JunD Represses Transcription and Translation of the Tight Junction Protein Zona Occludens-1 Modulating Intestinal Epithelial Barrier Function

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The AP-1 transcription factor JunD is highly expressed in intestinal epithelial cells, but its exact role in maintaining the integrity of intestinal epithelial barrier remains unknown. The tight junction (TJ) protein zonula occludens (ZO)-1 links the intracellular domain of TJ-transmembrane proteins occludin, claudins, and junctional adhesion molecules to many cytoplasmic proteins and the actin cytoskeleton and is crucial for assembly of the TJ complex. Here, we show that JunD negatively regulates expression of ZO-1 and is implicated in the regulation of intestinal epithelial barrier function. Increased JunD levels by ectopic overexpression of the *junD* gene or by depleting cellular polyamines repressed ZO-1 expression and increased epithelial paracellular permeability. JunD regulated ZO-1 expression at the levels of transcription and translation. Transcriptional repression of ZO-1 by JunD was mediated through cAMP response element-binding protein-binding site within its proximal region of the ZO-1-promoter, whereas induced JunD inhibited ZO-1 mRNA translation by enhancing the interaction of the ZO-1 3'-untranslated region with RNA-binding protein T cell-restricted intracellular antigen 1-related protein. These results indicate that JunD is a biological suppressor of ZO-1 expression in intestinal epithelial cells and plays a critical role in maintaining epithelial barrier function.

INTRODUCTION

JunD is a member of the Jun family of proteins, which are primary components of the activator protein 1 (AP-1) transcription factor (Hirai *et al.*, 1989; Ryder *et al.*, 1989). Jun proteins (c-Jun, JunB, and JunD) can form either AP-1 homo- or heterodimers among themselves or with Fos family members (c-Fos, FosB, Fra1, and Fra2) and directly bind to their target promoters at specific DNA elements such as TGAGTCA (classical AP-1 site) and TGACGTCAT (Liu *et al.*, 2004; Hock *et al.*, 2007; Xiao *et al.*, 2007a). Although the different AP-1 dimers exhibit rather similar DNA binding specificities, they differ in their transactivation efficiencies. Studies of AP-1 functions in vitro as well as in vivo reveal that there are distinct roles for each member (Wisdom *et al.*, 1999; Wenger, 2002; Shaulian and Karin, 2002; Gerald *et al.*, 2004; Tsuji, 2005; Hilfiker-Kleiner *et al.*, 2006; Xiao *et al.*, 2007a; Hock *et al.*, 2007). Although *c-jun* and *junB* behave as immediate early response genes and enhance the G1 to S phase transition of the cell cycle upon mitogenic stimulation

(Ryder *et al.*, 1989; Bakiri *et al.*, 2000), *junD* expression remains almost invariant, and its product is partially degraded upon serum-induced reentry into the cell cycle (Pfarr *et al.*, 1994; Pillebout *et al.*, 2003; Hilfiker-Kleiner *et al.*, 2006). Overexpression of JunD inhibits cell proliferation, whereas immortalized cells lacking *junD* (*junD*^{-/-}) display higher proliferation rates than their wild-type counterparts (Weitzman *et al.*, 2000), suggesting that JunD is a negative regulator of the cell division cycle. Our previous studies show that *junD* expression is tightly regulated in intestinal epithelial cells (IECs) and plays a critical role in maintaining intestinal mucosal tissue homeostasis under physiological conditions (Patel and Wang, 1999; Li *et al.*, 2002; Xiao *et al.*, 2007a). However, the exact role and mechanism of JunD action as a regulator of the intestinal epithelial barrier function remain unknown.

Epithelial cells line the intestinal mucosa and form an important barrier that protects the subepithelial tissue against a wide array of noxious substances, allergens, and luminal microbial pathogens. The effectiveness and stability of this epithelial barrier depends on the activity of junctional complexes that include tight junctions (TJs), adherens junctions, desmosomes, and gap junctions (Guo *et al.*, 2003; Van Itallie and Anderson, 2004; Schneeberger and Lynch, 2004; Funke *et al.*, 2005). The TJ is the apical-most element of the junctional complex and seals epithelial cells together in a way that prevents even small molecules from leaking between cells (Van Itallie and Anderson, 2004; Matter *et al.*, 2005; Shin *et al.*, 2006). To date, four classes of TJ-transmembrane proteins and >30 TJ-membrane-associated proteins are identified in mammalian epithelial and endothelial

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cells (Fanning, 2006). At the ultrastructural level, the TJ seems as highly organized strands that encircle the apical-lateral boundary and primarily consist of transmembrane proteins such as occludin, tricellulin, and one or more members of the claudin family. These transmembrane proteins are, in turn, associated with a cytosolic plaque of proteins that closely links to the cortical cytoskeleton. Despite considerable efforts from many laboratories, the precise mechanisms underlying TJ assembly and the coordination of signals and barrier activity in response to stress are poorly understood.

The zona occludens (ZO)-1 protein is the best characterized member of a large family of membrane-associated scaffolding and signaling molecules known as the membrane-associated guanylate kinase homologues (MAGUKs). MAGUK proteins, characterized by a core motif of conserved protein-binding domains including one or more postsynaptic density 95/disc-large/zona occludens domains, a Src homology 3 domain, and a guanylate kinase-like domain, are highly organized within a complicated network of interconnecting cytoskeleton with TJ-transmembrane proteins (Funke *et al.*, 2005; Fanning, 2006). Much effort has been made to define the function of ZO-1 and has shown that it is one of key regulators of TJ assembly (Fanning *et al.*, 1998; Reichert *et al.*, 2000; Ryeom *et al.*, 2000; Wittchen *et al.*, 2000; Fanning *et al.*, 2007a). Umeda *et al.* (2006) have recently reported that ZO-1 and its homologue ZO-2 independently determine where claudins are polymerized in tight junction strand formation and that cells are unable to assemble tight junctions when both ZO-1 and ZO-2 are down-regulated. ZO-1 is proposed to function as a multidomain scaffold that coordinates the assembly of transmembrane and cytosolic proteins into the TJ and/or regulates the activity of these proteins once assembled (Utepergenov *et al.*, 2006; Fanning *et al.*, 2007a,b). In support of this notion, ZO-1 is shown to bind most of TJ-transmembrane proteins including occludin, claudins, and the CTX (Ig) superfamily (Schneeberger and Lynch, 2004; Fanning, 2006). In addition, ZO-1 also binds >10 cytoplasmic proteins and various components of the cortical cytoskeleton.

