Supporting Information

Badura et al. 10.1073/pnas.1203853109

SI Materials and Methods

Cell Culture. Cell lines included MCF10A, HBT-20, MDA-MB-231, and MCF-7, obtained from American Type Culture Collection and cultivated under recommended guidelines. Cells were transfected with DNA plasmids using *Trans*IT LT1 Transfection Reagent as described by the manufacturer (Mirus).

Antibodies and Immunoblot Analysis. Rabbit polyclonal antiserum directed to the N terminus of eIF4G2 was a gift of N. Sonenberg (McGill University, Montreal, Canada). Mouse monoclonal anti-eIF4A antibody was provided by W. Merrick (Case Western Reserve University, Cleveland, OH). HRP-conjugated secondary antibodies were from GE Healthcare. All other antibodies were from Cell Signaling Technology. Enhanced chemiluminescence (ECL) system (Amersham) was used for detection. Following treatments, cells were washed twice in ice-cold PBS and lysed in 0.5% Nonidet P-40 lysis buffer at 4 °C or 0.5% SDS lysis buffer. Nonidet P-40 lysates were clarified by centrifugation at 13,000 × *g* for 10 min, and protein concentrations were determined for each sample by Bradford assay (Bio-Rad). Protein concentrations for SDS lysates were determined by the DC Protein Assay (Bio-Rad).To determine the total levels and phosphorylation status of specific proteins, equal amounts of protein were resolved by SDS/PAGE and analyzed by protein immunoblotting with specific antibodies. The phosphorylation status of most proteins was determined by immunoblotting membrane first with phospho-specific antibody and then stripping the membranes using Restore Western blot stripping buffer (Pierce), followed by reprobing the membranes with non–phospho-specific antibodies.

Flow Cytometry. After treatment, cells were trypsinized in Trypsin/EDTA (Cellgro), centrifuged at 1,000 × *g* for 4 min, and resuspended in PBS. Cells were fixed by adding drop wise to 5 mL of 100% ethanol. For cell cycle studies, cells were centrifuged out of solution and resuspended in PBS with 1% FBS, 200 mg/mL propidium iodide in 38 mM sodium citrate, and 2 mg/mL RNase A. Cells were incubated at 37 °C for 2 h and stored at 4 °C until DNA histogram acquisition on a FACSCalibur flow cytometer (Becton Dickinson) and analysis using FlowJo (Tree Star).

Immunofluorescence. Cells were plated onto collagen-coated coverslips and treated. At indicated times posttreatment, coverslips were washed twice with PBS and fixed in 4% paraformaldehyde for 12 min while shaking at room temperature. Coverslips were then washed twice in PBS and permeabilized in 0.25% TritonX-100 in PBS for 12 min while shaking at room temperature. Cells were again washed twice in PBS and then blocked in 10% BSA in PBS with azide overnight at 4 °C. Cells were next washed in PBS and then incubated with primary antibody at the manufacturer's suggested dilution in 3% BSA in PBS for 2 h at room temperature. Coverslips were again washed and then incubated with TRITC- or FITC- conjugated secondary antibody in 3% BSA for 1 h. Coverslips were washed three times in PBS, once with dH_2O, and allowed to dry in the dark at room temperature. Coverslips were then inverted and coated with mounting media (Vector Laboratories) and then fixed to microscope slides with nail polish.

Analysis of eIF4E/4E-BP1 Interaction. Equal amounts of protein from Nonidet P-40 lysates were incubated with 7-Methyl GTP-Sepharose beads (Pierce; per the manufacturer's instructions) and incubated at equal volumes while rotating at 4 °C. Beads were pelleted at 5,000 rpm for 5 min in an Eppendorf micro-centrifuge, and 10% of the supernatant was removed for flow-through analysis. Samples were washed in 1 mL of lysis buffer four times, and the retentates were released from the beads by addition of 6× SDS sample buffer and boiling at 100 °C for 5 min. Retentates and supernatant flow-through samples were subjected to SDS/PAGE and analyzed by immunoblotting.

