MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response

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Introduction

Cells are highly sensitive to conditions that disrupt the environment of the ER or that increase demand on its machinery for synthesis, maturation, and transport of secretory cargo. Under such conditions of ER stress, cells launch the unfolded protein response (UPR) to balance client protein load with the folding capacity of the ER. Three distinct signaling pathways comprise the mammalian UPR and are initiated by the ER transmembrane sensor protein kinase RNA activated–like ER kinase (PERK), activating transcription factor 6 (eIF2α), and inositol-requiring enzyme 1 (IRE1; Ron and Walter, 2007). Activated PERK phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2-α), effectively down-regulating protein synthesis (Harding et al., 2000b). Proteolytic processing of ATF6 yields an active transcription factor (Haze et al., 1999; Ye et al., 2000) that up-regulates expression of ER resident quality control proteins, including chaperones and ER-associated degradation (ERAD) components (Wu et al., 2007; Yamamoto et al., 2007; Adachi et al., 2008). Upon activation of IRE1, its endoribonuclease activity initiates an unconventional cytosolic splicing of XBP1 mRNA, resulting in a translational frameshift that generates XBP1(S), a basic leucine zipper transcription factor (Shen et al., 2001; Yoshida et al., 2001; Calfon et al., 2002). XBP1(S) enhances a variety of ER and secretory pathway processes by up-regulating expression of genes involved in protein entry into the ER, protein folding and maturation, ERAD, and vesicular trafficking (Lee et al., 2003; Shaffer et al., 2004). If ER stress is not sufficiently alleviated by these adaptive mechanisms, the UPR can commit the damaged cell to death (Tabas and Ron, 2011).

XBP1 is subject to transcriptional, posttranscriptional, and posttranslational controls (Chen and Qi, 2010; Lee et al., 2011; Wang et al., 2011; Yanagitani et al., 2011; Majumder et al., 2012), indicating that the activity of this crucial UPR transcription factor is carefully balanced. MicroRNAs (miRNAs), ~22-nt single-stranded RNAs that typically exert posttranscriptional control of gene activity (Bartel, 2009), represent a sizeable class of regulators, which outnumbers kinases and phosphatases (Leung and Sharp, 2010). A few ER stress-inducible miRNAs have been identified and shown to hinder translation of various secretory pathway proteins (Bartoszewski et al., 2011; recently designated miR-30c-2-3p), is induced by the protein kinase RNA activated–like ER kinase (PERK) pathway of the UPR and governs expression of XBP1 (X-box binding protein 1), a key transcription factor that augments secretory capacity and promotes cell survival in the adaptive UPR. These data provide the first link between an miRNA and direct regulation of the ER stress response and reveal a novel molecular mechanism by which the PERK pathway, via miR-30c-2*, influences the scale of XBP1-mediated gene expression and cell fate in the UPR.

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Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; ATF6, activating transcription factor 6; CHOP, CCAAT enhancer-binding protein homologous protein; ERAD, ER-associated degradation; IRE1, inositol-requiring enzyme 1; MEF, mouse embryonic fibroblast; miRNA, microRNA; MUT, mutant; NF-κB, nuclear factor κB; PERK, protein kinase RNA activated–like ER kinase; qRT-PCR, quantitative RT-PCR; Tg, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response; UTR, untranslated region.

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Results and discussion

miR-30c-2* is a potential regulator of XBP1 expression

Using two computational algorithm programs, TargetScan (Lewis et al., 2005) and MicroCosm (Krek et al., 2005), we searched for miRNAs with potential base pair complementarities to conserved sequences in the XBP1 mRNA 3’ untranslated region (UTR). This survey predicted a target site, featuring attributes of functional miRNA, for miR-30c-2* (recently designated miR-30c-2-3p) in the XBP1 3’ UTR (Fig. 1 A, left). First, the 7-nt sequence in the XBP1 3’ UTR exhibiting Watson–Crick pairing to positions 2–8, the “seed” region (Lewis et al., 2005), of miR-30c-2* is conserved across the three species assessed (Fig. 1 A, right). Second, miR-30c-2* includes a conserved 5’ U (Fig. 1 A, left). Sequence analysis of miRNA* strand populations has revealed a strong disfavor for 5’ G, a feature avoided by recognized miRNA regulatory strands (Frank et al., 2010; Yang et al., 2011). Finally, the sequences of both miR-30c-2* and its guide strand, miR-30c (recently designated miR-30c-2-5p; Fig. 1 B), are identical across numerous species, including Homo sapiens and Mus musculus (Fig. 1 C). Cross-species conservations of the miRNA sequence, the seed region, and a 5’ U are all key characteristics of endogenous functional miRNA (Lai, 2002; Krek et al., 2005; Lewis et al., 2005). Notably, our bioinformatics analysis did not reveal a target site in the XBP1 3’ UTR for the corresponding guide strand miR-30c.

