Supporting Information

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SI Materials and Methods

Cell Culture. HEK 293T and HCT116 cells were grown in DMEM and RPMI, respectively, supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin in 5% (vol/vol) CO_2 at 37 °C. Transfections with siRNA were performed using Lipofectamine 2000 (Invitrogen).

Abs. Abs for tubulin (mouse; Sigma), Flag (mouse; F3156, Sigma), Myc (mouse, sc-40; Santa Cruz), YY1 (mouse, sc-7341; Santa Cruz), eukaryotic initiation factor-2 subunit-α (eIF2α) (rabbit, sc-11386; Santa Cruz), p-eIF2α (rabbit, #9721S; Cell Signaling), general control nonrepressed-2 (GCN2) (rabbit, ab70214; Abcam), p-GCN2 (rabbit, #3301; Cell Signaling), p-Ser (rabbit, ab9332; Abcam), and p-Thr (rabbit, #9381; Cell Signaling) were used to carry out Western blot, immunoprecipitation (IP), and immunofluorescence assays. Abs for MRS (mammalian methionyl-tRNA synthetase), EPRS (glutamyl-prolyl-tRNA synthetase), and aminoacyl-tRNA synthetase-interacting multifunctional protein-3 (AIMP3) were previously described (1). Polyclonal rabbit Ab specific to p-Ser662 of MRS was produced using synthetic peptide containing p-Ser662 (GMFV{pSer}KFFGGYVPEC) as an immunogen (Genscript).

Cloning and Detection of $tRNA_i^{Met}$ and $tRNA_e^{Met}$, and siRNA Sequences. Human genomic DNA sequences around Met-charged initiator tRNA ($tRNA_i^{Met}$) and $tRNA_e^{Met}$ were PCR-amplified with primer pairs, 5′-AAAGTGCAGTGACTACAGGCGTGA-3′ and 5′-ATGACACTTGGGTGTCCATGA-3′ for $tRNA_i^{Met}$ and 5′-TTGACCCTGGGTTTGTTCCTGTGA-3′ and 5′-TACACGTGCTCTTTCCTGGACACT-3′ for $tRNA_e^{Met}$, and cloned into the pGEM-T-Easy vector (Promega). Primers used for RT-PCR and PCR to produce Northern probe were 5′-CTGGGCCCATAACCCAGAG-3′ and 5′-TGGTAGCAGAGGATGGTTTC-3′ for $tRNA_i^{Met}$, and 5′-CTCGTTAGCGCAGTAGGTAGC-3′ and 5′-GGATCGAACTCACGACCTTC-3′ for $tRNA_e^{Met}$, respectively. Sequences of siRNAs specific to GCN2 and the 3′UTR of endogenous MRS were 5′-GCAUAAGGUCCUGAGUGCAUCUAAU-3′ and 5′-UUUAUUACUGUCCCUAUCU-3′, respectively.

In Vitro Pull-Down Assay. GST- and maltose-binding protein (MBP)-fusion proteins were expressed in *Escherichia coli* BL21 (DE3) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h at 30 °C. Harvested cells were lysed by sonication, and lysates were incubated with glutathione Sepharose 4B (GE Healthcare) or amylose resin (New England BioLabs) in the lysis buffer (PBS containing 0.5% Triton X-100 and protease inhibitor) at 4 °C for 4 h. Radiolabeled AIMP3 and MRS were synthesized by in vitro translation with the TNT-coupled translation kit (Promega) and incubated with immobilized GST- or MBP-fusion protein at 4 °C for 4 h in binding buffer (lysis buffer containing 1 mM DTT, and 5 mM EDTA). The beads were washed three times with binding buffer. Eluted proteins were separated by SDS/PAGE and detected by autoradiography.

Yeast Two-Hybrid Assay. Full-length (F) and deletion domains (D) of MRS and AIMP3 were cloned into pLexA and pJG4-5 (B42). LexA- and B42-fusion proteins (LexA-MRS F and B42-AIMP3 Ds or LexA-AIMP3 F and B42-MRS Ds) were expressed in the yeast strain EGY. Interactions were determined by induction of reporters, LEU2, and lacZ. To detect interactions, colonies were picked and streaked onto Ura^-, His^-, Trp^-, Leu^-/glucose media and Ura^-, His^-, Trp^-/galactose media containing X-Gal and raffinose.

