

Activation of the Esophagin Promoter during Esophageal Epithelial Cell Differentiation

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Esophagin is a member of the small proline-rich protein family of cell envelope precursor proteins, which are expressed during squamous cell differentiation. Esophagin is expressed at high levels in normal esophageal epithelium, but its expression is absent from esophageal squamous cell carcinomas and adenocarcinomas. Moreover, loss of esophagin expression is present in areas of dysplasia or normal mucosa adjacent to carcinomas, suggesting that absence of esophagin may constitute a harbinger of early esophageal malignant transformation. A greater understanding of transcriptional control of esophagin may provide valuable insights into esophageal malignancy. Therefore, this study was undertaken in order to isolate and carry out initial characterization of a functional promoter for esophagin. A genomic clone containing esophagin was isolated and sequenced, including 2.7 kb of the esophagin promoter region. Esophagin expression was studied in response to various treatments of primary cultured human esophageal epithelial cells and squamous cell carcinoma cell lines. Calcium was the strongest inducer of the endogenous esophagin promoter, with induction occurring at 12–72 hours. In primary cultured esophageal epithelial cells, a region spanning 116 bp upstream of the transcriptional start site to 45 bp downstream was sufficient to direct low, basal, *in vitro* esophagin expression. However, responsiveness of primary esophageal cells to calcium required inclusion of promoter elements 1688 bp upstream of the transcriptional start site. Site-directed mutagenesis studies suggested a putative role for C/EBP- β , OCT-1, and OCT-3 transcription factor binding sites in the minimal promoter region. In conjunction with published human *in vivo* studies, these data support the hypothesis that esophagin is a biomarker of esophageal squamous cell differentiation and provide an *in vitro* model to evaluate regulatory factors involved in this differentiation process.

INTRODUCTION

Phenotypic evidence of impaired cellular differentiation is a defining characteristic of cancer cells. Many genes have been identified whose protein products are markers for cell differentiation in specific cell types or tissues. Loss of expression of these genes in human cancers can be assessed and may afford diagnostic and/or prognostic significance. Altered expression of cellular differentiation genes, however, is only an indicator of a more fundamental perversion present in neoplastic cells: disruption of signal transduction pathways controlling the balance between cellular differentiation and proliferation. In stratified squamous epithelia such as the epidermis and esophagus, terminal differentiation involves a highly coordinated sequence of events that is reflected in the

histological appearance of several distinct layers of epithelial cells [1,2]. As part of the normal growth of stratified squamous tissues, terminally differentiated cells are continuously sloughed off [3]. In this context, epithelial cell renewal is dependent on a small population of stem cells that possess an intrinsic ability to divide and produce committed progenitor cells that will repopulate the mature cell layers [4]. Failure of a progenitor cell to fully differentiate as a result of genetic or epigenetic influences may preserve a state of enhanced cell proliferation that may predispose to the accumulation of mutations in cancer genes and, thus, to eventual neoplastic transformation [5].

The esophagin gene (*SPRR3*) is a member of the multi-gene family encoding small proline-rich proteins (*SPRRs*) that are expressed in stratified squamous epithelia during

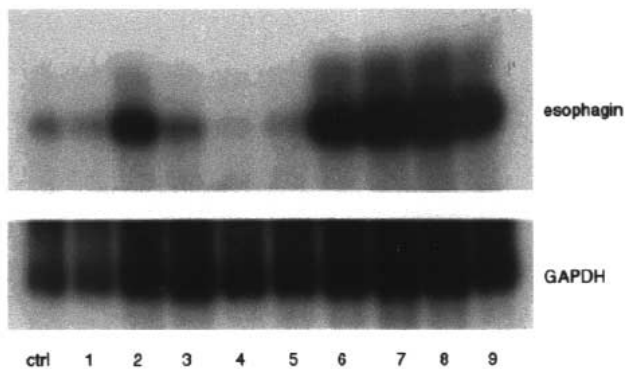


FIG. 1. Northern blot analysis of induction of esophagin expression in primary human esophageal epithelial cells in response to mediators of squamous differentiation. Lane 1, UV irradiation (35 J/m²); lane 2, PMA (20 ng/ml); lane 3, IL-1 α (5 U/ml); lane 4, IFN- γ (20 ng/ml); lane 5, IL-1 α (5 U/ml) + IFN- γ (20 ng/ml); lane 6, 0.15 mM CaCl₂; lane 7, 0.15 mM CaCl₂ + 10⁻⁷ M vitamin D₃; lane 8, 1.0 mM CaCl₂; lane 9, 1.0 mM CaCl₂ + 10⁻⁷ M vitamin D₃.

provide evidence supporting the involvement of binding sites for the transcription factors Oct-1 and C/EBP- β located in the minimal promoter region.

