Supplementary Table 1. The list of ribosomal proteins and eukaryotic translation initiation factors from rapamycin-treated and untreated cells.

Accession	Gene	Discription	Score	Coverage	Rapamycin-treated/untreated
P15880	RPS2	40S ribosomal protein S2	341.11	57.00	1.04
P23396	RPS3	40S ribosomal protein S3	1108.38	79.84	1.03
P61247	RPS3A	40S ribosomal protein S3a	596.05	72.35	1.03
P62753	RPS6	40S ribosomal protein S6	138.59	42.17	1.02
P62241	RPS8	40S ribosomal protein S8	331.75	52.88	1.02
P46781	RPS9	40S ribosomal protein S9	99.05	61.86	1.04
P46783	RPS10	40S ribosomal protein S10	189.69	50.30	1.09
P08708	RPS17	40S ribosomal protein S17	189.90	45.93	1.03
P42677	RPS27	40S ribosomal protein S27	122.23	41.67	1.04
P26373	RPL13	60S ribosomal protein L13	180.02	43.13	1.08
E7EPB3	RPL14	60S ribosomal protein L14	47.71	24.19	0.97
P61313	RPL15	60S ribosomal protein L15	282.96	53.92	1.07

M0R3D6	RPL18A	60S ribosomal protein L18a	192.85	46.10	1.12
J3QR09	RPL19	60S ribosomal protein L19	172.40	34.72	1.08
P62750	RPL23A	60S ribosomal protein L23a	231.20	53.21	1.06
C9JXB8	RPL24	60S ribosomal protein L24	77.73	52.07	1.04
P61254	RPL26	60S ribosomal protein L26	67.90	39.31	1.12
P46779	RPL28	60S ribosomal protein L28	112.26	45.99	1.11
P49207	RPL34	60S ribosomal protein L34	143.16	35.90	1.08
P05388	RPLP0	60S ribosomal protein P0	595.03	44.48	0.98
P05387	RPLP2	60S ribosomal protein P2	338.79	48.70	1.03
Q9BY44	EIF2A	Eukaryotic translation initiation factor 2A	102.50	31.97	1.02
H3BRV0	EIF3C	Eukaryotic translation initiation factor 3 subunit C	339.70	28.68	1.08
P60228	EIF3E	Eukaryotic translation initiation factor 3 subunit E	222.86	34.61	1.00
P23588	EIF4B	Eukaryotic translation initiation factor 4B	117.85	39.12	1.09
Q04637-3	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	427.97	27.90	1.04

H0Y3P2	EIF4G2	Eukaryotic translation initiation factor 4 gamma 2	147.39	24.05	1.05
O60841	EIF5B	Eukaryotic translation initiation factor 5B	123.73	18.20	1.06

Supplementary Table 2. The list of mTOR-mediated phosphopeptides from ribosomal proteins.

Gene	**Discription**	**Phosphosite**	**Sequence**	**Light/Heavy ratio**	**Predicted kinase**
RPS2	40S ribosomal protein S2	S77	IKpSLEEIYLFSLPIK	0.68	NA
RPS2	40S ribosomal protein S2	S264	ETVFTKpSPYQEFTDHLVK	0.68	mTOR
RPS3	40S ribosomal protein S3	T221	DEILPTpTPISEQK	0.54	mTOR
RPS3A	40S ribosomal protein S3a	S236	LMELHGEGpSSSGK	0.64	NA
RPS6	40S ribosomal protein S6	S148	KLFNLpSKEDDVR	0.04	NA
RPS6	40S ribosomal protein S6	S235	RLpSSLRASTSK	0.03	RSK
RPS6	40S ribosomal protein S6	S236	LSpSLRApSTSK	0.04	NA
RPS6	40S ribosomal protein S6	S240	LSpSLRApSTSK	0.04	RSK
RPS8	40S ribosomal protein S8	S160	ISpSLLEEQFQQGK	0.51	NA