Maintenance of dynamic level of ZO-1 protein is critical for normal function of the epithelial barrier, but little is known about the mechanism by which ZO-1 expression is regulated at the molecular level. By computational analysis, the ZO-1-promoter was shown to contain several potential AP-1 and cAMP response element-binding protein-binding (CREB) sites, suggesting that it is a putative target of JunD. In this study, we sought to directly investigate the role of JunD in regulating ZO-1 expression. The data presented here indicate that overexpression of JunD represses ZO-1 transcription, in turn causing an increase in epithelial paracellular permeability. Experiments using different ZO-1 promoter mutants revealed that the transcriptional repression of ZO-1 by JunD is mediated through CREB binding sites within the proximal region of the ZO-1 promoter. Unexpectedly, however, we also found that JunD also inhibited ZO-1 translation, likely by enhancing the interaction of the ZO-1 3'-untranslated region (3'-UTR) with the RNA-binding protein T cell-restricted intracellular antigen 1-related protein (TIAR). This study provides new insights into the mechanisms underlying ZO-1 expression and shows that JunD is implicated in the regulation of the epithelial barrier function through modulation of ZO-1.

MATERIALS AND METHODS

Chemicals and Supplies

Tissue culture medium and dialyzed fetal bovine serum were from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma-Aldrich (St. Louis, MO). The antibodies recognizing JunD, TIAR, HuR, and AUF1 were from Santa Cruz Biotechnology (Santa Cruz, CA), whereas antibodies against ZO-1, occludin, and E-cadherin were purchased from Invitrogen. The secondary antibody conjugated to horseradish peroxidase was from Sigma-Aldrich. α -Difluoromethylornithine (DFMO) was from Genzyme (Cambridge, MA). 14 C-labeled mannitol was purchased from Amersham Pharmacia Biotech (Chalfont St. Giles, United Kingdom). The 12-mm Transwell filters (0.4- μ m pore size; clear polyester) were obtained from Corning Life Sciences (Lowell, MA).

Cell Culture

Caco-2 cells (a human colon carcinoma cell line) were obtained from the American Type Culture Collection (Manassas, VA) at passage 16. They were maintained in Eagle's minimal essential medium with 10% heat-inactivated fetal bovine serum and 50 μ g/ml gentamicin, and passages 18–23 were used for the experiments as described previously (Li *et al.*, 2002). The stable Cdx-2-transfected IEC-6 cells (IEC-Cdx2L1 cells) were developed from IEC-6 cells (Quaroni *et al.*, 1979) that were derived from normal rat intestinal crypt cells (Suh and Traber, 1996). The LacSwitch System (Stratagene, La Jolla, CA) was used for directing the conditional expression of Cdx2, and isopropyl β -D-thiogalactoside (IPTG) was used to induce gene expression. Stock stable Cdx-2-transfected IEC-6 cells were grown in DMEM as described previously (Rao *et al.*, 2002; Guo *et al.*, 2005). Before experiments, cells were grown in DMEM containing 4 mM IPTG for 16 d to induce cell differentiation.

Plasmid Construction and Transfection

The plasmid clone (pRSV-hjD) containing the human *junD* gene was obtained from American Type Culture Collection. Two polymerase chain reaction (PCR) primers (sense, TACCGCTAGCGGAGGATGGAAACACCCTTC; antisense, GTCAGGTACCCTCAGTACGCCGGGACCTG) were used to amplify the complete open reading frame of *junD* from pRSV-hjD. The resulting PCR product was sequenced to confirm that no mutations were introduced by PCR and then cloned into an expression vector pcDNA3.1(+) (Invitrogen) for expression directed by the cytomegalovirus promoter. The full-length ZO-1 promoter was cloned from human genomic DNA, and various constructs of the ZO-1 promoter luciferase (Luc) reporter were generated, including full-length ZO-1 promoter Luc reporter, F-Luc (~2.8-kb regulatory region upstream of the ZO-1 gene fused to the Luc reporter gene), and its deletion mutants, 22-Luc, 10-Luc, and 5-Luc. The 5'-deletion ZO-1 promoter construct of the 10-Luc and the cAMP response element-binding protein (CREB) point mutants of the ZO-1 promoter were generated using QuikChange site-directed mutagenesis kit and performed according to the manufacturer's instructions (Stratagene). Mutations of CREB binding site within the ZO-1 promoter were verified by DNA sequencing. Transient transfection was performed with Lipofectamine reagent (Invitrogen). All these ZO-1-promoter constructs are from firefly luciferase reporters. The promoter constructs were transfected into cells along with pRL-null, a *Renilla* luciferase control reporter vector from Promega (Madison, WI), to monitor transfection efficiencies. The transfected cells were lysed for assays of promoter activity using the dual-luciferase reporter assay system (Promega). The luciferase activity from individual constructs was normalized by *Renilla*-driven luciferase activity in every experiment.

RNA Interference

Silencing RNA duplexes were designed to specifically cleave JunD mRNA, and they were transfected into cells as described previously (Pfäfflin *et al.*, 2006). The sequence of small interfering RNA (siRNA) specifically targeting the coding region of JunD mRNA (siJunD) was AGCATGCTGAAGAAA-GACGC, whereas the sequence of control siRNA (C-siRNA) was ATGAT-CAGCCTTTCAGCTC. The siRNA sequence targeting TIAR (siTIAR) was AAGGGCTAATTTCATTGTGTCAGA; and the C-siRNA was AATTCTCCGA-ACGTGTCACGT (Mazan-Mamczarz *et al.*, 2006). For each 60-mm cell culture dish, 15 μ l of the 20 μ M stock duplex siJunD, siTIAR, or C-siRNA was mixed with 300 μ l of Opti-MEM (Invitrogen). This mixture was gently added to a solution containing 15 μ l of Lipofectamine 2000 in 300 μ l of Opti-MEM. The solution was incubated for 20 min at room temperature and gently overlaid onto monolayers of cells in 3 ml of medium, and cells were harvested for various assays after 48-h incubation.

Western Blot Analysis

Whole-cell lysates were prepared using 2% SDS, sonicated, and centrifuged (12,000 rpm) at 4°C for 15 min. The supernatants were boiled for 5 min and size-fractionated by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide). After transferring proteins onto nitrocellulose filters, the blots were incubated with primary antibodies recognizing ZO-1, occludin, E-cadherin,

TIAR, AUFI, or HuR; following incubations with secondary antibodies, immunocomplexes were developed by using chemiluminescence.