To test the capacity of miR-30c-2* to exert regulatory activity via its putative target site in the XBP1 3’ UTR, we constructed reporter vectors containing a single copy of either the wild-type target sequence or an altered seed region (mutant [MUT]; Fig. 2 A) positioned 3’ of a firefly luciferase gene. Overexpression of miR-30c-2* in NIH-3T3 fibroblasts reduced the activity of the luciferase reporter containing the wild-type target sequence but not of the luciferase reporter containing the MUT target site (Fig. 2 A). Therefore, miR-30c-2* is functionally competent and recognizes the predicted cognate XBP1 3’ UTR target site in a sequence-specific manner. Next, we asked whether miR-30c-2* can alter the expression of endogenous XBP1. Overexpression of miR-30c-2* in HeLa cells attenuated induction of both XBP1 mRNA (Fig. 2 B) and XBP1(S) protein (Fig. 2 C) in response to tunicamycin (Tm), an inhibitor of N-linked glycosylation that triggers the UPR. In agreement with these data, the induction of XBP1-dependent, ER stress-responsive genes (Lee et al., 2003; Adachi et al., 2008) SEC23B (Fig. 2 D), which encodes a cargo receptor involved in vesicle trafficking, and DNAJB9 (Fig. S1 A), which encodes the ER chaperone cofactor Erdj4, in response to Tm was severely impaired in cells overexpressing miR-30c-2*. In contrast, we observed normal induction of the XBP1-independent UPR target gene DDIT3 (encodes CCAAT enhancer-binding protein homologous protein [CHOP]; Fig. S1 B; Lee et al., 2003), indicating the presence of an intact UPR in this system. These data establish that miR-30c-2* has the capacity to limit induction of XBP1 mRNA, XBP1(S) protein, and XBP1-dependent target genes. XBP1(S) positively regulates XBP1 gene transcription (Yoshida et al., 2001); hence, miR-30c-2* could regulate XBP1 expression by impeding translation of XBP1(S) and/or promoting degradation of XBP1 transcripts (Huntzinger and Izaurralde, 2011).

ER stress-mediated induction of miR-30c-2* involves the PERK pathway and nuclear factor-κB (NF-κB)

As a potential regulator of XBP1, we reasoned that expression of miR-30c-2* might be modulated during the UPR. We found that treatment of cells with either Tm or thapsigargin (Tg), an inhibitor of the ER Ca2+ ATPase and a strong inducer of the UPR, up-regulates expression of miR-30c-2* (Fig. 3 A). Using gene knockout mouse embryo fibroblasts (MEFs) and their wild-type counterparts, we then determined that ER stress-induced expression of miR-30c-2* is dependent on the PERK pathway of the UPR, whereas ATF6-α and IRE1-α are dispensable for this event (Fig. 3 B).

