Supplemental Data

Activated MNK1 pathway maintains protein synthesis in rapalog-treated gliomas

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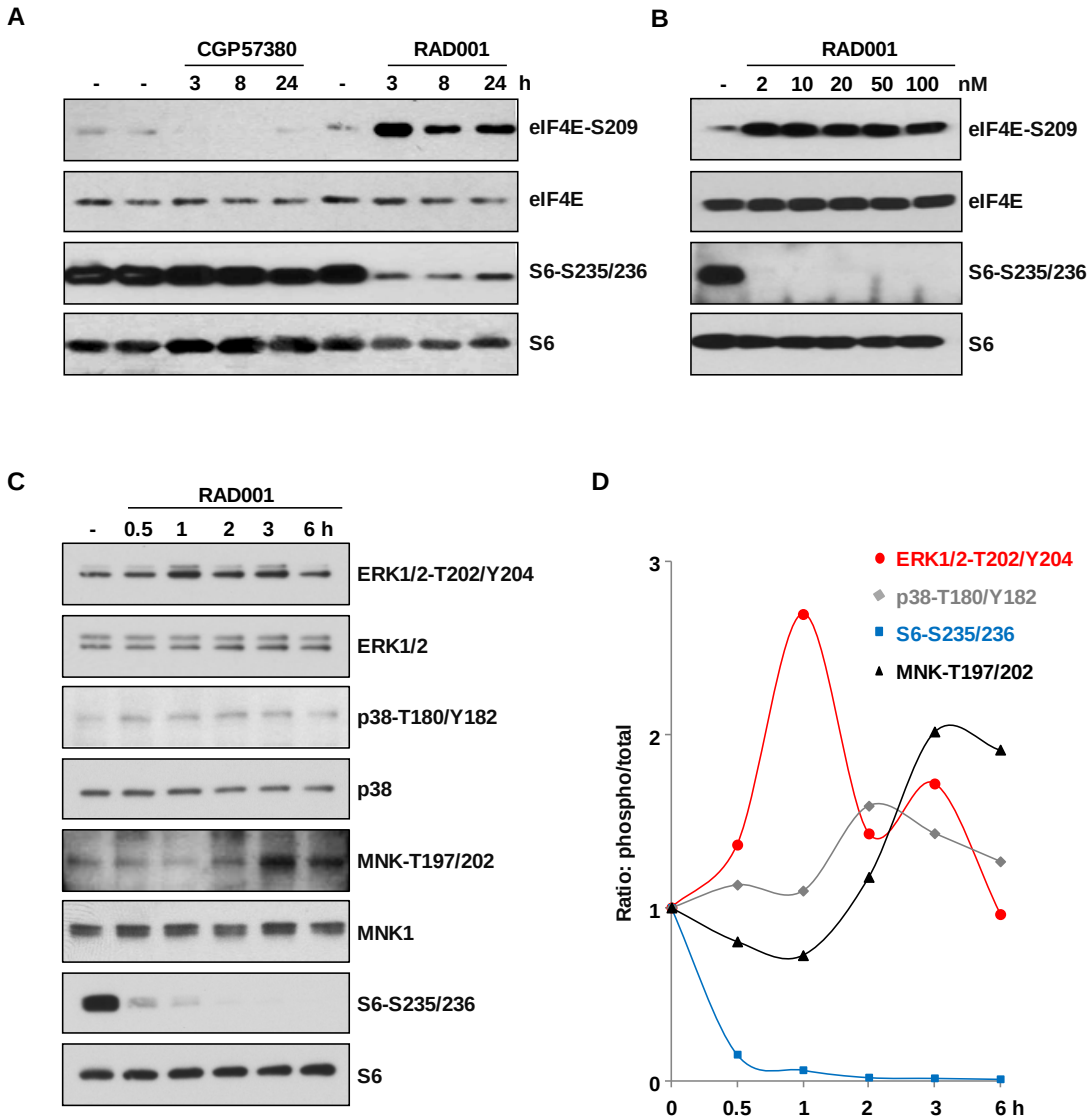
Symbol	Accession No / Protein Name	CGP57380			RAD001		CGP57380 and RAD001	
		Exp1 [H/L]	Exp2 [L/H]	Exp3* [H/L]	Exp1 [H/L]	Exp2 [L/H]	Exp1 [H/L]	Exp2 [L/H]
4EBP1	(Q13541) Eukaryotic translation initiation factor 4E-binding protein 1	2.56	2.45	1.97	4.94	7.81	9.60	12.19
4EBP2	(Q13542) Eukaryotic translation initiation factor 4E-binding protein 2	nd	1.52	nd	6.03	8.33	5.14	4.11
4ET	(Q9NRA8) Eukaryotic translation initiation factor 4E transporter	1.14	0.88	0.99	1.07	0.84	0.70	0.96
ATX2L	(Q8WWM7) Ataxin-2-like protein	0.87	1.06	0.94	0.84	0.88	0.59	0.75
CQ085	(Q53F19) Uncharacterized protein C17orf85	1.01	0.91	0.87	0.96	1.02	0.92	0.96
CV028	(Q9Y3I0) UPF0027 protein C22orf28	1.146	0.99	1.07	1.26	1.02	1.07	0.90
DDX1	(Q92499) ATP-dependent RNA helicase DDX1	1.17	1.04	1.12	1.29	0.72	1.14	1.02
DDX5	(P17844) Probable ATP-dependent RNA helicase DDX5	1.15	0.82	nd	1.20	1.03	0.99	0.92
EIF3B	(P55884) Eukaryotic translation initiation factor 3 subunit B	1.05	1.11	0.96	0.97	0.98	0.96	0.97
EIF3C	(Q99613) Eukaryotic translation initiation factor 3 subunit C	1.03	1.03	nd	0.99	1.01	0.97	0.87
EIF3D	(O15371) Eukaryotic translation initiation factor 3 subunit D	1.11	0.95	0.96	0.70	1.13	1.26	0.92
EIF3G	(O75821) Eukaryotic translation initiation factor 3 subunit G	1.1	1.04	1.04	1.17	1.15	1.15	1.06
EIF3H	(O15372) Eukaryotic translation initiation factor 3 subunit H	nd	1.15	nd	0.94	0.97	0.84	1.16
EIF3I	(Q13347) Eukaryotic translation initiation factor 3 subunit I	1.10	1.04	1.04	1.06	1.00	1.00	0.95