RESULTS

Isolation and Analysis of the Esophagin Genomic Clone

Based on the esophagin cDNA sequence [13], PCR primers were used to generate a 1.3-kb PCR product containing the sole intron, because all SPRR genes sequenced so far consist of only two exons and a single intron [10]. Screening of a human placental genomic library using the PCR product as a probe identified an approximately 3-kb fragment that was shown by Southern hybridization, DNA sequencing, primer extension, and ribonuclease protection assays to contain exon 1 and adjacent upstream genomic sequence. This 3-kb fragment was subcloned and sequenced in its entirety to yield 2.7 kb of promoter sequence. Consensus binding sites for known transcription factors were identified by computer database searches using MatInspector 2.1 [14] and the TRANSFAC 3.2 Matrices [15]. Consensus binding sites for several transcription factors implicated in squamous cell differentiation, including AP-1, OCT-1, C/EBP- β , the ETS family, and STAT, were identified.

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Previously, we reported that the loss of expression of esophagin is observed in primary esophageal squamous cell carcinomas and adenocarcinomas, as well as in esophageal tumor cell lines [13]. In addition, decreased expression of esophagin was often apparent in areas of cellular dysplasia and normal mucosa adjacent to cancers, suggesting that loss of esophagin expression occurs early in neoplastic transformation [13].

Squamous cell carcinoma (SCCA) and adenocarcinoma account for similar numbers of esophageal cancers worldwide [10]. Although several molecular genetic alterations have been implicated in the development of SCCA, including mutation of the *TP53* and *CDKN2A* tumor suppressor genes, *MYC* and cyclin D1 oncogene amplification, and epidermal growth factor (EGF) and EGF receptor overexpression, the progression of events that occurs during tumorigenesis is poorly defined [11,12].

The determination of promoter elements controlling esophagin expression during terminal esophageal squamous cell differentiation can enhance our understanding of intracellular regulatory pathways controlling this process. Promoter studies may identify regulatory factors that are involved in dysregulated gene expression in esophageal cancer. Here, we report the cloning, sequencing, and functional analysis of 2.7 kb of the esophagin promoter. We define a minimal promoter region required for expression of esophagin in cultured primary esophageal cells, and we identify several distal regulatory regions involved in calcium-induced esophagin expression. We also demonstrate activity of our cloned promoter in response to calcium, ultraviolet light (UV) irradiation, and treatment with phorbol ester, which correlates well with endogenous promoter activity. Finally, we

Esophagin Expression during Calcium Treatment of Primary Esophageal Epithelial Cells

To study the regulation of esophagin expression by calcium, normal primary epithelial cells from freshly resected human esophagus were subcultured according to our established protocols. More than 80% of these cells retained a basaloid phenotype throughout several cell passages. Induction of terminal differentiation by increasing extracellular calcium to 0.15 mM or 1 mM or by treatment with phorbol myristyl acetate (PMA) or UV irradiation resulted in an increase in esophagin expression as determined by northern blot analysis (Fig. 1).

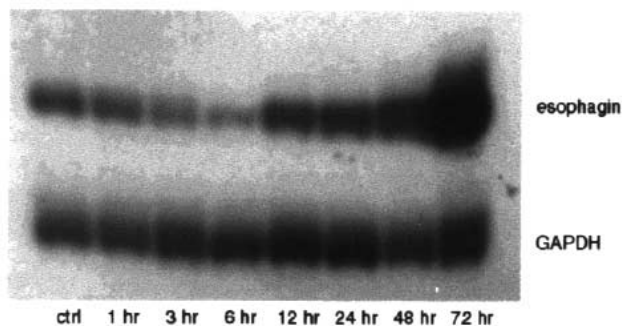


FIG. 2. Northern blot analysis of induction of esophagin expression in primary human esophageal epithelial cells in response to calcium. The extracellular calcium concentration was increased from 0.05 mM to 0.15 mM and total RNA was prepared at the indicated times.