Bimolecular Fluorescence Complement and Immunofluorescence Assays. AIMP3 and MRS were cloned into bimolecular fluorescence complement (pBiFC)-VN173 (Flag tag) and pBiFC-VC155 (HA tag). HeLa cells were cotransfected with the pBiFC-VN173-AIMP3 and pBiFC-VC155-MRS. The next day, the cells were UV-irradiated (60 J/m^2) and recovered. These cells were fixed with 100% methanol for 15 min at room temperature and incubated with blocking solution (PBS containing 1% (vol/vol) BSA and 0.05% Triton X-100) for 1 h at room temperature. After blocking, the cells were stained with primary Ab and Alexa555-conjugated secondary Ab for 1 h. DAPI was used for nuclear staining. HeLa cells transfected with pEGFP-AIMP3 were UV-irradiated and recovered with serum free (SF) medium. At various time points, the cells were harvested, fixed, and stained with propidium iodide. After mounting, MRS and AIMP3 interaction and cellular localization of AIMP3 was observed by fluorescence and confocal microscopy.

Cell Fractionation. HeLa cells were UV-irradiated and recovered with SF medium. After harvest, irradiated cells were lysed with buffer A (10 mM Hepes pH 7.4, 10 mM KCl, 100 μM EDTA, 1 mM DTT, 0.4% Nonidet P-40, and protease inhibitor mixture). Cell lysates were centrifuged at 15,000 × *g* for 3 min, and the supernatants were transferred into a new tube. The pellets were washed twice with 1× cold PBS and resuspended in buffer B (10 mM Hepes pH 7.4, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (vol/vol) Glycerol, and protease inhibitor mixture). After incubation for 1 h, the resuspensions were centrifuged at 15,000 × *g* for 5 min to obtain the nuclear fraction.

Two-Dimensional PAGE. Phosphorylation detection with 2D-PAGE was carried out as previously described (2). Briefly, 500 μg proteins were rehydrated in resolubilization buffer (7 M urea, 2 M thiourea, 2% ASB-14, 0.5% Triton X-100, 1% (vol/vol) ampholyte, 1% (vol/vol) tributylphosphine, and 0.1% bromophenol blue). Protein solutions were absorbed into 7-cm pH-gradient strips and subjected to isoelectric focusing.

In Vitro Kinase Assay. MBP-fusion MRS deletion domains D1 (residues 1–266), D2 (267–597), and D3 (598–900) were purified from *E. coli* BL21 (DE3) using amylose resin. Flag-tagged GCN2 WT or the GCN2 K618R mutant expressed in 293T cells was immunoprecipitated and incubated with each purified MBP-MRS domain in kinase buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 250 μM ATP, and 10 μCi [γ-^{32}P]ATP) at 30 °C.

For the peptide kinase assay, GST-GCN2 kinase domain (KD) WT and the K618R mutant expressed from *E. coli* BL21 (DE3) were immobilized with glutathione Sepharose 4B beads. N-terminal biotinylated peptides were chemically synthesized (GL Biochem). Each peptide [25 μM of MRS Ser662 (RAGMFVSKFFGG), MRS S662A (RAGMFVAKFFGG), GCN2 Thr899 (PSGHLTGMVGT), and eIF2α Ser51 (ELSRRRIR)] was reacted with the GST-GCN2 KD at 30 °C for 30 min. Reaction mixtures were centrifuged at 1,000 × *g* for 3 min and the soup was recovered and filtered through a streptavidin-coated matrix biotin-capture (SAM) membrane (3) using a 96-well Minifold filtration apparatus. The SAM membrane was washed according to Schaefer and Guimond (3) and exposed for autoradiography.

Nano-LC-MS/MS Analysis. Protein bands cut from SDS/PAGE gels were trypsin digested, and nano-LC-MS/MS analysis was performed on an Agilent 1100 series nano-LC and LTQ-mass spectrometer (Thermo Electron).