RPS9	40S ribosomal protein S9	S153	LDpSQKHIDFSLR	0.64	ATM
RPS10	40S ribosomal protein S10	S146	KAEAGAGpSATEFQFR	0.72	NA
RPS17	40S ribosomal protein S17	S113	LLDFGpSLSNLQVTQPTVGMNFK	0.69	NA
RPS27	40S ribosomal protein S27	S27	LVQpSPNSYFMDVK	0.57	NA
RPL13	60S ribosomal protein L13	S77	AGRGFpSLEELR	0.51	NA
RPL13	60S ribosomal protein L13	S106	NKpSTESLQANVQR	0.58	RSK
RPL13	60S ribosomal protein L13	S139	KGDpSSAEELKLATQLTGPVMPVR	0.59	CKII
RPL14	60S ribosomal protein L14	S139	AALLKApSPK	0.52	mTOR
RPL15	60S ribosomal protein L15	S97	FARpSLQSVAEER	0.56	NA
RPL18A	60S ribosomal protein L18a	S71	SSGEIVYCGQVFEKpSPLRVK	0.57	mTOR
RPL18A	60S ribosomal protein L18a	S123	AHpSIQIMK	0.67	NA
RPL19	60S ribosomal protein L19	S13	LASpSVLR	0.63	NA
RPL23A	60S ribosomal protein L23a	S43	KIRTpSPTFR	0.58	mTOR
RPL24	60S ribosomal protein L24	S86	AITGApSLADIMAK	0.42	NA

RPL26	60S ribosomal protein L26	T139	GKYKEEpTIEK	0.69	NA
RPL28	60S ribosomal protein L28	S115	RApSAILR	0.64	RSK
RPL34	60S ribosomal protein L34	S12	RLpSYNTASNK	0.45	RSK
RPLP0	60S ribosomal protein P0	S304 S307	VEAKEEpSEEpSDEDMGFGLFD	0.40	CKII
RPLP2	60S ribosomal protein P2	S17	YVASYLLAALGGNSpSPSAK	0.47	mTOR
RPLP2	60S ribosomal protein P2	S102 S105	KEEpSEEpSDDDMGFGLFD	0.46	CKII

Supplementary Table 3. The list of mTOR-mediated phosphopeptides from eukaryotic translation initiation factors.

Gene	**Discription**	**Phosphosite**	**Sequence**	**Light/Heavy ratio**
EIF2A	Eukaryotic translation initiation factor 2A	S506	SDKpSPDLAPTPAPQSTPR	0.66
EIF3C	Eukaryotic translation initiation factor 3 subunit C	S39	QPLLLpSEDEEDTKR	0.71
EIF3E	Eukaryotic translation initiation factor 3 subunit E	S399	LGHVVMGNNAVpSPYQQVIEK	0.30
EIF4B	Eukaryotic translation initiation factor 4B	S498	SQSpSDTEQQpSPTSGGGk	0.48
EIF4B	Eukaryotic translation initiation factor 4B	S504	SQSpSDTEQQpSPTSGGGk	0.48
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	S1187	SFpSKEVEER	0.20

EIF4G2	Eukaryotic translation initiation factor 4 gamma 2	T508	TQpTPPLGQTPQLGLK	0.50
EIF5B	Eukaryotic translation initiation factor 5B	S113	QSFDDNDpSEELEDKDSK	0.37
EIF5B	Eukaryotic translation initiation factor 5B	S135 S137	VEMYpSGpSDDDDDFNKLPK	0.50
EIF5B	Eukaryotic translation initiation factor 5B	S214	NKPGPNIEpSGNEDDDASFK	0.67

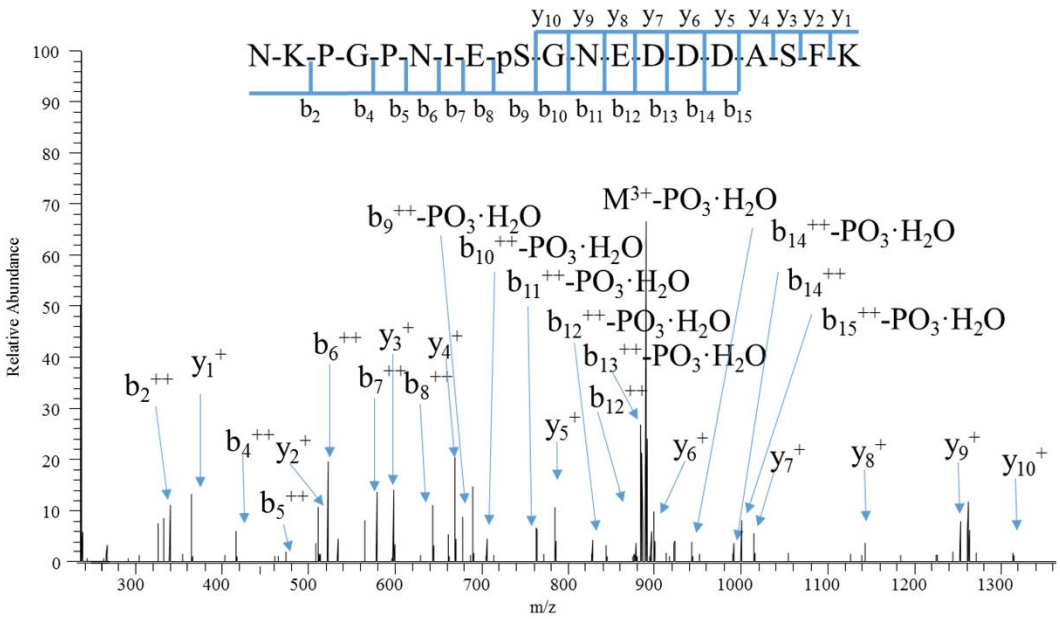
Supplementary Table 4. The list of binding partners of wildtype eIF5B and two mutants (eIF5B-S214A and eIF5B-S214E).