Reverse Transcription (RT)-PCR and Real-Time PCR Analysis

Total RNA was isolated by using RNeasy mini kit (QIAGEN, Valencia, CA) and used in reverse transcription and PCR amplification reactions as described previously (Guo *et al.*, 2005). The specific sense and antisense primers for ZO-1 included 5'-GCCTCTGCAGTAAAGCAT-3' and 5'-AAGAGCTG-GCTGTTTAA-3', and the expected size of ZO-1 fragments was 249 base pairs. The levels of β -actin PCR product were assessed to monitor the even RNA input in RT-PCR samples. Real-time quantitative PCR (qRT-PCR) was performed using 7500-Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA) with specific primers, probes, and software (Applied Biosystems). The levels of ZO-1, occludin, and E-cadherin mRNAs were quantified by qRT-PCR analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Chromatin Immunoprecipitation (ChIP)

Caco-2 cells were transfected with the JunD expression vector or control vector (Null) for 48 h and then fixed with 1% formaldehyde to cross-link chromatin. ChIP analysis was performed using the ChIP-IT kit (Active Motif, Carlsbad, CA), following the manufacturer's recommendations with minor modification, as described previously (Xiao *et al.*, 2007a). Briefly, cells were suspended in lysis buffer and gently dounced on ice with 10 strokes to aid in nuclei release. After centrifugation, the nuclear pellet was suspended in digestion buffer and the chromatin was sheared with Enzymatic Shearing Cocktail. The sheared DNA samples were centrifuged, and the supernatants were collected and precleared with protein G beads. The precleared DNA samples were then incubated with the anti-JunD or control immunoglobulin G (IgG) antibodies overnight with constant rotation. The immunocomplexes were captured by addition of protein G beads, and the immunoprecipitated DNA was collected from the beads using ChIP elution buffer. DNA-protein cross-links were reversed and deproteinized, and DNA was recovered and amplified by PCR. Primers to amplify the proximal region of the ZO-1 promoter containing a CREB binding site were 5'-CTTGAGGTCTAATGTGGGGTG-3' and 5'-CATGGCTTTCATCTCCGAG-3'. Primers to amplify the proximal region of the GAPDH promoter (a negative control) were 5'-TAC-TAGCGGTTTTACGGGCG-3' and 5'-TCGAA-CAGGAGGAGCAGAGAGCGA-3'. The DNA isolated through IgG ChIP was used as a negative control. The input DNA, obtained from chromatin that was processed (cross-linked and reversed) similarly to the samples, served as a positive control for PCR effectiveness.

Translation Assays

ZO-1 translation was measured by chimeric firefly luciferase reporter assays (Vasudevan and Steitz, 2007) and polysome analysis (Kawai *et al.*, 2006). The 545-base pairs AU-rich element (ARE) fragment from the human ZO-1 3'-UTR was amplified and subcloned into the pGL3-Luc plasmid (Promega) at the XbaI site to generate the chimeric pGL3-Luc-ZO1-3'UTR. The sequence and orientation of the fragment in the luciferase reporter were identified by DNA sequencing and enzyme digestion. Luciferase activity was measured using the dual-luciferase assay system following the manufacturer's instruction. The firefly-to-*Renilla* luciferase ratio was further normalized for RNA levels.

Polysome analysis was performed as described previously (Kawai *et al.*, 2006). For the preparation of different molecular weights of polysomal fractions, 70% confluent cells were transfected with the JunD expression vector or Null plasmid for 48 h before the collection. Gradient analysis was performed essentially as described previously (Pillai *et al.*, 2005). Briefly, cycloheximide was added to 100 μ g/ml concentration 15 min before lysis and was also incubated in solution used for cell washing and lysis. After lysis with the PEB lysis buffer (0.3 M NaCl, 15 mM MgCl₂, 15 mM Tris-HCl, pH 7.6, 1% Triton X-100, 1 mg/ml heparin, and 100 μ g/ml cycloheximide), nuclei were pelleted at 10,000 $\times$ g for 10 min, and the resulting supernatant was fractionated through 10 to 50% linear sucrose gradient. The eluted fractions were prepared with a fraction collector (Brandel, Gaithersburg, MD), and their quality was monitored at 254 nm using a UV-6 detector (ISCO, Lincoln, NE). RNA extracted from gradient fractions and input lysates was used for qPCR analysis.

Preparation of Synthetic RNA Transcripts

cDNA from Caco-2 cells was used as a template for PCR amplification of the coding region (CR) and 3'-UTR of ZO-1. The 5'-primers contained the T7 RNA polymerase promoter sequence (T7): 5'-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3'. To prepare the CR of ZO-1, oligonucleotides (T7)5'-CGGTGGTCTGAGCCTGTAAG-3' and 5'-GGATCTACATGCGACGACAA-3' were used. To prepare the ZO-1 3'-UTR template (spanning position 5659–7186), oligonucleotides (T7)5'-CCCGAGATCCAAATTATCTCG-3' and 5'-GCAAA-CAGACCAAGCCAGCAC-3' were used. PCR-amplified products were used as templates to transcribe biotinylated RNAs by using T7 RNA polymerase in the

presence of biotin-cytidine 5'-triphosphate as described previously (Mazan-Mamczarz *et al.*, 2006).

RNA-Protein Binding Assays

For biotin pull-down assays, biotinylated transcripts (6 μ g) were incubated with 120 μ g of cytoplasmic lysate for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Invitrogen) and analyzed by Western blot analysis. To assess the association of endogenous TIAR with endogenous ZO-1 mRNA, immunoprecipitations (IPs) of TIAR-mRNA complexes were performed as described previously (Xiao *et al.*, 2007). Twenty million Caco-2 cells were collected per sample, and lysates were used for IP for 4 h at room temperature in the presence of excess (30 μ g) IP antibody (IgG or anti-TIAR). RNA in IP materials was used in RT followed by PCR analysis to detect the presence of ZO-1 mRNA.

Immunofluorescence Staining

Immunofluorescence was performed as described previously (Guo *et al.*, 2003). Cells were fixed using 3.7% formaldehyde, and the rehydrated samples were incubated overnight at 4°C with primary antibody in blocking buffer and then incubated with secondary antibody conjugated with Alexa Fluor-594 (Invitrogen) for 2 h at room temperature. After rinsing, slides were mounted, and viewed through a confocal microscope (model LSM410; Carl Zeiss, Thornwood, NY). Images were processed using Photoshop software (Adobe Systems, San Jose, CA).

Paracellular Tracer Flux Assay and Transepithelial Electrical Resistance Measurement

Flux assays were performed on the 12-mm Transwell as described in our previous publications (Guo *et al.*, 2003). [¹⁴C]Mannitol (mol. wt. 184), a membrane-impermeable molecule, served as the paracellular tracer, and the basal medium was collected 2 h after addition of [¹⁴C]mannitol for measurement using a liquid scintillation counter (Beckman Coulter, Fullerton, CA). Transepithelial electrical resistance (TEER) was measured with an epithelial voltmeter under open circuit conditions (WPI, Sarasota, FL) as described previously (Wang *et al.*, 2005), and the TEER of all monolayers was normalized to that of control monolayers in same experiment.

Polyamine Analysis

The cellular polyamine content was analyzed by high-performance liquid chromatography analysis as described previously (Wang and Johnson, 1991). Briefly, after 0.5 M perchloric acid was added, the cells were frozen at -80°C until ready for extraction, danylation, and high-performance liquid chromatography analysis. The standard curve encompassed 0.31–10 μ M. Values that fell >25% below the curve were considered undetectable. The results are expressed as nanomoles of polyamines per milligram of protein.

