Nuclear factor of activated T-cell c3 inhibition of mammalian target of rapamycin signaling through induction of regulated in development and DNA damage response 1 in human intestinal cells

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ABSTRACT The nuclear factor of activated T-cell (NFAT) proteins are a family of transcription factors (NFATc1–c4) involved in the regulation of cell differentiation. We identified REDD1, a negative regulator of mammalian target of rapamycin (mTOR) through the tuberous sclerosis complex (TSC1/2 complex), as a new molecular target of NFATc3. We show that treatment with a combination of phorbol 12-myristate 13-acetate (PMA) plus ionophore A23187 (Io), which induces NFAT activation, increased REDD1 mRNA and protein expression and inhibited mTOR signaling; pretreatment with the calcineurin inhibitor cyclosporin A (CsA), an antagonist of NFAT signaling, decreased REDD1 induction and mTOR inhibition. Knockdown of NFATc3, not NFATc1, NFATc2, or NFATc4, attenuated PMA/Io-induced REDD1 expression. Treatment with PMA/Io increased REDD1 promoter activity and increased NFATc3 binding to the REDD1 promoter. Overexpression of NFATc3 increased REDD1 mRNA and protein expression and increased PMA/Io-mediated REDD1 promoter activity. Treatment with PMA/Io increased expression of the goblet cell differentiation marker MUC2; these changes were attenuated by pretreatment with CsA or knockdown of REDD1 or NFATc3. Overexpression of NFATc3 increased, while knockdown of TSC2 decreased, MUC2 expression. We provide evidence showing NFATc3 inhibits mTOR via induction of REDD1. Our results suggest a role for the NFATc3/REDD1/TSC2 axis in the regulation of intestinal cell differentiation.

INTRODUCTION The mammalian intestinal mucosa undergoes a process of continual renewal, characterized by active proliferation of stem cells localized near the base of the crypts, progression of these cells up the crypt–villus axis with cessation of proliferation, and subsequent differentiation into one of the four primary cell types (i.e., absorptive enterocytes, mucin-producing goblet cells, Paneth cells, and hormone-secreting enteroendocrine cells). In the process of differentiation, enterocytes and goblet and enteroendocrine cells migrate toward the lumen of the gut. MUC-2, which is the predominant structural component of the intestinal mucus layer, is exclusively and abundantly expressed by goblet cells in the colon (Garg et al., 2007). MUC-2 is secreted into the lumen and forms the mucus layer, which acts as a barrier between the luminal contents and the epithelial surface to protect the epithelium from pathogens. The mechanisms by which committed cells are allocated to the different cell lineages of the intestine are poorly understood. Various in vitro culture models have proven to be useful in addressing interesting questions related to intestinal cell differentiation (Simon-Assmann \textit{et al.}, 2007). Owing to the poor survival of primary cells, primary intestinal cell cultures are limited for the study of differentiation. Some colon
cancer cell lines, such as HT29 and Caco-2, exhibit an absorptive enterocyte phenotype (both HT29 and Caco-2) or a mucin-producing goblet cell phenotype (HT29) based on the experimental conditions. Although by no means a perfect system, these cell lines have served as useful models for delineating potential pathways leading to different differentiation phenotypes. Delineating the molecular factors regulating intestinal proliferation and differentiation is crucial to our understanding of not only normal gut development and maturation, but also aberrant gut growth.

