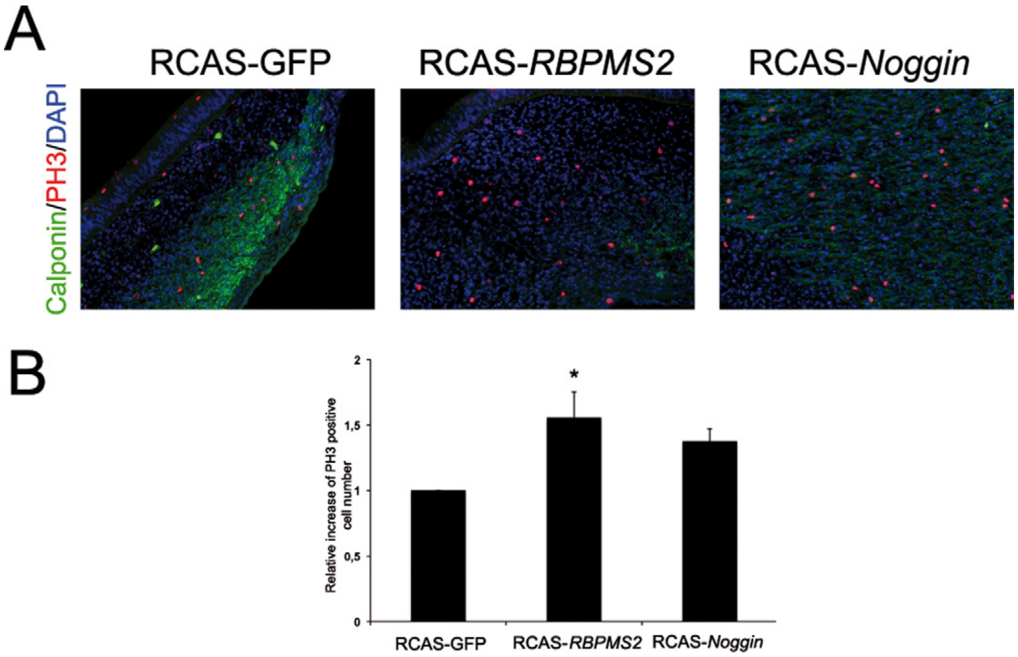
Supplementary Figure 1. Efficiency and trophism of avian retroviruses on differentiated SMC primary cultures. (*A*) Enteric neurons are not targeted by the retroviral misexpression system used in this study. Immunofluorescence analysis of SMC primary cultures prepared from E15 gizzard muscles infected with Myc-tagged *RBPMS2* (RCAS-*RBPMS2*) or RCAS-empty retroviruses for 3 days. *RBPMS2* retroviruses targeted exclusively visceral SMCs as indicated by Myc expression. Ectopic *RBPMS2* expression had no indirect effect on the formation of the enteric plexus and on neuron differentiation as observed using anti-HuC/D antibodies. *Scale bar*, 20 μm. (*B*) Immunofluorescence analysis of primary cultured SMCs infected with high titers of RCAS-*RBPMS2* or RCAS-empty retroviruses for 3 days. Nuclei were visualized with Hoechst. Anti-Myc antibodies were used to identify cells infected by RCAS-*RBPMS2* retroviruses. *Scale bar*, 20 μm.