EWS	(Q01844) RNA-binding protein EWS	1.03	0.97	nd	0.99	0.89	1.03	1.04
FUS	(P35637) RNA-binding protein FUS	1.10	1.01	0.91	1.19	1.02	1.12	1.07
GEMI5	(Q8TEQ6) Gem-associated protein 5	1.15	1.03	0.87	1.01	0.81	0.83	0.85
GRP75	(P38646) Stress-70 protein, mitochondrial	1.17	1.11	1.01	1.01	1.27	1.27	1.30
GRP78	(P11021) 78 kDa glucose-regulated protein	1.15	1.04	0.98	0.49	0.92	0.75	0.84
HNRPU	(Q00839) Heterogeneous nuclear ribonucleoprotein U	1.06	1.01	nd	0.97	1.33	1.03	1.19
HSP7C	(P11142) Heat shock cognate 71 kDa protein	1.09	0.95	1.13	0.87	nd	0.87	nd
IF4A1	(P60842) Eukaryotic initiation factor 4A-I	0.97	1.16	nd	nd	1.05	0.82	0.90
IF4E	(P06730) Eukaryotic translation initiation factor 4E	1.02	1.02	0.98	0.99	0.63	1.01	1.09
IF4G1	(Q04637) Eukaryotic translation initiation factor 4 gamma 1	1.05	1.11	0.97	0.86	0.86	0.83	0.88
IF4G3	(O43432) Eukaryotic translation initiation factor 4 gamma 3	1.10	0.98	1.08	1.13	0.88	1.05	1.16
IMA2	(P52292) Importin subunit alpha-2	1.08	0.81	nd	0.84	0.77	0.68	0.53
IMA4	(O00629) Importin subunit alpha-4	1.04	1.05	0.85	0.87	1.06	0.87	1.06
LSM12	(Q3MHD2) Protein LSM12 homolog	0.91	nd	nd	1.00	0.89	0.71	0.69
NCBP1	(Q09161) Nuclear cap-binding protein subunit 1	1.07	1.01	1.11	1.18	1.30	1.02	1.13
NCBP2	(P52298) Nuclear cap-binding protein subunit 2	1.23	0.99	1.12	1.29	1.18	1.26	1.01
NUFP2	(Q7Z417) Nuclear fragile X mental retardation-interacting protein 2	0.96	0.82	nd	0.68	nd	nd	0.65
PABP1	(P11940) Polyadenylate-binding protein 1	0.86	nd	nd	0.81	nd	0.53	nd
ROA2	(P22626) Heterogeneous nuclear ribonucleoproteins A2/B1	0.98	0.82	1.01	1.04	nd	1.06	1.04
SRRT	(Q9BXP5) Serrate RNA effector molecule homolog	1.11	nd	nd	1.02	nd	nd	1.28