PERK-mediated down-regulation of global protein synthesis leads, paradoxically, to increased translation of ATF4 (Harding et al., 2000a), a factor that drives expression of a variety of targets, including the proapoptotic transcription factor CHOP, enzymes that reduce oxidative stress, and proteins that function in amino acid metabolism (Harding et al., 2003). In addition, the PERK pathway activates NF-κB, a dimer of Rel family proteins that regulates a myriad of genes involved in inflammation, stress responses, cell growth, and apoptosis (Karin et al., 2002; Li and Verma, 2002). In its inactive state, NF-κB is sequestered in the cytoplasm bound to proteins known as inhibitors of NF-κB (IκB; Baeuerle and Baltimore, 1988). PERK-mediated repression of protein synthesis depletes the cytosolic pool of IκB, freeing NF-κB to enter the nucleus and activate target genes (Jiang et al., 2003; Deng et al., 2004). Bioinformatics analysis revealed a potential NF-κB binding site (5’-GGGGG-GTCTTAT-3’) ~1.8 kb upstream of the mapped miR-30c-2* chromosomal location. This candidate NF-κB binding site, exhibiting a 2-nt mismatch with the NF-κB consensus sequence (5’-gagRNAAYCC-3’; the lowercase letters indicate the most common nucleotide in a variable position), was previously implicated as a functional NF-κB enhancer element in the tumor...
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We then used chromatin immunoprecipitation (ChIP) to determine whether NF-κB binds to the predicted motif upstream of miR-30c-2* during the UPR. The analysis revealed a greater than eightfold enrichment of NF-κB at this region after 6 h of Tm treatment (Fig. 3 D). These data (Fig. 3, B–D) suggest that NF-κB, downstream of PERK, plays a critical role in up-regulating expression of miR-30c-2* in the Tm-induced UPR.

necrosis factor α promoter (Shakhov et al., 1990). Additional searches for transcription factor binding sites upstream of miR-30c-2* revealed no known or predicted binding sites for either ATF4 or CHOP. Therefore, we tested whether ER stress-induced expression of miR-30c-2* involves NF-κB. Overexpression of a constitutively active, dominant-negative IκB-α MUT (Brockman et al., 1995) attenuated induction of miR-30c-2* in response to Tm (Fig. 3 C). We then used chromatin immunoprecipitation (ChIP) to determine whether NF-κB binds to the predicted motif upstream of miR-30c-2* during the UPR. The analysis revealed a greater than eightfold enrichment of NF-κB at this region after 6 h of Tm treatment (Fig. 3 D). These data (Fig. 3, B–D) suggest that NF-κB, downstream of PERK, plays a critical role in up-regulating expression of miR-30c-2* in the Tm-induced UPR.
peaked at ~6 h of Tm treatment (Fig. 4 A). Interestingly, miR-30c-2* was moderately induced as early as 2 h of Tm treatment and was maximal around 6 h (Fig. 4 A). A similar expression profile was observed in Tm-treated NIH-3T3 cells (Fig. S2). The concomitant up-regulation of miR-30c-2* and XBP1 mRNA suggested that miR-30c-2* might influence XBP1 expression as the UPR proceeds. If so, we reasoned that inhibiting miR-30c-2* expression could affect XBP1 expression and the magnitude of XBP1-mediated gene transcription.

We next sought to determine whether endogenous miR-30c-2* indeed targets XBP1 mRNA. We treated HeLa cells with Tm for 2–18 h and assessed the kinetics of induction for both miR-30c-2* and XBP1. As expected, XBP1 was induced early (2 h) and peaked at ~6 h of Tm treatment (Fig. 4 A). Interestingly, miR-30c-2* was moderately induced as early as 2 h of Tm treatment and was maximal around 6 h (Fig. 4 A). A similar expression profile was observed in Tm-treated NIH-3T3 cells (Fig. S2). The concomitant up-regulation of miR-30c-2* and XBP1 mRNA suggested that miR-30c-2* might influence XBP1 expression as the UPR proceeds. If so, we reasoned that inhibiting miR-30c-2* expression could affect XBP1 expression and the magnitude of XBP1-mediated gene transcription.
accumulation during the UPR would result in increased levels of XBP1 mRNA and XBP1(S) protein. To test this hypothesis, we stably expressed an miRNA inhibitor specific for miR-30c-2* in HeLa cells. In cells expressing anti–miR-30c-2*, the accumulation of miR-30c-2* in response to Tm was ablated at peak induction times (4 and 6 h; Fig. 4 B). Conversely, when treated with Tm, cells expressing anti–miR-30c-2* exhibited greater induction of XBP1 mRNA (Fig. 4 C), XBP1(S) protein (Fig. 4 D) and XBP1-dependent, ER stress-responsive genes (Lee et al., 2003; Adachi et al., 2008) SEC23B, DNAJB9, SRP54A, which encodes a subunit of the signal recognition particle, and EDEM1, which encodes an ERAD component (Fig. S3, A–C). Again, we observed normal induction of the XBP1-independent UPR target gene DDIT3 (Fig. S3 D). These findings demonstrate that endogenous miR-30c-2* regulates expression of XBP1 during the UPR and, in turn, modulates the magnitude of XBP1(S)-mediated gene transcription.