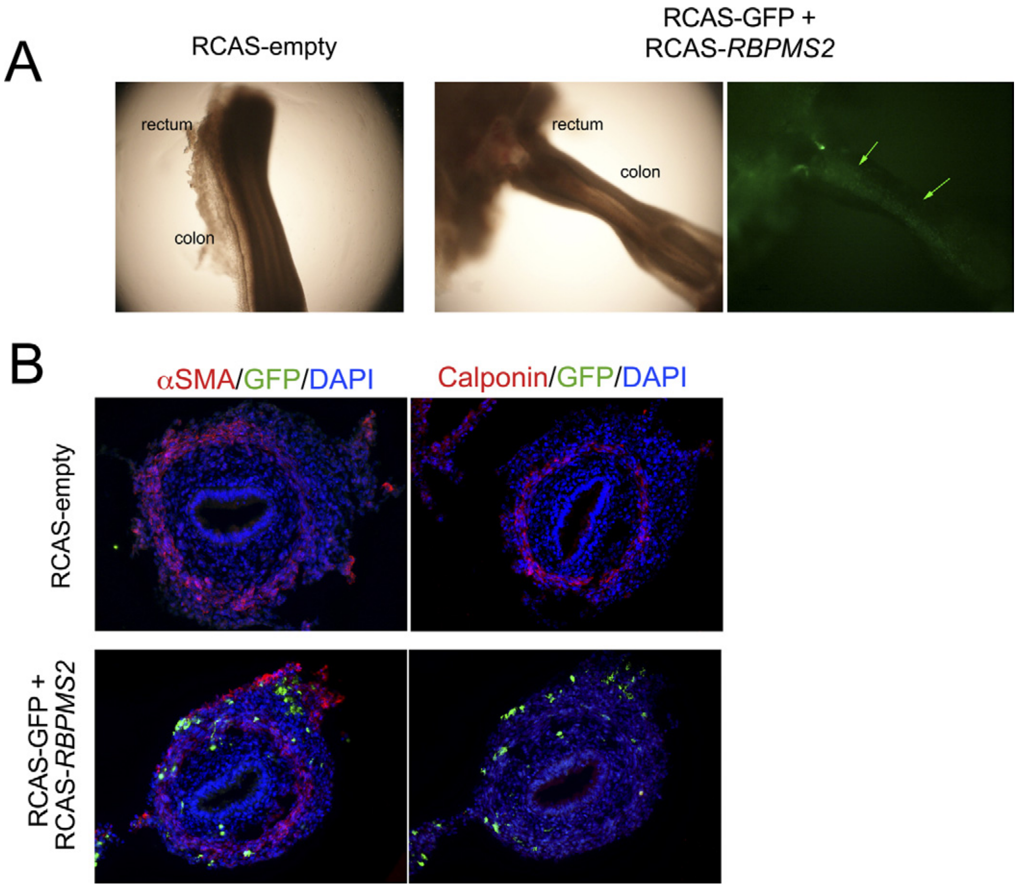
Supplementary Figure 2. α*SMA* expression in the chick gastrointestinal system. Whole-mount in situ hybridization of dissected E5, E6, and E9 chick gastrointestinal tracts using an anti-sense riboprobe directed against α*SMA*. α*SMA* is expressed in the whole mesenchymal gastrointestinal tract with no difference along the anteroposterior axis in the developing and differentiated visceral smooth musculature.



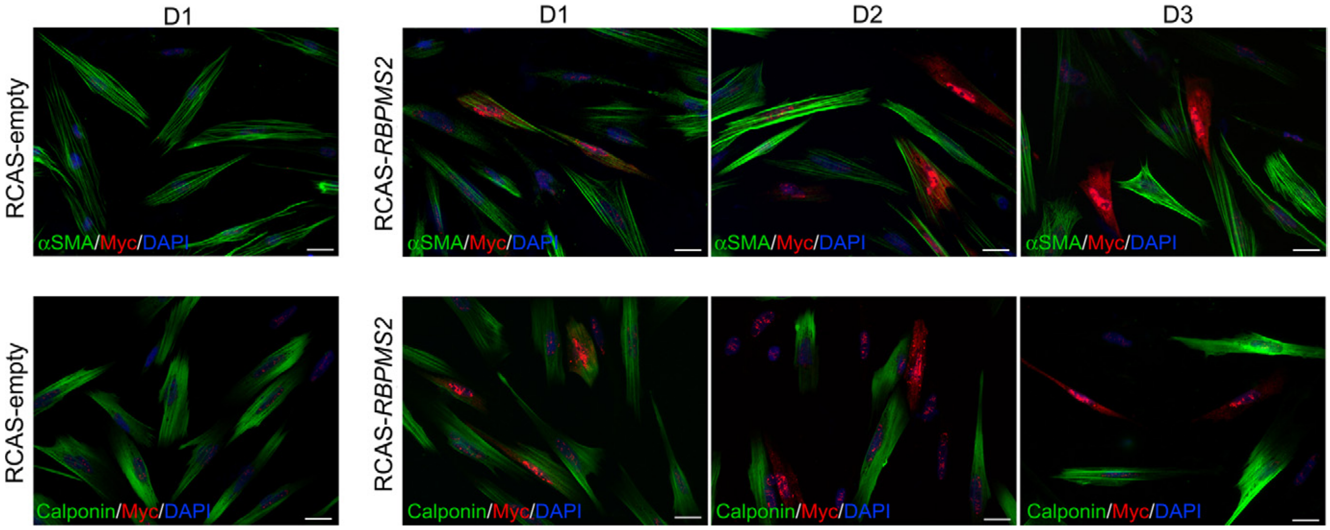
Supplementary Figure 3. Sustained *RBPMS2* expression strongly inhibits calponin expression. Western blot analysis of protein extracts from RCAS-Myc-*RBPMS2* misexpressing stomachs (E7) compared with control organs (RCAS-GFP). A total of 10 μg of whole protein extracts were processed for each condition. Chick calponin was detected with anticalponin antibodies (Sigma-Aldrich). The *right panel* shows the quantification of the Western blot data.