Statistics

Values are means $\pm$ SE from three to eight samples. Immunoblotting and immunofluorescence staining results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using Duncan's multiple range test (Harter, 1960).

RESULTS

Overexpression of JunD Inhibits ZO-1 Expression and Disrupts Epithelial Barrier Function

To determine the involvement of JunD in the regulation of the intestinal epithelial barrier, we examined the effect of overexpressing the wild-type *junD* gene on the expression of the TJ protein ZO-1 in Caco-2 cells. This cell line was chosen for analysis because it provides a unique in vitro model for epithelial barrier studies (Chen *et al.*, 2007; Halbleib *et al.*, 2007; Schwarz *et al.*, 2007; Subramanian *et al.*, 2007) and can be efficiently transfected (Li *et al.*, 2002; Xiao *et al.*, 2007a). As shown in Figure 1A, transient transfection with the JunD vector dramatically increased JunD protein expression levels (by ~6-fold at 24 h and by ~14-fold at 48 and 72 h after transfection) compared with JunD levels in the population transfected with the control vector. Increased JunD was associated with a potent inhibition in ZO-1 expression, as indicated by a decrease in levels of ZO-1 mRNA (Figure 1Ba) and protein (Figure 1C). The inhibitory effect of JunD was specific for ZO-1 because JunD overexpression did not alter

Figure 1. Effect of ectopic expression of the *junD* gene on ZO-1 expression. (A) Representative immunoblots of JunD protein. Caco-2 cells were grown in minimum essential medium containing 10% fetal bovine serum and transfected using the expression vector containing human *junD* cDNA (JunD) or control vector lacking *junD* cDNA (Null) by using Lipofectamine. Whole-cell lysates were harvested at the indicated times after transfection, and JunD protein (~40 kDa) was measured by Western blot analysis. After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. (B) Changes in mRNA levels in cells described in A. a, ZO-1; b, occludin; and c, E-cadherin. Total cellular RNA was isolated, and levels of ZO-1, occludin, and E-cadherin mRNAs were measured by real-time quantitative PCR analysis. Values are means $\pm$ SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with the Null for 48 h. C, representative immunoblots of ZO-1, occludin, and E-cadherin proteins as measured by Western blot analysis in cells described in A. Data are representative from three independent experiments showing similar results.

the expression levels of occludin or the adherens junction E-cadherin (Figure 1B, b and c, and C).

Consistently, ZO-1 immunostaining decreased significantly after ectopic expression of the *junD* gene (Figure 2, A and B), although there were no differences in the levels of occludin or E-cadherin immunostaining (Figure 2, C–F) between controls and cells overexpressing JunD. Transient transfection with the JunD vector also disrupted the intestinal epithelial barrier function. TEER values decreased (Fig-

Figure 2. Cellular distribution of ZO-1, occludin, and E-cadherin proteins and changes in epithelial barrier function after JunD overexpression. For immunostaining studies, Caco-2 cells were plated in a four-well chamber slide for 2 d and then transfected with the JunD expression vector (JunD) or control vector (Null) for 72 h. Cells were fixed; permeabilized; incubated with the specific antibody against ZO-1, occludin, or E-cadherin; and then with anti-IgG conjugated with fluorescein isothiocyanate. (A) ZO-1 in Null-transfected cells. (B) ZO-1 in JunD-transfected cells. (C) Occludin in Null-transfected cells. (D) Occludin in JunD-transfected cells. (E) E-cadherin in Null-transfected cells. (F) E-cadherin in JunD-transfected cells. Original magnification, 1000 $\times$. Three experiments were performed that showed similar results. (G) Changes in TEER in control cells and cells transfected with JunD or Null for 72 h. TEER assays were performed on 12-mm Transwell filters as described in *Materials and Methods*. Values are means $\pm$ SE of data from eight samples. * $p < 0.05$ compared with control cells and cells transfected with the Null. (H) [¹⁴C]Mannitol permeability. The membrane-impermeable trace molecule [¹⁴C]mannitol was added to the insert medium, and the entire basal medium was collected 2 h thereafter for paracellular trace flux assays. Values are means $\pm$ SE of data from eight samples. * $p < 0.05$ compared with control cells and cells transfected with the Null.

ure 2G), whereas the levels of paracellular flux of [¹⁴C]mannitol increased (Figure 2H) after JunD overexpression. Neither ZO-1 expression nor the epithelial barrier function was affected in the control vector transfection group. In addition, the decreases in ZO-1 expression and epithelial barrier function in cells overexpressing JunD did not result simply from decreased cell growth, because growth inhibition triggered by the removal of serum for 72 h did not alter ZO-1 levels or epithelial barrier function in Caco-2 cells (data not shown). These results indicate that forced expression of the *junD* gene represses ZO-1 expression and results in dysfunction of the epithelial barrier.

JunD Represses ZO-1 Transcription through a CREB Binding Site within Its Proximal Promoter Region

To define the mechanism by which JunD represses ZO-1 expression, we examined the effect of increased JunD on

Figure 3. Effect of ectopic expression of the *junD* gene on ZO-1 promoter activity after mutation of AP-1 and CREB binding sites. (A) Schematic representation of deletion of ZO-1-promoter Luc reporter constructs. AP-1 and CREB indicate the relative locations of AP-1 and CREB binding sites within the ZO-1-promoter. (B) Basal activity of various deletion mutants of the ZO-1 promoter in Caco-2 cells without JunD overexpression. Cells were transfected with different deletion mutants of ZO-1-promoter, and levels of the luciferase reporter activity were detected 48 h after the transfection. Results were normalized relative to *Renilla*-driven luciferase activity and expressed as means $\pm$ SE of data from three separate experiments. (C) Changes in luciferase reporter activity of deletion constructs after JunD overexpression. Cells were cotransfected with different ZO-1 promoter luciferase reporter deletion constructs and the JunD expression vector or Null, and luciferase activity was assayed 48 h after transfection. Values are means $\pm$ SE of data from three separate experiments. * $p < 0.05$ compared with cells transfected with the Null. (D) Luciferase reporter activity of CREB point mutant of ZO-1-promoter. a, schematic representation of CREB point mutants of the ZO-1 promoter. Mutagenic oligonucleotides were designed to hybridize to the ZO-1-promoter construct to create the mutant where the CREB consensus site was eliminated by making a base change. b, levels of reporter gene activity. Values are means $\pm$ SE of data from three separate experiments. * $p < 0.05$ compared with Null-transfected cells. (E) Binding activity as measured by ChIP analysis. The association of JunD with a proximal region of ZO-1 promoter (between -620 and -446) was determined using an anti-JunD antibody (Ab); IgG was used as a negative control. Cross-linked chromatin isolated from cells transfected with either JunD-overexpressing or empty control vectors was immunoprecipitated by using an anti-JunD antibody and the associated chromosomal DNA fragments were amplified by PCR using ZO-1 promoter-specific primers and GAPDH promoter-specific primers as described in *Materials and Methods*. The expected size of the PCR product was 175 base pairs. Chromosomal DNA input was subject to the same procedures and served as a positive control. Three experiments were performed that showed similar results.