miR-30c-2* influences cell fate under conditions of ER stress

Expression of XBP1(S) and its downstream target genes is considered to be proadaptive in the UPR. Therefore, to further investigate the impact of endogenous miR-30c-2* on the overall cellular response to ER stress, we assessed the fate of HeLa cells expressing either the anti–miR-30c-2* or the inhibitor control after an extended period of UPR activation. At 0, 24, and 30 h of Tm treatment, cells were stained with 7-aminoactinomycin D (7-AAD), a fluorescent DNA intercalator dye that penetrates the compromised membranes of late-stage apoptotic or necrotic cells, and analyzed by flow cytometry. Indeed, the percentage of cells scoring as 7-AAD permeable was attenuated by anti–miR-30c-2* at all intervals tested (Fig. 5, A and B, 7-AAD Pos). To determine whether this anti–miR-30c-2* effect was in fact XBP1 dependent, we performed similar experiments in wild-type and XBP1-deficient MEFs.
Figure 4. Endogenous miR-30c-2* negatively regulates XBP1 expression in the UPR. (A) qRT-PCR analysis of miR-30c-2* and XBP1 mRNA in HeLa cells treated with Tm for the indicated intervals; data are plotted as fold change in treated versus untreated cells (set at 1). (B–E) Analysis of HeLa cells stably expressing either a miR-30c-2*–specific inhibitor (anti–miR-30c-2*) or a control scrambled inhibitor (Inh Ctrl) and either left untreated or treated with Tm for the indicated intervals. (B, C, and E) qRT-PCR analysis of miR-30c-2* (B), XBP1 mRNA (C), and SEC23B mRNA (E); data are plotted as fold change in treated versus untreated cells (set at 1). (D) Immunoblot analysis of XBP1(S) and β-actin (top) and the corresponding quantitative data plotted as fold change in XBP1(S) protein, normalized to β-actin, in treated versus untreated cells (set at 1; bottom) are from a single representative experiment out of three repeats. Data are means ± SD. *, P < 0.05; **, P < 0.03.
Recent studies have unveiled ER stress-inducible miRNAs that negatively regulate translation of certain secretory pathway proteins (Bartoszewski et al., 2011; Behrman et al., 2011), affording cells another means of balancing protein load with ER capacity. In contrast, our finding that ER stress-inducible miR-30c-2* as expected, XBP1-deficient MEFs exhibited heightened sensitivity to Tm-induced toxicity as compared with wild-type MEFs (Fig. 5 C). Importantly, expression of anti–miR-30c-2* protected wild-type, but not XBP1-deficient, MEFs against Tm-induced death (Fig. 5, C and D).

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regulates expression of XBP1 is the first discovery of a miRNA that directly modulates a UPR effector. Moreover, our data reveal a novel regulatory interface between the PERK and IRE1–XBP1 pathways that involves NF-κB and miR-30c-2* (Fig. 5 E). It seems counterintuitive that miR-30c-2* exists to compromise cellular stress tolerance by extinguishing XBP1. Rather, we reason that overzealous expression of XBP1(S) might be deleterious depending on the nature, intensity, and duration of physiological conditions that increase demands on the ER. By buffering the level of XBP1(S), miR-30c-2* could contribute to the delicate balance between pro- and maladaptive outcomes in the UPR. Interestingly, a recent study revealed that XBP1(S) mRNA is stabilized early in the UPR and then becomes increasingly labile (Majumder et al., 2012). In light of our data, it is intriguing to speculate that the accumulation of miR-30c-2* accelerates the turnover of XBP1(S) mRNA as the UPR progresses.