The nuclear factor of activated T-cells (NFAT) proteins are a family of transcription factors whose activation is controlled by calcineurin, a calcium-dependent phosphatase. Four distinct genes encoding closely related NFATc proteins (NFATc1–4) have been identified and are involved in multiple biological processes ranging from lymphocyte activation and development to cardiac hypertrophy. NFAT, which exists in a highly phosphorylated form in the cytoplasm, translocates into the nucleus upon dephosphorylation by the phosphatase calcineurin in response to increases in intracellular calcium. Once there, it binds to enhancer elements of specific genes leading to transcriptional activation. Cyclosporin A (CsA), a potent and specific inhibitor of calcineurin, is often used to inhibit NFAT transcriptional activity. NFAT has been shown to regulate cell differentiation and development in a number of cell types. For example, NFAT regulates the development of the cardiovascular system (de la Pompa et al., 1998). Primary keratinocyte cell differentiation is associated with nuclear localization of NFAT; this effect is blocked by CsA (Santini et al., 2001). NFAT also plays a role in adipocyte differentiation and stimulation of myogenic differentiation via activation of calcineurin (Delling et al., 2000). NFAT signaling is required for neuregulin-regulated Schwann cell differentiation (Kao et al., 2009). Recently we showed that activation of NFATc1 and NFATc4 increases PTEN (the tumor suppressor protein phosphate and tensin homologue deleted in chromosome ten) and p27kip1 (the cyclin-dependent kinase inhibitor protein) expression and decreases Akt phosphorylation and that NFAT activation is required for sodium butyrate-mediated differentiation of HT29 colon cancer cells to an enterocyte-like phenotype (Wang et al., 2011a). In addition, we have found that NFATc1 regulates the expression of TRAIL (TNF-related apoptosis-inducing ligand; Wang et al., 2011b), which contributes to intestinal cell differentiation.

Mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol kinase–related kinase family and regulates protein translation, cell cycle progression, and cell proliferation. The TOR signaling events are essential for epithelial growth, morphogenesis, and differentiation in the vertebrate intestine. mTORC1 phosphorylates p70 S6 kinase and eukaryotic initiation factor 4E-binding protein-1 (4E-BP-1) and increases the translation of mRNAs with long, highly structured 5′-untranslated regions, such as c-Myc. REDD1 (RTP801/Dig2/DDIT4) protein recently was identified in REDD1 induction in intestinal cells. In this paper, we show that REDD1 is regulated by NFATc3 and contributes to MUC2 expression. Moreover, our study identifies a novel NFATc3/REDD1/TSC2 axis in the regulation of intestinal cell differentiation.

RESULTS

NFAT activation increases REDD1 expression in intestinal cells

Previously we have shown that the mTOR kinase regulates tumorigenesis, proliferation, and metastasis of human colorectal cancer cells (Shao et al., 2004). Moreover, we found that NFAT activation is required for sodium butyrate–mediated enterocyte-like differentiation (Wang et al., 2011a). In our current study, we investigated the cellular mechanisms regulating mTOR repressor REDD1 expression in these intestinal-derived cell lines. HT29 cells were pretreated over a time course with phorbol 12-myristate 13-acetate (PMA; 100 nM) plus ionophore A23187 (Io; 2.5 μM), pharmacological agents that activate NFAT in intestinal cell types (Duque et al., 2005). Whole-cell lysates were analyzed by Western blotting using anti-REDD1 antibody (Figure 1A); induction of REDD1 expression was noted at 1–2 h after PMA/Io treatment.

To determine whether REDD1 induction requires RNA transcription, we treated HT29 cells with actinomycin D (10 μg/ml), which inhibits transcription, in combination with PMA/Io for 1.5 h; total protein was extracted; and Western blotting was performed (Figure 1B). Induction of REDD1 by PMA/Io was completely blocked by actinomycin D. REDD1 mRNA has a very short half-life in fibroblasts (T1/2 = 20.4 min; Otulakowski et al., 2009). Similarly, we found that the REDD1 mRNA half-life in HT29 cells is 19.6 min.

To determine whether PMA/Io treatment increased REDD1 mRNA expression, we pretreated HT29 cells with CsA, an inhibitor of calcineurin (Martinez-Martinez and Redondo, 2004), and subsequently treated with PMA (100 nM) plus Io (2.5 μM) in the presence or absence of CsA for 1.5 h. Total RNA was extracted, and REDD1 expression was determined by real-time reverse transcription PCR (RT-PCR; Figure 1C). PMA/Io treatment resulted in the induction of REDD1 mRNA expression compared with control cells treated with vehicle (i.e., Me2SO); this induction was attenuated by pretreatment with CsA. Therefore these results suggest a role for NFAT activation in transcriptional REDD1 induction in HT29 cells.