Gene	**Discription**	**LFQ intensity**			
		Flag-control	**Flag-eIF5B**	**Flag-eIF5B S214A**	**Flag-eIF5B S214E**
ATAD3A	ATPase family AAA domain-containing protein 3A	0	2.05E+06	1.84E+06	7.40E+05
DDX18	ATP-dependent RNA helicase DDX18	3.44E+06	3.63E+07	2.18E+07	2.13E+07
DDX27	ATP-dependent RNA helicase DDX27	5.08E+05	1.04E+07	0	8.09E+06
DDX50	ATP-dependent RNA helicase DDX50	4.83E+05	1.15E+07	2.26E+06	6.62E+06
DDX54	ATP-dependent RNA helicase DDX54	1.48E+06	1.18E+07	3.68E+05	1.05E+07
CKMT1A	Creatine kinase U-type, mitochondrial	0	7.84E+05	4.08E+05	0

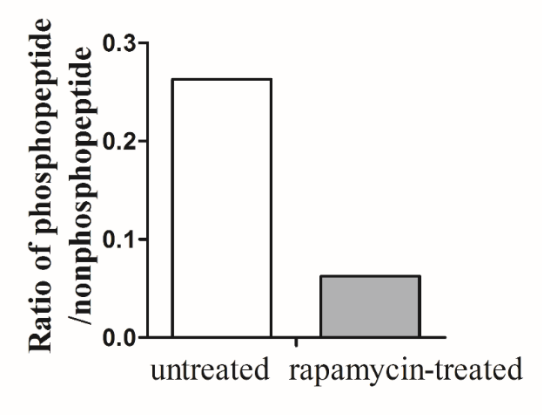
POLRMT	DNA-directed RNA polymerase, mitochondrial	2.45E+06	1.83E+07	2.10E+07	1.95E+07
STAU1	Double-stranded RNA-binding protein Staufen homolog 1	6.45E+05	5.50E+06	4.01E+06	1.18E+06
TRIM25	E3 ubiquitin/ISG15 ligase TRIM25	4.53E+05	1.21E+07	2.35E+06	2.26E+06
EIF2S2	Eukaryotic translation initiation factor 2 subunit 2	2.44E+07	1.27E+08	1.40E+08	5.18E+07
EIF2S3	Eukaryotic translation initiation factor 2 subunit 3	2.69E+07	1.81E+08	1.41E+08	9.80E+07
EIF5	Eukaryotic translation initiation factor 5	0	5.05E+07	1.95E+07	3.08E+06
EXOS6	Exosome complex component MTR3	1.25E+06	7.84E+06	1.09E+07	3.99E+06
EXOS10	Exosome component 10	8.19E+05	2.01E+07	2.44E+06	1.73E+07
FMR1	Fragile X mental retardation protein 1	0	1.90E+06	3.59E+05	0
FXR1	Fragile X mental retardation syndrome-related protein 1	1.07E+07	7.06E+07	3.87E+07	4.46E+07
FXR2	Fragile X mental retardation syndrome-related protein 2	4.09E+06	3.62E+07	6.70E+06	2.17E+07
GRWD1	Glutamate-rich WD repeat-containing protein 1	8.29E+05	6.85E+06	6.42E+06	1.60E+06
GNL3	Guanine nucleotide-binding protein-like 3	2.37E+06	1.39E+07	1.01E+07	1.51E+07
DKC1	H/ACA ribonucleoprotein complex subunit 4	0	1.57E+06	3.14E+06	0