[^{35}S]methionine Incorporation Assay. Cells were labeled with 50 μCi of [^{35}S]methionine/cysteine per mL (Easytag Express Protein Labeling Mix; Perkin-Elmer) in Met/Cys-free DMEM with 5% FBS for 20 min, lysates prepared, and specific activity of [^{35}S] incorporation was determined by trichloroacetic acid (TCA) precipitation onto GF/C filters and liquid scintillation counting.

Single-Step Real-Time Quantitative RT-PCR. qRT-PCR analysis of mRNAs was performed using the SYBR Green QRT-PCR Kit (Sigma) in a Lightcycler instrument (Roche). Reverse transcription was carried out at 61 °C for 20 min, denaturation at 95 °C for 30 s, followed by 45 cycles of amplification: 95 °C for 2 s for denaturation, 59 °C for 5 s for annealing, and 72 °C for 10 s for amplification and acquisition. Primer sets used for specific gene quantification were designed using the Roche RT-PCR primer design tool (www.roche-applied-science.com) and are available upon request.

Polysome-Associated mRNA Isolation. Polysome isolation was performed by separation of ribosome-bound mRNAs in sucrose gradients. Beckman Ultra-Clear centrifuge tubes were loaded with 5.5 mL of 50% sucrose in low-salt buffer (LSB) [200 mM Tris (pH7.4) in DEPC H_2O, 100 mM NaCl, 30 mM $MgCl_2$] with 1:1,000 RNasin (Fermentas) and 100 μg/mL cycloheximide (CHX) in ethanol and incubated at 4 °C horizontally overnight. Media were removed from cell cultures and replaced with media containing 100 μg/mL CHX, and cells were incubated at 37 °C for 10 min to halt protein synthesis and trypsinized in trypsin/EDTA containing 100 μg/mL CHX. Cells were washed twice in PBS containing CHX, RNasin, and Roche complete EDTA-free protease inhibitor tablet and lysed in LSB with CHX and RNasin. Lysates were added to a Dounce homogenizer and incubated on ice for 3 min before addition of Triton detergent buffer (1.2% Triton N-100, 0.2 M sucrose in LSB) and homogenization. Samples were transferred to cold sterile Eppendorf centrifuge tubes and centrifuged at 13,000 rpm for 10 min at 4 °C. Postnuclear supernatants were transferred to centrifuge tubes containing 100 μL of Heparin solution (10 mg/mL heparin, 1.5 M NaCl in LSB) with RNasin and CHX, applied to a sucrose gradient. Gradients were micro centrifuged at 36,000 rpm at 4 °C for 2 h in an SW41 rotor, and supernatants recovered with an ISCO-UV fractionator. Samples were recovered into Eppendorf centrifuge tubes containing 40 μL of RNase-free 0.5 M EDTA and kept on ice. One volume acidic phenol-chloroform was added to each sample, which were then mixed and centrifuged at 10,000 × *g* for 15 min. The acidic phenol-chloroform extraction was repeated with the aqueous phase, which was then extracted twice via chloroform extraction. RNA was precipitated at −20 °C overnight by addition of isopropanol with 0.1 vol of sodium acetate (NaOAc) (pH5.2). The following day, RNA pellets were recovered by centrifugation, washed with 70% (vol/vol) ethanol, air-dried, and resuspended in sterile H_2O. RNA from fractions was then pooled based on their relative rate of translation, with fractions containing four or more ribosomes considered heavily translated and used for gene chip analysis. The RNA quality was examined with the Agilent Technologies kit.

