The endoplasmic reticulum stress transducer OASIS is involved in the terminal differentiation of goblet cells in the large intestine

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Running title: Physiological UPR during goblet cell maturation

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Background: OASIS is an ER stress sensor expressed in the large intestine.
Results: Mature goblet cells are decreased in *Oasis*-/- mice. Knockdown of the *Oasis* transcript impairs the maturation of goblet cells.
Conclusion: OASIS plays crucial roles in terminal differentiation of goblet cells.
Significance: OASIS signaling during the ER stress response is involved in cell differentiation and maturation.

OASIS is a bZIP transmembrane transcription factor localized in the endoplasmic reticulum (ER) that is cleaved in its transmembrane region in response to ER stress. This novel ER stress transducer has been demonstrated to express in osteoblasts and astrocytes and promote terminal maturation of these cells. Additionally, OASIS is highly expressed in goblet cells of the large intestine. In this study, we investigated the roles of OASIS in goblet cell differentiation in the large intestine. To analyze the functions of OASIS in goblet cells, we examined morphological changes and the expression of goblet cell differentiation markers in the large intestine of *Oasis*-/- mice. By disrupting the *Oasis* gene, the number of goblet cells and production of mucus were decreased in the large intestine. *Oasis*-/- goblet cells showed abnormal morphology of mucous vesicles and rough ER. The expression levels of mature goblet cell markers were lower, and conversely those of early goblet cell markers were higher in *Oasis*-/- mice, indicating that differentiation from early to mature goblet cells is impaired in *Oasis*-/- mice. To determine the association of OASIS with other factors involved in goblet cell differentiation, in vitro experiments using cell culture model were performed. We found that OASIS was activated in response to mild ER stress which is induced in differentiating goblet cells. Knockdown of the *Oasis* transcript perturbed goblet cell terminal differentiation. Together, our data indicate that OASIS plays crucial roles in promoting the differentiation of early goblet cells to mature goblet cells in the large intestine.

The endoplasmic reticulum (ER) is a central cellular organelle responsible for the synthesis, folding and posttranslational modifications of proteins destined for the secretory pathway. A number of cellular stress conditions lead to the accumulation of unfolded or misfolded proteins in the ER lumen. These conditions, which are collectively termed ER stress, have the potential to induce cellular damage (1, 2). The ER responds to these perturbations by activating an integrated signal transduction pathway through the ER stress transducers, which is called the unfolded protein response (UPR) (3-5). The UPR involves at least three distinct components; 1) translational attenuation to decrease the demands made on the organelle (6); 2) transcriptional induction of genes encoding ER-resident chaperones to facilitate protein folding (7, 8); and 3) ER-associated degradation (ERAD) to degrade the unfolded proteins accumulated in the ER (9, 10). If these strategies fail, cells undergo ER stress-induced apoptosis (11, 12). The UPR was originally described as a system by which cells evade damage in response to acute ER perturbation. However, recent advances have revealed that the UPR also provides important signals for regulating cell differentiation and maturation, or the maintenance of basal cellular homeostasis (13-16).

Previously, we identified OASIS (old astrocyte specifically induced substance) as a novel ER stress transducer (17). OASIS is basic leucine zipper (bZIP) transcription factor that belongs to the cyclic AMP-responsive element (CRE)-binding protein/activating transcription factor (ATF) family. Although OASIS is localized to the ER membrane under normal conditions, it is cleaved at the membrane in response to ER stress. Consequently, its cleaved N-terminal cytoplasmic domain, which contains the bZIP domain, translocates into the nucleus where it activates the transcription of target genes (18, 19). High expression of OASIS was observed in the osteoblasts of osseous tissues and the astrocytes of the central nervous system (20-22). From the analysis of knockout mice, OASIS has been demonstrated to be involved in terminal differentiation and osteoblasts (23-25) and astrocytes (submitted by Saito).

The intestinal epithelium is composed of four distinct cell types, including the absorptive enterocytes and the goblet, Paneth and enteroendocrine secretory cell lineages (26, 27). Stem cells are committed to generate these lineages by the Wnt and Notch signaling cascades: Wnt signaling is required for the generation of the secretory lineages, whereas Notch signaling is necessary for the differentiation of enterocytes (28, 29). However, the molecular mechanisms underlying the differentiation of intestinal epithelial cells are incompletely defined. Recently, the ETS (E26 transformation-specific)-domain transcription factor, SPDEF (SAM-pointed domain-containing ETS-like factor) (30, 31), has been reported to act downstream of ATOH1 (32), which is an essential determinant of secretory lineages downstream of β-catenin (33, 34). Maturation of Paneth and goblet cells has been shown to be impaired in *Spdef* deficient mice, whereas immature secretory progenitors accumulate in the intestine (35). These observations suggest that SPDEF promotes the terminal differentiation of a secretory progenitor pool into Paneth and goblet cells. Interestingly, the expression of CREB4, an ER stress transducer that is structurally similar to OASIS, is completely abolished in *Spdef*-deficient Paneth and goblet cells (35). These observations suggest that the expression of CREB4 is controlled at downstream of SPDEF, and that ER stress response signaling through ER stress transducers such as CREB4 mediates the differentiation and maturation of secretory cell lineages in the intestinal epithelium. In this study, we investigated the roles of the ER stress transducer OASIS, which is expressed in the crypt base of the large intestinal epithelium, in the differentiation of goblet cells.

Consequently, we demonstrated that OASIS plays crucial roles in promoting the terminal differentiation of goblet cells in the large intestine.

EXPERIMENTAL PROCEDURES

Animals – 3-week old C57BL/6 mice or *Oasis*-/- mice were used in this study. The *Oasis*-/- mice were previously established in our laboratory (22-24). In all studies comparing wild-type and *Oasis*-/- mice, sex-matched littermates derived from the mating of *Oasis*+/- mice were used. The experimental procedures and housing conditions for animals were approved by the Committee of Animal Experimentation, Hiroshima University.

Cell culture, treatments and transfection – LS174T cells were cultured in Eagle's minimal essential medium supplemented with 10% FCS and 1% non-essential amino acids. LS174T cells were cultured overnight in 9.2 cm^2 dish for RNA or protein isolation, and in a Lab–Tek Chamber Slide (Nalge Nunc) for periodic acid-Schiff (PAS) staining. Subsequently, cells were treated with 2 mM sodium butyrate, which induces LS174T cells to differentiate to mature goblet cells. For *Oasis* knockdown experiments, LS174T cells were transfected with 1 μg *Oasis* siRNA (predesigned siRNA pool targeting *Oasis*: AAAAGAAGGUGGAGACAUU, GGGACCACCUGCAGCAUGA, GAAGGAGUAUGUGGAGUGU, and CAGGAGAGCCGUCGUAAGA; Thermo Scientific Dermacon, M-008579-01-0005) or control siRNA (Silencer Cy3 labeled negative control No.1 siRNA; Life Technologies, AM4621). Transfection was performed 12 hrs before treatment with sodium butyrate using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocols. 24 hrs after treatment with sodium butyrate, PAS staining was performed as described previously (36). The PAS-stained slide was counterstained with hematoxylin solution.

RNA isolation and RT-PCR – Total RNA was isolated from the large intestine of 3-week old mice or LS174T cells using ISOGEN (Wako) according to the manufacturer's protocol. First-strand cDNA was synthesized in a 20 μl reaction volume using a random primer (Takara) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was performed using each specific primer set in a total volume of 30 μl containing 0.8 μM of each primer, 0.2 mM dNTPs, 3 units of *Taq* polymerase, and 10× PCR buffer (Agilent). Primer sequences are summarized in Supplementary Information, Table S1. The PCR products were resolved by electrophoresis on a 4.8% acrylamide gel. The density of each band was quantified using the Adobe Photoshop Elements 2.0 Program (Adobe Systems Incorporated).