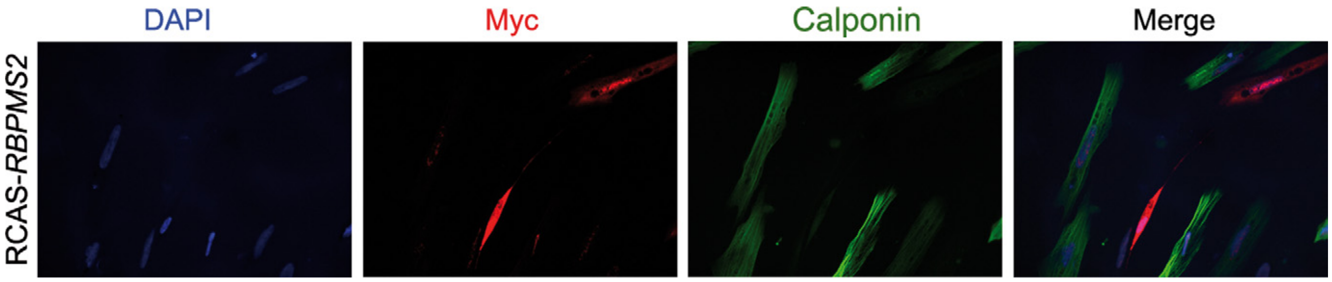
Supplementary Figure 4. Impact of *RBPMS2* and *Noggin* sustained expression on visceral SMC differentiation and proliferation in vivo. (*A*) Serial transversal sections from E9 chick stomachs infected with RCAS-GFP, RCAS-*RBPMS2*, or RCAS-*Noggin* retroviruses were analyzed by immunofluorescence. Differentiated SMCs were identified with anticalponin antibodies and mitotic cells with anti-PH3 antibodies. Sustained *RBPMS2* and *Noggin* expression inhibit calponin expression. (*B*) Quantification of mitotic cells using the mitotic marker PH3 in E9 chick stomachs infected with RCAS-GFP, RCAS-*RBPMS2*, or RCAS-*Noggin* retroviruses by immunofluorescence. Values are the mean ± SEM of 3 independent experiments. Statistical analysis using the Student *t* test confirmed the significant increase (1.55-fold; $^*P < .05$) of PH3-positive cells upon *RBPMS2* misexpression. The 1.37-fold induction after *Noggin* misexpression was not statistical significant.



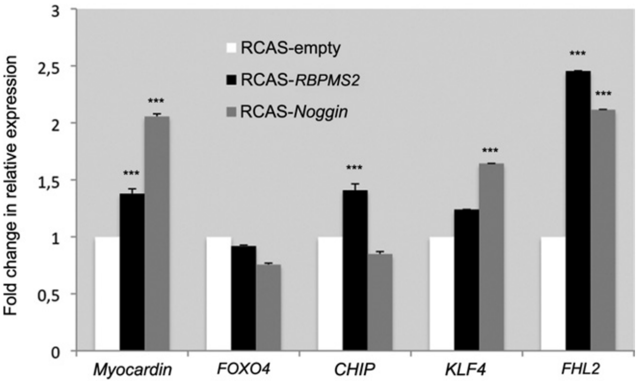
Supplementary Figure 5. Sustained *RBPMS2* expression alters chick colonic SMC differentiation. (*A*) E7 colons after retroviral misexpression of RCAS-empty (control) (*left panel*) or of RCAS-*RBPMS2* and RCAS-GFP (*right panels*). The presence of retroviruses was confirmed by direct observation of GFP expression (*right panel*; *green arrows* indicate the colon). (*B*) Serial cross-sections of E7 colons infected with RCAS-empty (control; *upper panels*) or RCAS-*RBPMS2* retroviruses (*lower panels*) analyzed by immunofluorescence. Nuclei were visualized with Hoechst. Antibodies directed against αSMA and calponin were used. Anti-GFP antibodies were used to confirm infection of the colon musculature. Sustained expression of *RBPMS2* specifically in the colon resulted in no morphologic alterations in comparison with controls. In addition, *RBPMS2* misexpression did not affect the determination of colon SMCs because αSMA-positive cells still were observed in the smooth muscle layer. (*B*) Conversely, it inhibited calponin expression in comparison with control colons, indicating that *RBPMS2* misexpression hinders SMC differentiation also in the developing colon.