To test whether induction of REDD1 is associated with the inhibition of mTOR signaling pathway, we treated HT29 cells with PMA plus Io in the presence or absence of CsA. Whole-cell lysates were analyzed by Western blotting using anti-REDD1 antibody. Treatment with PMA/Io increased REDD1 expression and, as a result of mTOR inhibition, treatment with PMA/Io decreased the expression of c-Myc and the phosphorylation of S6 and 4E-BP-1 compared with control cells treated with vehicle (i.e., Me2SO; Figure 1D); these decreases were attenuated by pretreatment with CsA (Figure 1D). Therefore NFAT activation increased REDD1 expression and inhibited the mTOR signaling pathway.

To determine whether this induction occurs in other colon cancer cells, we analyzed REDD1 expression in the human colon cancer cell lines Caco-2, SW480, and HCT116 after treatment with PMA/Io for various times. PMA/Io induced REDD1 expression and decreased S6 phosphorylation in all three cell lines compared with control (Figure 1E). Together our results suggest a role for NFAT activation in REDD1 induction in intestinal cells.

NFATc3 regulates REDD1 expression in intestinal cells

Four isoforms of NFAT have been identified. To determine which of the NFAT isoforms are involved in REDD1 regulation, we silenced
was attenuated by knockdown of NFATc3. The efficiency of knockdown of individual NFAT isoforms was confirmed by real-time RT-PCR and Western blotting as shown in Figure 2, B and C. The results indicate that NFATc3 is important for PMA/Io-induced REDD1 expression in human intestinal cells.

To better delineate the role of NFATc3 in REDD1 regulation, we transfected HT29 cells with a plasmid encoding NFATc3 or siRNA targeting NFATc3. Overexpression of NFATc3 (Figure 3A, left) increased REDD1 protein expression and decreased mTOR and S6 phosphorylation. Knockdown of NFATc3 (Figure 3A, right) decreased REDD1 protein expression and increased mTOR and S6 phosphorylation. Overexpression or knockdown of NFATc3 was confirmed using anti-NFATc3 antibody. To address whether REDD1 mRNA induction paralleled the increase in REDD1 protein, we used real-time RT-PCR (Figure 3, B and C) on total RNA extracted from transfected HT29 cells; REDD1 mRNA induction was noted with NFATc3 overexpression (Figure 3B). In addition, a decrease in REDD1 mRNA was noted with NFATc3 knockdown (Figure 3C).

To confirm NFATc3-mediated REDD1 induction in other colon cancer cell lines, we transfected Caco-2, HCT116, and SW480 cells with NFATc3 plasmid or siRNA targeting NFATc3. Overexpression of NFATc3 (Figure 3D) increased REDD1 protein expression and decreased S6 phosphorylation in these cell lines. Knockdown of NFATc3 (Figure 3E) decreased REDD1 protein expression and increased S6 phosphorylation. These data demonstrate a role for NFATc3 in REDD1 induction in intestinal cells.

NFATc3 is a transcriptional activator of REDD1 in HT29 cells

Overexpression of NFATc3 increased REDD1 expression, while knockdown of NFATc3 decreased REDD1 expression. To determine whether NFAT induction of REDD1 expression was regulated at the promoter level, we first cloned the −2931/−97 base pair region (relative to the translation initiation site) upstream of REDD1 and inserted this promoter fragment into the pGL2m-luciferase reporter plasmid. HT29 cells were then transfected with the REDD1 promoter construct. Treatment with PMA/Io increased promoter activity, and this increase was attenuated by CsA (Figure 4A). Overexpression of NFATc3 increased basal and PMA/Io-mediated REDD1 promoter activity (Figure 4B).

To further determine the contribution of NFATc3 to REDD1 promoter activation, we next performed deletional analyses. Deletion of −2931 to −890 did not abrogate NFATc3 induction of the REDD1 promoter activity (unpublished data), suggesting that elements between −890 and −97 are important for NFATc3 induction of REDD1.
attenuated by treatment with CsA (Figure 1D). We next determined whether NFATc/REDD1 regulates c-Myc expression. HT29 cells stably transfected with the short hairpin RNA (shRNA) targeting REDD1 and the control shRNA were established. Knockdown of REDD1 was confirmed in HT29 cells transfected with NFATc3 shRNA by Western blotting (Figure 5A). Consistently, increased c-Myc protein expression and S6 phosphorylation levels were noted after knockdown of REDD1. To determine whether NFATc3 regulates c-Myc expression, we silenced NFATc3 by transfection of HT29 cells with the NFATc3 siRNA. As shown in Figure 5B, transfection of NFATc3 siRNA increased c-Myc protein expression. Treatment with PMA/Io decreased c-Myc, which was attenuated by knockdown of NFATc3. The efficiency of knockdown of NFATc3 was confirmed by Western blotting (Figure 5B, bottom panel). As expected, PMA/Io-induced REDD1 expression was attenuated by NFATc3 knockdown. To further confirm