DNA Microarray Data Analysis. DNA array analyses were performed by the NYU Genomics Facility. Samples were processed in duplicate for each experimental condition. The preparation of cRNA probes and hybridization to GeneChip HGU133A plus 2.0 arrays were as directed by the manufacturer (Affymetrix) and scanned on a GeneArray 3000 scanner. Raw data were normalized by model-based expression index algorithm using dChip (1) filtered on presence/absence calls using GeneSpring software version 7.2 (Silicon Genetics/Agilent). Differentially abundant mRNAs were identified in a cross-validation approach using TM4 analysis software (2) by combining *t* test with *P* value cutoff of 0.05 and/or Significance Analysis of Microarrays (SAM) (3) with false discovery rate (FDR) set to 5%. To group mRNAs based on translation activity, we collected all mRNAs from ≥four ribosomes as the heavy polysome set of sucrose gradient fractions, compared with pooled one to three ribosome sets and a parallel control for total cytoplasmic mRNA. To identity cellular functions, we used GO::Termfinder, defined by the GO consortium, for subsets of mRNAs that were differentially found in polysome free, light, and heavy polysomes with the different experimental conditions. In general, only if there were five or more mRNAs in a category with a twofold enrichment was it considered differentially regulated and a GO category. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (4) version 6.7 was used to interpret mRNA abundance data. Data were normalized to the untreated (non-IR), nonsilencing (NS) control samples.

1. Li C, Wong WH (2001) Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. *Proc Natl Acad Sci USA* 98(1):31–36.
2. Saeed AI, et al. (2003) TM4: A free, open-source system for microarray data management and analysis. *Biotechniques* 34(2):374–378.
3. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98(9):5116–5121.
4. Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57.

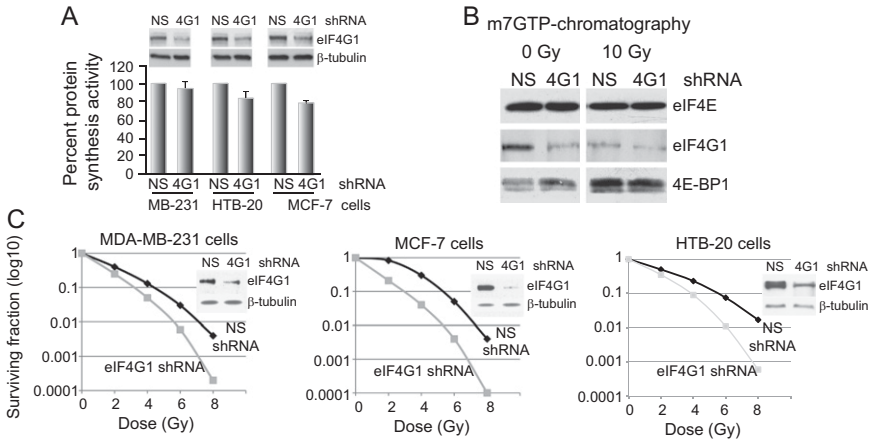

Fig. S1. Effect of eIF4GI silencing on overall protein synthesis and eIF4F integrity in breast cancer cell lines. (*A*) NS and eIF4G1-silenced MB-231, HTB-20, and MCF-7 cells were treated with 8 Gy IR or mock-treated. Protein synthesis rates were determined by [^{35}S]methionine/cysteine incorporation at each time indicated. Results were normalized to NS nonirradiated, NS control (0 h) and expressed as the mean with SEMs shown. Inset immunoblots demonstrate silenced levels of eIF4G1. (*B*) NS MCF10A cells were irradiated with 8 Gy IR, harvested, and lysed at 24 h. Equal amounts of protein extracts were subjected to m^{7}GTP-Sepharose cap chromatography, and retentates were recovered by elution with SDS, resolved by 10% SDS/PAGE, and immunoblotted with the indicated antibodies. (*C*) Clonogenic survival analysis studies identical to those described in Fig. 1*C* were conducted with MDA-MB-231, MCF-7, and HBT-20 breast cancer cell lines. Assays were performed in triplicate with SEMs shown.