Western blotting – For western blotting, proteins were extracted from LS174T cells using cell extraction buffer containing 10% SDS, 0.5 M EDTA (pH 8.0), 100 mM methionine, and a protease inhibitor mixture (MBL International). The lysates were incubated on ice for 45 min. After centrifugation at 16,000 × *g* for 15 min, the protein concentrations of the supernatants were determined. Protein-equivalent samples were loaded onto sodium dodecyl sulfate-polyacrylamide gels. Anti-β-actin (Sigma; 1:3000) and anti-OASIS (purified from a hybridoma as described previously (22, 23)) antibodies were used for western blotting. The density of each band was quantified using the Adobe Photoshop Elements 2.0 Program (Adobe Systems Incorporated).

Histological analysis and in situ hybridization – Large intestine from 3-week old mice was fixed overnight in 10% Formalin Neutral Buffer Solution (Wako). Samples were then dehydrated with ethanol, embedded in paraffin and sectioned (5 μm). Hematoxylin-eosin staining and PAS staining were performed

according to standard protocols. *In situ* hybridization was performed using digoxigenin-labeled cRNA probes (Supplementary information, Table S2). Antisense and sense probes were made by in vitro transcription in the presence of digoxigenin-labeled dUTP, using various cDNAs subcloned into the pGEM-Teasy vector (Promega) as templates. Large intestine isolated for *in situ* hybridization was frozen immediately and sectioned (6 μm). The frozen sections were fixed for 20 min with 4% formalin in phosphate-buffered saline (PBS, pH 7.4). The sections were then washed with PBS and treated with 0.1% proteinase K for 5 min. After washing with PBS, the sections were re-fixed for 20 min with 4% formalin in PBS, and treated with 0.1M triethanolamine, 2.5% anhydrous acetic acid for 10 min, followed by washing with PBS. Sections were prehybridized for 1 hr at 37°C in hybridization buffer (0.01% Dextran Sulfate, 0.01M Tris-HCl pH 8.0, 0.05 M NaCl, 50% Formamide, 0.2% Sarcosyl, 1× Denhardt's solution, 0.5 mg/ml Yeast tRNA, 0.2 mg/ml Salmon testis DNA), and then hybridized overnight at 55°C in hybridization solution with 100 ng/ml cRNA probe. After washing with 4× saline sodium citrate buffer for 20 min at 60°C, the sections were further washed 2× saline sodium citrate buffer, 50% formamide for 30 min at 60°C. Sections were treated RNaseA in RNase buffer (10 mM Tris-HCl pH7.4, 1 mM 0.5 M EDTA (pH 8.0), 0.5 M NaCl) for 30 min at 37°C to remove un-hybridized probe. After RNase treatment, sections were washed with 2× saline sodium citrate buffer, 50% formamide for 30 min at 60°C, and then blocked with 1.5% blocking reagent in 100 mM Tris-HCl pH 7.5, 150 mM NaCl for 1 hr at room temperature. For detection of digoxigenin-labeled cRNA probes, anti-digoxigenin antibody conjugated to alkaline phosphatase was used at a dilution of 1:500, and color was developed by incubation with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solution.

Electron microscopy – Large intestine from 3-week old mice was fixed in 1% glutaraldehyde in PBS for 15 min. After washing with distilled water, the tissues were post-fixed in 0.5% osmium tetroxide in 0.1 M cacodylate buffer for 30 min. Following dehydration, the tissues were embedded in EPON812, and ultra-thin sections were stained with uranyl acetate and lead citrate. Stained sections were visualized using a Hitachi 7100 electron microscope operated at 80 kV. The mean cell area was determined using ImageJ software (NIH).

Statistical analysis – Statistical comparisons were made using the unpaired-Student's-t-test. Statistical significance between two samples was determined by a P value of less than 0.05. P values of less than 0.05, 0.01, or 0.001 are described as *P < 0.05, **P < 0.01, or ***P<0.001, respectively.

RESULTS

OASIS was highly expressed in immature goblet cells of the large intestine – We previously reported that OASIS is expressed in osteoblasts and astrocytes (17, 20, 21, 23). To examine the tissue distribution of *Oasis* mRNA more precisely, we performed RT-PCR using mRNA isolated from various tissues of 3-week old mice. We detected strong *Oasis* mRNA signals in submandibular gland, lung, stomach, and in the large intestine where it was most intense (Fig.1A). *Oasis* mRNA was expressed highly in all portions of the large intestine except for the appendix. In contrast, the levels of *Oasis* mRNA were very low in the small intestine (Fig.1B). In the digestive tract, there are three distinct cell types, i.e. absorptive enterocytes, enteroendocrine cells, and goblet cells. To identify which of these cells expressed *Oasis* mRNA, we carried out *in situ* hybridization using *Oasis* cRNA probes. The

Oasis signals were focally detected in the base of the crypt, but not in the apical portion of the crypt (Fig.1C). In contrast, *Gapdh* mRNA was observed in all cells (both basal and apical) of the crypt. The cells expressing *Oasis* mRNA possessed vacuoles in their cytosol, indicating that OASIS is expressed in goblet cells. Moreover, the goblet cells at the base of the crypt are immature cells that are developing from intestinal stem cells (27, 37, 38). Thus, we concluded that the cells expressing OASIS were immature goblet cells.

The numbers of mature goblet cells decreased in Oasis-/- mice – To elucidate the functions of OASIS in immature goblet cells, we first performed histological analysis in the *Oasis*-/- mice that we generated previously (22-24). The mice were born at the expected Mendelian ratios and were fertile, but showed impaired bone formation. However, the general morphology of all other tissues and organs was normal, and the architecture of the intestinal tract was unaffected. Additionally, formation of the crypt and differentiation of enterocytes also appeared normal. However, the apparent numbers of goblet cells that contained abundant vacuoles were decreased in *Oasis*-/- mice (Fig.2A). PAS staining showed a marked decrease in the number of cells containing mucus both in the proximal and distal large intestine of *Oasis*-/- mice (Fig.2B-E). In contrast, immature cells that contain less mucus were increased in the crypt epithelium, suggesting that the differentiation or maturation of goblet cells was inhibited in *Oasis*-/- mice.

Next, we carried out electron microscopic examination of goblet cells in WT and *Oasis*-/- mice. Normal goblet cells have abundant cytoplasm containing large secretory vesicles (Fig.3A). In *Oasis*-/- mice, almost all goblet cells at the crypt base and in the lower portion of the crypt showed an abnormal ultrastructure in which both the number and size of vesicles were decreased, and the membranes of some vesicles were often fused (Fig.3B). Moreover, the rough endoplasmic reticulum was abnormally expanded in these cells (Fig.3D). In the upper portion of the crypt, cells with abnormal ultrastructure were also observed (Fig.3F). The other types of cells in the crypt epithelium, such as enteroendocrine cells and enterocytes, were morphologically intact in *Oasis*-/- mice (data not shown). Goblet cells are known to be differentiated from intestinal stem cells in the crypt base, and the mature goblet cells move to the apical portion of the crypt. We observed abnormal morphology of goblet cells from the base to the apical portion of the crypt in *Oasis*-/- mice, suggesting that deletion of the *Oasis* gene affected the differentiation or maturation of goblet cells from the intestinal cell lineage involving impairment of vesicle formation.