NFATc3/REDD1 regulates c-Myc expression in intestinal cells

We have shown that treatment with PMA/Io decreased the expression of c-Myc, an mTOR downstream target molecule, and this was attenuated by treatment with CsA (Figure 1D). We next determined whether NFATc/REDD1 regulates c-Myc expression. HT29 cells stably transfected with the short hairpin RNA (shRNA) targeting REDD1 and the control shRNA were established. Knockdown of REDD1 was confirmed in HT29 cells transfected with NFATc3 shRNA by Western blotting (Figure 5A). Consistently, increased c-Myc protein expression and S6 phosphorylation levels were noted after knockdown of REDD1. To determine whether NFATc3 regulates c-Myc expression, we silenced NFATc3 by transfection of HT29 cells with the NFATc3 siRNA. As shown in Figure 5B, transfection of NFATc3 siRNA increased c-Myc protein expression. Treatment with PMA/Io decreased c-Myc, which was attenuated by knockdown of NFATc3. The efficiency of knockdown of NFATc3 was confirmed by Western blotting (Figure 5B, bottom panel). As expected, PMA/Io-induced REDD1 expression was attenuated by NFATc3 knockdown. To further confirm
the NFATc3 regulation of c-Myc expression, we transfected Caco-2 and HCT116 cells with a plasmid overexpressing NFATc3 and determined c-Myc expression by Western blotting. As shown in Figure 5C, overexpression of NFATc3 increased c-Myc expression in these cells, suggesting that NFATc3 regulates c-Myc expression through the induction of REDD1 expression in intestine-derived cells.

**NFATc3/REDD1/TSC2 pathway is involved in the regulation of the goblet cell marker MUC2**

HT29 cells can differentiate into two major intestinal cell types: enterocytes (as shown by increased alkaline phosphatase activity and villin expression) and goblet cells (as shown by increased MUC2 expression). Recently we have shown that activation of NFATc1 and NFATc4 increases PTEN and p27kip1 expression and that NFAT activation is required for sodium butyrate–mediated intestinal cell differentiation (Wang et al., 2011a). In addition, we have found that NFATc1 regulates the expression of TRAIL (Wang et al., 2011b), which contributes to intestinal cell differentiation (Rimondi et al., 2006). However, knockdown of NFATc3 did not alter sodium butyrate–induced alkaline phosphatase activity, a differentiation marker of intestinal enterocytes (Qingding Wang, unpublished data). To test whether NFATc3/REDD1 regulates goblet cell differentiation, we transfected Caco-2 and HCT116 cells with a plasmid overexpressing NFATc3 and determined c-Myc expression by Western blotting.