Northern Blotting for tRNA^{Met} Detection. Total RNA (30 μg), isolated with the miRNeasy Mini Kit (Qiagen) or TRIzol (Invitrogen) under acidic condition, was separated by 6.5% acid-gel urea (8%) PAGE, and transferred onto a nylon membrane with a semidry transfer apparatus. DNA probes were PCR-amplified and biotinylated with the BrightStar Psoralen-Biotin kit (Ambion). Northern hybridization was performed using the BrightStar BioDetect kit (Ambion). For the analysis of charged tRNA, total RNA was run by acid-gel urea PAGE and subjected to Northern hybridization using initiator tRNA-specific probe (4).

Coexpression and Copurification of AIMP3 and MRS. AIMP3 gene was cloned into the His-tag multiple cloning site (MCS) of pACYCDuet (Novagen) to construct pKMJ101. The MRS gene was cloned into the S-tag MCS of pKMJ101 to construct pKMJ102. Site-directed mutagenesis was used to introduce the S662D mutation into pKMJ102. pKMJ101, pKMJ102, and pKMJ103 (the MRS S662D mutant) were transformed into *E. coli* BL21 (DE3), and His-AIMP3 was purified using Ni-NTA agarose. Copurification of MRS WT and the S662D mutant was confirmed by immunoblotting.

Circular Dichroism Spectrum. MBP-fusion MRS WT and S662D mutant were purified using amylase resin. MBP-tag proteins, eluted with lysis buffer containing 50 mM maltose at 4 °C overnight, were dialyzed with 10 mM potassium phosphate buffer (pH 7.4). The circular dichroism (CD) spectrum was analyzed with a Jasco J-815 CD spectrometer at 25 °C in the near UV range of 250–350 nm. Samples were loaded into a 0.1-cm path-length cuvettete. The results are presented as an average of three repeated scans after subtraction of buffer background.

Protein Digestion by Trypsin and Elastase. MBP-MRS WT and the S662D mutant (15 μg) were digested with 5 μg trypsin (HyClone) for 3 min at room temperature or 0.6 U of elastase (Sigma) for 30 min at 37 °C. Digested samples were separated by SDS/PAGE and stained with Coomassie brilliant blue.

Insertion of Peptide Linker. Insertion of the peptide linker with the sequence of Gly-Gly-Gly-Gly-Ser between the residues 233 and 234 of MRS WT and of the S662D mutant was performed with the QuikChange site-directed mutagenesis kit (Stratagene). Primer pairs, 5′-TCTGAGGAGGAGATTGGCGGCGGCGGCTCTG-CTATGGCTGTTACT-3′ and 5′-AGTAACAGCCATAGCA-GAGCCGCCGCCGCCAATCTCCTCCTCAGA-3′ were used for the mutational insertion into pGEX-4T-1 encoding MRS WT or the MRS S662D mutant.

Gel Shift and Filter Binding Assays. Initiator tRNA^{Met} was synthesized by in vitro transcription with [α-^{32}P]UTP (3,000 Ci/mmol; Izotop). Purified His-MRS proteins were mixed with the tRNA$_i^{Met}$ probes in the binding buffer (20 mM Tris-HCl pH 7.4, 75 mM KCl, 10 mM $MgCl_2$, and 5% (vol/vol) glycerol) and incubated at 30 °C for 30 min. Reaction mixtures were separated by 6% nondenaturing PAGE and dried. For the dot-blot assay, reaction mixtures were filtered through a nitrocellulose membrane using a 96-well Minifold filtration apparatus.