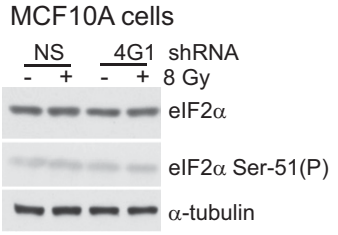

Fig. S2. eIF2α phosphorylation is not altered in IR treatment of breast cancer cells. NS MCF10A cells were irradiated with 8 Gy IR, harvested, and lysed at 24 h. Equal amounts of protein extracts were resolved by SDS/PAGE and subjected to immunoblot analysis with antibodies to proteins and phospho-specific protein as shown.

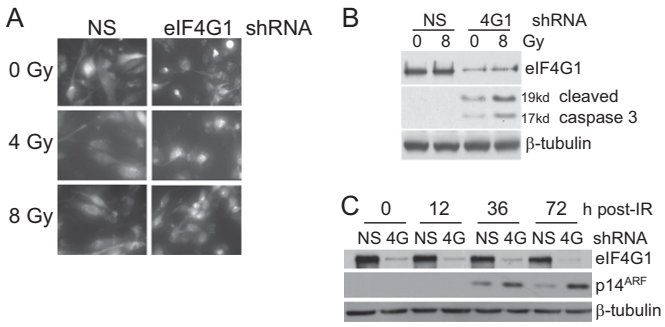

Fig. S3. Depletion of eIF4G1 sensitizes cells to IR-mediated autophagy, apoptosis, and senescence. (*A*) Cells were transfected with a vector expressing a GFP-tagged LC3 protein and then stably transfected with lentivirus expressing NS or eIF4G1 shRNAs for 3 d, followed by treatment with 4 or 10 Gy IR or mock-treated. Luminescence was observed and photographed at 24 h post-IR. Representative fields of three independent studies show increased clustering of GFP-LC3 at 12 h following 4 or 10 Gy IR treatment. (*B*) Caspase 3 cleavage increases in cells with reduced eIF4G1 levels. NS and eIF4G1-silenced cells at 0 or 8 Gy IR were lysed 24 h later, and equal protein amounts were resolved by SDS/PAGE and immunoblotted as shown. (*C*) Cells were irradiated with 8 Gy IR and harvested at the indicated time points into SDS lysis buffer, and immunoblot analysis was conducted using equal amounts of total protein lysates with the indicated antibodies. $p14^{ARF}$ is a marker of cellular senescence-like phenotype. Assays were performed in triplicate with representative immunoblots shown.