In addition to its link to the PERK pathway, NF-κB can be activated downstream of many signaling molecules, including IRE1 (Kaneko et al., 2003; Hu et al., 2006), Toll-like receptors (Kawai and Akira, 2010), and cytokine receptors (Li and Verma, 2002). This raises the interesting possibility that certain stimuli not obviously associated with ER stress, such as cytokines that induce NF-κB, might influence XBP1 via miR-30c-2*. We hypothesize that the relative contribution of miR-30c-2* to the “fine tuning” of XBP1 activity may vary in distinct tissue-, developmental-, and stress-specific settings in which the entire UPR or individual UPR pathways are engaged. It will be particularly interesting to investigate the degree to which miR-30c-2* influences gene expression, cell function, and cell fate in normal as well as pathophysiologic processes that involve XBP1, such as plasma cell differentiation (Iwakoshi et al., 2003), macrophage activation by Toll-like receptor signaling (Martinon et al., 2010), and tumor cell survival (Romero-Ramirez et al., 2004). Finally, our data add miR-30c-2* to a small but growing list of mammalian miRNA* species with defined regulatory activities (Yang et al., 2011), underscoring that miRNA* strands play critical roles in gene regulation.

RNA isolation and quantitative real-time RT-PCR
RNAs were extracted from cells using either the miRNeasy Mini Kit for miRNA analysis or the RNeasy Plus Mini Kit (Qiagen), and 300 ng total RNA was reverse transcribed using an RT-PCR system (miRCury LNA Universal RT microRNA PCR; Exiqon) for miRNA analysis and the reverse transcription system (ImProm-II; Promega) for mRNA analysis. Resulting cDNA from miRNA and mRNA were diluted 1:80 and 1:40, respectively. Real-time PCR was performed using a thermocycler (C1000; Bio-Rad Laboratories) with an optic module real-time detection system (CFX96; Bio-Rad Laboratories). Reactions were performed in triplicate using the SYBR green supermix (IQ; Bio-Rad laboratories). miR-30c-2* was amplified using primers (LNA; Exiqon). Forward and reverse primers used are as follows: 5′-GAACCCCGGATGCAAG-3′, and 5′-GAGGCTCGAATGAGTT-3′.

Materials and methods

Cell culture and transfections
NIH-3T3 fibroblasts, MEFs, and HeLa cells were cultured as previously described (Bonninassay et al., 2009). IRE1α−/−, XBP1−/−, and ATF6α−/− and corresponding wild-type MEF cell lines were provided by R. Kaufman (University of Michigan, Ann Arbor, MI). PERK−/− and corresponding wild-type MEF cell lines were provided by D. Ron (University of Cambridge, Cambridge, England, UK). Cells were transfected using either a calcium phosphate method or Lipofectamine 2000 (Invitrogen). For transient transcription, NIH-3T3 cells were seeded at either 7 × 104 cells/60-mm dish or 3 × 105 cells/well on 6-well plates, HeLa cells were seeded at either 104 cells/60-mm dish or 5 × 104 cells/well on 6-well plates, and MEFs were seeded at 105 cells/60-mm dish. To generate cell lines stably expressing miRNA, HeLa cells were seeded at 3 × 102 cells/100-mm dish, transfected with Lipofectamine 2000, and then selected in 3 μg/ml puromycin (MediTech) for 3 d after transfection. Death of all nontransfected control cells was achieved by day 5 after transfection. To induce ER stress, cells were treated with either 1 μg/ml Tm (Sigma-Aldrich) or 0.4 μM Tg (EMD) (MediaTech) for 7 d after transfection. Death of all nontransfected control cells was achieved by day 5 after transfection. To induce ER stress, cells were treated with either 1 μg/ml Tm (Sigma-Aldrich) or 0.4 μM Tg (EMD) (MediaTech) for 7 d after transfection.

Bioinformatic sequence analysis
miRNA sequences were retrieved from the miBase sequence database. Prediction of miRNA target sites in the XBP1 3′ UTR was conducted using two algorithm-based software programs, TargetScan (Whitehead Institute for Biomedical Research) and MicroCosm (European Bioinformatics Institute). Potential transcription factor binding sites upstream of the miR-30c-2* chromosomal location were identified using the NSITE program (Softberry) and the University of California, Santa Cruz Genome browser.