**FIGURE 3: NFATc3 regulated REDD1 expression in HT29, Caco-2, HCT116, and SW480 cells.**

(A) HT29 cells were transfected with control vector or NFATc3 (left) or control siRNA or siRNA targeting NFATc3 (right). After a 48-h incubation, REDD1, NFATc3, β-actin, phospho-mTOR (pS2448), mTOR, phospho-S6 (pS235/236), and S6 expression was determined by Western blotting. (B and C) HT29 cells were transfected with control vector or NFATc3 plasmid (B) or transfected with control siRNA or siRNA targeting NFATc3 (C). After a 48-h incubation, total RNA was extracted and REDD1 and NFATc3 mRNA levels were determined by real-time RT-PCR. (Data represent mean ± SD; *p < 0.01 vs. control as determined by two-sample t tests.) (D and E) Caco-2, HCT116, and SW480 cells were transfected with control vector or NFATc3 (D) or transfected with control siRNA or siRNA targeting NFATc3 (E). After a 48-h incubation, REDD1, NFATc3, β-actin, phospho-S6 (pS235/236), and S6 expression was determined by Western blotting.
assessed expression of MUC2, a goblet cell differentiation marker, by real-time RT-PCR. Treatment with PMA/io increased MUC2 expression (Figure 6A); this increase was attenuated by treatment with CsA. In addition, knockdown of NFATc3 decreased MUC2 mRNA (Figure 6B), while overexpression of NFATc3 increased MUC2 mRNA (Figure 6B). Moreover, knockdown of NFATc3 (Figure 6C) or REDD1 (Figure 6D) attenuated PMA/io-increased MUC2 expression. Because REDD1 inhibits mTOR through TSC2, we next determined whether TSC2 mediated the REDD1 regulation of MUC2 expression. HT29 cells were transfected with nontargeting control siRNA or siRNA targeting TSC2. As shown in Figure 6E, consistent with knockdown of REDD1, knockdown of TSC2 significantly decreased MUC2 expression in HT29 cells. Decreased TSC2 expression and increased S6 phosphorylation was confirmed by Western blotting (Figure 6E, right). Importantly, these results suggest the regulation of intestinal goblet cell differentiation by the NFATc3/REDD1/TSC2 axis.

**DISCUSSION**

We have previously shown that the mTOR kinase regulates tumorigenesis, proliferation, and metastasis of human colorectal cancer cells (Shao et al., 2004; Gulhati et al., 2011). In the present study, we demonstrate by complementary approaches (i.e., chemical inhibition, siRNA knockdown, and overexpression) that NFATc3 regulates REDD1 expression. Consistent with these results, activation of NFATc3/REDD1 increases and inhibition of NFATc3/REDD1 decreases mTOR downstream target c-Myc expression and MUC2 expression, which is a marker of goblet cell differentiation. Taken together, our results suggest that intestinal cell differentiation is regulated in part by NFATc3/REDD1 signaling.

We found that NFATc3 increased REDD1 mRNA and protein expression. In addition, we found that overexpression of NFATc3 increased REDD1 promoter activity, suggesting that the increased REDD1 expression may be mediated through regulation of transcription. Although NFATc3 binding to the REDD1 promoter was demonstrated by ChIP assay, computer analysis of REDD1 promoter sequences (-2931/-97) showed no typical NFAT binding sites located in this REDD1 promoter region, implying an indirect regulation of REDD1 expression by NFATc3. Several transcription factors, such as CCAAT/enhancer-binding protein (C/EBP) and Elk-1, mediate REDD1 expression (Lin et al., 2005). A cooperative interaction between NFAT and C/EBP regulating expression of the calcineurin regulatory proteins has been reported (Oh et al., 2010). Moreover, the NFAT/Elk-1 complex plays a role in the early stages of mitogen-stimulated proliferation in pancreatic cancer cells (Koenig et al., 2010). Hypoxia-inducible factor 1 (HIF-1) regulates REDD1 expression (Shoshani et al., 2011). Treatment with PMA/IO results in increased REDD1 expression in intestinal cells; however, PMA/io-induced REDD1 expression cannot be completely blocked by knockdown or inhibition of NFAT, suggesting PMA/io increased REDD1 expression by both NFAT-dependent and -independent mechanisms. In addition, although PMA/io induces NFAT signaling in various cell types, PMA/io also stimulates other signaling pathways. For example, PMA/io enhances Elk1 reporter activity in HEK293T cells (Hao et al., 2011). Treatment with PMA/io results in an increase in the level of HIF-1α protein in the HMC-1 cells (Walczak-Drzewiecka et al., 2008). It is possible that C/EBP, Elk1, or HIF-1α might be involved, along with NFATc3, in the regulation of REDD1 expression in human intestinal cells. We are currently investigating this possibility.