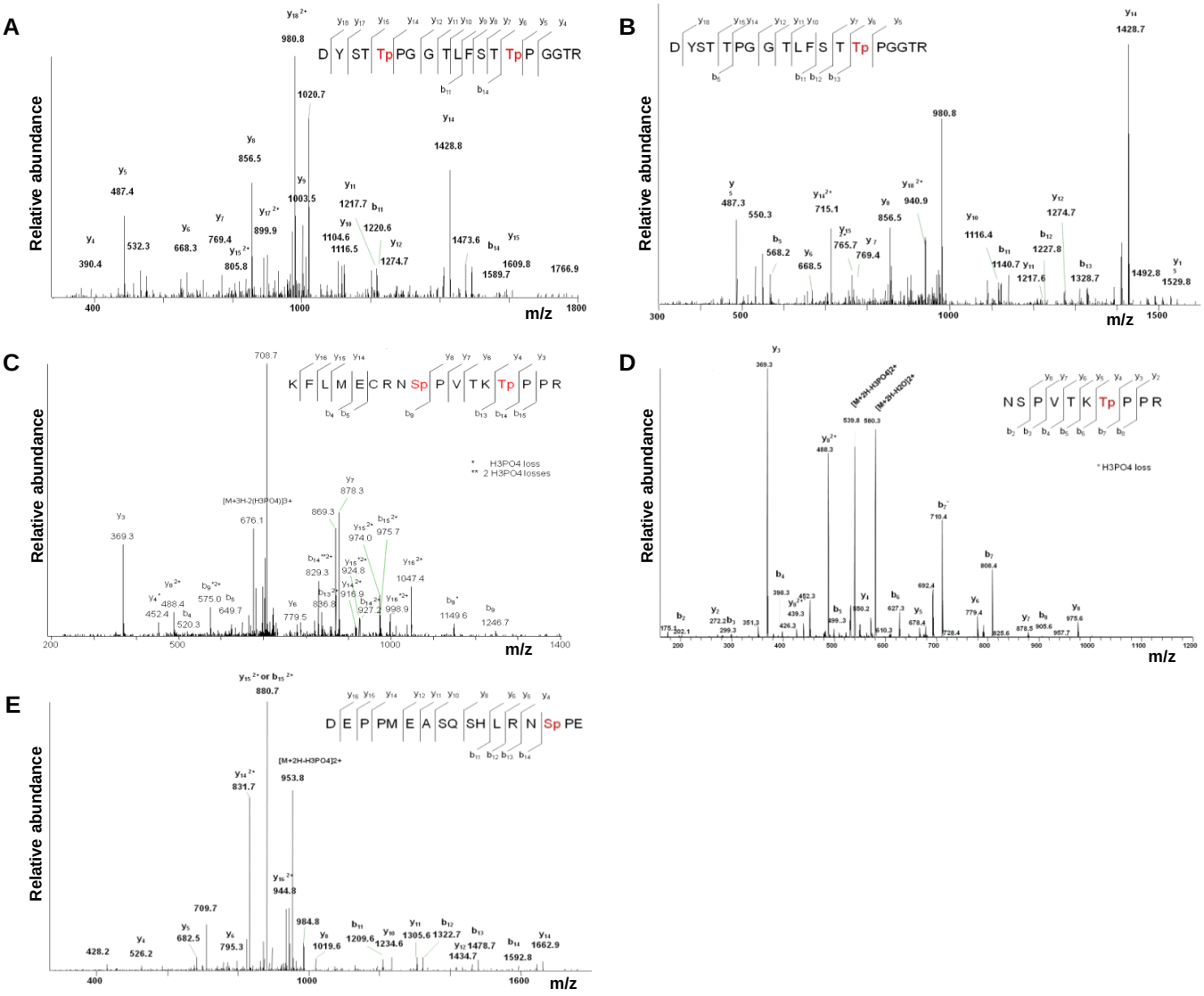
Supplemental Table 1

Translation initiation complexes isolated from CGP57380 or/and RAD001-treated U373 cells. Numbers represent fold changes of SILAC-labeled m^7GTP-precipitated proteins, analyzed after Trypsin or AspN* digestion by quantitative mass spectrometry. Precipitated proteins form inhibitor-treated cells were compared with proteins isolated from control cells incubated with DMSO. In experiments 1, 3 or 2 unlabelled light (L) or isotopically labeled heavy (H) cells were used, respectively, as a reference (control).



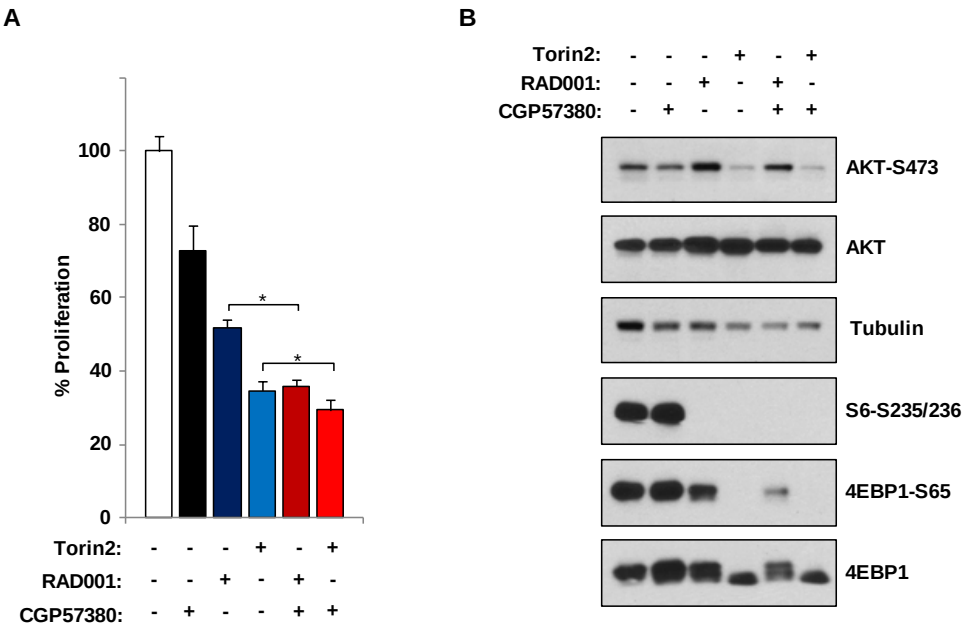
Supplemental Figure 1

Increased phosphorylation of eIF4E at Ser209 in RAD001-treated cells. LN229 (**A, B**) and U373 (**C**) cells were treated with 10 μM CGP5730 and 10 nM RAD001 (**A, C**) or with RAD001 at various concentrations (**B**) for the indicated time points or for 3 h (**B**). Whole protein lysates were prepared and phosphorylation of eIF4E, ERK1/2, p38, MNK and S6 ribosomal protein was monitored by immunoblotting using phospho-specific antibodies. Blots were stripped and re-probed with eIF4E , ERK1/2, p38, MNK1 and S6 as control. (**D**) Changes in phosphorylation levels are shown as the ratio of phospho/total signals for the indicated time points; ratios in controls were normalized to 1.0.