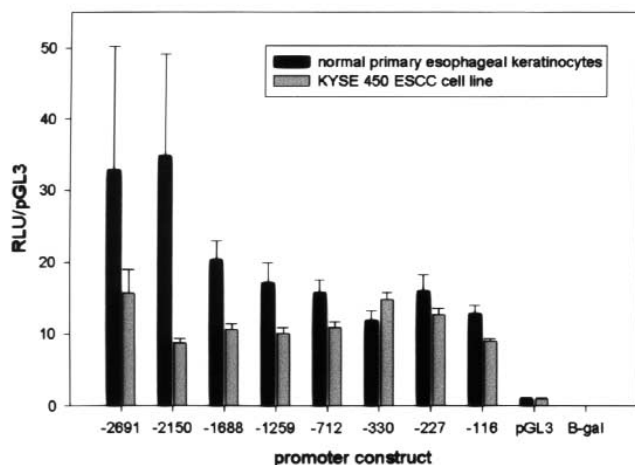


FIG. 3. Deletion mapping of the esophagin promoter in primary human esophageal epithelial cells and KYSE 450 cells. Luciferase activity is given relative to the activity of the promoterless pGL3 construct. Promoter constructs include the first exon (+45). Each assay was performed in triplicate; standard error bars are shown.

Esophagin expression was induced as early as 12 hours after increasing the extracellular calcium concentration in the medium and was maximal at 72 hours after induction (Fig. 2).

Promoter Deletion Analysis

A panel of eight different promoter deletions was constructed in the pGL3 luciferase reporter vector. The entire first exon of esophagin was included in all promoter constructs. Transfection of promoter constructs into either primary human esophageal epithelial cells or KYSE-450 esophageal cancer cells revealed detectable promoter activity significantly greater than empty pGL3 vector (Fig. 3). The region from -116 to +45 bp was sufficient to direct baseline expression ninefold greater than empty vector in untreated KYSE-450 cells. The region from -116 to -227 bp contributed an additional 40% in reporter activity, which was further enhanced 17% by the inclusion of sequence to -330 bp. With more distal deletions, luciferase reporter activity first decreased 26% (from -330 to -712 bp), remained 10-fold greater than the pGL3 vector (through -1688 bp), then decreased (with the construct from -2150 to +45 bp) to slightly less than the ninefold increase seen with the minimal -116/+45 construct. Reporter activity increased to 16-fold above pGL3 with the entire promoter construct (-2691 to +45 bp). Thus, maximal transcriptional activity was observed with the -330/+45 construct, implying the presence of one or more negative regulatory elements within the more distal promoter region from -330 to -2150 bp and a positive regulatory element in the region from -2150 to -2691 bp.

In unstimulated primary esophageal epithelial cells, the minimal -116/+45 promoter fragment directed a 13-fold increase in luciferase activity relative to pGL3 alone. Reporter activity increased by 25% with the addition of sequence to

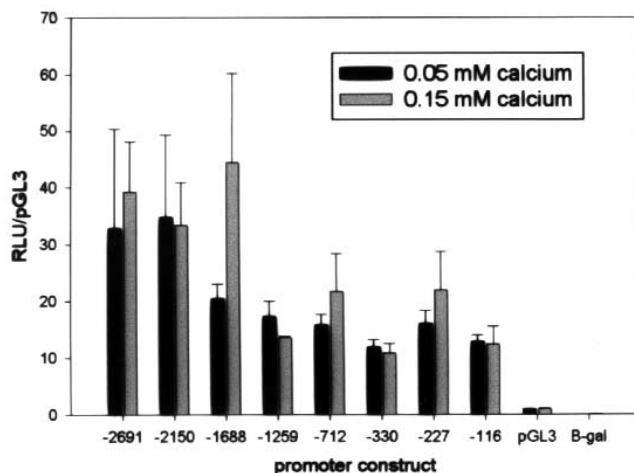


FIG. 4. Deletion mapping of the esophagin promoter in primary esophageal epithelial cells. Cells were grown in either low (0.05 mM) extracellular calcium or high (0.15 mM) extracellular calcium, which induces squamous differentiation. Luciferase activity is given relative to the activity of the promoterless pGL3 construct. Promoter constructs include the first exon (+45). Each assay was performed in triplicate; standard error bars are shown.