MUC2 produced by intestinal goblet cells is the major component of the intestinal mucus barrier that lubricates and protects the underlying intestinal epithelium. Endoplasmic reticulum stress-related mucin depletion could be a fundamental component in the pathogenesis of human colitis (Heazlewood et al., 2008). We show that NFATc3 increases REDD1, thus increasing MUC2 expression and suggesting that the NFATc3/REDD1/MUC2 axis might play a protective role in the intestinal mucosa. This supposition is supported by the fact that glutamine, an amino acid that is the primary fuel source for enterocytes and is essential for gut homeostasis and health, induces autophagy under basal and stressed conditions and prevents intestinal cell apoptosis under heat stress through its regulation of...
the mTOR kinase pathway (Sakiyama et al., 2009). Glutamine contributes to intestinal cell survival during physiologic stress by induction of autophagy (Sakiyama et al., 2009). In addition, REDD1 promotes cell survival through the induction of autophagy (Molitoris et al., 2011). Moreover, mice lacking NFATc3 show impaired development and increased apoptosis of T-cells (Oukka et al., 1998). The role of the NFATc3/REDD1/MUC2 axis in mucus barrier protection remains to be fully determined.

A considerable amount of evidence links REDD1 to tumor suppression. REDD1 is defined as a key metabolic regulator that suppresses tumorigenesis through distinct effects on mTORC1 activity and mitochondrial function. REDD1-mediated mTORC1 inhibition contributes to tumor suppression, and genetic ablation of REDD1 drives tumor formation (DeYoung et al., 2008). REDD1 is down-regulated in a subset of human carcinomas (DeYoung et al., 2008). In agreement with our current findings demonstrating NFATc3/REDD1 regulation of MUC2 expression in HT29 cells, inactivation of MUC2 causes intestinal tumor formation with spontaneous progression to invasive carcinoma, demonstrating that MUC2 is involved in the suppression of colorectal cancer (Velchik et al., 2002).

In addition, NFATc3 has also been shown to be a tumor suppressor gene. NFATc3 deficiency may contribute to the development of mammary gland adenocarcinoma in aging female mice (Lee et al., 2005). The lack of NFATc3 in mice leads to an increased susceptibility to lymphoma induced by virus infection (Glud et al., 2005). Together these findings suggest that the NFATc3/REDD1/MUC2 axis may contribute to the regulation of intestinal cell tumorigenesis.

The role of NFAT in differentiation of a variety of cell types has been shown (Delling et al., 2000; Santini et al., 2001; Kao et al., 2009; Wang et al., 2011). Our results demonstrate the regulation of intestinal goblet cell differentiation and c-Myc expression by the NFATc3/REDD1/TSC2 axis. Several pathways have been shown to play an important role in maintaining and regulating intestinal homeostasis, including Wnt and Notch (Yeung et al., 2011). One potential mechanism by which NFATc3/REDD1/TSC2 regulates goblet cell differentiation and c-Myc expression is through inhibition of Notch signaling, which is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancers (Sikandar et al., 2010). A combination of genetic and molecular data demonstrated that c-Myc is a direct transcriptional target of Notch1 participating as an indispensable downstream effector in intracellular domain of Notch1–induced tumorigenic action (Efstratiadis et al., 2007). Inhibition of Notch1 activation using pharmacological approaches or genetic manipulation results in the predominance of the goblet cell lineage in the intestine and colon. TSC loss promotes Rheb-dependent Notch activation in flies, rodents, and humans (Pear, 2010) although rigorous proof is lacking that inactivation of TSC2 mediates Notch1 activation in intestinal cells.

Another potential mechanism by which NFATc3/REDD1/TSC2 may regulate goblet cell differentiation is through Wnt signaling. In the intestine, the Wnt signaling pathway has been implicated in the regulation of the proliferation/differentiation balance. The c-Myc transcription factor is a potent inducer of proliferation and is required for Wnt/β-catenin signaling in intestinal epithelium. Wnt signals control the differentiation of the secretory cell lineage of the epithelium, as illustrated by the fact that overexpression of the Wnt-pathway inhibitor Dickkopf1 blocks differentiation of the Paneth, goblet, and enteroendocrine cell types (Pinto et al., 2003). Recently NFAT protein has been shown to repress canonical Wnt signaling via its interaction with dishevelled protein and to participate in regulating neural progenitor cell proliferation and differentiation (Huang et al., 2011). Moreover, the phosphatidylinositol 3-kinase–mTOR axis is required for Wnt-Myc–driven intestinal regeneration and tumorigenesis (Morton et al., 2011). Together these findings suggest that the NFATc3/REDD1/TSC2 axis likewise plays a role in intestinal cell homeostasis.