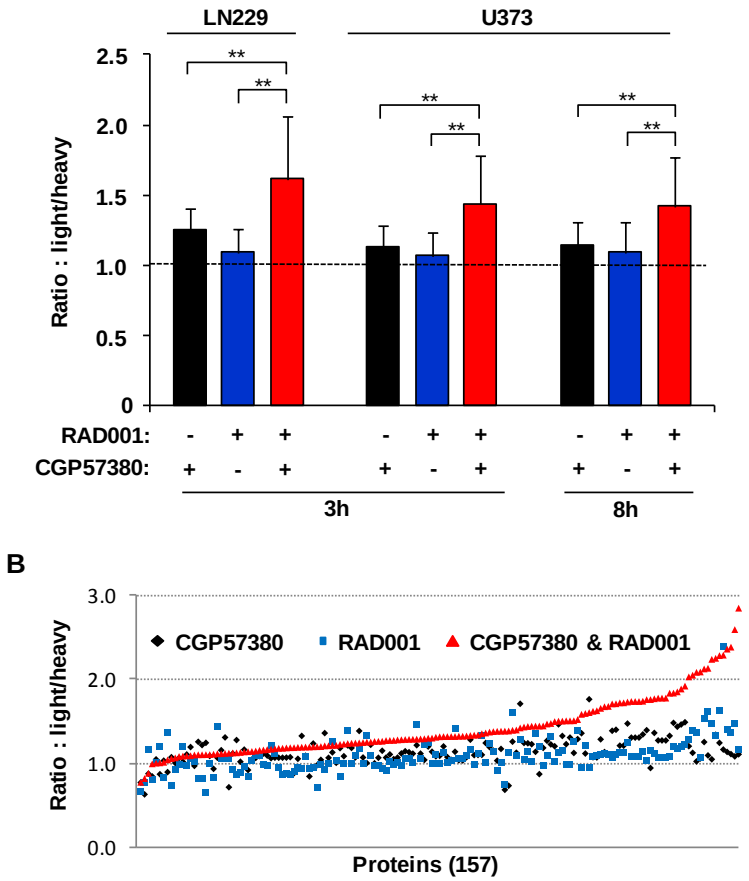
Supplemental Figure 2

MS2 spectra for identified and quantified 4EBP1 phosphopeptides. LN229 cells were treated with 10 μM CGP5730 and/or 10 nM RAD001 for 2 h and 4EBP1 was immunoprecipitated using an 4EBP1-specific antibody and analyzed by LC-MSMS. The fragments showing an H_3PO_4 loss are marked with an asterisk and detected y- and b-ions are indicated in the sequences covering 4EBP1 phosphorylation on Thr 37/46 (**A**), Thr46 (**B**), Ser65/Thr70 (**C**) Thr70 (**D**) and Ser101 (**E**).



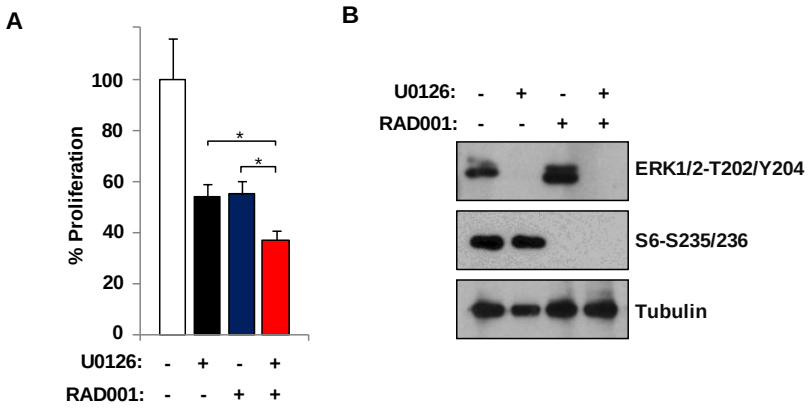
Supplemental Figure 3

Analysis of proliferation in CGP57380/RAD001- or Torin2-treated glioma cells. (**A**) An MTT-based assay for U373 cell proliferation 3 days after treatment with 10 μM CGP57380 and/or with 10 nM RAD001 or Torin2. Results were assayed in triplicate and are shown as % proliferation compared with control cells. Data represent mean ± SD. **P* <0.05. (**B**) Inhibition of AKT, S6 and 4EBP1 phosphorylation was monitored by WB analysis. Tubulin was used as a loading control.