HP1BP3	Heterochromatin protein 1-binding protein 3	0	5.93E+06	3.26E+06	4.98E+06
HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	0	4.75E+05	0	0
HMMR	Hyaluronan mediated motility receptor	0	4.09E+05	4.90E+05	6.53E+05
NAT10	N-acetyltransferase 10	5.26E+05	1.52E+07	2.06E+06	1.10E+07
NONO	Non-POU domain-containing octamer-binding protein	0	7.38E+05	1.42E+06	0
NEMF	Nuclear export mediator factor NEMF	0	2.74E+06	0	1.09E+06
YBX1	Nucleolar GTP-binding protein 1	3.24E+06	1.64E+07	1.38E+07	1.31E+07
NOP56	Nucleolar protein 56	2.79E+06	1.59E+07	8.59E+06	6.16E+06
NOP58	Nucleolar protein 58	1.53E+06	7.72E+06	4.45E+06	3.53E+06
PES1	Pescadillo homolog	0	5.13E+06	2.48E+06	2.16E+06
FARSB	Phenylalanine--tRNA ligase beta subunit	0	1.44E+06	2.51E+05	2.03E+05
SERBP1	Plasminogen activator inhibitor 1 RNA-binding protein	0	4.27E+05	0	0
PA2G4	Proliferation-associated protein 2G4	1.06E+06	7.00E+06	1.36E+07	2.72E+06
IQGAP1	Ras GTPase-activating protein-binding protein 1	1.20E+06	6.63E+06	4.39E+06	0

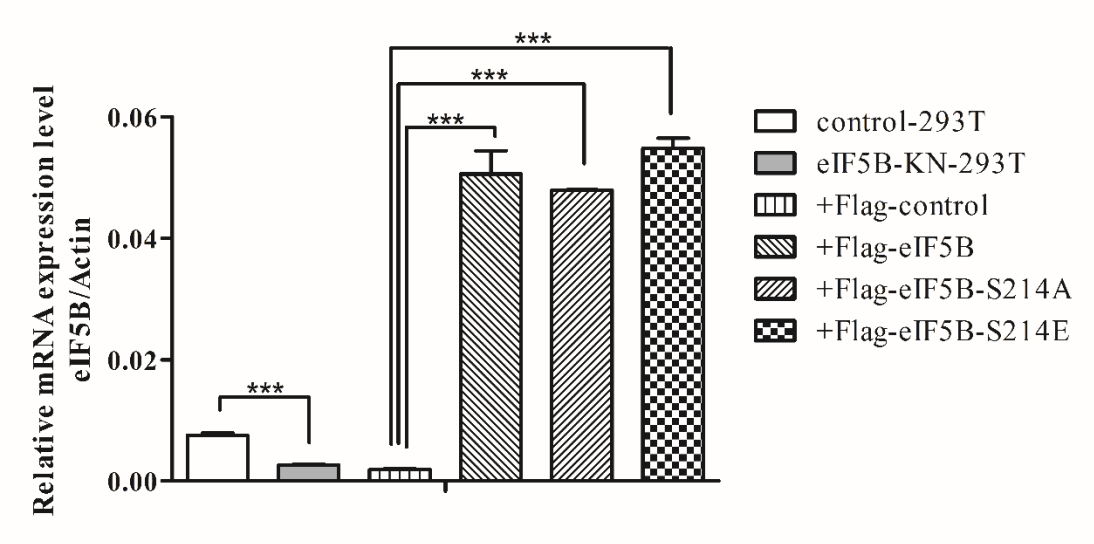
UPF1	Regulator of nonsense transcripts 3B	0	1.15E+06	1.94E+06	0
RSL1D1	Ribosomal L1 domain-containing protein 1	3.14E+06	1.57E+07	2.15E+07	1.54E+07
RRP1B	Ribosomal RNA processing protein 1 homolog B	5.62E+05	5.94E+06	2.45E+06	1.34E+06
RBM28	RNA-binding protein 28	3.27E+06	2.06E+07	0	1.56E+07
SRP68	Signal recognition particle subunit SRP68	7.13E+05	5.59E+06	3.68E+06	5.46E+06
SRP72	Signal recognition particle subunit SRP72	9.42E+05	5.69E+06	5.10E+06	3.57E+06
SND1	Staphylococcal nuclease domain-containing protein 1	0	1.54E+06	0	5.91E+05
SMCA5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	4.05E+06	2.04E+07	0	2.04E+07
NSUN2	tRNA (cytosine(34)-C(5))-methyltransferase	0	1.57E+06	0	3.70E+06
RTCB	tRNA-splicing ligase RtcB homolog	0	2.48E+06	2.21E+06	0
TUBB6	Tubulin beta-6 chain	0	3.69E+06	2.41E+06	1.24E+06