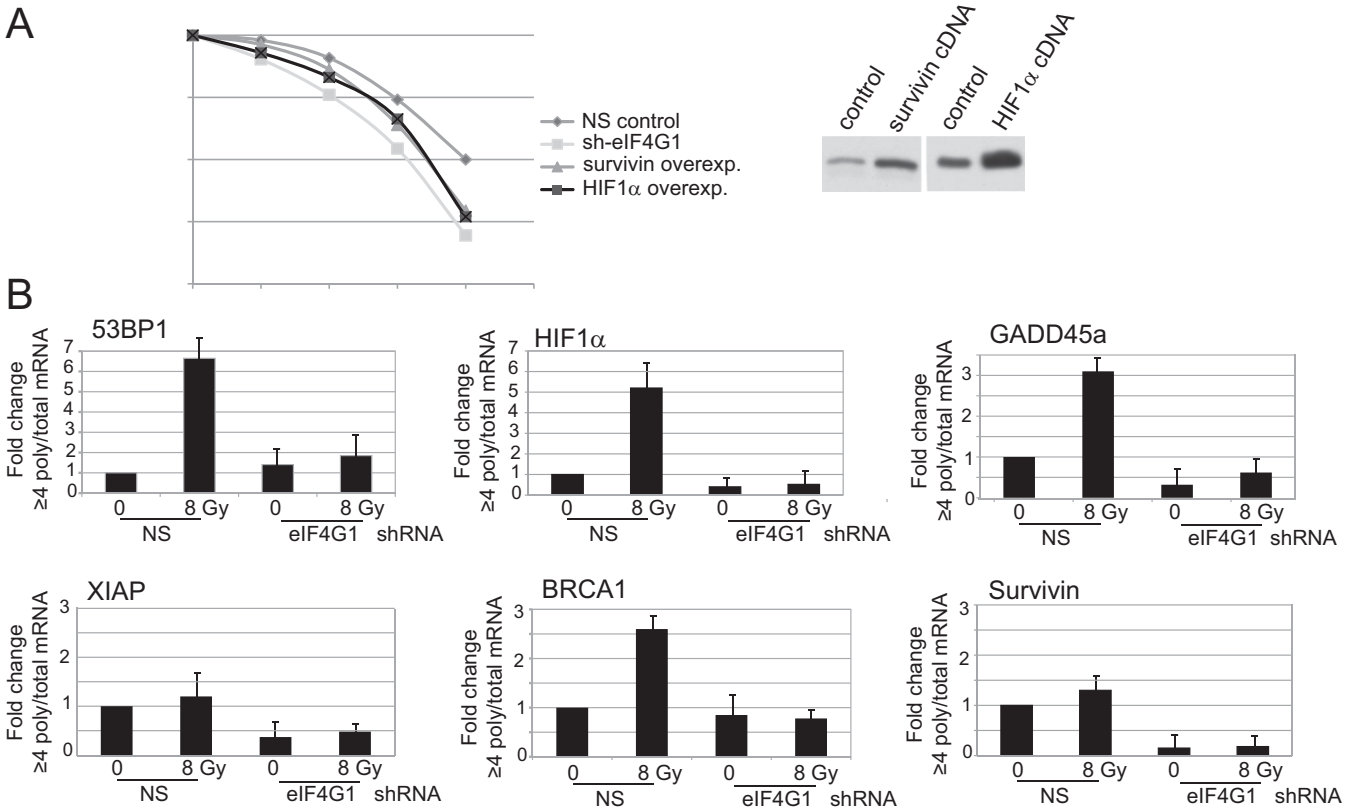

Fig. S4. eIF4G1 is required for the selective increased translation of DDR and survival mRNAs and increased cell survival. (*A*) Overexpression of survivin or HIF1α is not sufficient to compensate for loss of eIF4G1 overexpression in cell survival to IR. MCF10A cells were reduced in eIF4G1 expression by shRNA silencing and concurrently overexpressed either survivin or HIF1α by cDNA vectors. Cells were irradiated as shown, and clonogenic survival analysis was conducted. (*B*) eIF4G1 is required for the selective translation activation of DDR mRNAs. RNA isolated from ribosome free and heavy polysome fractions were subjected to qRT-PCR for the indicated mRNAs, normalized to total actin as an internal control. Results shown are the fold changes between the cycle threshold (C_t) values of each mRNA and actin, normalized to that of a total mRNA sample compared with the heavy polysome sample (≥four ribosomes) for 0 Gy of the NS control sample. Results express the mean of three independent experiments with SEM shown.