In conclusion, our results demonstrate that NFATc3 contributes to the regulation of the mTOR repressor REDD1 and mTOR downstream–targeted c-Myc expression. Furthermore, our study demonstrates a novel role for the NFATc3/REDD1/TSC2 axis in the regulation of goblet cell differentiation.

MATERIALS AND METHODS

Materials

PMA, calcineurin inhibitor CsA, and antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO). Io was from Alexis (San Diego, CA). Rabbit polyclonal anti-RED1 antibody was from Proteintech Group (Chicago, IL). Rabbit monoclonal anti-c-Myc antibody was from Epitomics (Burlingame, CA). Mouse monoclonal anti-NFATc1 antibody and rabbit anti-NFATc4 antibody were from Affinity BioReagents (Golden, CO). Rabbit polyclonal anti-NFATc3 antibody, used for Western blotting, was from Proteintech (Chicago, IL). Mouse monoclonal anti-NFATc2, mouse monoclonal anti-NFATc3 antibody (for the ChIP assay), and rabbit polyclonal anti-tuberin (TSC2;
that effectively eliminate nonfunctional siRNA (Reynolds et al., 2004).

siRNA SMARTpool, consisting of four siRNA duplexes, was purchased from Dharmacon (Lafayette, CO). The plasmid encoding human 4E-BP-1 (pT37/46), and anti–4E-BP-1 antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit anti–phospho-mTOR (Ser-2448), -actin, phospho-S6, and anti–β-actin antibodies (E).

FIGURE 6: NFATc3 and REDD1 regulation of MUC2 mRNA expression. (A) HT29 cells were pretreated with CsA for 30 min; this was followed by treatment with the combination of PMA/Io for 1.5 h. (B) HT29 cells were transfected with control siRNA or siRNA targeting NFATc3 (left panel) or transfected with control vector or NFATc3 plasmid (right panel). Transfected cells were incubated for 48 h. (C) HT29 cells were transfected with control siRNA or siRNA targeting NFATc3. After a 48-h incubation, transfected cells were treated with PMA/Io for an additional 1.5 h. (D) HT29 cells, stably transfected with control or REDD1 shRNA, were treated with PMA/Io for 1.5 h. (E) HT29 cells were transfected with control siRNA or siRNA targeting TSC2 and incubated for 48 h. Total RNA was extracted, and MUC2 mRNA levels were determined by real-time RT-PCR (A–E). (Data represent mean ± SD; *p < 0.01 vs. control or control siRNA or control shRNA or vector alone; #p < 0.01 vs. PMA/Io alone as determined by ANOVA.) Total protein was extracted and subjected to Western blotting using anti-TSC2, anti–phospho-S6 (pS235/236), anti-S6, and anti–β-actin antibodies (E).

for Western blotting) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti–phospho-mTOR (Ser-2448), anti-mTOR, anti–phospho-S6 (pS235/236), anti-S6, anti–phospho-4E-BP-1 (pT37/46), and anti–4E-BP-1 antibodies were purchased from Cell Signaling (Beverly, MA). The plasmid encoding human NFATc3 was from Addgene (Cambridge, MA). Human NFATc1, NFATc2, NFATc3, NFATc4, and nontargeting control siRNA SMARTpool were purchased from Dharmacon (Lafayette, CO). siRNA SMARTpool, consisting of four siRNA duplexes, was designed using an algorithm composed of 33 criteria and parameters that effectively eliminate nonfunctional siRNA (Reynolds et al., 2004).