Aminoacylation Assay. His-tagged MRS expressed in *E. coli* Rosetta (DE3) was purified using ProBond Resin (Invitrogen), following washing with lysis buffer containing 20 mM KH_2PO_4 and 500 mM NaCl, pH 7.8 and with lysis buffer containing 10% (vol/vol) glycerol and then changing the pH from 7.8, to 6 and 5.2, and back to 6 using 20 mM imidazole at the final step. His-MRS was eluted in the presence of 200 mM imidazole (pH 6.0) and dialyzed with PBS containing 20% (vol/vol) glycerol. Initiator tRNA^{Met} was synthesized by in vitro transcription. MRS aminoacylation activity was assayed at 37 °C in reaction buffer (30 mM Hepes, pH 7.4, 100 mM potassium acetate, 10 mM magnesium acetate, 2 mM ATP, 20 μM Met, 100 μg/mL tRNA$_i^{Met}$, and 25 μCi [^{35}S]Met (1,000 Ci/mmol; Izotop). Aminoacylation reactions were quenched on 3MM filter paper prewetted with 5% trichloroacetic acid containing 1 mM Met. After washing with 5% trichloroacetic acid and drying, radioactivity was detected by liquid scintillation counter (Wallac 1409).

Radiolabeled Met-tRNA^{Met} Isolation. Cells were incubated in Met-free media with 1 μCi [^{35}S]Met (1,175 Ci/mmol; Perkin-Elmer) for 45 min. Total RNA (30 μg) was purified under acidic conditions, and separated by acid-urea PAGE. Met-tRNA^{Met} was detected by autoradiography.

[^{35}S]Met Incorporation Assay. Cells were incubated in Met-free media containing 1 μCi [^{35}S]Met. After incubation, the cells were washed with cold PBS, and the amount of radioactive protein was measured by liquid scintillation. Count data were normalized to cell number.

1. Ko YG, Kang YS, Kim EK, Park SG, Kim S (2000) Nucleolar localization of human methionyl-tRNA synthetase and its role in ribosomal RNA synthesis. *J Cell Biol* 149: 567–574.
2. Han JM, et al. (2008) AIMP2/p38, the scaffold for the multi-tRNA synthetase complex, responds to genotoxic stresses via p53. *Proc Natl Acad Sci USA* 105:11206–11211.
3. Schaefer EM, Guimond S (1998) Detection of protein tyrosine kinase activity using a high-capacity streptavidin-coated membrane and optimized biotinylated peptide substrates. *Anal Biochem* 261:100–112.
4. Köhrer C, Rajbhandary UL (2008) The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs and aminoacyl-tRNA synthetases. *Methods* 44: 129–138.

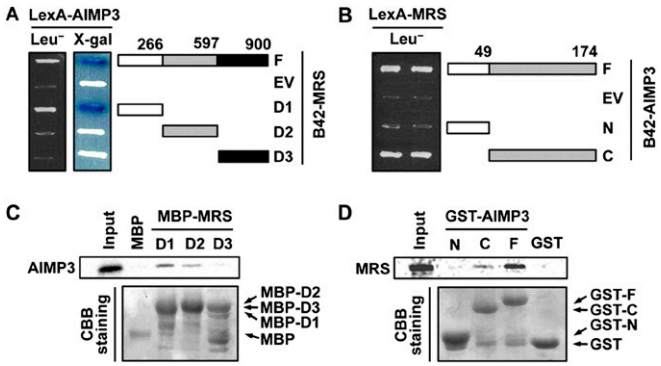

Fig. S1. MRS and AIMP3 interact each other via GST-like domains. (*A*) Binding ability of B42-MRS, D1, D2, and D3 to LexA-AIMP3 was determined by cell growth on LEU⁻ and blue colony formation on X-Gal plates. (*B*) Interaction of B42-AIMP3 domains (N: residues 1–49 and C: residues 50–174) with LexA-MRS was determined. (*C*) Radioactively labeled AIMP3 was incubated with different domains of MBP-MRS. After stained with Coomassie brilliant blue (CBB), gel was dried and exposed for autoradiography. (*D*) Radioactively labeled MRS was incubated with the F (full), N, and C domains of GST-AIMP3. Coprecipitation of MRS was detected by autoradiography.