ZO-1 gene transcription. The ZO-1 promoter fragment was cloned from human genomic DNA, which contained several potential AP-1 and CREB sites as illustrated in Figure 3A (Supplemental Figure 1). To map the JunD-responsive region of the ZO-1-promoter, different reporter constructs were prepared containing deletions in the ZO-1-promoter. As shown in Figure 3B, the elements that contained a 2787-base pair region of the ZO-1-promoter were required for basal and regulatory ZO-1 expression, as the 5'-deletions of the ZO-1-promoter gradually decreased to basal levels of reporter gene activity. The results presented in Figure 3C further show that ectopic expression of the *junD* inhibited ZO-1 promoter activity and that this repression was mediated through a CREB binding site within its proximal promoter region. The ZO-1 promoter activity decreased signif-

icantly after JunD overexpression when cells were transfected with either the F-Luc construct (full-length promoter), 22-Luc (-2227-base pair promoter fragment containing three CREB sites but without AP-1 site), or 10-Luc (-1007-base pair promoter fragment containing only one CREB site). However, this inhibition completely disappeared when cells were transfected with the 5-Luc (-501-base pairs promoter fragment containing no CREB site). To further determine the exact function of this proximal CREB site within the ZO-1 promoter region, the CREB site was eliminated by a two-base change, as indicated in Figure 3Da. As shown in Figure 3Db, the JunD-induced ZO-1 repression was prevented when this CREB site within the proximal region of the ZO-1 promoter was point-mutated. Under these conditions, there were no significant differences in

ues are means $\pm$ SE of data from three separate experiments. * $p < 0.05$ compared with controls and cells exposed to DFMO plus Put. (D) [^{14}C]Mannitol permeability in cells described in A. After cells were grown in control cultures or cultures containing DFMO or DFMO plus Put for 4 d, and then they were trypsinized, plated at confluent density on the insert, and maintained under the same culture conditions for additional 48 h. [^{14}C]Mannitol was added to the insert medium, and the entire basal medium was collected 2 h thereafter for paracellular trace flux assays. Values are means $\pm$ SE of data from eight samples. * $p < 0.05$ compared with control and DFMO + Put.

levels of ZO-1 promoter activity between controls and cells overexpressing JunD.

ChIP analysis was used to examine the *in vivo* association of JunD with a proximal region of the ZO-1 promoter. Nuclear fractions were immunoprecipitated using a specific anti-JunD antibody in cells transfected with the JunD overexpression or control vectors, and the associated DNA was purified. Using specific PCR primers, a 174-base pair PCR product was obtained which matched the sequence of a proximal region of ZO-1 promoter from -620 to -446 (containing the CREB site) relative to the transcriptional start site. JunD was bound to the ZO-1 promoter *in vivo*, as shown using an anti-JunD antibody in cells overexpressing JunD (Figure 3E, top, lane 5). This association was specific for JunD, because no PCR product was detectable in JunD-transfected cells when using a nonspecific antibody (IgG; Figure 3E, top lane 6) or when using primers to an unrelated promoter such as the GAPDH promoter (Figure 3E, bottom, lane 5). These results indicate that JunD represses ZO-1 transcription by interacting with CREB binding site within the proximal region of the ZO-1 promoter.

Increased Levels of Endogenous JunD by Depleting Cellular Polyamines Is Associated with ZO-1 Inhibition and Impairment of the Epithelial Barrier Function

To examine the implication of JunD-modulated ZO-1 expression in maintaining the epithelial barrier integrity under biological conditions, we examined the changes in ZO-1 expression levels and intestinal epithelial paracellular permeability after an increase in endogenous JunD abundance by depleting cellular polyamines in differentiated IEC-Cdx2L1 cells. As reported previously (Suh and Traber, 1996; Rao *et al.*, 2002; Guo *et al.*, 2005), IEC-Cdx2L1 cells grown in the presence of 4 mM IPTG for 16 d showed a well-differentiated phenotype. These differentiated IEC-Cdx2L1 cells were polarized, showed lateral membrane interdigitations and microvilli at the apical pole, and also exhibited a well-

developed barrier function as indicated by an increase in TEER and a decrease in paracellular tracer flux compared with their parental IEC-6 cells (data not shown). Consistent with our previous studies (Rao *et al.*, 2002; Guo *et al.*, 2005), exposure of differentiated IEC-Cdx2L1 cells to 5 mM DFMO (specific inhibitor of polyamine biosynthesis) for 6 d completely inhibited ornithine decarboxylase (ODC) enzyme activity and almost totally depleted cellular polyamines. The levels of putrescine and spermidine were undetectable on day 6 after treatment with DFMO, and spermine had decreased by ~65% (data not shown).

As shown in Figure 4A, polyamine depletion by DFMO increased the steady-state levels of JunD protein, which was prevented by exogenous putrescine (10 μM) given together with DFMO. The increase in endogenous JunD levels in the polyamine-deficient cells was also associated with an inhibition in ZO-1 promoter activity (Figure 4B, left) when cells were transfected with the 10WT-Luc (with the CREB site), but this repression was abolished when the CREB site was mutated (Figure 4B, right). The decreased levels of ZO-1 promoter activity were accompanied by reductions of ZO-1 mRNA (Figure 4Ca) and protein (Figure 4Cb) in polyamine-deficient cells. Furthermore, the reduced ZO-1 levels in cells with elevated JunD through polyamine depletion was also associated with dysfunction of the epithelial barrier, as indicated by an increase in levels of paracellular flux of [^{14}C]mannitol (Figure 4D). In the presence of DFMO, exogenous putrescine not only prevented induced JunD but also returned ZO-1 expression to near normal level. Consequently, the epithelial barrier function was also returned to normal when putrescine was administered together with DFMO.