Cell culture, transfection, and treatment
The human colon cancer cell lines HT29 and HCT116 cells were maintained in McCoy's 5A supplemented with 10% fetal calf serum (FCS). SW480 and Caco-2 were maintained in DMEM supplemented with 10% FCS and MEM supplemented with 15% FCS, respectively. HT29 cells were transfected with the siRNA duplexes (100 nM) and plasmids by electroporation (Gene Pulser; Bio-Rad, Hercules, CA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively, as we have described previously (Wang et al., 2006). HT29, HCT116, Caco-2, and SW480 cells were transiently transfected with plasmid encoding human NFATc3 using Lipofectamine 2000. Renilla reporter pRL-null, an internal control to normalize for variation in transfection efficiency, was cotransfected with REDD1 promoter constructs using Lipofectamine 2000, and the luciferase activity was determined and normalized to the Renilla activity as we have described previously (Kim et al., 2004).

Western blot analysis
Total protein was resolved on a 4–10% gradient polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated for 1 h at room temperature in blotting solution. REDD1, c-Myc, phospho-mTOR (pS2448), mTOR, phospho-S6 (pS235/236), S6, phospho-4E-BP-1 (pT37/46), 4E-BP-1, TSC2, NFATc1, NFATc2, NFATc3, NFATc4, and β-actin were detected with specific antibodies following blotting with a horseradish peroxidase–conjugated secondary antibody and visualized using an enhanced chemiluminescence detection system.

Quantitative real-time RT-PCR analysis
Total RNA was extracted and DNase-treated (RQ1; Promega, Madison, WI). Synthesis of cDNA was performed with 1 μg of total RNA using the reagents in the TaqMan Reverse Transcription Reagents Kit from Applied Biosystems (Foster City, CA; #N8080234). The TaqMan probe and primers for human REDD1, NFATc1, NFATc2, NFATc3, NFATc4, MUC2, and glyceraldehyde 3-phosphate dehydrogenase were purchased from Applied Biosystems. Quantitative real-time RT-PCR analysis was performed with an Applied Biosystems Prism 7000HT Sequence Detection System using TaqMan universal PCR Master Mix as we have described previously (Kim et al., 2004).

ChIP assay
The ChIP assay was performed using the ChIP-IT Express Enzymatic Kit from Active Motif (Carlsbad, CA) according to the manufacturer's protocol. PCR of the human REDD1 promoter region was performed using total (input) or immunoprecipitated chromatin with

for Western blotting) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti–phospho-mTOR (Ser-2448), anti-mTOR, anti–phospho-S6 (pS235/236), anti-S6, anti–phospho-4E-BP-1 (pT37/46), and anti–4E-BP-1 antibodies were purchased from Cell Signaling (Beverly, MA). The plasmid encoding human NFATc3 was from Addgene (Cambridge, MA). Human NFATc1, NFATc2, NFATc3, NFATc4, and nontargeting control siRNA SMARTpool were purchased from Dharmacon (Lafayette, CO). siRNA SMARTpool, consisting of four siRNA duplexes, was designed using an algorithm composed of 33 criteria and parameters that effectively eliminate nonfunctional siRNA (Reynolds et al., 2004).
the following pair of oligonucleotide primers: 5′-CTCAGGGATC-CCTTAAACCTA-3′ and 5′-GAACTGCTAAGCAAAGTGCG-3′.

**shRNAs and generation of stable cell lines**

MISSION control shRNA Lentiviral Particles with shRNAs were purchased from Sigma-Aldrich. The control shRNA (Non-Target shRNA Control Transduction Particles, # SHC002V) contains four base-pair mismatches within the short-hairpin sequence to any known human or mouse genes. shRNAs to human REDD1 (NM_019058; Sigma-Aldrich) constructed in pLKO.1-puro vector and purchased from Sigma-Aldrich were used; the sequence was as follows: CCAGGTAGCCTAAGGGCTAATCAAATCGTTGAGCATCATTTTG (TRCN0000062421). The lentiviruses-mediated delivery of shRNA was carried out as we have previously described (Li et al., 2011). HT29 cells were infected with the control shRNA or shRNA to human REDD1 lentiviruses particles and stably expressing cells were selected with puromycin at a concentration of 2 μg/ml.

**Statistical analysis**

Descriptive statistics (mean ± SD) were calculated and bar graphs were generated to summarize data across different cell-culture experimental conditions. For two-group comparisons, a two-sample t test was used; for multiple-group comparisons, analysis of variance (ANOVA) was utilized, and contrasts were generated to perform specific pairwise comparisons.

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