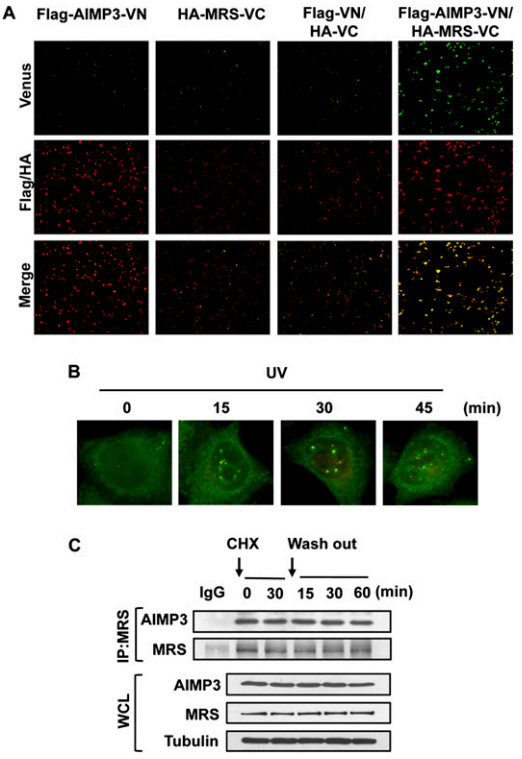

Fig. S2. AIMP3 dissociates from MRS and translocates to the nucleus following UV irradiation but not by cycloheximide treatment. (*A*) AIMP3 and MRS were cloned into pBiFC-VN173 and pBiFC-VC155, respectively, resulting expression of Flag-AIMP3-VN (nonfluorescent N-terminal fragment of Venus) and HA–MRS-VC (nonfluorescent C-terminal fragment of Venus) fusion proteins. HCT116 cells were transfected with single or both of fusion proteins and reconstitution of Venus fluorescence by close proximity of VN and VC was observed by fluorescence microscopy (200x). Control vectors, pBiFC-VN173 and pBiFC-VC155, were also tranfected into HCT116 cells together as a negative control. Venus fluorescence was observed only in the cells cotransfected with Flag–AIMP3-VN and HA–MRS-VC (green color). Autofluorescence from Flag-VN and HA-VC, Flag–AIMP3-VN, or HA–MRS-VC was weak and could be differentiated from reconstituted green fluorescence. Flag or HA (only for single transfection of HA–MRS-VC) was observed by red fluorescence using Alexa Fluor 555-conjugated anti-Flag or HA Ab for transfection control. (*B*) HeLa cells transfected with GFP-AIMP3 were irradiated by UV and cells were fixed and stained with propidium iodide. Cellular localization of GFP-AIMP3 was monitored by fluorescence microscopy (600x). (*C*) HeLa cells were treated with cycloheximide (CHX) for 30 min. After washing, the cells were harvested at the indicated times. MRS was immunoprecipitated and bound proteins were detected by immunoblotting. Dissociation of AIMP3 and MRS was not observed by CHX treatment.