JunD Silencing Restores ZO-1 Expression and Promotes the Barrier Function

We used siRNA targeting the JunD mRNA (siJunD) to reduce JunD levels (Pfäfflin *et al.*, 2006) and thus directly

Figure 5. Effect of JunD silencing on ZO-1 expression and paracellular permeability in polyamine-deficient cells. Cells were initially treated with 5 mM DFMO for 4 d and then transfected with siJunD or C-siRNA in the presence of DFMO for additional 48 h. (A) Representative immunoblots of JunD and ZO-1 proteins. (B) Changes in levels of paracellular permeability in JunD-knockdown cells described in A. After cells were transfected with siJunD or C-siRNA, they were plated at confluent density on the insert, and maintained in the presence of DFMO for additional 48 h. Levels of paracellular permeability were measured 2 h after addition of [^{14}C]mannitol. Values are means $\pm$ SE of data from eight samples. * $p < 0.05$ compared with control cells; +, $p < 0.05$ compared with cells treated with DFMO alone or DFMO-treated cells transfected with C-siRNA.

examine its putative role in the observed changes in ZO-1 reduction after polyamine depletion. With >95% cells transfected (data not shown), siJunD potently silenced JunD expression in polyamine-deficient cells (Figure 5A). To verify the specificity of siJunD used in this study, the membranes were reprobbed with anti-JunB antibody and showed that levels of JunB protein were not affected by siJunD (data not shown). Inhibition of JunD expression by transfection with the siJunD prevented the repression of ZO-1 protein levels in polyamine-deficient cells. When DFMO-treated cells were exposed to siJunD for 48 h, ZO-1 protein expression returned to near normal level. JunD silencing also promoted function of the epithelial barrier in polyamine-deficient cells as indicated by a decrease in levels of paracellular flux of [^{14}C]mannitol (Figure 5B). In contrast, transfection with C-siRNA at the same concentration had no effect on the expression of JunD and ZO-1 proteins, and it did not alter paracellular permeability. Because polyamine depletion also increased the levels of other transcription factors such as p53 (Zhang *et al.*, 2007), we examined the effect of p53 silencing on ZO-1 expression and found that inhibition of p53 expression by transfection with siRNA targeting the p53 mRNA did not prevent the repression of ZO-1 expression or the barrier dysfunction in polyamine-deficient cells (data not shown). These results indicate that ZO-1 is a downstream target of JunD after polyamine depletion and that increased levels of endogenous JunD in polyamine-deficient cells re-

sults in dysfunction of the epithelial barrier as a result of repression of ZO-1 expression.

JunD Induces Relocalization of ZO-1 mRNA to Low-Molecular-Weight Polysomes

Quantitative analysis of the changes in ZO-1 mRNA and protein levels (Figure 1) indicated a 50% decrease in ZO-1 mRNA levels after ectopic expression of *junD*, whereas ZO-1 protein levels were decreased by >95%. These findings suggest that JunD also regulated ZO-1 protein stability, translation, or both processes. To test these possibilities, two experiments were performed. First, we examined changes in the half-life of ZO-1 protein after JunD overexpression. Our results (Supplemental Figure 2) revealed that increased JunD failed to alter the stability of ZO-1 protein, because there were no significant differences in the degradation rates of ZO-1 protein between controls and cells transfected with the JunD vector after protein synthesis was inhibited by administration of cycloheximide.

Second, we determined the effect of induced JunD on ZO-1 translation by examining the relative distributions of ZO-1 mRNA and RNA-binding proteins (HuR, TIAR, and AUF1) on individual fractions from polysome gradients. Polysome distribution profiles were prepared in control cells and in cells transfected with the JunD vector as described previously (Bhattacharya *et al.*, 2006; Lal *et al.*, 2006; Vasudevan and Steitz, 2007). In this study, fractions 1–3 made up mRNAs that were not associated with components of the translation machinery or cosedimented with ribosome subunits (monosomes); hence, they were not considered to be translated. Fractions 4–6 made up mRNAs that bound to single ribosomes or formed polysomes of low molecular-weight and they were considered to be translated at low-to-moderate levels. Fractions 7–9 made up the mRNAs that were associated with polysomes of high molecular weight, and they were thus considered to be actively translated. As shown in Figure 6A, ectopic expression of the *junD* caused a global inhibition of translation as indicated by a decrease in the high-molecular-weight polysome component. Western analysis of fractions across the profiles showed that HuR was abundant in low-molecular-weight fractions (2–5), but there were no significant differences in HuR abundance in sucrose density gradients from control and JunD-transfected cells. The levels of TIAR and AUF1 in both controls and JunD-transfected cells were predominantly located in monosomes and ribosome subunits (1–3). Interestingly, the levels of TIAR detected in fractions 1–2 were increased after JunD overexpression. When the distribution of the ZO-1 mRNA levels in fractions across the profiles in these two groups was compared, ZO-1 mRNA was shown to shift toward non-translating or low-translating gradient fractions (3–4) after JunD overexpression (Figure 6B). These observations are in keeping with the general trend in polysome levels and strongly support the possibility that increased JunD also inhibits ZO-1 translation.

JunD Increases the Interaction of ZO-1 3'-UTR with RNA-binding Protein TIAR

To define the exact role of JunD in regulating ZO-1 translation, we studied whether JunD levels altered the interaction of ZO-1 mRNA with RNA-binding proteins. Based on the fact that there are stretches of U- and AU-rich regions in the ZO-1 3'-UTR (Figure 7A) and that transcripts bearing such sequences generally show affinity for TIAR, AUF1, and HuR, we postulated that these RNA-binding proteins associated with the ZO-1 3'-UTR and further hypothesized that these interactions would be regulated by JunD. To test these

Figure 6. Polysomal distributions of RNA-binding proteins (HuR, TIAR, and AUF-1) and ZO-1 mRNA in cells overexpressing JunD. After cells were transfected with Null (control) or the JunD expression vector (JunD) for 48 h, nuclei were pelleted, and the resulting supernatant was fractionated through a 10 to 50% linear sucrose gradient. (A) Distribution of HuR, TIAR, and AUF1 proteins in various polysomal fractions prepared from control cells (a) or cells transfected with the JunD (b). Proteins obtained from individual fractions were analyzed by Western blot analysis with the specific antibody against HuR, TIAR, or AUF1; and three experiments were performed that showed similar results. (B) quantification of ZO-1 mRNA levels in extracts from controls and JunD-transfected cells. Left, relative distribution of ZO-1 mRNA along the gradients. Total RNA was isolated from each of fractions, and levels of ZO-1 mRNA were measured by real-time RT-PCR analysis. Right, quantitative data of ZO-1 mRNA in fractions of monosomes unbound to ribosomal material (UN), fractions of low molecular weight (LMW), and fractions of high molecular weight (HMW). Values are means $\pm$ SE from three separate experiments. * $p < 0.05$ compared with controls.

possibilities, we first used biotinylated transcripts spanning the ZO-1 3'-UTR in RNA pull-down assays using cell lysates prepared from controls and cells overexpressing JunD. As shown in Figure 7B, the ZO-1 3'-UTR transcript readily associated with cytoplasmic AUF1 and HuR, as detected by Western blot analysis of the pull-down material, but there were no changes in the binding intensity between controls and cells transfected with the JunD vector for 24 and 48 h. In contrast, ZO-1 3'-UTR binding to TIAR increased significantly when using lysates prepared from JunD-transfected cells. Importantly, transcripts corresponding to the coding region (CR) of ZO-1 did not bind to AUF1, HuR, and TIAR (Figure 7Bb).