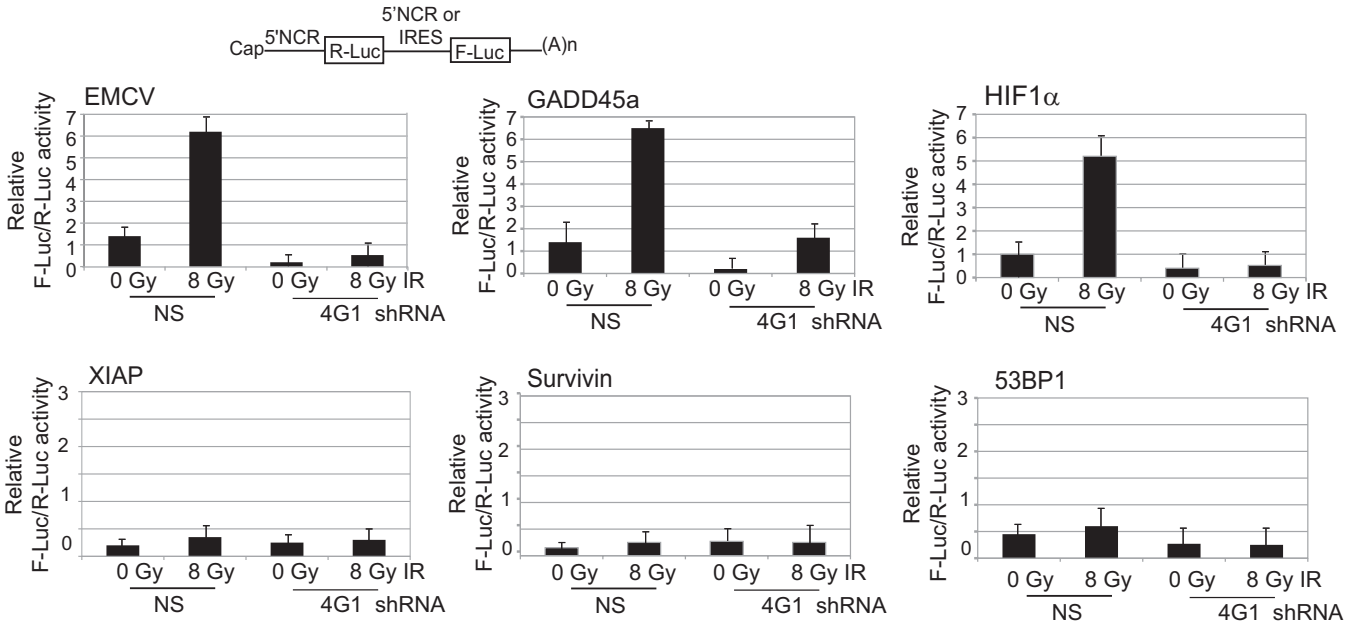

Fig. S5. Cap (eIF4E) dependence and comparative eIF4G1 dependence in mRNA translation of IR-induced mRNAs. MCF10A cells were transfected with bicistronic luciferase reporter vectors (diagram) expressing a single mRNA with a cap-dependent 5′ UTR containing the Renilla luciferase coding region, followed by the 5′ UTR of the respective test mRNA or, in the case of EMCV, the known IRES, driving the translation of the Firefly luciferase–coding region. Cells silenced for NS or eIF4G1 were subjected to 8 Gy IR and tested for luciferase activities at 24 h. Data are the mean of three independent experiments with SEM shown, expressed as the ratio of internal (Renilla luciferase) to 5′ cap–dependent (Firefly luciferase).

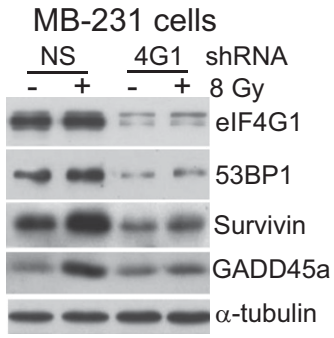

Fig. S6. Protein levels in irradiated and eIF4G1-silenced MB-231 cells. NS and eIF4G1-silenced MB-231 cells were irradiated with 8 Gy IR, harvested, and lysed at 24 h. Equal amounts of protein extracts were resolved by SDS/PAGE and subjected to immunoblot analysis with antibodies as shown.

