SUPPLEMENTAL FIGURE LEGENDS

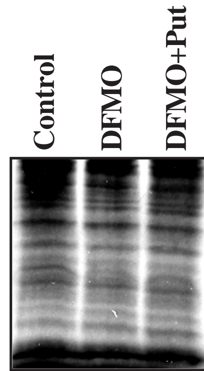
Supplemental Figure A1. Changes in levels of global protein synthesis after polyamine depletion by DFMO (A) and increased polyamines by ODC overexpression (B). After incubation of cells with L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min, whole-cell lysates were subjected to SDS/PAGE, and then visualized with a PhosphorImager. Three experiments were performed that showed similar results.

Supplemental Figure A2. Distribution of HuR proteins in various polysomal fractions prepared from control IEC-6 cells and cells treated with DFMO alone or DFMO plus Put for 4 days. Proteins obtained from individual fractions were analyzed by Western blot analysis to detect HuR. Three experiments were performed that showed similar results.

Supplemental Figure A3. Levels if HuR and Chk2 proteins in the complex immunoprecipitated (IP) by the anti-Chk2 antibody in IEC-6 cells following polyamine depletion. Cells were grown in control medium or in medium containing 5 mM DFMO alone or DFMO plus 10 μM putrescine (Put) for 4 days, and then whole-cell lysates were harvested for IP assays. After cell lysates (500 μg each) were immunoprecipitated by using a specific antibody (Ab) against Chk2, IP samples were subjected to electrophoresis by 10% SDS-PAGE. The levels of HuR and Chk2 proteins were then assessed by Western blot analysis.

Supplemental Figure A4. c-Myc protein stability in cells overexpressing HuR or Chk2. (**A**) Representative Western blot analysis of c-Myc levels in: ***a***), control cells; ***b***), cells overexpressing HuR; and ***c***), cells overexpressing Chk2. After cells were transfected with either the empty control vector, Chk2-expression vector or HuR-expression vector for 48 h, cycloheximide (CHX, 50 μg/ml) was added and whole-cell lysates were harvested at the times indicated. The levels of c-Myc protein proteins were assayed by Western blot analysis; β-Actin served as the loading control. ***B***: Western blotting signals in panel **A** were quantified by densitometry and plotted. Values are means ± SE of data from three separate experiments; c-Myc intensity signals were corrected by densitometric measurement of β-actin.

A



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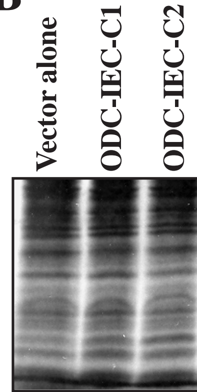
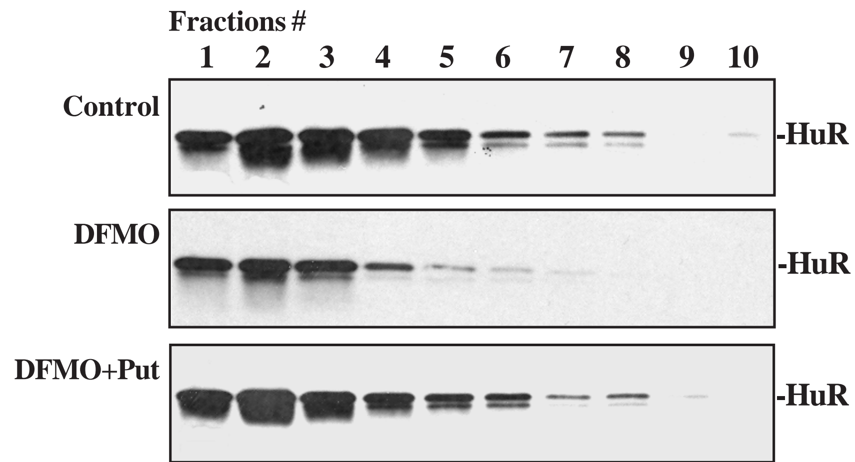
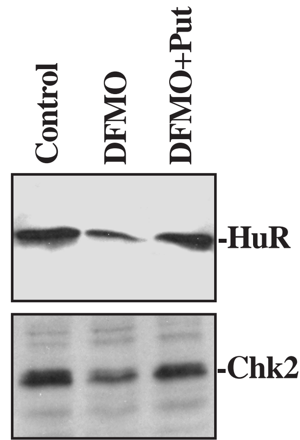