OASIS functions in the terminal differentiation of goblet cells – We next focused on the roles of OASIS in goblet cell differentiation. Intestinal epithelial stem cells are determined to become cells of the secretory lineage such as goblet cells and enteroendocrine cells by Notch ligand Delta-like 1 (Dll1)(39), whereas absorptive enterocytes are specified by Notch and the transcription factor Hairy and enhancer of split 1 (HES1) (40). Subsequently, secretory progenitor cells are specified to become goblet cells by the transcription factors, Atonal homolog 1 (ATOH1) (33, 34) and Growth factor independent 1 (GFI1) (41, 42). We examined the expression levels of various differentiation markers of the goblet cell lineages in the large intestine of *Oasis*-/- mice by RT-PCR. All genes involved in the primary differentiation from stem cells to early goblet cells or absorptive enterocytes were normally expressed in *Oasis* -/- mice (Fig.4A). Therefore, intestinal epithelial stem cells are correctly specified to both the goblet cell and absorptive enterocyte lineages. Interestingly, the expression of *Trefoil factor 3* (*Tff3*), an early goblet cell marker, was significantly increased in *Oasis*-/- mice (Fig.4A). Conversely, the mature goblet cell markers, *Muchin 2* (*Muc2*), *Anterior gradient 2* (*Agr2*), and *Resistin like*

beta (*Retnlb*), were markedly decreased (Fig.4B). *In situ* hybridization revealed *Tff3* mRNA signals were observed diffusely in the crypt: the intensity was very faint in WT mice, but elevated in *Oasis*-/- mice (Fig.4D). *Muc2* mRNA signals were robustly observed throughout the crypt epithelium in WT mice, but were almost abolished in *Oasis*-/- mice (Fig.4E). These results suggest that differentiation from early to mature goblet cells is impaired in the large intestine of *Oasis*-/- mice.

Recently, the Ets-domain transcription factor, SPDEF, was reported to be required for the terminal differentiation of goblet cells (35). In the large intestine, *Spdef*-/- mice showed a similar phenotype to that of *Oasis*-/- mice, i.e. goblet cells are correctly specified but the terminal differentiation of early to mature goblet cells was impaired. CREB4 is an ER resident CREB/ATF family transcription factor that is structurally very similar to OASIS. Interestingly, the expression of CREB4 was reported to be down-regulated in the intestine of *Spdef*-/- mice, indicating that CREB4 could be a direct target of SPDEF. We therefore examined whether the SPDEF-CREB4 pathway is related to the OASIS signaling pathway. The expression of *Spdef* mRNA was not altered in the large intestine of *Oasis* -/- mice (Fig.4C and 4F), implying that OASIS does not regulate the expression of the *Spdef* gene. In contrast, the expression of CREB4 was significantly decreased in *Oasis* -/- mice (Fig.4C). These data suggest that the expression of CREB4 is regulated by OASIS. Combined with the published data, CREB4 is therefore a common downstream target of OASIS and SPDEF, but the expression of CREB4 is regulated independently. Furthermore, CREB4 may be a marker for the terminal differentiation of goblet cells, and may function together with the OASIS signaling pathway in the maturation of goblet cells.

Activation of OASIS during terminal differentiation of goblet cells – To investigate the roles of OASIS in the terminal differentiation of goblet cells, we examined the expression and activation of OASIS in the human colon cancer LS174T cell line. LS174T cells are secretory progenitors that can be induced to differentiate into goblet cells by treatment with sodium butyrate (36). In LS174T cells treated with 2 mM sodium butyrate for 24 hrs, PAS-positive secretory granules were produced (Fig. 5A), and the expression of mature goblet cell markers such as *Muc2* and *Agr2* was gradually induced from 6 hrs after treatment (Fig.5B). Western blotting showed that the expression of full-length OASIS increased after 6 hrs treatment with sodium butyrate, peaking at 12 hrs following which it gradually decreased. The cleaved fragment of OASIS, p50 OASIS (OASIS N-terminus), also increased in synchrony with the expression of full-length OASIS, indicating that OASIS is activated during the differentiation of LS174T cells to mature goblet cells (Fig.5C). Because OASIS is activated in response to ER stress (17), it is possible that ER stress occurred during goblet cell differentiation. We examined the expression of ER stress markers after treatment with sodium butyrate. Messenger RNAs for the ER stress markers, *Bip* and *Chop,* were slightly but significantly up-regulated in accordance with the pattern of OASIS activation (Fig.5D). These findings suggest that mild ER stress is induced during goblet cell differentiation, and that OASIS is subjected to regulated intramembrane proteolysis in response to the mild ER stress. When differentiating into mature goblet cells, early goblet cells gradually start to produce abundant proteins such as MUC2, and these proteins are overloaded into the ER. Thus, mild ER stress during maturation of goblet cells could be derived from a high demand for synthesis and secretion of mucus materials.

Our findings in *Oasis* -/- mice described above suggest that CREB4 could be expressed as a common downstream target of OASIS and SPDEF during differentiation of early to mature goblet cells. We therefore examined the

expression of *Creb4* and *Spdef* mRNAs after treatment of LS174T cells with sodium butyrate. The expression of *Creb4* mRNA was induced 6 hrs after treatment, the same time point at which expression of p50 OASIS (OASIS N-terminus) began (Fig.5E). *Creb4* mRNA expression then gradually decreased from 12 hrs after treatment. In contrast, although *Spdef* was also induced from 6 hrs, the expression level was maintained until 24 hrs after treatment (Fig.5E), indicating that SPDEF is not downstream of OASIS.

OASIS is required for the differentiation of goblet cells – To determine whether OASIS is indispensable for the differentiation of goblet cells, we examined the effects of *Oasis* knockdown using *Oasis* siRNA on the differentiation of LS174T cells. We confirmed that *Oasis* siRNA successfully and significantly suppressed the expression of OASIS in LS174T cells (Fig.6A). PAS-staining showed that secretory granules were significantly decreased in LS174T cells transfected with *Oasis* siRNA following treatment with sodium butyrate (Fig.6B). Furthermore, the mature goblet cell markers, *Muc2* and *Agr2*, were dramatically down-regulated (Fig.6C). These data suggest that inhibition of OASIS expression impaired the terminal maturation of LS174T cells to goblet cells.

Next, to analyze the relationship between the OASIS and SPDEF pathways, we checked the expression of SPDEF and CREB4 in LS174T cells treated with sodium butyrate. Even when the expression of OASIS was suppressed by siRNA, the up-regulation of *Spdef* mRNA following treatment with sodium butyrate was not affected. However, induction of CREB4 by sodium butyrate was significantly inhibited by transfection of LS174T cells with *Oasis* siRNA (Fig.6D). These findings support our hypothesis that CREB4, but not SPDEF, is downstream of OASIS.

The expression of ER stress markers was slightly up-regulated during differentiation of LS174T cells to mature goblet cells (Fig.6E). However, the slight elevation of these genes was not affected by knockdown of *Oasis* mRNA, indicating that mild ER stress occurs prior to OASIS activation during the maturation of goblet cells. Taken together, our data suggest that OASIS plays an essential role in goblet cell differentiation following its activation by mild ER stress (Fig.7).

DISCUSSION

We previously demonstrated that OASIS promotes the differentiation of osteoblasts and is involved in bone formation (23-25). In this study, we found that *Oasis* is highly expressed in goblet cells at the crypt base in the large intestine. Goblet cells synthesize and secrete large amounts of mucus proteins, including mucin, and are similar to osteoblasts in that abundant proteins are loaded into the ER. Therefore, ER stress is easily induced in secretory goblet cells (43, 44). However, immature goblet cells do not produce mucus and do not undergo ER stress. During differentiation from secretory progenitor cells to mature goblet cells, immature cells develop the machinery for dealing with abundant proteins in the ER, including the UPR system. We did in fact demonstrate that ER stress is induced during goblet cell differentiation, and is followed by the induction of genes expressed in mature goblet cells. Thus, it is possible that ER stress is a trigger for the maturation of goblet cells. Similar phenomena are also seen in plasma cell differentiation. For the full development of antibody secretion, activation of the UPR is essential (45, 46). Taken together, previous data and our findings suggest that activation of the UPR by ER stress is required for the maturation of immature cells to "professional" secretory cells.