Second, we examined the association of endogenous ZO-1 mRNA with endogenous TIAR following increased JunD through IP of TIAR under conditions that preserved its association with target mRNAs in ribonucleoprotein (RNP) complexes. The RNP complexes immunoprecipitated using anti-TIAR antibody did contain endogenous ZO-1 mRNA, as measured by conventional PCR and real-time (quantitative PCR or Q-PCR) analyses (Figure 7C). The association of endogenous ZO-1 mRNA with endogenous TIAR increased significantly after ectopic expression of the *jund*. Importantly, the ZO-1 mRNA was undetectable in nonspecific IgG1 IP materials (Figure 7Ca, right). In this study, GAPDH was also examined as a negative control, because this highly abundant mRNA is present as a low-level contaminant in the IP materials, thus serving to monitor the equal input of lysate as reported (Lal *et al.*, 2006; Mazan-Mamczarz *et al.*, 2006). In addition, we also examined the effect of increased JunD on the expression of TIAR, AUF1, HuR, and demonstrated that JunD overexpression did not alter levels of total

TIAR, AUF1, and HuR proteins (Supplemental Figure 3). Together, these findings indicate that increased JunD promotes the interaction of ZO-1 3'-UTR with RNA-binding protein TIAR.

TIAR Silencing Prevents the JunD-mediated Repression of ZO-1 Translation

Levels of TIAR were reduced by transfection with siTIAR as described previously (Mazan-Mamczarz *et al.*, 2006), and the firefly luciferase reporter containing ZO-1 3'-UTR were constructed (Figure 8A) and used for an *in vivo* assays for ZO-1 translation as described previously (Vasudevan and Steitz, 2007). To distinguish translational output from mRNA turnover, most luciferase assays were normalized to luciferase-reporter RNA levels to obtain the translation efficiency. As shown in Figure 8B, overexpression of JunD inhibited ZO-1 translation as indicated by a decrease in ZO-1 ARE luciferase reporter gene activity. TIAR silencing by transfection with siTIAR totally prevented the JunD-induced inhibition of ZO-1 translation. The levels of ZO-1 translation efficiency in siTIAR-transfected cells overexpressing JunD were similar to those observed in control cells. Consistently, in TIAR-silenced cells, the decrease in levels of ZO-1 protein induced by JunD was also abolished (Figure 8, C and D). In contrast, transfection of control siRNA did not reduce TIAR expression and failed to alter the inhibitory effect of increased JunD on ZO-1 translation and its protein levels. Together, these results indicate that increased JunD represses ZO-1 translation by enhancing the interaction of TIAR with the ZO-1 3'-UTR in intestinal epithelial cells.

Figure 7. Binding of cytoplasmic TIAR, AUF1, and HuR to ZO-1 transcripts in cells described in Figure 6. (A) Schematic representation of ZO-1 mRNA and the AU-rich sequences in its 3'-UTR. (B) Representative immunoblots of TIAR, AUF1, and HuR using the pull-down materials from the 3'-UTR (a) or CR (b). Cytoplasmic lysates (120 μ g each) prepared from either controls and JunD-transfected cells were incubated with 6 μ g of biotinylated ZO-1 3'-UTR or CR for 30 min at 25°C, and the resulting RNP complexes were pulled down by using streptavidin-coated beads. Levels of TIAR, AUF1, and HuR proteins in the pull-down materials were measured by Western blot analysis. Three experiments were performed that showed similar results. (C) Association of endogenous TIAR with endogenous ZO-1 mRNA after ectopic expression of the *junD* gene. a, lysates from controls and JunD-transfected cells were used for IP in the presence of anti-TIAR antibody or nonspecific IgG1. RNA in the IP material was used in RT-PCR reactions to detect the presence of ZO-1 mRNA and the resulting PCR products (~249 base pairs) were visualized in agarose gels. b, quantification of ZO-1 mRNA levels in the IP materials as measured by real-time PCR analysis. Values are the means $\pm$ SE of data from three separate experiments. * $p < 0.05$ compared with controls.

DISCUSSION

JunD critically regulates barrier function and homeostasis in the intestinal epithelium (Patel and Wang, 1999; Li *et al.*, 2002; Guo *et al.*, 2003; Xiao *et al.*, 2007a). The inhibitory influence of JunD on proliferation is particularly important

because the intestinal epithelium has the most rapid turnover rate of any tissue in the body (Wang *et al.*, 1991; Hall *et al.*, 1994; Radtke and Clevers, 2005). In the present study, we highlight a new function of JunD in the regulation of ZO-1 expression and therefore in barrier function of the intestinal epithelium, thus advancing our understanding of the functions of JunD transcription factor in the intestinal mucosa. The results presented here provide insight into the molecular regulation of ZO-1 gene expression and show that the mechanism underlying ZO-1 repression by JunD operates at the levels of transcription and translation.

The results reported in the present study clearly indicate that there is a functional CREB binding site within the proximal region of the ZO-1 promoter and that JunD represses ZO-1 transcription through direct interaction with this CREB site. Several pieces of evidence from the current studies demonstrate that this CREB sequence is crucial for ZO-1 repression by JunD in IECs. First, deletion of this proximal CREB sequence from the ZO-1-promoter (5-Luc) almost prevented JunD-induced repression of ZO-1 promoter activity. Second, ZO-1 repression by overexpression of JunD was abolished when this CREB site within its proximal promoter region was eliminated by making a two-base change. Third, ChIP assay revealed that JunD bound to the proximal region of the ZO-1 promoter in vivo. Finally, CREB point mutation of the ZO-1 promoter also prevented the repression of ZO-1 transcription by increased endogenous JunD after polyamine depletion. The CREB binding site differs from the classical AP-1 binding site by insertion of a single base pair in the middle, and it primarily interacts with CREB-binding proteins (Montminy *et al.*, 1986). However, it was reported that some CREB-binding proteins such as CREB-II can form heterodimers with Jun proteins but not Fos proteins and that these heterodimers share DNA binding specificity with CREB homodimers and interact with the CREB but not with the AP-1 site (MacGregor *et al.*, 1990). Although the exact mechanism underlying the interaction of JunD with CREB within the ZO-1 promoter remains unknown, these experiments establish that the sequence located at proximal region of the ZO-1 promoter is a functional CREB binding site and that JunD represses ZO-1 through this CREB.