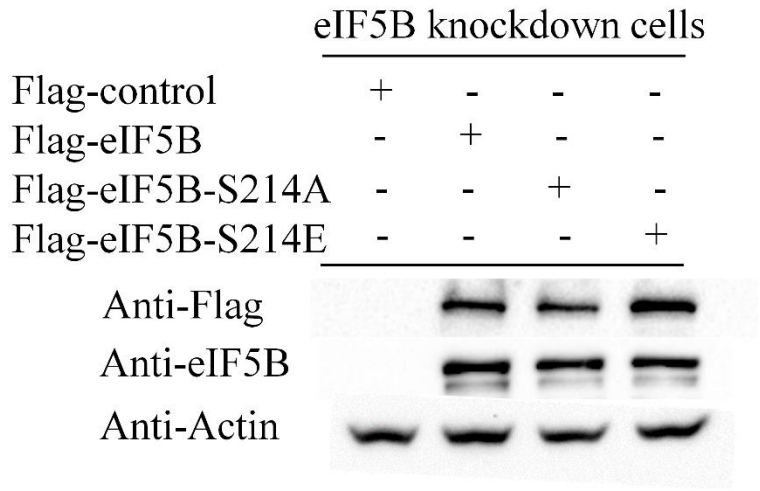
Supplementary Figure 1. A MS/MS spectrum of the peptide containing phosphorylated Ser214 of eIF5B.



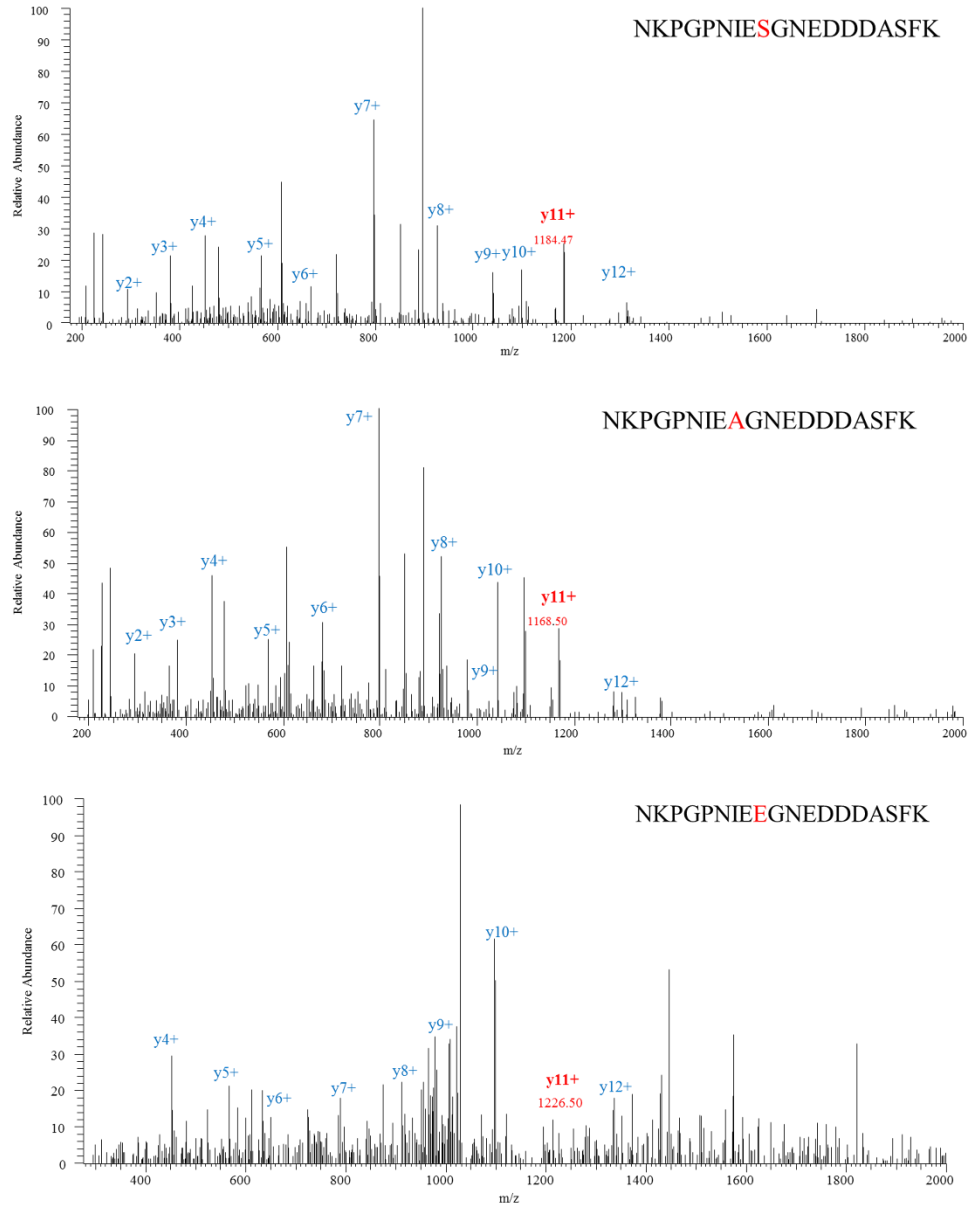
Supplementary Figure 2. The ratio of the phosphorylated Ser214-containing peptide to unphosphorylated peptide from untreated and rapamycin-treated samples.