In the large intestine of *Oasis*-/- mice, the number of mature goblet cells that contain abundant secretory granules was dramatically reduced. We found that *Tff3*, an early goblet

cell marker, was up-regulated, while mature goblet cell markers such as *Muc2*, *Agr2*, and *Retnlb,* were conversely down-regulated. Genes involved in the primary differentiation of immature goblet cells were not affected, indicating that intestinal stem cells are correctly specified to both secretory progenitors and absorptive enterocyte lineages. Thus, we concluded that the abnormal architectures observed in the crypt epithelium of *Oasis*-/- mice are caused by impaired terminal maturation of goblet cells. The anomalies in the large intestine of *Oasis*-/- mice are also reminiscent of the phenotype seen in *Spdef*-/- mice, which showed a profound loss of mature goblet cells (35). In this study, the authors used microarray analyses to show that OASIS is not down-regulated to any extent in *Spdef* -/- colon. Furthermore, we observed that the expression of SPDEF was not altered in the large intestine of *Oasis*-/- mice. These findings suggest that both genes are involved in the terminal differentiation of goblet cells, but by independent pathways. Interestingly, the expression of CREB4 was significantly down-regulated both in *Oasis*-/- and *Spdef*-/- large intestine, indicating that CREB4 is a common downstream target of OASIS and SPDEF that may be crucial for the terminal differentiation of goblet cells (Fig.7). CREB4 is an ER-resident transmembrane transcription factor that is structurally similar to OASIS and contains a bZIP domain in its cytoplasmic region. Because OASIS also possesses a bZIP domain, CREB4 may form a heterodimer with OASIS that promotes the expression of target genes required for the differentiation of goblet cells. Previous characterization of *Creb4* knockout mice revealed anomalies in spermatogenesis (47), but a detailed analysis of the gut has not conducted. Because CREB4 could be a key molecule for the maturation of goblet cells, further examination of *Creb4*-/- mice may enable further understanding of the molecular mechanisms involved in goblet cell differentiation.

Goblet cells in the large intestine produce and secrete abundant mucus, which provides a protective barrier against bacteria and other harmful stimuli. Defects in this barrier contribute to chronic diseases, including colitis and cancer. *Muc2*-/- mice have been observed to develop spontaneous colitis (48). Similarly, intestinal trefoil factor-deficient mice exhibit increased susceptibility to dextran sodium sulfate-induced colitis (49). Therefore, impairment of goblet cell maturation may be linked to inflammation of the intestinal mucosa. Because mature goblet cells in *Oasis*-/- mice are drastically decreased, it is possible that the mice are also sensitive to colitis. Targeted deletion of the ER stress response transcription factor, XBP1, and the ER stress sensor, IRE1β, causes sensitivity to experimental colitis (50-52). However, the mechanisms of which are sensitive to colitis are not consistent with the case of mice deleted goblet cell differentiation-related genes such as *Muc2* or *Intestinal trefoil factor*. Loss of *Xbp1* or *Ire1β* induces severe ER stress in goblet cells, and leads to apoptosis and chronic inflammation in the large intestine. We observed abnormally expanded rough ER in the goblet cells of *Oasis*-/- mice, suggesting that the function of the ER in *Oasis*-/- goblet cells may be impaired due to sensitivity to ER stress. Thus, OASIS may play roles not only in the differentiation of goblet cells, but also in protecting these cells from ER stress. Indeed, overexpression of OASIS was reported to protect against ER stress-induced cell death (17). Although further examination into the relationship between OASIS function and the onset of colitis is required, it is possible that OASIS is a novel drug target for inflammatory bowel diseases such as ulcerous colitis.

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FOOTNOTES

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The abbreviations used are: bZIP, basic leucine zipper; ER, endoplasmic reticulum; UPR, unfolded

protein response; OASIS, old astrocyte specifically induced substance; BBF-2, box B-binding factor-2; BBF2H7, BBF2 human homolog on chromosome 7; CREB, cyclic AMP-response element-binding protein; CREB4, cyclic AMP-response element-binding protein 4; BiP, immunoglobulin heavy chain-binding protein; ERAD, ER-associated degradation; Col1, type I collagen; SPDEF, SAM-pointed domain-containing ETS-like factor; Atoh1, atonal homolog 1; Hes1, hairy and enhancer of split 1; Dll1, delta-like 1; Gfi1, growth factor independent 1; Muc2, muchin 2; Agr2, anterior gradient 2; Retnlb, restin like beta; Tff3, trefoil factor 3; XBP1, X-box binding protein 1; IRE1, inositol requiring 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

FIGURE LEGENDS

Fig. 1. The expression of *Oasis* mRNA in the large intestine. A, RT-PCR analysis of *Oasis* mRNA in various tissues from 3-week old mice. *Oasis* mRNA is highly expressed in the large intestine. B, The expression of *Oasis* mRNA in each region of the large intestine. Right; quantitative analysis of *Oasis* mRNA. C, Hematoxylin-Eosin staining (HE) in the large intestine. Many goblet cells with mucus-containing vacuoles are observed in the crypt. D and E, in situ hybridization analysis of *Oasis* mRNA (D) and *Gapdh* mRNA (E) in the large intestine. *Oasis* mRNA is expressed in the lower portion of the crypt.

Fig. 2. Anomalies of goblet cells in the large intestine of *Oasis*-/- mice. A, HE staining; B, PAS staining. The numbers of goblet cells with vacuoles are severely decreased in *Oasis*-/- mice. C and D, PAS-Hematoxylin counter staining of WT and *Oasis*-/- large intestine. C, proximal large intestine; D, distal large intestine. E, The percentages of PAS-positive goblet cells in the crypt of the large intestine in WT and *Oasis*-/- mice. Data are mean ± s.d., n=5, ***P < 0.001, unpaired Student's t-test.

Fig. 3. Electron microscopy of goblet cells in the large intestine. A and B, images of goblet cells at the crypt base in the large intestine. C and D, high magnification of the rough ER in panels A and B. Note that rough ER in *Oasis*-/- goblet cells displays aberrant expansion. E and F, goblet cells in the upper portion of the crypt. The numbers of mucus vesicles are decreased and the membrane of some vesicles are often fused in goblet cells of *Oasis*-/- large intestine. Scale bars, 4μm (A, B, E, and F), 1.6 μm (C and D)

Fig. 4. The expression of goblet cell markers in the large intestine. A, RT-PCR of genes involved in primary differentiation from intestinal stem cells and an early goblet cell marker, *Tff3*. Note that the expression of *Tff3* is significantly elevated in *Oasis*-/- mice. B, RT-PCR of mature goblet cell markers. C, RT-PCR analysis of *Spdef* and *Creb4*. Data are mean ± s.d., n=3, *P < 0.05, **P < 0.01, unpaired Student's t-test. D-F, *in situ* hybridization of *Tff3* (D), *Muc2* (E), and *Spdef* (F) in the large intestine.