Reporter and expression vectors
The pmir-XBP1 Wt and pmir-MIR-XBP1 Mut luciferase reporter vectors were constructed using oligonucleotides [40 base pairs] containing a single copy of either the wild-type or MUT putative miR-30c-2* target sequence present in the human XBP1 3′ UTR [Integrated DNA Technologies]. The MUT fragment includes target-abolishing substitutions in nucleotides 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 of the miR-30c-2* seed region. Both the wild-type and MUT fragment contained a BlpI site used in screening transformants. Fragments were ligated into the Spel–HindIII sites of the pmirREPORT vector (Applied Biosystems), with firefly luciferase as the primary reporter gene. The pCMV–Renilla luciferase vector (Promega) provides constitutive expression of Renilla luciferase. The pCMV-miR-30c-2 and pCMV-miR-empty vector ( OriGene) contain a cassette encoding GFP. pCMV-miR-30c-2 provides constitutive expression of both miR-30c and miR-30c-2*. The miArrest vectors [GeneCopoeia] pEZXAM02-anti-mir-30c-2* and pEZXAM02 inhibitor control contain cassettes encoding puromycin resistance and mCherry. The pEZXAM02-anti-miR-30c-2* provides constitutive expression of an miR-30c-2*–specific inhibitor, and the pEZXAM02 inhibitor control yields a scrambled, nonspecific anti-miRNA. The posttranscriptional processing of the anti-miRNA expressed from miArrest miRNA inhibitor vectors yields a structure that hybridizes with two molecules of the target miRNA, thereby trapping the miRNA and preventing it from exerting regulatory activity. The pCDNA3.1-bcαΔN vector, provided by W. Lin (University of South Alabama, Mobile, AL), encodes a truncated bcαΔN lacking the amino-terminal 36 amino acids required for signal-induced degradation (Brockman et al., 1995).

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Preparation of cell extracts and immunoblotting

Cell lysates were prepared using lysis buffer (0.25 M Tris-HCl/0.2% SDS, pH 6.8, 20% glycerol, 4% SDS, 10 mM β-glycerolphosphate, and 1 µl/ml protease inhibitor cocktail [Sigma-Aldrich]). Clarified lysates were assayed for protein content using a protein assay [DC, Bio-Rad Laboratories] and bovine serum albumin as standards. Equivalent amounts of protein were added to an equal volume of 2x sample buffer (125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 20% glycerol, 4% SDS, and 0.02% bromophenol blue) and separated by electrophoresis in 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to membranes (Immobilon-P; Millipore) using a 3-cyclohexylamino-1-propanesulfonic acid–buffered system and placed in blocking buffer [PBS, pH 7.4, 5% nonfat milk, and 0.1% Tween 20 (PBST)]. Immunoblotting was performed using a mouse anti-human XBP1 [S1] antibody (catalog no. 647502, BioLegend), a mouse anti–β-actin antibody [Sigma-Aldrich], a rabbit horseradish peroxidase–conjugated anti–mouse IgG antibody (Cell Signaling Technology), and enhanced chemiluminescence substrate (Super Signal West Dura; Thermo Scientific) as described in the manufacturer’s protocol and subjected to a ChIP assay (Fisher Scientific) chemiluminescence reagents. Signals were captured using an imaging system [LAS-1000, Fujifilm] and quantified using Image Gauge V4.0 software (Fujifilm).

ChIP assay

Chromatin was prepared using the enzymatic ChIP kit [ChIP-IT Express, Active Motif] as described in the manufacturer’s protocol and subjected to immunoprecipitation using an anti-NF-kB p65(ReA) antibody [catalog no. 17-1000, Millipore] and an IgG control antibody. The recovered DNA was subjected to PCR using a forward primer, 5′-ATACAGGACCCT-TCAATGGC4C3-3′, and reverse primer, 5′-AAGCATCACAAAGCT-TCCTG3′-3′, to amplify a 131-base pair segment including the putative NF-kB p65(ReA) binding site. Fold enrichment was determined by first solving for the DNA quantity of the NF-kB p65(ReA) ChIP and IgG samples and then calculating the fold enrichment of the NF-kB p65(ReA) ChIP relative to the IgG sample. As controls, successful immunoprecipitation of NF-kB p65(ReA)-associated DNA fragments was verified by quantitative RT-PCR (qRT-PCR) using ChIP primers specific for the NF-kB p65(ReA) binding site. Fold enrichment was determined by first immunoprecipitation using an anti–NF-kB p65(ReA) antibody [Active Motif] as described in the manufacturer’s protocol and subjected to a ChIP assay (Fisher Scientific) chemiluminescence reagents. Signals were captured using an imaging system [LAS-1000, Fujifilm] and quantified using Image Gauge V4.0 software (Fujifilm).