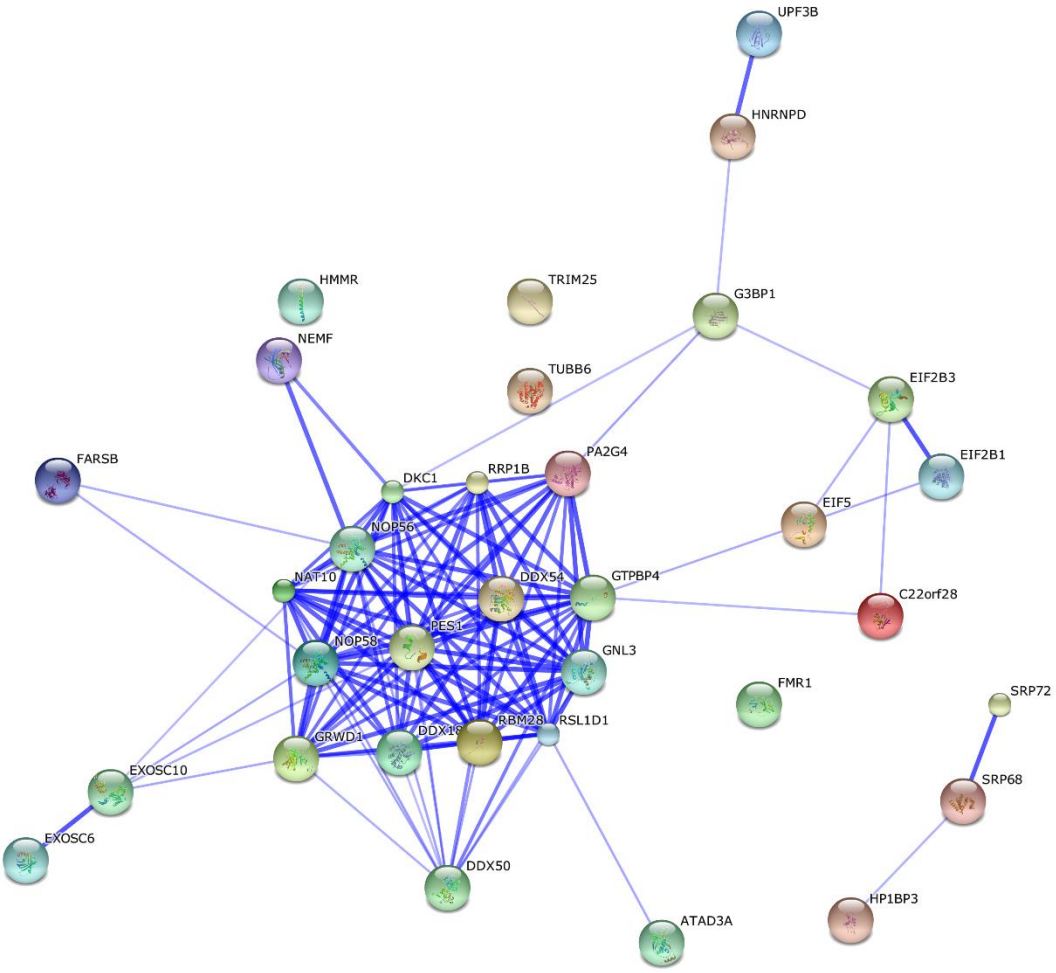
Supplementary Figure 3. The qPCR analysis of eIF5B mRNA expression levels in eIF5B-knockdown cells and the eIF5B, eIF5B-S214A and eIF5B-S214E overexpression cells.



Supplementary Figure 4. Western blotting of eIF5B in eIF5B knockdown cells and the eIF5B, eIF5B-S214A and eIF5B-S214E overexpression cells.



Supplementary Figure 5. The MS/MS spectra of the Ser214-, Ala214- and Glu214- containing peptide of eIF5B from the eIF5B, eIF5B-S214A and eIF5B-S214E overexpression cells.