-227 bp, then decreased by 26% with the addition of sequence to -330 bp. Luciferase activity then increased steadily with each successive increase in promoter length to reach a maximum activity with the -2150/+45 fragment, which directed a 35-fold increase in reporter activity over background pGL3 levels.

To evaluate the esophagin promoter for potential calcium-responsive elements, primary esophageal epithelial cells were transfected with promoter deletion reporter constructs and then treated with 0.15 mM extracellular calcium. No significant response to calcium was seen with five different constructs spanning the -1259 to +45 bp region; however, a twofold increase in luciferase activity occurred with the addition of sequence to -1688 bp (Fig. 4).

Identification of Promoter Regulatory Elements

Based on the results of a MatInspector 2.1 [14] database screen of the promoter sequence and on our identification of the minimal promoter region at -116 to +45 bp, several potentially important DNA promoter elements were identified. C/EBP- β , OCT-1, and OCT-3 sites were found in the minimal promoter, and are potential regulators of esophagin expression. The overlapping C/EBP- β and OCT-3 sites at -35 bp, the C/EBP- β site at -53 bp, and the OCT-1 site at -60 bp were mutated by the introduction of a single-base transversion within each core consensus sequence in the -116/+45 bp promoter construct. In primary cultured human esophageal epithelial cells, mutation of any single site resulted in a significant decrease in reporter activity (Fig. 5A). For example, the OCT-3 (-35 bp) and C/EBP- β (-53 bp) mutants resulted in 58% and 43% reductions, respectively, in the response of the promoter to calcium relative to the wild-type minimal promoter (Fig. 5A).

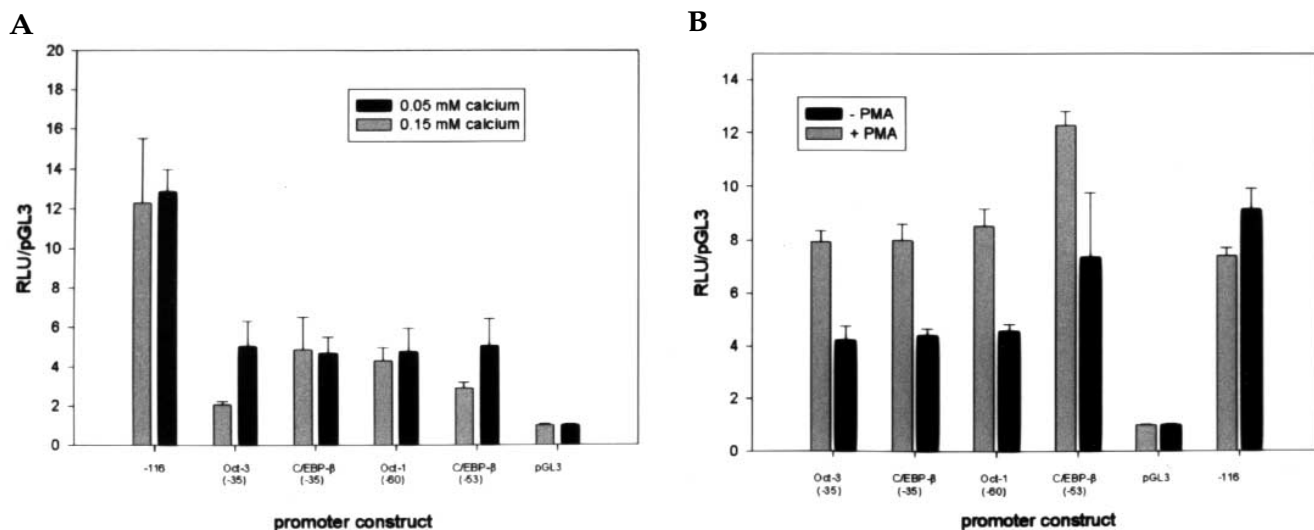


FIG. 5. Esophagin promoter analysis. We carried out mutational analysis of consensus transcription factor binding sites for OCT-1, OCT-3, and C/EBP- β within the proximal esophagin promoter (-116/+45) in calcium-induced primary esophageal epithelial cells (A) and PMA-treated KYSE-450 squamous cell carcinoma cells (B). Luciferase activity is given relative to the activity of the promoterless pGL3 construct. Each assay was performed in triplicate; standard error bars are shown.