Fig. 5. Gene expression and OASIS activation during goblet cell maturation. A, PAS staining of LS174T cells 24 hrs after treatment with sodium butyrate. LS174T cells are completely differentiated to mature goblet cells containing PAS-positive mucus 24 hrs following treatment. B, RT-PCR of mature goblet cell markers in LS174T cells treated with sodium butyrate. The expression levels of mature goblet cell markers are increased after treatment with sodium butyrate. C, Western blot analysis of OASIS in LS174T cells treated with sodium butyrate (left). Quantification of the amounts of OASIS N-terminus (right). D, RT-PCR of ER stress markers during LS174T cell differentiation. E, RT-PCR of *Spdef* and *Creb4* mRNAs in LS174T cells treated with sodium butyrate. Data are mean ± s.d., n=5.

Fig. 6. OASIS is required for the terminal differentiation of goblet cells. A, RT-PCR of *Oasis* mRNA in LS174T cells 12 hrs after transfection with *Oasis* siRNA. The expression of *Oasis* mRNA is significantly suppressed by the siRNA. B, PAS staining of LS174T cells 24 hrs after treatment with sodium butyrate. *Oasis* knockdown results in inhibition of goblet cell maturation. C-E, RT-PCR of mature goblet cell markers (C), *Spdef* and *Creb4* (D), and ER stress markers (E) in LS174T cells treated with sodium butyrate following transfection with *Oasis* siRNA. Data are mean ± s.d., n=5. *P < 0.05, **P < 0.01, unpaired Student's t-test.

Fig.7. Putative roles of OASIS in the differentiation of goblet cells in the large intestine.

SUPPLEMENTAL TABLE LEDEND

Table 1. RT-PCR was performed using each specific primer set.
Table2. Digoxigenin-labeled probes for each gene.

Figure 1

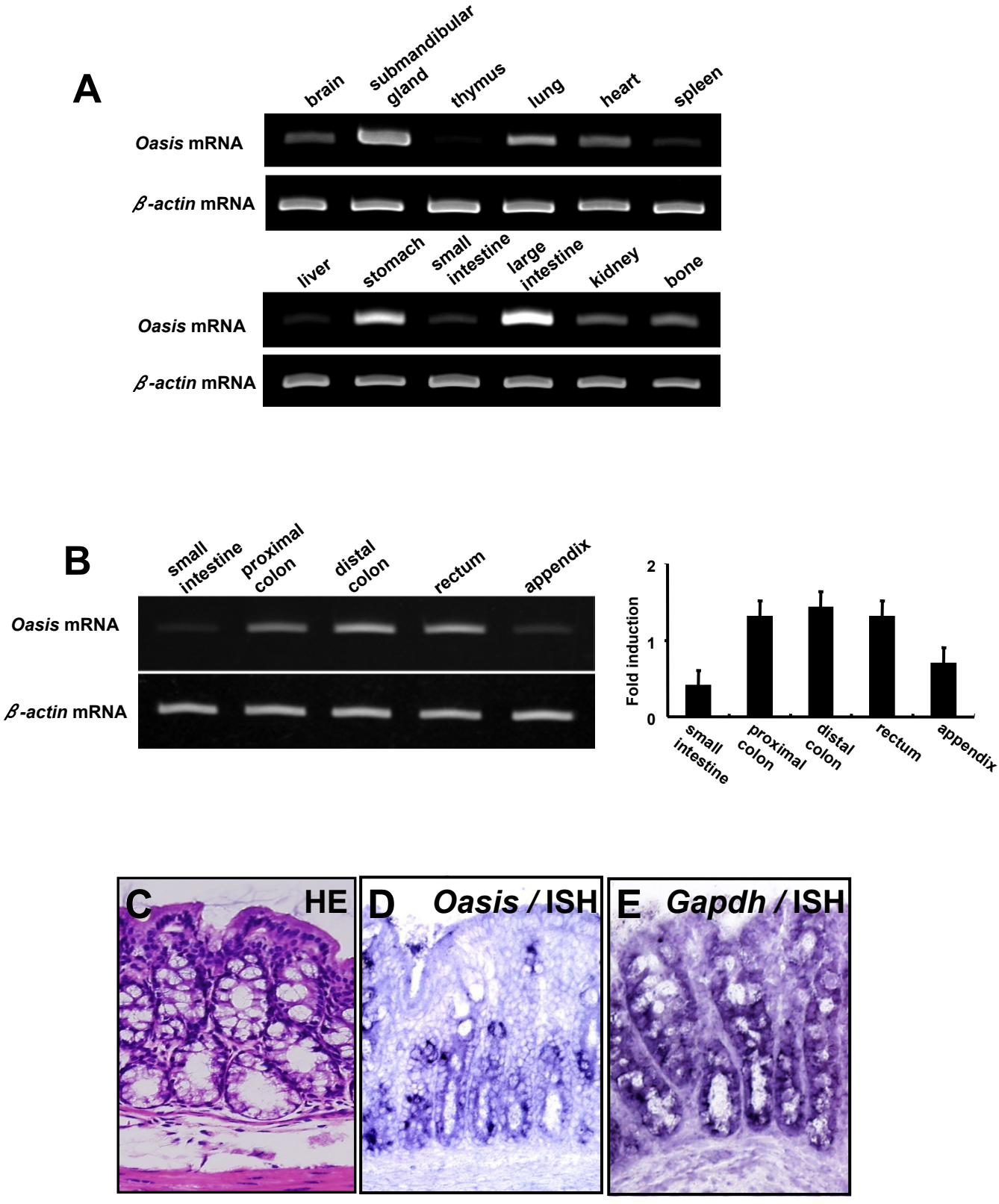

A
brain
submandibular gland
thymus
lung
heart
spleen
Oasis mRNA
β-actin mRNA
liver
stomach
small intestine
large intestine
kidney
bone
Oasis mRNA
β-actin mRNA
B
small intestine
proximal colon
distal colon
rectum
appendix
Oasis mRNA
β-actin mRNA
Fold induction
2
1
0
small intestine
proximal colon
distal colon
rectum
appendix
C HE
D Oasis / ISH
E Gapdh / ISH