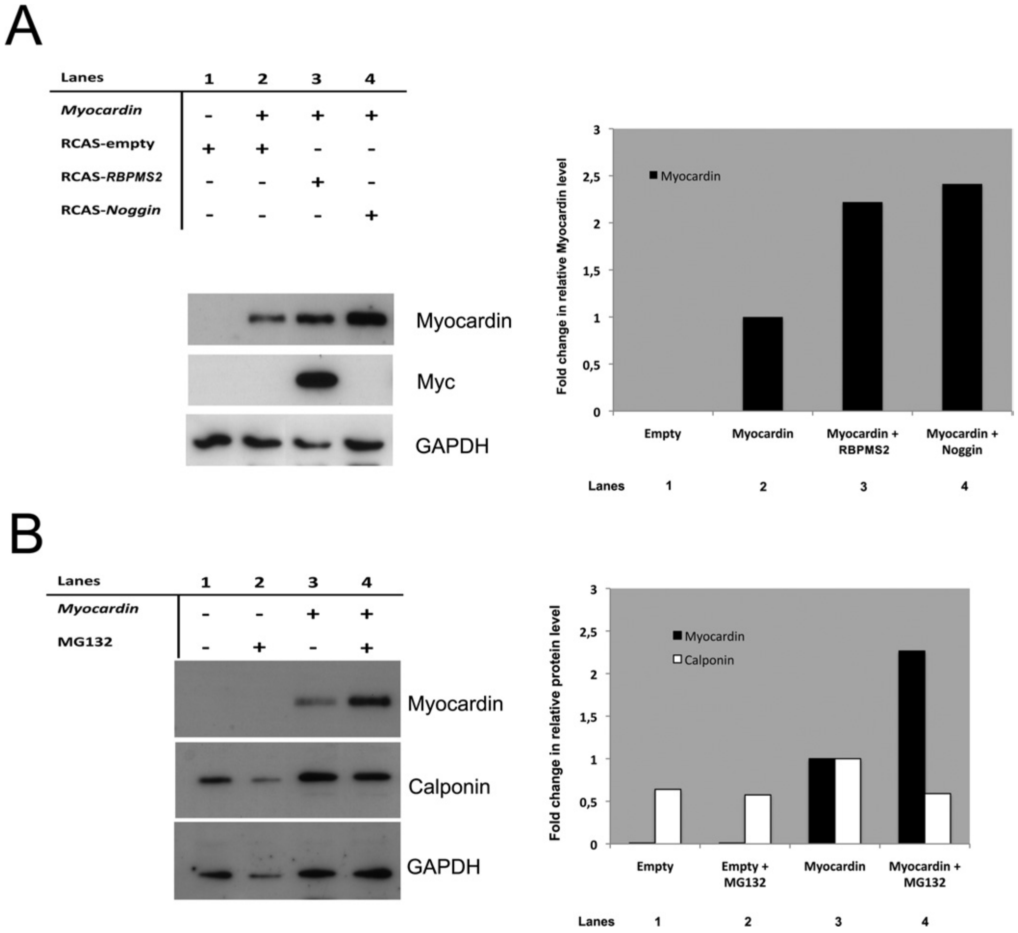
Supplementary Figure 6. Time-line study of *RBPMS2* overexpression in primary cultures of differentiated SMCs. Immunofluorescence analysis of primary SMCs infected with RCAS-empty (*left panels*) or RCAS-*RBPMS2* (*right panels*) retroviruses for 1 (D1), 2 (D2), and 3 (D3) days. Nuclei were visualized with Hoechst. Antibodies directed against SMC markers: αSMA (*upper panels*) and calponin (*lower panels*) were used. Anti-Myc antibodies were used to identify cells infected by RCAS-*RBPMS2* retroviruses. *RBPMS2* misexpression inhibited first the expression of calponin (after 2 days), and after 3 days αSMA expression also was down-regulated. *Scale bar*, 20 μm.