A similar analysis was performed in KYSE-450 esophageal cancer cells. While the C/EBP- β (-53 bp) mutant exerted only a minimal effect on reporter activity, with a 20% reduction in luciferase activity compared with the wild-type minimal promoter, the OCT-3 (-35 bp), C/EBP- β (-35 bp), and OCT-1 (-60 bp) mutants all demonstrated a 50–54% reduction in luciferase activity (Fig. 5B). However, while PMA stimulation resulted in a small decrease in luciferase activity when the wild-type sequence was present, PMA treatment of the OCT-3 (-35 bp), C/EBP- β (-35 bp), and OCT-1 (-60 bp) mutants resulted in an increase in reporter activity slightly greater than the level achieved with PMA stimulation of the wild-type sequence. Furthermore, PMA stimulation of the C/EBP- β (-53 bp) mutant resulted in an increase in luciferase activity 68% greater than that of both the untreated C/EBP- β (-53 bp) mutant and the PMA-treated wild-type sequence.

DISCUSSION

We report here that the endogenous promoter for esophagin, a gene that we earlier showed to be transcriptionally silenced in esophageal tumors [13], is upregulated by extracellular calcium in primary esophageal epithelial cells. Furthermore, we show that treatment of these cells with calcium induces the activity of a cloned exogenous promoter. Calcium is known to induce differentiation in other epithelial cell systems [16–18]. Our earlier study showed that esophagin expression is greatest in cells that are the most differentiated, becoming lost in normal basal undifferentiated cells or as cells evolve toward a malignantly transformed (that is, dedifferentiated) phenotype [13]. These findings support the hypothesis that calcium functions as a differentiating agent in the esophagus.

Available evidence supports the involvement of esophagin in squamous cell differentiation [19–21]. Moreover, our studies suggest that studying the induction of esophagin by calcium and other factors may provide a simple *in vitro* model of esophageal squamous cell differentiation. With respect to various known inducers of differentiation, we saw a weaker response to PMA, UV irradiation, and IL-1 α or interferon- γ (Fig. 1). These findings conflict with some other studies of these agents in inducing differentiation, but are consistent with others. For example, UV irradiation induces the proline-rich proteins SPRR1 and SPRR2, which are associated with keratinocyte differentiation [22,23].

The chromosomal colocalization of esophagin with the other SPRR genes and the squamous differentiation cluster on 1q21 further support esophagin's potential involvement in esophageal epithelial differentiation. In fact, the human SPRR family includes multiple genes clustered within a single 300-kb genomic DNA region close to the lorcrin and involucrin genes in a locus that has been named the "epidermal differentiation complex" [24–26]. Expression of these genes may be intimately coordinated. The tissue-specific expression profile of individual SPRRs, however, suggests that a level of independent transcriptional regulation must also exist. Putative roles for several specific transcription factors have been proposed in regulating SPRR expression [27–32]. A study of the putative SPRR3 promoter directed reporter gene expression in response to calcium in cultured keratinocytes, suggesting that the normal regulatory apparatus that regulates tissue-specific expression of SPRR3 is inactive in cultured keratinocytes, permitting aberrant SPRR3 expression [33]. In addition, an AP-1 binding site (-164) and an ATF binding site (-148) seem to be required for calcium-mediated expression of SPRR3 in cultured keratinocytes.

Like the endogenous esophagin promoter, the 2.7-kb promoter that we cloned and studied also exhibits induction by calcium. However, the degree of induction of this promoter by calcium was only two- to fourfold, in contrast to the 1000-fold induction seen with the endogenous promoter (Fig. 1). This finding suggests that additional upstream or downstream transcriptional regulatory elements may be required for the full physiologic response to calcium. In fact, our promoter deletion studies showed the greatest increase in basal and induced expression when the most distal promoter elements were included (Figs. 3 and 4). Thus, it is possible that elements further upstream of the region we studied exert an even greater degree of influence on esophagin expression. Similarly, enhancer elements within the gene's single intron or within and outside of the gene *per se* may account for the induction of the endogenous gene by calcium. Nevertheless, our detailed mutagenesis analyses suggest definite, albeit modest, roles for specific transcriptional activators in the regulation of esophagin expression (Fig. 5). For example, one-base mutations within the consensus binding sequences for OCT-1, OCT-3, and two CEBP- β binding sites resulted in two- to threefold reductions in expression relative to the unmutated minimal promoter (-116 to +45 bp). These results suggest that although these Oct-1 and CEBP sites may play finite roles in expression control, major transcriptional control sites are located outside the minimal promoter region.