Figure 2

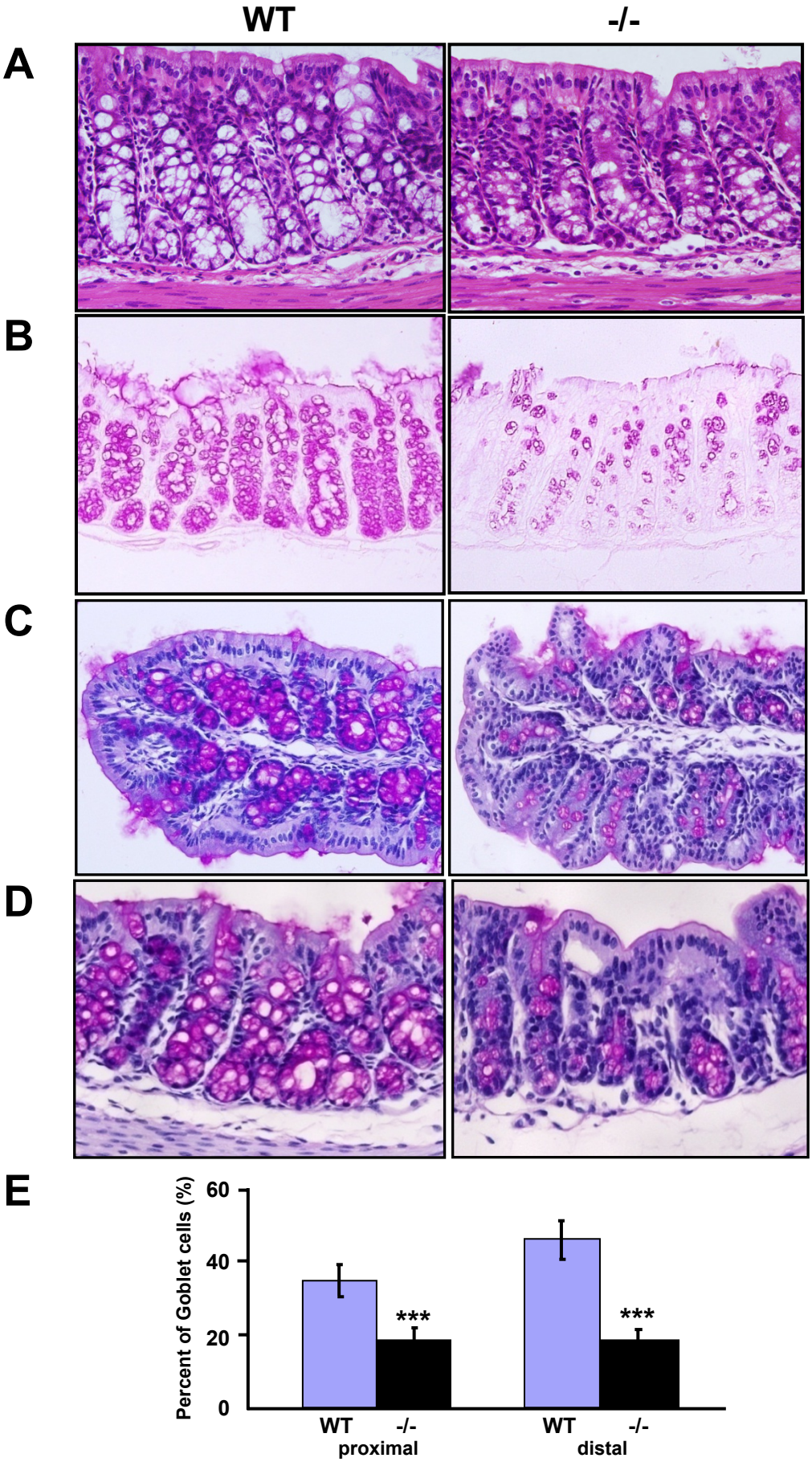

WT
-/-
A
B
C
D
E
Percent of Goblet cells (%)
60
40
20
0
***
***
WT
-/-
WT
-/-
proximal
distal