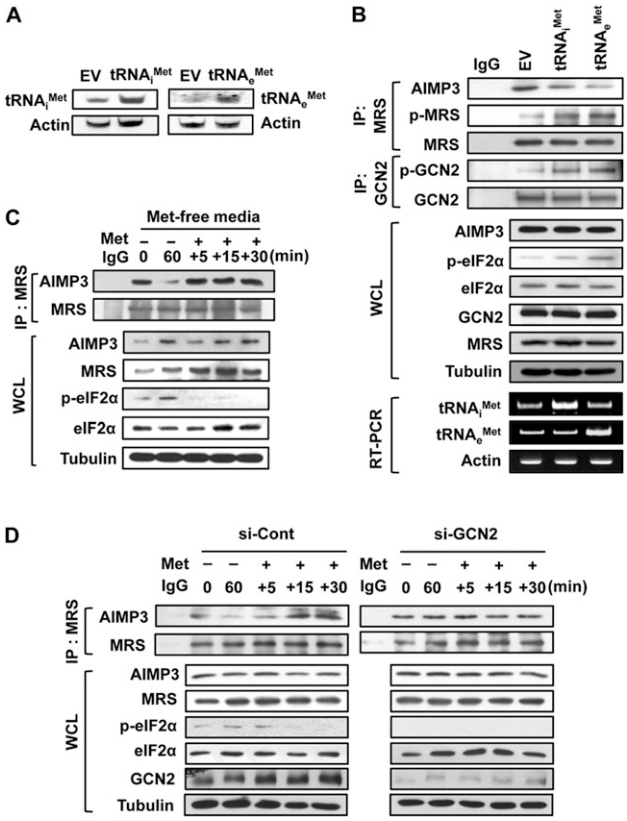

Fig. S3. MRS phosphorylation and AIMP3 dissociation were mediated by GCN2. (*A*) HeLa cells were transfected with pGEM-T-Easy vector encoding initiator and elongator tRNA^{Met}. After 24 h, total RNA was purified, and tRNA expression levels were analyzed by Northern blotting using a biotinylated probe to confirm increased expression of transfected initiator and elongator tRNA^{Met}. Actin was used as a loading control. (*B*) HeLa cells transfected with pGEM-T-Easy vector encoding initiator and elongator tRNA^{Met} were lysed after 24-h incubation. Empty vector (EV)-transfected cells were used as a control. MRS and GCN2 were immunoprecipitated. Co-IP AIMP3 and phosphorylated MRS (p-MRS) and p-GCN2 were determined by specific Abs. Expression of each tRNA was assayed by RT-PCR. (*C*) HeLa cells were incubated in Met-free media for 1 h and then Met was added in the media. Cells were harvested and the association between MRS and AIMP3 was monitored. (*D*). Met-dependent interaction between AIMP3 and MRS was monitored as described in *C*, using HeLa cells transfected with si-Control (Cont) or si-GCN2.

