METHODS

Cell Culture and Antibodies. Human colorectal cancer HCT-116 cells, human embryonic kidney 293 cells, mouse embryonic fibroblast NIH 3T3 cells and rat fibroblast cell line Rat1 cells were cultured as recommended by ATCC. All cell culture reagents were purchased from Mediatech Inc. (Herndon, VA). Antibodies against GAPDH, c-Myc (9E10), Ubc9, eIF4E were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SUMO-1 was purchased from Zymed Laboratory (San Francisco, CA). Anti-eIF4G was purchased from Bethyl Laboratories (Montgomery, TX). Anti-ODC was purchased from Enzo Life Sciences International (Plymouth Meeting, PA). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Plasmids and Transfection. The expression plasmid of wild type HA-eIF4E has been described previously (Hu et al., 2007). The HA tagged eIF4E SUMO mutants, phosphorylation mutant (serine 209 mutated to alanine, S209A) and phosphorylation mimic (Serine 209 mutated to glutamic acid, S209E) were generated by PCR. The retroviral vectors expressing non-tagged eIF4E or eIF4E mutants were generated by excising eIF4E cDNA fragments from pcDNA vectors and subsequently cloning into the retroviral vector pMX-IRES-BSR using EcoRI and XhoI restriction enzyme sites. The shRNA Ubc9 plasmid was generated by cloning the Oligonucleotide GCAGAGGCCTACACGATTTAC (Integrated DNA Technologies, Inc.) into the pSilencer™ 4.1-CMV neo vector (Ambion). Negative control pSilencer vector that expresses a scrambled control siRNA was from Ambion. The mammalian expression vector of Ubc9 was purchased from OriGene (Rockville, MD). Transient transfections
were performed using Fugene™ (Roche Applied Science, Indianapolis, IN) or PolyJet™ (SignaGen Laboratories, Ijamsville, MD) according to the protocols provided. The stable cell lines and their individual control cell lines were generated by G418 selection. For transient transfection, the cells were in general harvested 48 hours after transfection. For shRNA knockdown of Ubc9, cells were harvested 72 hours after transfection. For viral vector infection, the retroviral vectors were transiently transfected into a potent retrovirus packaging cell line named platinum-E (Plat-E) according to an established protocol (Pear et al., 1993). 48 hours post transfection infectious retrovirus were collected and used to infect NIH-3T3 cells, Rat1 and HCT-116 cells. The stable cell lines were established by Blasticidin S selection.

**m7GTP Pull-down Assay.** 7-Methyl-GTP Sepharose™ 4B beads from Amersham (Piscataway, NJ) was used for the pull-down assay. To specifically elute cap-bound eIF4E, precipitated proteins were eluted from the resin with 0.1 mM m7GDP (Jena Bioscience GmbH, Jena Germany) in IP lysis buffer. The eluted proteins were separated by SDS-PAGE gels and analyzed by immunoblotting using antibodies indicated in the figure legends. For loading control, in general, 10% amount of input lysate was resolved by SDS-PAGE and immunoblotted with anti-eIF4E.

**Reverse Transcription and PCR (RT-PCR).** The PARIS kit (Ambion) was used to isolate cytosolic mRNAs. The RT-PCR assays were conducted as described earlier (Hu et al., 2007).
Polysome Fractioning. The polysome fractioning was performed according to protocol described in earlier report (Rousseau et al., 1996) with modification. Briefly, the Rat1 cell lines overexpressing control vector, eIF4E or eIF4E-S5 mutant from fifteen 150-mm tissue culture plates (70-80% confluency, 5 plates for each cell line) were treated with 100 μg/ml of cycloheximide for 5 minutes. The cells were then washed twice with cold PBS containing 100 μg/ml of cycloheximide. Subsequently, the cells were scraped using cell lifter and cells from 5 plates were collected into one 50 ml tube. The cells were then pelleted by centrifugation. The cell pellets were then transferred to a new 1.5 ml eppendorf tube and resuspended in 0.2 ml of low salt buffer (10 mM Tris-HCl, pH7.4, 15 mM NaCl, 12.5 mM MgCl2, 500 μg/ml heparin and 100 μg/ml Cyclohexamide). After 2 minutes incubation on ice, 0.16 ml of cytoplasmic lysis buffer (20 mM Tris-HCl (pH 7.2), 130 mM KCl, 30 mM MgCl2, 1% NP40, 0.05% Sodium Deoxycholate, 0.2 mg/ml Heperin, 100 μg/ml Cyclohexamide and 1 mM dithiothreitol, 200 units of recombinant RNase Inhibitor (New England Biolabs) and complete protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN)) was added to each tube and the cell lysates were incubated on ice for 3 minutes with several vortex. The lysates were then spun down at 4°C for 2 min at 10,000 g. The supernatant (cytoplasmic extract) were collected into new tubes. 40 μL of the supernatants from each sample were collected for total mRNA measurement. The equal optical density units (260 nm) of cytoplasmic extracts from each cell lines were then layered over 10-45% linear sucrose gradients (in 10 mM Tris-HCl (pH 7.2), 60 mM KCl, 10 mM MgCl2, 0.1 mg/ml Heparin Sulfate and 1 mM DTT) and centrifuged at 36,000 rpm in a Beckman SW41 rotor for 4h at 4°C. Gradients were fractionated using an ISCO density gradient fractionator equipped with an
absorbance monitor (254 nm) and 3 fractions with volume of about 4ml were collected in a 50 ml tube. The 3 fractions represent short polysome/monosome pool, intermediate polysomes and heavy polysomes. The RNA from each pool was extracted and mRNAs coding for ODC, c-Myc, surviving, Bcl-2 and β-actin were analyzed by reverse transcription polymerase chain reaction (RT-PCR).