Supplemental Figure A1



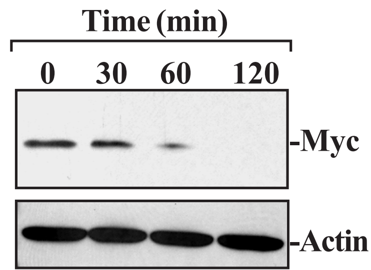
Supplemental Figure A2



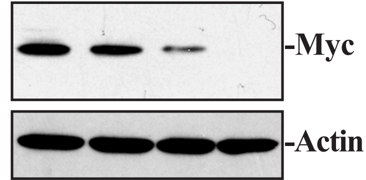
Supplemental Figure A3

A

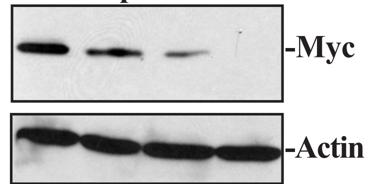
a. **Control**



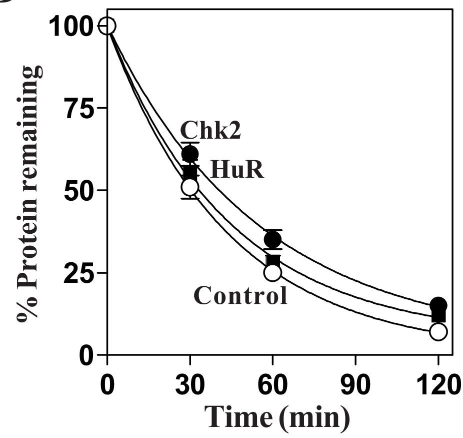
b. **HuR overexpression**



c. **Chk2 overexpression**



B



Supplemental Figure A4

SUPPLEMENTAL-5: EXPERIMENTAL PROCEDURES

Chemicals and Supplies

Tissue culture medium and dialyzed fetal bovine serum were from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma (St. Louis, MO). The antibodies against c-Myc, Chk2, β-tubulin, and lamin B, and α-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signal (Danvers, MA); Zymed (San Francisco, CA), and BD Bioscience (San Jose, CA). The secondary antibody conjugated to horseradish peroxidase was purchased from Sigma, and DFMO (α-difluoromethylornithine) was from Genzyme (Cambridge, MA). L-[^{35}S]methionine and L-[^{35}S]cysteine (Easy Tag EXPRESS) were purchased from NEN/Perkin-Elmer (Boston, MA).

Cell Culture

The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13 and used at passages 15-20 for the current experiments. IEC-6 cells were derived from normal rat intestinal crypt cells as judged by morphological and immunological criteria (Quaroni *et al*., 1979). They are non-tumorigenic and retain the undifferentiated characteristics of normal intestinal crypt cells. ODC-overexpressing IEC-6 (ODC-IEC) cells were developed from IEC-6 cells as described in our previous studies (Liu *et al*., 2006) and expressed a more stable ODC variant with full enzyme activity (Ghoda *et al*., 1989). Caco-2 cells were obtained from ATCC at passage 16, and passages 18-23 were used for the current studies. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, 10 μg/ml insulin, and 50 μg/ml gentamicin.

Plasmid Construction, Luciferase Reporters and Transfection

The complete open reading frame of Chk2 cloned from human genomic DNA was sequenced and then cloned into an expression vector pcDNA3.1(+) (Invitrogen) for expression directed by the cytomegalovirus promoter. The vectors expressing wild-type HuR-TAP fusion proteins or point-mutated HuR-TAP fusion proteins were generated as described previously (Abdelmohsen *et al*., 2007), in which HuR S88, S100, and T118 amino acid residues were changed individually to alanine by site-directed mutagenesis in plasmid pGEX2T-HuR. Cells were transfected by using the Lipofectamine™ Reagent and performed as recommended by the manufacturer (Invitrogen), while levels of expressing proteins were assayed 24 and 48 h after transfections.

The chimeric firefly luciferase reporter construct of the c-Myc 3'-UTR was generated as described previously (Liao *et al*., 2007). The 403-bp ARE fragment from the c-Myc 3'-UTR was amplified and subcloned into the pGL3-Luc plasmid (Promega) at the XbaI site to generate the chimeric pGL3-Luc-c-Myc-3'UTR. The sequence of the c-Myc 3'UTR segment linked to the luciferase plasmid corresponds to nucleotides 7290-7693 of the human c-Myc gene (GenBank X00364, J00120). This ~400-bp region contains the ARE and spans both polyadenylation sites. 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 manufacturers' instruction. The Firefly to *Renilla* luciferase ratio was further normalized for RNA levels.