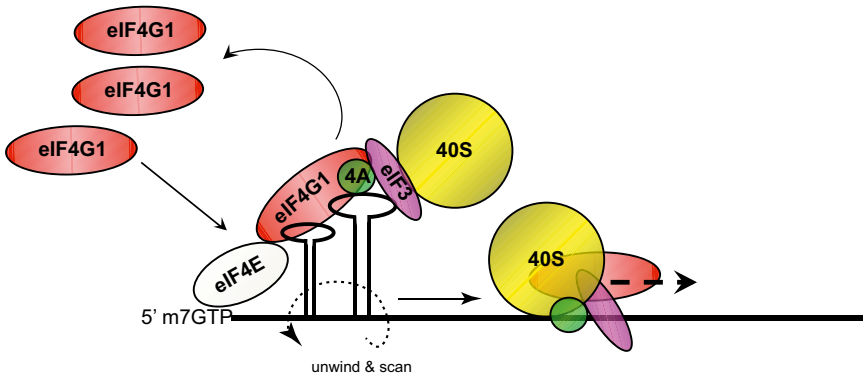

Fig. S7. Model for eIF4G1 enhanced translation of mRNAs with increased 5′-UTR secondary structure near the m^7GTP cap. This model posits that although eIF4E binds to the 5′-capped end of the mRNA, eIF4G1 also participates in mRNA-specific interactions via its RNA-binding domains with structural hairpin elements near the cap. eIF4G1 then recycles with the pool of overexpressed free eIF4G1, bringing in eIF4A, eIF3, and its associated 40S ribosome subunit, tertiary complex (not shown), and other eIF3-associated factors that participate in initiation. Tread milling of eIF4G1 through the cap-initiation complex would therefore increase translation initiation without the need for increased expression of eIF4E and would not benefit from it. Recycling of eIF4G1 might be facilitated by alteration of eIF4G1 or eIF4E phosphorylation events that weaken the eIF4E-eIF4G1 interaction. Thereafter, unwinding and 5′-to-3′ scanning of the 40S ribosome to the translation initiation codon takes place.

Table S1. Biological classifications of mRNAs in heavy polysomes in eIF4G1 silenced cells compared with WT cells with 8 Gy IR

Modulation and category	ID	Term	Gene count	%*	$P^{\dagger}$
UP					
GO BP	GO:0007155	Cell adhesion	46	14.46540881	2.006×10^{-13}
	GO:0009611	Response to wounding	29	9.119496855	6.485×10^{-7}
	GO:0006955	Immune response	28	8.805031447	2.154×10^{-4}
	GO:0016337	Cell–cell adhesion	26	8.176100629	6.146×10^{-11}
	GO:0016477	Cell migration	16	5.031446541	2.109×10^{-4}
	GO:0030855	Epithelial cell differentiation	12	3.773584906	5.047×10^{-5}
GO CC	GO:0031012	Extracellular matrix	23	7.232704403	2.185×10^{-6}
	GO:0015629	Actin cytoskeleton	19	5.974842767	9.828×10^{-6}
	GO:0005912	Adherens junction	10	3.144654088	4.430×10^{-3}
GO MF	GO:0005178	Integrin binding	7	2.201257862	7.777×10^{-4}
KEGG pathway	hsa04510	Focal adhesion	14	4.402515723	1.200×10^{-3}
DOWN					
GO BP	GO:0007049	Cell cycle	161	23.60703812	4.165×10^{-76}
	GO:0051301	Cell division	79	11.58357771	4.113×10^{-44}
	GO:0006260	DNA replication	60	8.797653959	9.894×10^{-38}
	GO:0051726	Regulation of cell cycle	51	7.478005865	4.539×10^{-17}
	GO:0006974	Response to DNA damage stimulus	51	7.478005865	8.032×10^{-15}
	GO:0006281	DNA repair	43	6.304985337	4.254×10^{-14}
	GO:0030163	Protein catabolic process	41	6.011730205	9.840×10^{-4}
	GO:0007010	Cytoskeleton organization	39	5.718475073	2.144×10^{-6}
	GO:0008283	Cell proliferation	38	5.571847507	5.433×10^{-6}
	GO:0007346	Regulation of mitotic cell cycle	25	3.66568915	3.565×10^{-9}
	GO:0006261	DNA-dependent DNA replication	24	3.519061584	4.142×10^{-18}
	GO:0000075	Cell cycle checkpoint	22	3.225806452	2.304×10^{-11}
	GO:0006310	DNA recombination	19	2.785923754	8.992×10^{-8}
	GO:0010564	Regulation of cell cycle process	19	2.785923754	3.261×10^{-7}
	GO:0006302	DSB repair	13	1.906158358	3.425×10^{-6}
	GO:0031497	Chromatin assembly	12	1.759530792	4.846×10^{-4}
	GO:0000077	DNA damage checkpoint	10	1.46627566	7.512×10^{-5}
	GO:0006270	DNA replication initiation	9	1.319648094	4.429×10^{-8}
	GO:0000724	DSB repair via homologous recombination	8	1.173020528	4.012×10^{-6}
	GO:0000725	Recombinational repair	8	1.173020528	4.012×10^{-6}
GO CC	GO:0000785	Chromatin	19	2.785923754	1.899×10^{-4}
GO MF	GO:0004674	Protein serine/threonine kinase activity	41	6.011730205	4.823×10^{-8}
KEGG pathway	hsa04110	Cell cycle	38	5.571847507	3.500×10^{-24}
	hsa03030	DNA replication	20	2.93255132	1.990×10^{-18}
	hsa04115	p53 signaling pathway	13	1.906158358	7.144×10^{-6}