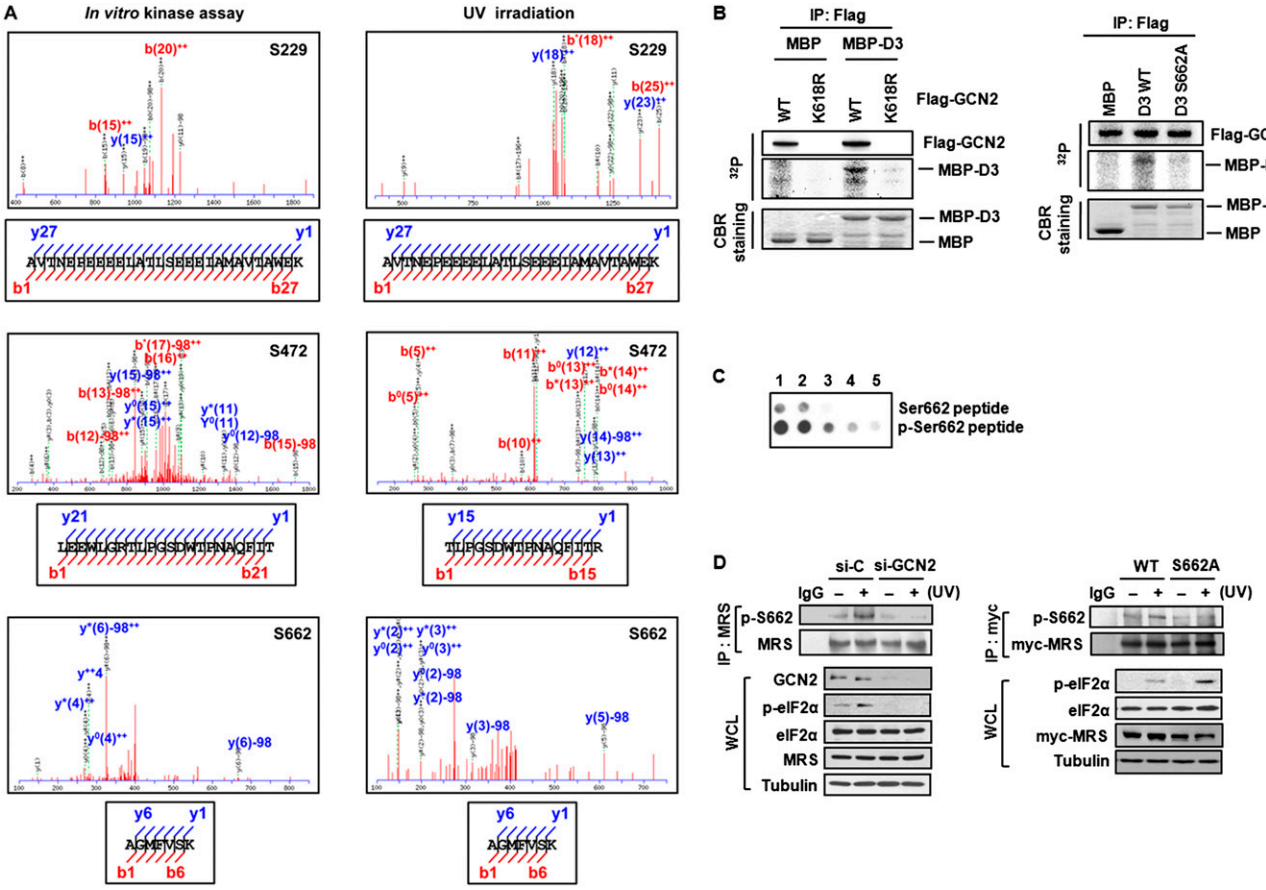

Fig. S4. Determination of GCN2-induced phosphorylation sites of MRS. (*A*) MRS proteins, which were phosphorylated by in vitro kinase reaction and immunoprecipitated from UV-treated HeLa cells, were processed for nano LC-MS/MS analysis as described in *Materials and Methods*. Phosphorylation at S229, S472, and S662 was detected in both cases. Detected peptide sequences spanning the phosphorylation site and the peaks for the equivalent fragment ions are shown ($P < 0.05$). (*B*) MBP-MRS D3 WT was incubated with GCN2 WT or the GCN2 K618R inactive mutant immunoprecipitated from 293T cells (*Left*) and MRS D3 WT and the S662A mutant were subjected to GCN2 kianse assay for in vitro kinase assay (*Right*). Phosphorylation signal was detected by autoradiography. (*C*) Specificity of p-Ser662–specific Ab to peptide harboring p-Ser662 was analyzed. MRS peptides (GMFVSKFFGGYVPEC) with or without phosphoserine residue were serially diluted, spotted onto PVDF membrane, and then immunoblotted using p-Ser662–specific Ab. Concentrations of peptides used were 5 μM (lane 1), 2.5 μM (lane 2), 1.25 μM (lane 3), 0.625 μM (lane 4), and 0.313 μM (lane 5). (*D*) MRS was immunoprecipitated from HeLa cells transfected with si-control (si-C) or si-GCN2 and p-MRS was detected using p-Ser662–specific Ab (*Left*). Exogenous MRS was immunoprecipitated from stable HeLa cells expressing MRS WT or S662A mutant and subjected to immunoblotting using p-Ser662–specific Ab (*Right*).

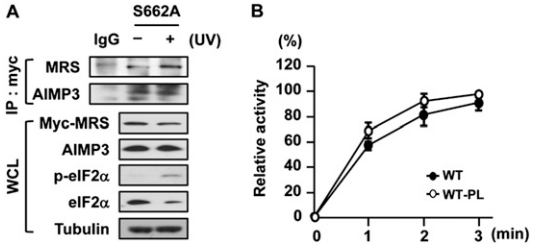

Fig. S5. Effects of Ala substitution or peptide linker insertion on MRS function. (*A*) The MRS S662A mutant was immunoprecipitated from stable HeLa cells and the bound AIMP3 upon UV was detected by immunoblotting. WCL, whole-cell lysates. (*B*) The aminoacylation assay was performed with purified His-MRS with or without peptide linker insertion. Results are presented as mean as mean ± SD ($n = 3$).