RNA Interference

The silencing RNA duplexes that were designed to specifically cleave HuR mRNA were synthesized and purchased from Invitrogen. The sequence of small interfering RNA (siRNA)

that specifically targets the coding region of HuR mRNA (siHuR) was GGAUGACAUUGGGAGAACGAAUUUA, whereas the sequence of control siRNA (Con-siRNA) was AAGTGTAGTATCACCAGGC. The siRNA that specifically targets Chk2 mRNA (siChk2) and its Con-siRNA were synthesized and purchased from Dharmacon. For each 60-mm cell culture dish, 3 µl of the 100 µM stock duplex siHuR, siChk2, or Con-siRNA was mixed with 500 µl of Opti-MEM medium (Invitrogen). This mixture was gently added to a solution containing 10 µl of LipofectAMINE 2000 in 500 µl 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 incubation for 48 h.

Assay for ODC Enzyme Activity and Polyamine Analysis

ODC activity was determined by radiometric technique in which the amount of $^{14}CO_2$ liberated from L-[1-^{14}C]ornithine was estimated. Sample collection and the assay procedure were carried out as described in our previous publications (Li *et al*., 1999; Liu *et al*., 2006). Enzymatic activity was expressed as picomoles of CO_2 per milligram of protein per hour. The cellular polyamines content was analyzed by high-performance liquid chromatography (HPLC) analysis as previously described (Wang and Johnson, 1991). Briefly, after 0.5 M perchloric acid was added, the cells were frozen at -80°C until ready for extraction, dansylation, and HPLC analysis. The standard curve encompassed 0.31-10 µM. Values that fell >25% below the curve were considered undetectable. The results are expressed as nanomoles of polyamines per milligram of protein.

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 (Zou *et al*., 2006; Liu *et al*., 2006). PCR primers for rat c-Myc were TCCGTTCAAGCAGATGAGCA and CCTATGTACACCGGAAGATT, yielding a 385-bp fragment. The levels of β-actin PCR product were assessed to monitor the even RNA input in RT-PCR samples. Real-time quantitative PCR (Q-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 c-Myc, XIAP, and occludin mRNAs were quantified by Q-PCR analysis and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Preparation of Synthetic RNA Transcripts

cDNA from IEC-6 cells was used as a template for PCR amplification of the coding region (CR) and 3'-UTR of c-Myc. The 5'-primers contained the T7 RNA polymerase promoter sequence (T7): 5'-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3'. To prepare the CR of c-Myc (Spanning positions 537-1898), oligonucleotides (T7)5'-TCTGCGACGAGGAAGAGAAT-3' and 5'-TGCTCATCTGCTTGAACGGA-3' were used. To prepare the c-Myc 3'-UTR template (spanning positions 1899-2355), oligonucleotides (T7)5'-ACTTACTGAGGAAACGGCGA-3' and 5'-TAAGAGAAGGCTCAATTATATTT-3' were used. To prepare the c-Myc 3'-UTR fragment-A (F-A) template (spanning positions 1899-2071), oligonucleotides (T7)5'-TGC ATAAACTGACCGGAAGTGAGGA-3' and 5'- AGTTCTTTTATGCCTTAACTTTGAGGCA-3' were used. To prepare the c-Myc 3'-UTR fragment-B (F-B) template (spanning positions

2044-2212), oligonucleotides (T7)5'-TGCCTCAAAGTTAAGGCATAAAAGAACT-3' and 5'-ATCTTGTATAACTGTTATAAACGTTTTATTAAAGT-3' were used. To prepare the c-Myc 3'-UTR fragment-C (F-C) template (spanning positions 2205-2355), oligonucleotides (T7)5'-TACAAGATTTTAAGACATGTATGATAAACCATAA-3' and 5'-TAAGAGTTGGCTCAAT TATATTTTTTCCA-3' were used. PCR-amplified products were used as templates to transcribe biotinylated RNAs by using T7 polymerase in the presence of biotin-cytidine 5'-triphosphate as described (Xiao *et al*., 2007).

RNA-Protein Binding Assays

For biotin pull-down assays, biotinylated transcripts (6 μg) were incubated with 120 μg of cytoplasmic lysate for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway) and analyzed by Western blot analysis. To assess the association of endogenous HuR with endogenous c-Myc mRNAs, immunoprecipitations (IP) of HuR-mRNA complexes were performed as described (de Silanes *et al*., 2004; Zou *et al*., 2005). Twenty million IEC-6 cells were collected per sample, and lysates were used for IP for 4 h at room temperature in the presence of excess (30 μg) IP antibody (IgG, or anti-HuR). RNA in IP materials was used in RT followed by PCR analysis to detect the presence of c-Myc mRNA.