Figure 3

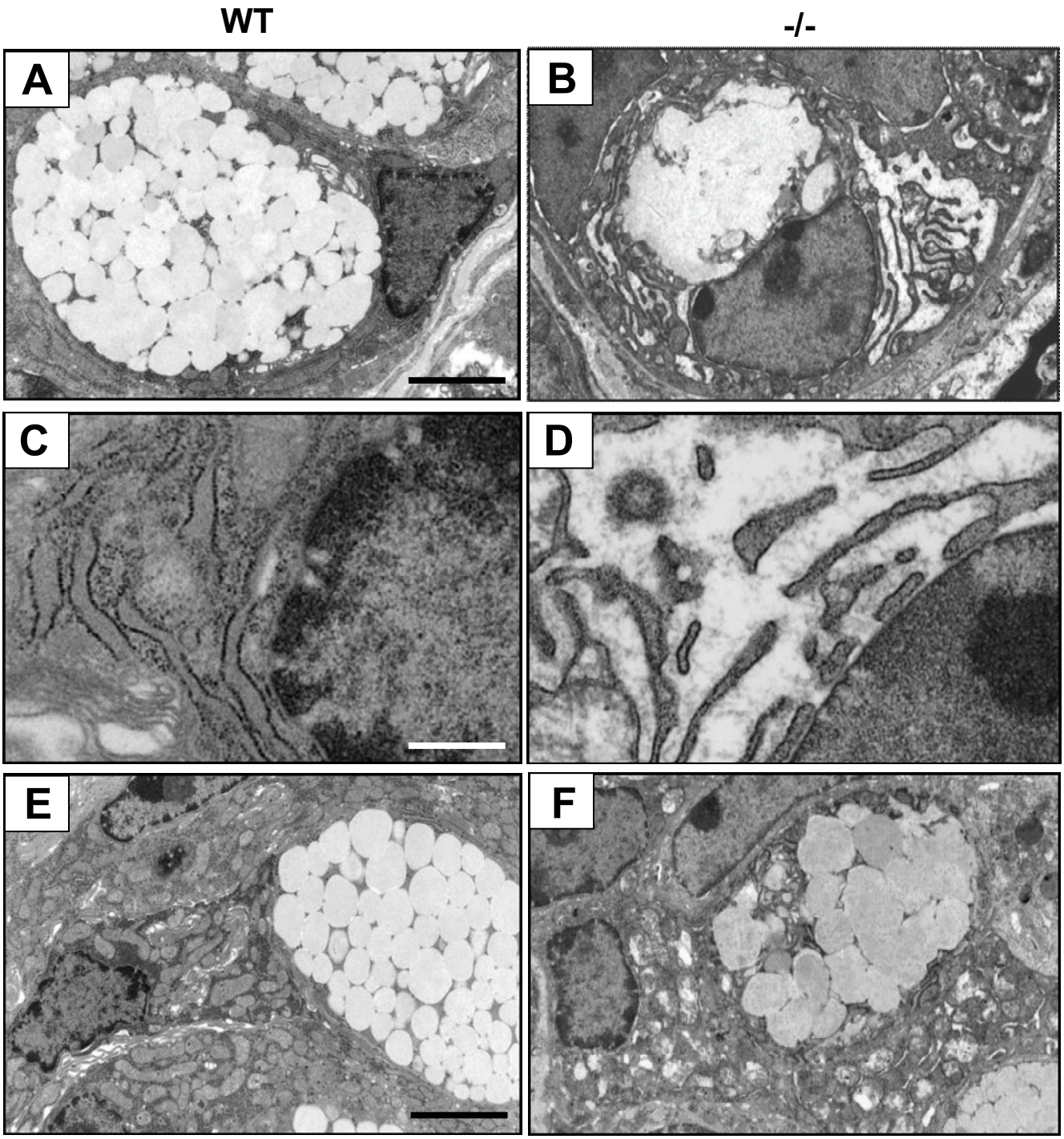


Figure 4

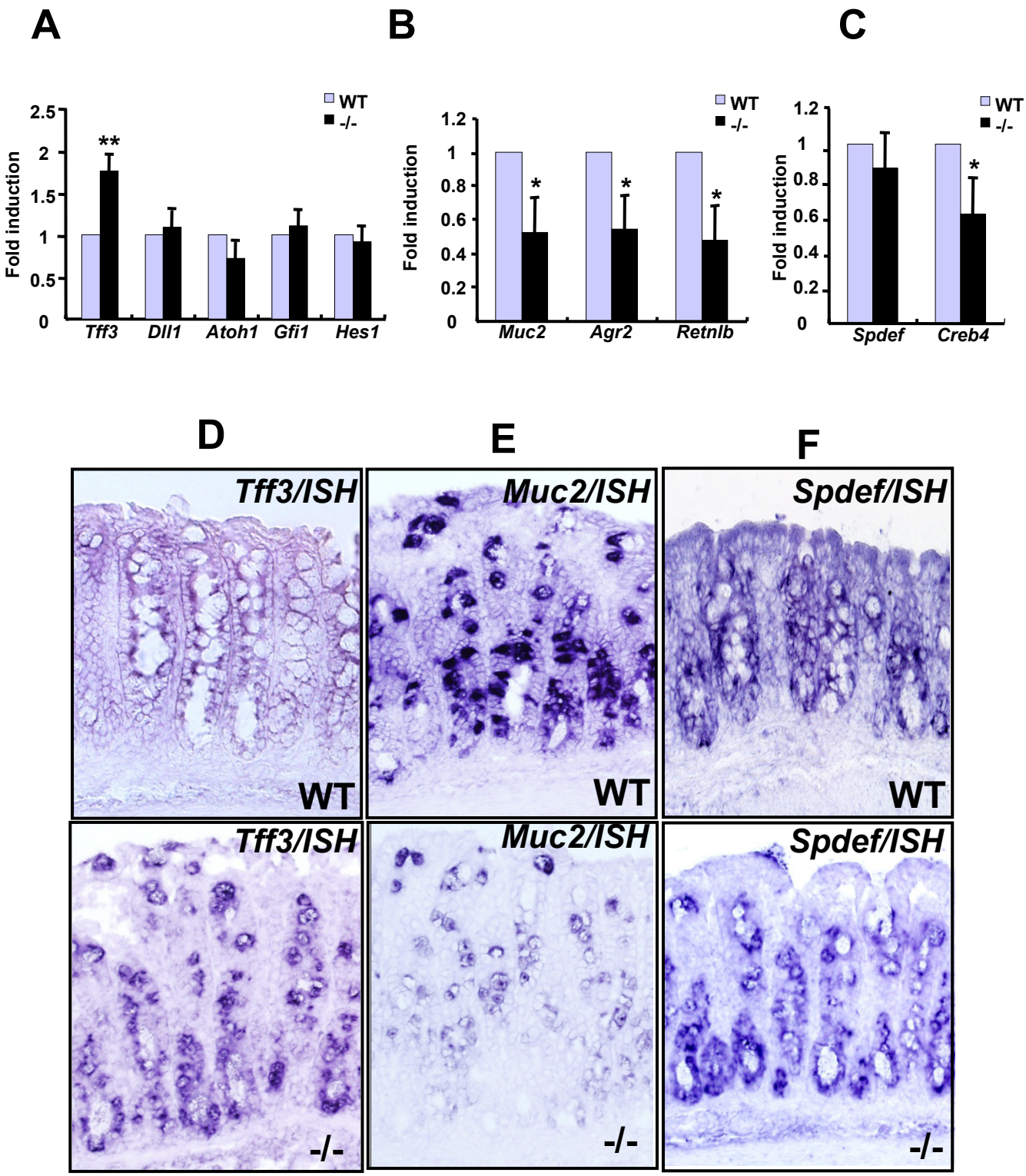

A
B
C
WT
-/-
Fold induction
2.5
2
1.5
1
0.5
0
**
Tff3
Dll1
Atoh1
Gfi1
Hes1
1.2
0.8
0.6
0.4
0.2
*
Muc2
Agr2
Retnlb
Spdef
Creb4
D
E
F
Tff3/ISH
Muc2/ISH
Spdef/ISH
WT
-/-

Figure 5

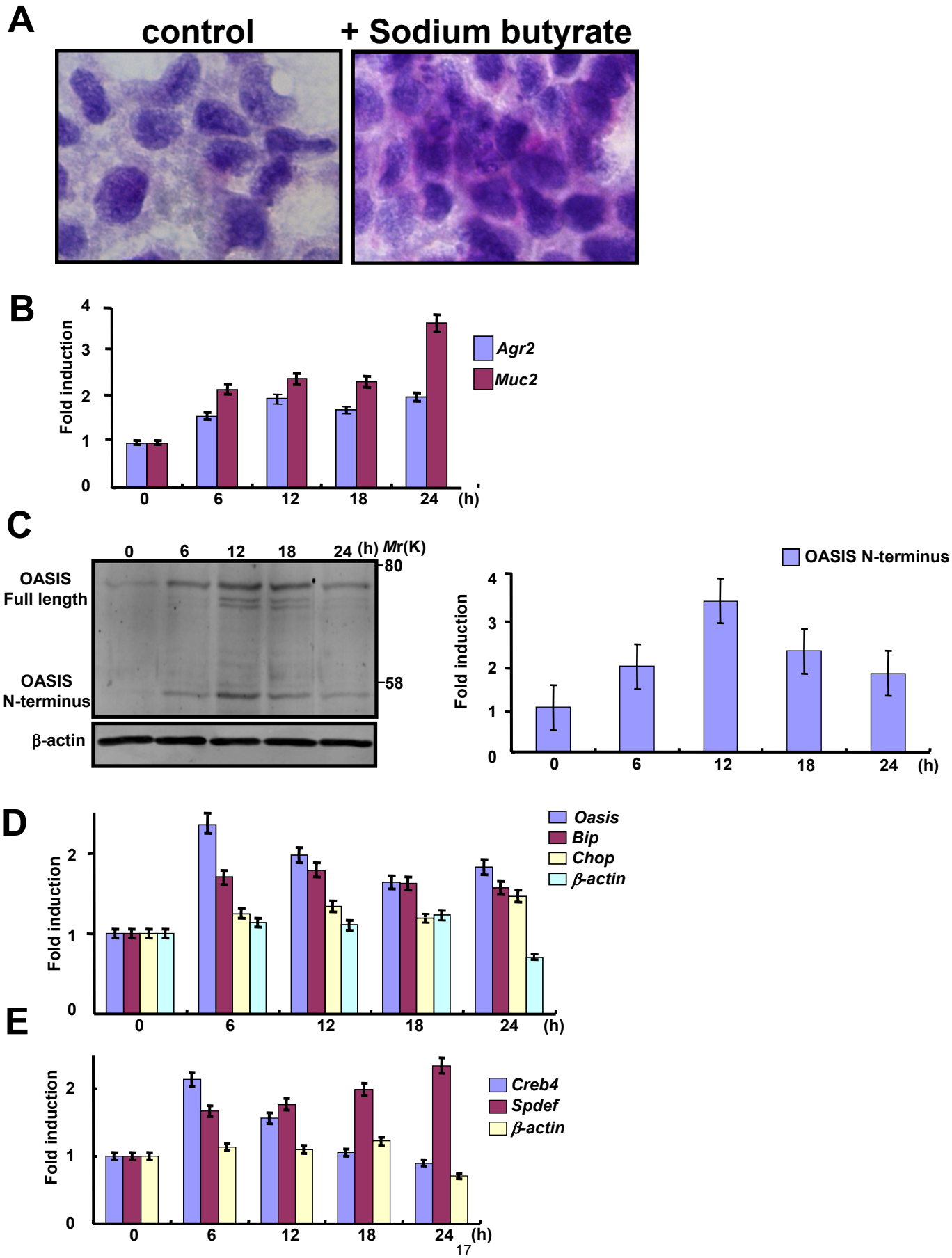

A
control
+ Sodium butyrate
B
Fold induction
Agr2
Muc2
0
6
12
18
24
(h)
C
0
6
12
18
24
(h)
Mr(K)
80
58
OASIS
Full length
OASIS
N-terminus
β-actin
OASIS N-terminus
Fold induction
D
Oasis
Bip
Chop
β-actin
E
Creb4
Spdef
β-actin