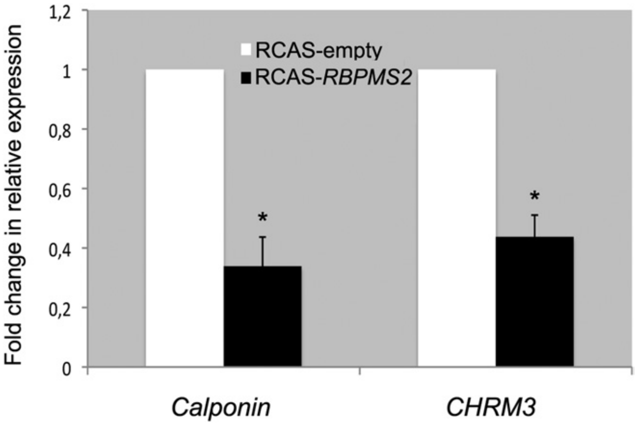
Supplementary Figure 7. Immunofluorescence analysis of SMC primary cultures prepared from E15 gizzard muscles and infected with Myc-tagged *RBPMS2* (RCAS-*RBPMS2*) retroviruses for 3 days. In *RBPMS2*-overexpressing cells, calponin expression is inhibited.



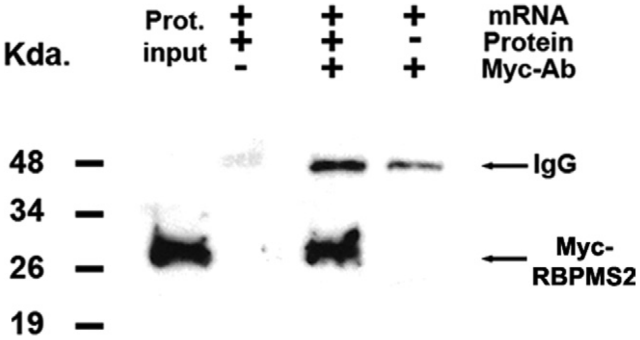
Supplementary Figure 8. Expression of mRNA levels coding for different protein partners of myocardin/SRF that modulate myocardin/SRF function during visceral SMC differentiation analyzed by QPCR in primary cultured SMCs infected with RCAS-empty, RCAS-*RBPMS2*, or RCAS-*Noggin* retroviruses for 7 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-*RBPMS2* and RCAS-*Noggin* vs RCAS-empty). We confirmed the up-regulation of *Myocardin* mRNAs upon *RBPMS2* and *Noggin* overexpression. We then found that *Noggin* overexpression increased *Kruppel-like factor 4* (*KLF4*) mRNA expression by 1.5-fold. Conversely, *Noggin* and *RBPMS2* overexpression did not affect *Forkhead box O4* (*FOXO4*) and *C terminus of Hsc70-Interacting Protein (CHIP)* expression, whereas it increased by more than 2-fold the level of *FHL2* mRNA. ***$P < .001$.



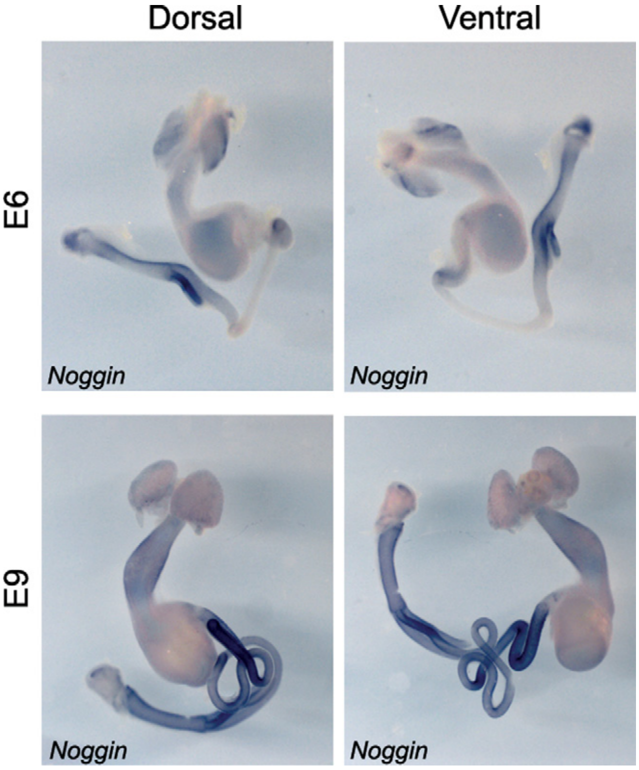
Supplementary Figure 9. Accumulation of myocardin protein in visceral SMC inhibits its function. (*A*) Western blot analysis of primary SMC cultures after *Myocardin* and *RBPMS2* or *Noggin* ectopic expression. Primary differentiated SMCs were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and cotransfected with JetPEI (Polyplus, France) with 2.5 μg of a mouse myocardin construct and 2.5 μg of empty or *RBPMS2* or *Noggin* retroviral constructs and analyzed after 48 hours. A total of 15 μg of whole protein extracts were processed for each condition. Myocardin (95 kilodaltons) was detected with the H300 antibody. Co-expression of Myocardin and *RBPMS2* or *Noggin* results in accumulation of Myocardin (*lanes 3 and 4*). *Right panel* shows the quantification of the Western blot data. (*B*) Western blot analysis of primary SMC cultures after proteasomal inhibition with 5 μmol/L MG132. Primary differentiated SMCs were grown in DMEM supplemented with 10% FBS and transfected, or not, with 5 μg of a mouse myocardin construct and analyzed after 48 hours. MG132 (proteasome inhibitor) was added to the medium for 12 hours after 36 hours of transfection. A total of 15 μg of whole protein extracts were processed for each condition. SMCs cultured in 10% FBS express low level of calponin (*lane 1*) that is not changed by incubation with MG132 (*lane 2*). Ectopic expression of Myocardin induces an increase of calponin expression (*lane 3*). Incubation with MG132 leads to myocardin accumulation, but reduces calponin expression (*lane 4*). *Right panel* shows the quantification of the Western blot data.