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.7 was used to interpret mRNA abundance data (*Materials and Methods*). The Modulation column refers to higher or lower abundance of mRNAs, indicated as UP (color-coded in red), or DOWN (color-coded in blue), respectively. BP, biological process; CC, cellular compartment; gene count, no. of genes mapping to each category; ID, the category identifier in GO or KEGG; KD, knockdown; KEGG, Kyoto Encyclopedia of Genes and Genomes database; MF, molecular fraction; WT, wild-type.

*Percentage of relative enrichment of mapped vs. submitted genes.

$^{\dagger}P$ values are significance measures of category enrichment using modified Fisher exact test P value [equals the NIH DAVID Expression Analysis Systematic Explorer (EASE) score].

Table S2. Features of 5′-UTR sequences for IR-induced, high eIF4GI-dependent mRNAs without an IRES

Gene	5′-UTR length (nt)	Total ΔG kcal/mol	uORFs (AUGs)	GC content (%)	Stable stems near cap*	Large single-stranded loop
p53[†,‡]	252	−73	0	47	2 (∼−20 to −25 kcal/mol)	1
53BP1[†]	114	−47	0	67	2 (∼−25 and −30 kcal/mol, 75% GC)	1
XIAP[‡]	160	−65	0	68	2 (−30 kcal/mol, 80–90% GC)	0
Survivin[†,‡]	121	−63	1	73	2 (−25 kcal/mol, 76% GC)	1
PARP[‡]	170	−75	0	72	2 (∼−30 kcal/mol, 90% GC)	0
BRCA1	232	−84	0	54	>4 (∼−30 kcal/mol, 70–90% GC)	2
BRCA2[‡]	227	−97	0	59	3–4 (>−35 kcal/mol, 70% GC)	2
Chk1[†,‡]	896	−261	4	58	>4 (>−25 kcal/mol, ∼70% GC)	1–2
ATM	385	−143	5	62	2–3 (>−25 kcal/mol, ∼70% GC)	1–2
MRE11[†]	190	−60	1	50	2 (−25 to >−35 kcal/mol, 50% GC)	0
Rad50	401	−184	0	68	2 (−25 to >−35 kcal/mol)	0
Rad51[†]	299	−135	0	69	>4 (−25 to >−35 kcal/mol, 60–80% GC)	1
Rad54[†]	131	−46	0	58	3 (−20 to −30 kcal/mol)[‡]	2

*Within 30 nt of cap; M-fold analysis of reported 5′ UTR sequence.
[†]Stable hairpin adjacent (5′) to initiating AUG.
[‡]Stable stem immediately adjacent (3′) to cap.