Figure 6

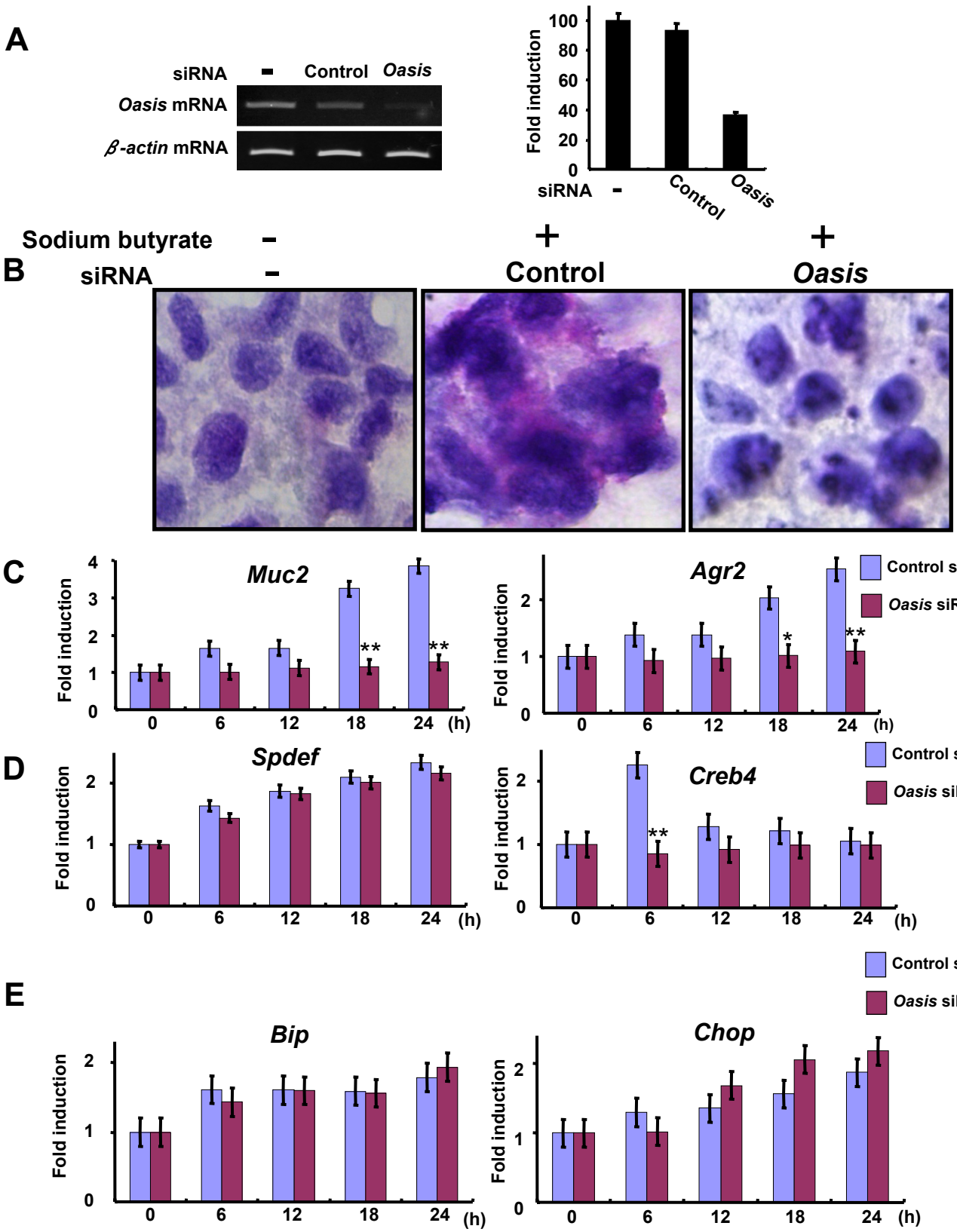
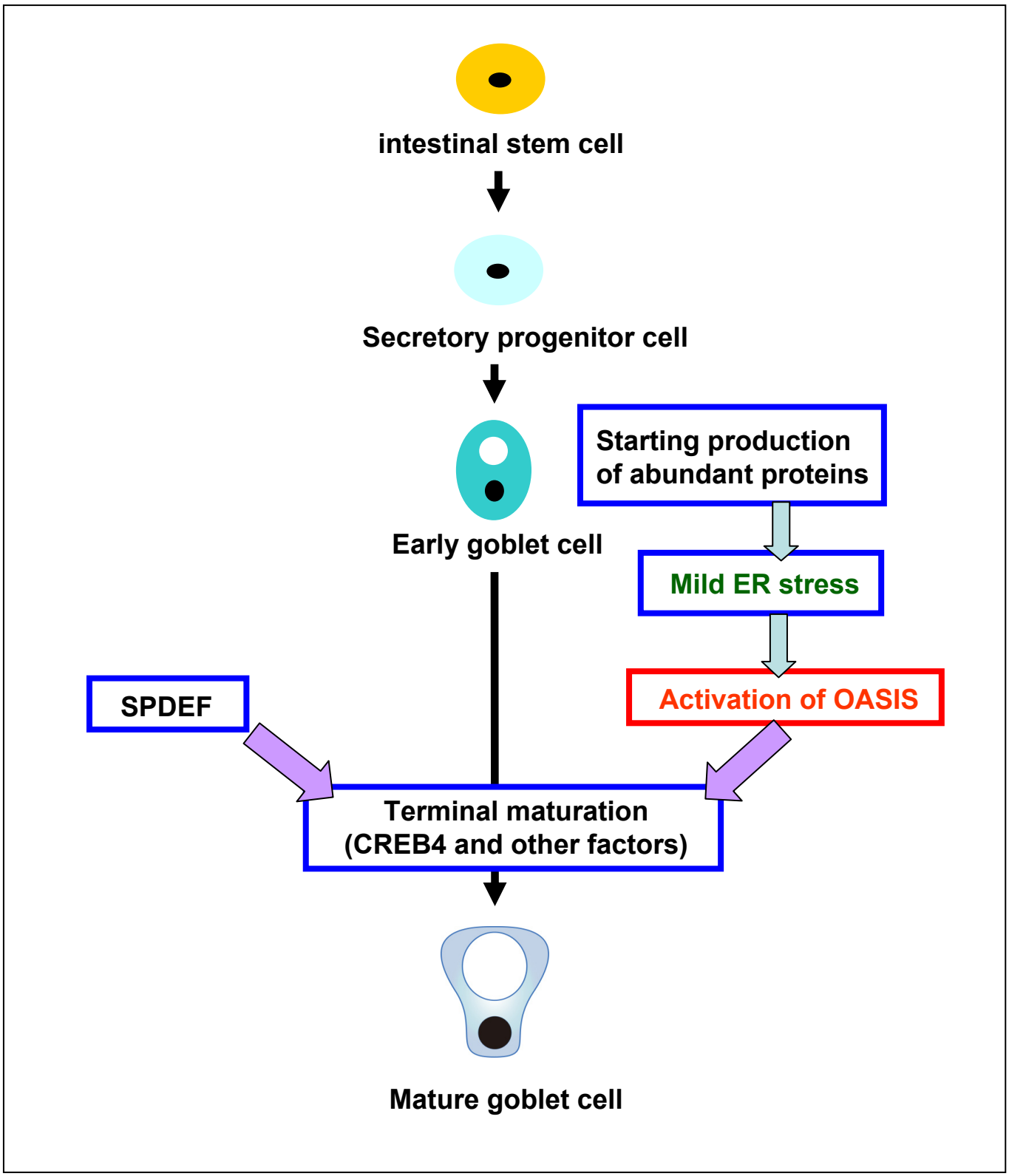

A
siRNA
-
Control
Oasis
Oasis mRNA
β-actin mRNA
Fold induction
siRNA
-
Control
Oasis
Sodium butyrate
-
+
+
B
siRNA
-
Control
Oasis
C
Muc2
Agr2
Control si RNA
Oasis siRNA
Fold induction
(h)
D
Spdef
Creb4
E
Bip
Chop

Figure 7


intestinal stem cell
Secretory progenitor cell
Early goblet cell
Starting production of abundant proteins
Mild ER stress
Activation of OASIS
SPDEF
Terminal maturation (CREB4 and other factors)
Mature goblet cell