Our results also indicate that JunD regulates ZO-1 expression posttranscriptionally, by inhibiting ZO-1 mRNA translation. Overexpression of JunD not only caused a shift of ZO-1 mRNA toward nontranslating or low-translating fractions of polysome profiles (Figure 6B), but it also inhibited the translation of a heterologous ZO-1 3'-UTR reporter (Figure 8B). In addition, increased JunD also caused a global inhibition of translation (Figure 6A). To the best of our knowledge, this is the first report showing that JunD plays an important role in translational regulation. A rapidly growing body of literature demonstrates the essential contribution of posttranscriptional events in gene expression programs, particularly by altering mRNA turnover and translation, two processes that critically contribute to maintaining intestinal mucosal homeostasis (Patel *et al.*, 1998; Li *et al.*, 2001, 2002; Zou *et al.*, 2006; Xiao *et al.*, 2007b). Such control mechanisms typically involve the association of transcripts with specific RNA-binding proteins (RBPs). Among the RBPs that regulate gene expression posttranscriptionally are RBPs that modulate mRNA turnover (HuR, NF90, AUF1, BRF1, TTP, and KSRP) and RBPs that modulate translation (TIAR, TIA-1, HuR, and NF90). The best characterized *cis*-acting elements regulating mRNA stability and translation are U- and AREs that are usually located in the 3'-UTR of many labile mRNAs (Garneau *et al.*, 2007). Likewise, different RBPs selectively recognize and bind to AREs of target

Figure 8. Effect of TIAR silencing on ZO-1 3'-UTR-regulated translation in cells overexpressing JunD. (A) Schematic of plasmids. a, control (pGL3-Luc); b, chimeric firefly Luc-ZO-1 3'UTR (pGL3-Luc-ZO-1ARE). (B) Changes in ZO-1 translation efficiency after JunD overexpression. After Caco-2 cells were transfected with the Null vector (control) or JunD expression vector (JunD) for 24 h, they were transfected with siTIAR or C-siRNA for additional 24 h and then transfected with the pGL3-Luc or pGL3-Luc-ZO-1ARE. Transfected cells were harvested and assayed for luciferase activity 24 h after transfection with the pGL3-Luc (served as negative control) or pGL3-Luc-ZO-1ARE. Data were normalized to the pGL3-Luc and *Renilla*-driven luciferase activity and expressed as the means $\pm$ SE of data from three separate experiments. * $p < 0.05$ compared with controls. +, $p < 0.05$ compared with cells transfected with JunD alone or JunD plus C-siRNA. (C) Representative immunoblots of TIAR and ZO-1 proteins in cells described in B. Levels of TIAR and ZO-1 proteins were measured by Western blot analysis, and equal loading was monitored by immunoblotting of actin. (D) Quantitative analysis derived from densitometric scans of immunoblots of ZO-1 as described in C. Values are the means $\pm$ SE from three separate experiments, and relative levels of ZO-1 protein were corrected for protein loading as measured using densitometry of actin. * $p < 0.05$ compared with controls. +, $p < 0.05$ compared with cells transfected with JunD alone or JunD plus C-siRNA.

mRNAs, leading to changes in transcript half-life and translation rate (Zou *et al.*, 2006; Mazan-Mamczarz *et al.*, 2006; Abdelmohsen *et al.*, 2007; Xiao *et al.*, 2007b).

Our results also show that JunD represses ZO-1 translation by increasing the interaction of TIAR with the ZO-1 3'-UTR that contains AU- and U-rich stretches, as shown in Figure 7. Our observations are consistent with studies demonstrating that TIAR binds to AU-rich elements commonly found in the 3'-UTRs of labile mRNAs and plays a general role as translational repressor in response to various intracellular and extracellular stress (Gueydan *et al.*, 1999; Kawai *et al.*, 2006; Mazan-Mamczarz *et al.*, 2006). Although HuR and AUF1 also bind to the ZO-1 3'-UTR, these interactions were not altered by JunD overexpression. How JunD influences TIAR binding to the ZO-1 3'-UTR remains an open question, but it is not due to changes in whole-cell TIAR levels (Supplemental Figure 3). We have preliminary evidence that supports the involvement of importin $\alpha 1$, an adaptor protein that transports RBPs from the cytoplasm to the nucleus (Zou *et al.*, 2008). Increased JunD inhibited expression of importin $\alpha 1$ and increased TIAR abundance in the cytoplasm, thus leading to an increase in TIAR binding to ZO-1 mRNA. The exact mechanisms by which JunD modulates importin $\alpha 1$ expression and TIAR subcellular distribution are the focus of our ongoing studies.

The translation data presented in Figure 8 indicate that TIAR levels critically influence repression of ZO-1 translation by JunD. Although the exact mechanism by which TIAR represses mRNA translation remains to be elucidated, TIAR is shown to promote the association of noncanonical preinitiation complexes onto mRNAs (Gueydan *et al.*, 1999; Anderson and Kedersha, 2002; Yu *et al.*, 2003; Kandasamy *et al.*, 2005). In response to stress, it is proposed that stress-activated kinases phosphorylate eukaryotic initiation factor (eIF) 2 α and thereby reduce the eIF2-GTP-tRNA^{Met} that is required to load tRNA^{Met} onto the 40S ribosomal subunit to initiate protein synthesis. Under such conditions, TIAR is

assumed to enhance the assembly of translationally inactive pre-initiation complexes (containing the small ribosomal subunit but lacking eIF2 and eIF5) and aggregate in stress granules (Anderson and Kedersha, 2002).

The data obtained in the present study further suggest that the JunD-mediated repression of ZO-1 expression is physiologically significant because increasing the levels of endogenous JunD by depleting cellular polyamines has a similar effect on ZO-1 expression and epithelial barrier function. Polyamines are important for maintaining intestinal epithelial integrity and their intracellular levels are tightly regulated under physiological and pathological conditions (Wang and Johnson, 1991). Polyamine depletion disrupts epithelial barrier function (Guo *et al.*, 2003; 2005), but the exact mechanisms by which polyamine depletion increases paracellular permeability remain poorly understood. The results presented in Figures 4 and 5 show that depletion of cellular polyamines increased JunD but inhibited ZO-1, whereas JunD silencing restored ZO-1 to near normal levels and promoted the barrier function in polyamine-deficient cells. These findings provide novel evidence that polyamine depletion impairs intestinal epithelial barrier function in part by repressing ZO-1 expression through JunD.

In summary, these results indicate that JunD negatively regulates ZO-1 expression and is implicated in controlling the intestinal epithelial barrier function. Increased JunD not only represses ZO-1 transcription but also inhibits its translation, thus leading to an increase in epithelial paracellular permeability. A search for mechanisms whereby JunD modulates ZO-1 transcription revealed that there is a functional CREB binding site within the proximal region of the ZO-1 promoter and that mutation of this CREB site totally blocks ZO-1 repression by JunD. The present study also indicates that overexpression of JunD increases TIAR binding to the ZO-1 3'-UTR, although it does not alter the whole-cell levels of TIAR protein. The silencing of TIAR prevented the JunD-induced inhibition of ZO-1 translation, suggesting that JunD

regulates ZO-1 translation by modulating the abundance of [TIAR-ZO-1 mRNA] complexes. Because JunD expression is highly regulated under physiological conditions, these findings suggest that the repression of ZO-1 is a highly dynamic process underlying the function of the intestinal epithelial barrier.

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