Supplementary Figure 6. Interactome analysis using the STRING software.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle medium (DMEM), normal and dialyzed fetal bovine serum (FBS and D-FBS) and penicillin/streptomycin were purchased from Wisent (Saint-Jean-Baptiste, CA).

SILAC DMEM medium, isotope labeling $^{13}C_6{}^{15}N_2$-Lysine, $^{13}C_6$-Arginine, $^{12}C_6{}^{14}N_2$-Lysine, $^{12}C_6$-Arginine and protein A/G agarose were purchased from Thermo (Waltham, MA). Dithiothreitol (DTT) and BCA protein assay kit were purchased from Solarbio (Beijing, China). Iodoacetamide (IAA) and the Flag M2 Affinity Gel were purchased from Sigma (St Louis, MO). Sequencing grade trypsin was purchased from Promega (Fitchburg, WI). TiO_2 beads were purchased from GL Sciences (Tokyo, Japan). The TMT labeling reagent was purchased from Thermo-Pierce Biotechnology (Rockford, IL). Anti-eIF5B and anti-Nat10 antibodies were purchased from Proteintech (Wuhan, China). Anti-actin antibody was purchased from Abmart (Shanghai, China). Anti-mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Boston, MA). The Total RNA Isolation System and Reverse Transcription kit was purchased from TIANGEN (Beijing, China).

Cell Culture and SILAC Labeling

Human embryonic kidney 293T cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2. For SILAC labeling, cells were culture in SILAC culture medium, which was made by mixing SILAC DMEM medium with 10% D-FBS, 1% penicillin/streptomycin, 146 mg/L isotope labeling $^{13}C_6{}^{15}N_2$-Lysine, and 84 mg/L

$^{13}C_6$-Arginine. 293T cells were grown for 10 passages in SILAC medium and all proteins were fully incorporated.

Sample Preparation and Ribosome isolation

Cells grown in light medium ($^{12}C_6{}^{14}N_2$-Lysine and $^{12}C_6$-Arginine, K^0R^0) were treated with 200 nM rapamycin for 2 hours, while cells grown in heavy medium ($^{13}C_6{}^{15}N_2$-Lysine and $^{13}C_6$-Arginine, K^8R^6) were untreated. Cells were washed twice with PBS and lysed using 8 M urea in PBS. The whole cell lysate was centrifuged at 14,000 × g for 30 minutes at 4°C. For ribosome isolation, cells were lysed in ice-cold extraction buffer (50 mM Tris–HCl, 100 mM KCl, 5 mM $MgCl_2$, 0.7% NP-40, 0.1 mg/ml cycloheximide, 1 mM DTT, protease inhibitor and phosphatase inhibitor). Cell lysates were centrifuged at 17 000 × g at 4°C for 20 minutes to remove nuclei, mitochondria and cell debris. The supernatant was loaded on 1 M sucrose cushion prepared in extraction buffer and centrifuged at 250 000 × g at 4°C for 4 hours (Beckman Optima MAX-XP, TLA-100.3 rotor). The ribosomal pellet was washed with extraction buffer and resuspended in 8 M urea in PBS. Protein concentrations were determined with the BCA method.

Quantitative Phosphoproteomic Analysis

Equal amounts of proteins from the whole lysate or the ribosomal fraction from untreated and rapamycin-treated cells were mixed, digested using trypsin, desalted with Sep-Pak C18 Vac cartridges (Waters, Milford, MA) and fractionated by off-line high-pH chromatography (HpH) before subsequent titanium dioxide (TiO_2) enrichment and LC-MS/MS analysis (Batth et al., 2014). The generated MS/MS spectra were searched using a Sequest HT Algorithm of Proteome Discoverer software (version 1.4, Thermo Scientific, USA). In the search criteria, SILAC $^{13}C_6{}^{15}N_2$-Lysine and $^{13}C_6$-Arginine (+8.014 Da at lysine and +6.020 Da at arginine) and phosphorylation (STY) were set as the variable modification and two missed cleavages were allowed. The PhosphoRS algorithm was used to calculate the probability of the phosphorylation sites. When the PhosphoRS probability was above 75%, the phosphorylation site was considered to be true. The experiment was carried out in two biological replicates.