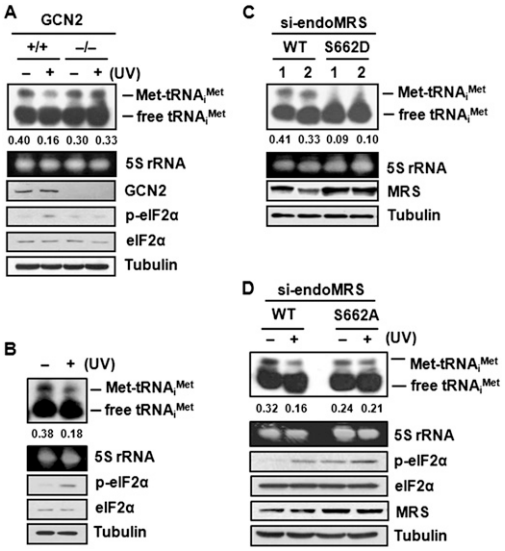

Fig. S6. Analysis for the charged initiator tRNA. Levels of charged $tRNA_i^{Met}$ in UV-irradiated *GCN2* mouse embryonic fibroblasts (*A*) or HeLa (*B*) were analyzed by acid-gel urea PAGE and Northern blotting. rRNA was used as a loading control. (*C*) Stable HeLa cells expressing MRS WT or the MRS S662D mutant were treated with siRNA specific to endogenous MRS, and levels of charged $tRNA_i^{Met}$ were analyzed. (*D*) After endogenous MRS was knocked down, stable cells expressing MRS WT or the MRS S662A mutant were UV-irradiated and analyzed for the levels of charged Met-$tRNA_i^{Met}$. Elevated p-eIF2α in the S662A-expressing cells implies that translational control via eIF2α could be enhanced when p-MRS–mediated translation control is not available, perhaps as a compensatory mechanism. The relative ratio of charged initiator tRNA to free initiator tRNA was quantified and presented.

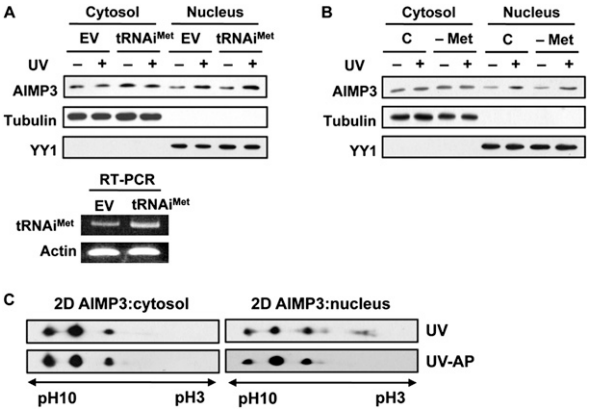

Fig. S7. Posttranslational modification of AIMP3 is needed for nuclear translocation. (*A*) HeLa cells transfected with initiator tRNA were UV-irradiated, and nuclear AIMP3 was analyzed by immunoblotting. EV-transfected cells were used as a control. (*B*) HeLa cells starved with Met for 1 h were irradiated by UV and fractionated for the analysis of nuclear AIMP3 by immunoblotting. Untreated cells were used as a control. (*C*) UV-irradiated HeLa cells were fractionated and analyzed by 2D-PAGE. Shift of protein spots to the acidic side, disappeared by alkaline phosphatase (AP) treatment was observed in nucleus.

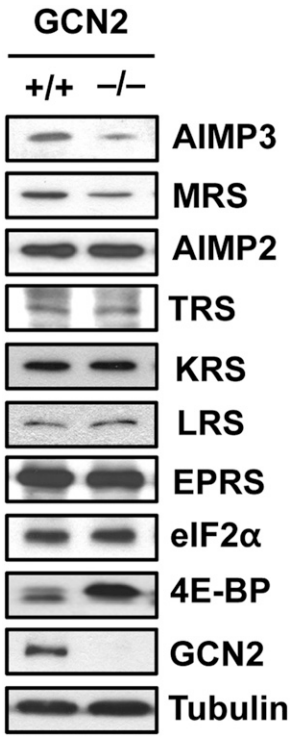

Fig. S8. Expression of translation-related proteins in *GCN2* mouse embryonic fibroblasts. Expression level of several translational components was analyzed by immunoblotting.