Supplementary Figure 10. Analysis of *cholinergic receptor muscarinic 3* (*CHRM3*) and *calponin* mRNA expression by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 7 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-*RBPMS2* vs RCAS-empty). **P* < .05. *CHRM3* mRNA level was decreased by 56% and *calponin* mRNA level by 67% in *RBPMS2*-overexpressing SMCs compared with control SMCs.



Supplementary Figure 12. Western blot analysis with anti-Myc antibodies after immunoprecipitation to assess the interaction between RBPMS2 and *Noggin*. Myc-tagged RBPMS2 in infected DF-1 cells was immunoprecipitated with anti-Myc antibodies bound to protein A Sepharose beads in the presence of total RNA from E6 chick gut. Fifty percent of beads were analyzed by Western blotting to detect Myc-tagged proteins. In the input and positive experiment lines a 26- kilodalton band corresponding to Myc-tagged RBPMS2 was observed.



Supplementary Figure 11. *Noggin* expression in the chick gastrointestinal (GI) system. Whole-mount in situ hybridization of dissected E6 and E9 chick GI tracts using an antisense riboprobe directed against *Noggin*. Early expression of *Noggin* is observed in the mesenchyme of the GI tract at E6.

Supplementary Table 1. Gene Specific Chick (c) Primers Used for QPCR

Targets	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon, bp
caSMA	CTG TAT GCT TCT GGG CG	GCA GTG GTC ACA AAG GAG	188
cCalponin	GAC GAA GGG CAA CAA CG	CAA GGG CTG GTC TGT CC	202
cSM22	TGA GCA GGG ATG TCC AGT	AGC CAA TGA TGT TCT TGC C	501
cMyocardin	CTT CTG TCA GCA ACA CCC	AAG ACT GCG ACT GGT AAC	300
cSRF	CAG GCA CCA CAG CAA AC	CAG CCG AGA CTG GGA TT	328
cNoggin	ACC CGG ACC CTA TCT TTG	CAG CCC GTC GTA GAA CTC	218
cUbiquitin	GGG ATG CAG ATC TTC GTG AAA	CTT GCC AGC AAA GAT CAA CCT T	147
cKLF4	GCT GCG GAT GGA AGT TTG	GCT GGC ACT GGA AAG GG	84
cFHL2	CAC AAA CCA ATC AGT GGT C	TTC AGG GCA GAG GAT GTC A	151
cCHIP	CTC ACT TCT TCC TGG GGC	TCG TTC TCT TGG TTG ATC CG	193
cFOXO4	AGC CGA ACT CTT CTG CTT CTA	ACT GGA GCC TTC AAA CTG TTA	180
cCHRM3	ACT TAC ATA CAG AGC CAA ACG A	ATG ATA GGT TCA CTT AGA AAC TGG A	174

CHRM3, cholinergic receptor muscarinic 3.