**Apoptosis Assay.** Serum starvation-induced apoptosis was assessed by (a) flow cytometric analysis of quantitate Annexin V–positive and propidium iodide–positive cells, and (b) ELISA based quantitation of cytoplasmic histone associated DNA fragmentation. (a) The NIH-3T3 cells were infected with retroviral vectors expressing WT eIF4E, eIF4E SUMO mutant (SUMO-deficient mutant eIF4E-S5), eIF4E phosphorylation mutant (eIF4E-S209A) or empty vector. The stable cell lines were established by Blasticidin-S selection. The stable cell lines were incubated in DMEM containing 0.1% fetal calf serum for 36 hours. After serum starvation, the attached and floating cells were harvested. The cells were first stained with propidium iodide and then with Annexin V-Alexa Fluor® 488 conjugate (Invitrogen) according to the manufacturer's instructions. Flow cytometry was used to quantitate Annexin V–positive and propidium iodide–positive cells. A minimum of 30,000 cells per measurement and at least triplicate samples were analyzed for each experiment. (b) The HCT-116 cells were transfected with HA-eIF4E or HA-eIF4E single mutants (K36R, K49R, K162R, K206R or K212R). 24 hours after transfection, the cells were treated with 0.2% FBS for 72 hours. After serum starvation, the fractions of apoptotic cells were evaluated by measuring cytoplasmic histone associated DNA fragmentation using a commercially
available kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer’s recommendations.

**Foci formation Assay and Soft Agar Assay.** The Rat1 cell lines expressing WT eIF4E, eIF4E-S209A and SUMO deficient mutant (eIF4E-S5) were plated at $1 \times 10^5$ cells in 100-mm dishes and grew in complete medium with addition of fresh medium every two days. 7 days later, the cells were fixed and stained with Giemsa and photographed under microscope. The soft agar assay was performed according to our previous report (Hu et al., 2004).

**LEGENDS TO SUPPLEMENTARY FIGURES**

**Supplementary Fig 1** | eIF4E is modified by SUMO-1 but not SUMO-2 conjugation. (A) shRNA knockdown of Ubc9 inhibits sumoylation of eIF4E in HCT-116 cells. The HCT-116 cell lines expressing control shRNA or shRNA Ubc9 were used for the study. Sumoylation was evaluated by IP with anti-eIF4E and subsequent IB with anti-SUMO-1. (B) eIF4E is not sumoylated by SUMO-2 conjugation. *In vitro* Sumoylation assay was performed using *in vitro* synthesized eIF4E and *in vitro* sumoylation kit as described in experimental procedures.

**Supplementary Fig 2** | Serum stimulation does not affect cellular SUMO-1 level. The HCT-116 cells were serum starved (0.2% FBS) for 22 hours then stimulated with or
without 20% FBS for additional 2 hours. To better compare the total cellular level of SUMO-1, the cells were lysed in the absence of NEM.

**Supplementary Fig 3** | Single mutation does not affect *in vitro* sumoylation of eIF4E. *In vitro* sumoylation assay was performed using *in vitro* synthesized HA-eIF4E or HA-eIF4E single mutants and *in vitro* sumoylation kit as described in the Methods. Sumoylated and non-sumoylated eIF4E was detected by IB with anti-HA.

**Supplementary Fig 4** | Phosphorylation of eIF4E is required for its sumoylation. (A) Lack of eIF4E phosphorylation prevents its sumoylation. The whole cell lysates from the HCT-116 cell lines expressing retroviral empty vector, wild type eIF4E, eIF4E phosphorylation mutant (labeled as S209A) or eIF4E SUMO-deficient mutant (labeled as S5) were used for the assay. Sumoylation of eIF4E was evaluated by IP with anti-eIF4E and subsequent IB with anti-SUMO-1. (B) Phosphorylation of sumoylated eIF4E. The HCT-116 cells were starved with 0.2% FBS for 22 hours then stimulated with 20% FBS for additional 2 hours. After treatment, the cells were lysed and the whole cell lysates were used for the experiments.

**Supplementary Fig 5** | Single mutation of eIF4E sumoylation site does not affect eIF4E’s anti-apoptosis property. The HCT-116 cells were transfected with HA-tagged wt eIF4E or its single mutants as labeled in the figure. 24 hours after transfection, the cells were starved with 0.2% FBS for 72 hours. Serum starvation-induced apoptosis was assessed by ELISA based quantitation of cytoplasmic histone associated DNA
fragmentation. Statistics were performed by one-way ANOVA followed by Tukey’s multiple comparison test using the data obtained from triplicate samples.

REFERENCES


Supplementary Figure 1

A

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SUMO-eIF4E

Light Chain

eIF4E input

B

eIF4E

E1/Ubc9

SUMO-2

IB: eIF4E

eIF4E
Supplementary Figure 2

FBS 0.2% 20%

IB: SUMO-1  SUMO-1
### Supplementary Figure 3

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**IB: HA**

**SUMO-HA-eIF4E**

**HA-eIF4E**
Supplementary Figure 4

A

B

25KDa 50KDa 75KDa 250KDa

IB: p-eIF4E(S209)

IP: SUMO-1

IB: eIF4E

IB: eIF4E eIF4E input

1 2 3 4

SUMO-p-eIF4E

Heavy Chain

Light Chain
Supplementary Figure 5

DNA Fragmentation (Absorbance [A405nm-A490nm])

VC  WT  K36R  K49R  K206R  K162R  K212R

IB: HA  HA-eIF4E  GAPDH

P< 0.01