These studies suggest that esophagin expression is induced by extracellular calcium, and that promoter elements within the 2.7 kb proximal to the structural gene account, at least in part, for this induction and response. Furthermore, when considered in the context of previously published studies, these results suggest that by evaluating factors that regulate the esophagin promoter, we may identify factors that are important in normal and abnormal esophageal cell differentiation. Experiments are currently underway to assess the use of an anti-esophagin antibody in screening esophageal biopsy samples as an early marker of cancer. Ultimately, this information may aid in the development of diagnostic and therapeutic strategies to reduce the morbidity and mortality of diseases marked by abnormal esophageal differentiation, such as cancer or premalignant syndromes.

MATERIALS AND METHODS

Genomic library screening. A genomic DNA clone containing the entire esophagin open reading frame was cloned from a human placental genomic library constructed in λ FIX II (Stratagene, La Jolla, CA). The λ FIX II library was screened using a PCR-generated probe derived from genomic DNA. The PCR product was cloned into pT7Blunt (Novagen, Madison, WI) according to the manufacturer's instructions to generate pT7-Int. A total of 1×10^6 λ FIX II phage was screened by plating at a density of 50,000 plaques per 137-mm LB plate with overnight incubation at 37°C. Phage DNA was extracted by the Lambda DNA Purification kit (Stratagene, La Jolla, CA). Recombinant plasmids were progressively sequenced using an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA).

Cell culture. The esophageal squamous cell carcinoma cell lines KYSE 70, KYSE 110, KYSE-450, and KYSE 520 were a generous gift from Yutaka Shimada

(Kyoto University, Kyoto, Japan) [34]. Cells were cultured in Ham's F12/RPMI medium (Gibco BRL, Rockville MD), 1% penicillin-streptomycin, and 2% fetal bovine serum (Gemini Bioproducts, Woodland, CA) at 37°C under 5% CO₂. Primary cultures of esophageal epithelial cells were prepared by modification of established methods [35,36]. Cells were incubated at 37°C under 5% CO₂ and the culture medium was changed every 24–48 hours until cells were 75% confluent, after which the cells were trypsinized and re-seeded at a density of 3.5×10^3 per ml. Treatment with UV irradiation at 35 J/m² was performed in a Stratlinker (Stratagene, La Jolla, CA) after removal of tissue culture medium; fresh medium was added to cells immediately after irradiation.

Northern blot hybridization. Total RNA was prepared from esophageal carcinoma cell lines and primary esophageal epithelial cells using Trizol (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. For northern blot analysis, 5 μ g total RNA was electrophoresed on 1.0% agarose, transferred overnight to Hybond-N nylon membranes (Amersham, Piscataway, NJ), and hybridized using the NorthernMax kit (Ambion, Austin, TX) according to the manufacturer's protocol.

Plasmid constructs. A panel of promoter deletion constructs was generated by PCR cloning of promoter regions into the TOPO-TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). Promoter constructs were excised from the recombinant pCR2.1 vectors by *SacI/XmaI* digestion, agarose gel-purified using GeneClean Spin (BIO101, Carlsbad, CA), and ligated into the pGL3-Basic promoterless luciferase reporter vector (Promega, Madison, WI) using T4 ligase. Plasmid construct sequences were verified by cycle sequencing using SequiTherm EXCEL as per the manufacturer's instructions (Epicentre, Madison, WI). Site-directed mutagenesis was performed using the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications.

Transfections and reporter assays. Endotoxin-free plasmids were purified using the Endofree Maxi Prep Kit (Qiagen, Valencia, CA). We plated 2×10^5 cells per well in six-well tissue culture plates 18–24 hours before transfection. Each purified pGL3 plasmid (2 μ g) was cotransfected with 2 μ g purified pSV- β gal plasmid (Clontech, Palo Alto, CA) to control for transfection efficiency using 10 μ l Superfect (Qiagen, Valencia, CA) as per the manufacturer's instructions. At 24–36 hours after transfection, cells were lysed and luciferase activity was assayed using the Luciferase Reporter System (Promega, Madison, WI) and a Turner Designs Model 20-e luminometer according to the manufacturer's protocol. β -Galactosidase activity was assayed using the compatible β -galactosidase Reporter System (Promega) as per the manufacturer's instructions.

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