Protein Quantitation by Parallel Reaction Monitoring (PRM)

PRM is a method to quantify multiple targeted peptides with the high sensitivity and specificity, in which the target precursor ion is isolated by the quadrupole mass filter and the fragment ions are detected in the Orbitrap mass analyzer. In the present experiment, equal amounts of proteins from untreated and rapamycin-treated cells were separated by 1D SDS-PAGE, respectively. The gel bands of eIF5B were excised and digested as previously described (Hu et al.,

2014). Peptides from untreated cells labeled by TMT^6-127 and peptides from rapamycin-treated cells labeled by TMT^6-128 were mixed. The targeted peptides were synthesized and added as standard. 5 ng nonphosphorylated peptide (NKPGPNIESGNEDDDASFK) and 1 ng phosphorylated peptide (NKPGPNIEpSGNEDDDASFK) were labeled by TMT^6-126, added into the mixture and analyzed by Thermo Scientific Q Exactive mass spectrometer in PRM mode. The PRM analysis was performed with the resolution of 17,500, the target AGC values of 1×10^6, individual isolation window of 2 Th window and maximum fill times within 100 ms. Fragmentation was performed with a normalized collision energy of 25. The absolute amount of the targeted peptide was quantified with the help of the standard peptide. Then the ratio of the phosphopeptide vs the nonphosphopeptide was calculated. This method was also used to detect the phosphorylation ratio of the Nat10-immunoprecipated eIF5B and the eIF5B from 293T cells.

Establishment of eIF5B knockdown cells

The CRISPR/cas9 technology was used to knockdown the eIF5B gene in 293T cells as previously reported (Ran et al., 2013). The eIF5B-specific gRNA sequence was 5'-GAGCGCCATTGACAAGCAATGGG-3' and non-silencing scrambled gRNA sequence was 5'-ACGATACAAGGCTGTTAGAGAG-3'. The clone with decreased eIF5B expression was selected as the eIF5B knockdown cells.

Isolation of the binding partners of the wild type eIF5B, eIF5B-S214A and eIF5B-S214E

The human eIF5B cDNA was synthesized from the total RNA of 293T cells. The recombinant eIF5B with flag tag was cloned into the plasmid pcDNA3.1 to create the pcDNA3.1-Flag-eIF5B vector. Then the Site-Directed Mutagenesis Kit (Thermo Scientific, USA) was used to generate two mutant plasmids: pcDNA3.1-Flag-eIF5B-S214A and pcDNA3.1-Flag-eIF5B-S214E according to the manufacturer's instruction.

Transient transfections of plasmids containing the Flag-only, Flag-eIF5B, Flag-eIF5B-S214A and Flag-eIF5B-S214E were performed with Lipofectamine 2000 from Invitrogen according to the manufacturer's instruction. Cells were harvested after 48 hours and lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium pyrophosphate, protease inhibitor and phosphatase inhibitor) at 4°C for 30 minutes. The supernatants collected by centrifugation at 14,000×g at 4°C for 20 minutes were incubated with the Flag M2 Affinity Gel at 4°C for 4 hours. After incubation, the gels were washed four times with lysis buffer and bound proteins were eluted with SDS-PAGE sample buffer.

Protein separation by 1D SDS-PAGE and mass spectrometry analysis

The samples of isolated complexes were separated by 1D SDS-PAGE, digested with trypsin and analyzed using an LTQ-Orbitrap mass spectrometer as previously described (Hu et al., 2014). The generated MS/MS spectra were searched against the human.fasta database downloaded from Uniprot using the MaxQuant software. All proteins were taken for label free quantification (LFQ) analysis. Through comparing the LFQ intensity of eIF5B immunoprecipitated complex with the FLAG-only immunoprecipitated complex, the binding partners of eIF5B were identified.

Immunoprecipitation

Equal amounts of 293T cell lysates were incubated at 4 °C overnight with 10 µl anti-eIF5B antibody, 10 µl anti-Nat10 antibody or without antibody, respectively, followed by 2 hours incubation with 30 µl 50% (v/v) protein A/G agarose. Pellets were washed five times in RIPA buffer and proteins were eluted with SDS-PAGE sample buffer. Then immumoblot analysis was carried out with the eIF5B and Nat10 antibodies. The Nat10-immumoprecipiated complex was also separated by 1D SDS-PAGE and analyzed by the PRM-based MS as described above.

Real-Time Quantitative PCR (qPCR) and Western Blotting

The expression of eIF5B in cells was detected using qPCR and western blotting as previously described (Hu et al., 2014). The primers of eIF5B using in qPCR analysis were listed as follows:

sense strand 5’- TGAAGGCTTCAGTGATGTTGGA -3’ and antisense strand 5’-AACTCCTAAACTATCAGCCATTTCTTGT -3’.

Statistical Method

Statistical analysis was performed with GraphPad Prism 5.0 software by using Student’s t test. The *P* values less than 0.05 were considered statistically significant.