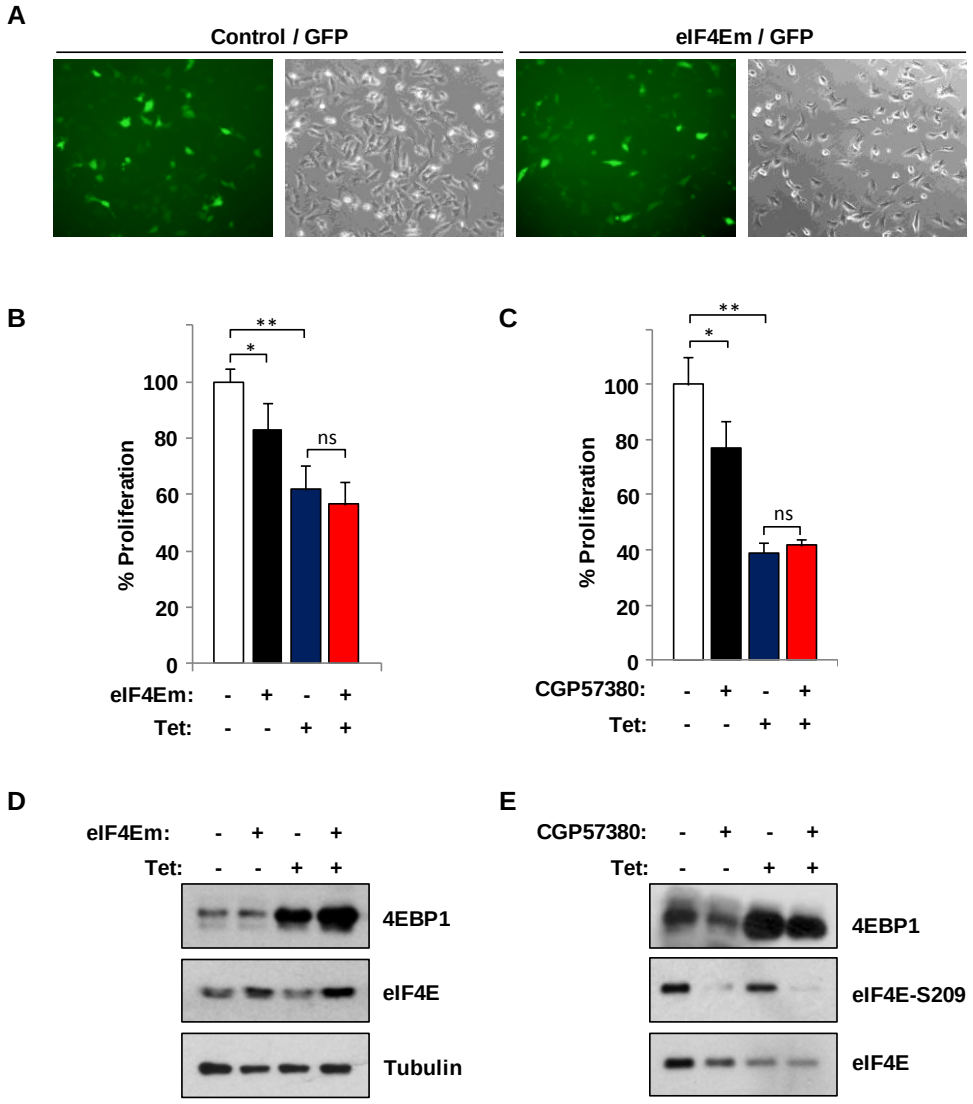
Supplemental Figure 4

Concomitant targeting mTORC1 and MNK1 increases inhibition of protein synthesis. Bulk protein synthesis in LN229 and U373 cells incubated with 10 μM CGP5730 and/or with 10 nM RAD001, as measured by heavy lysine and arginine incorporation during a 3- or 8-h and MS-based analysis. (**A**) Bars represent averages ± SD of light to heavy ratios for all identified proteins. Ratios in control DMSO-treated cells were set to 1. ***P* <0.01. (**B**) Points represent ratios of light to heavy labeled proteins for each identified protein obtained from U373-treated cells and a SILAC labeling period of 8-h as described above.



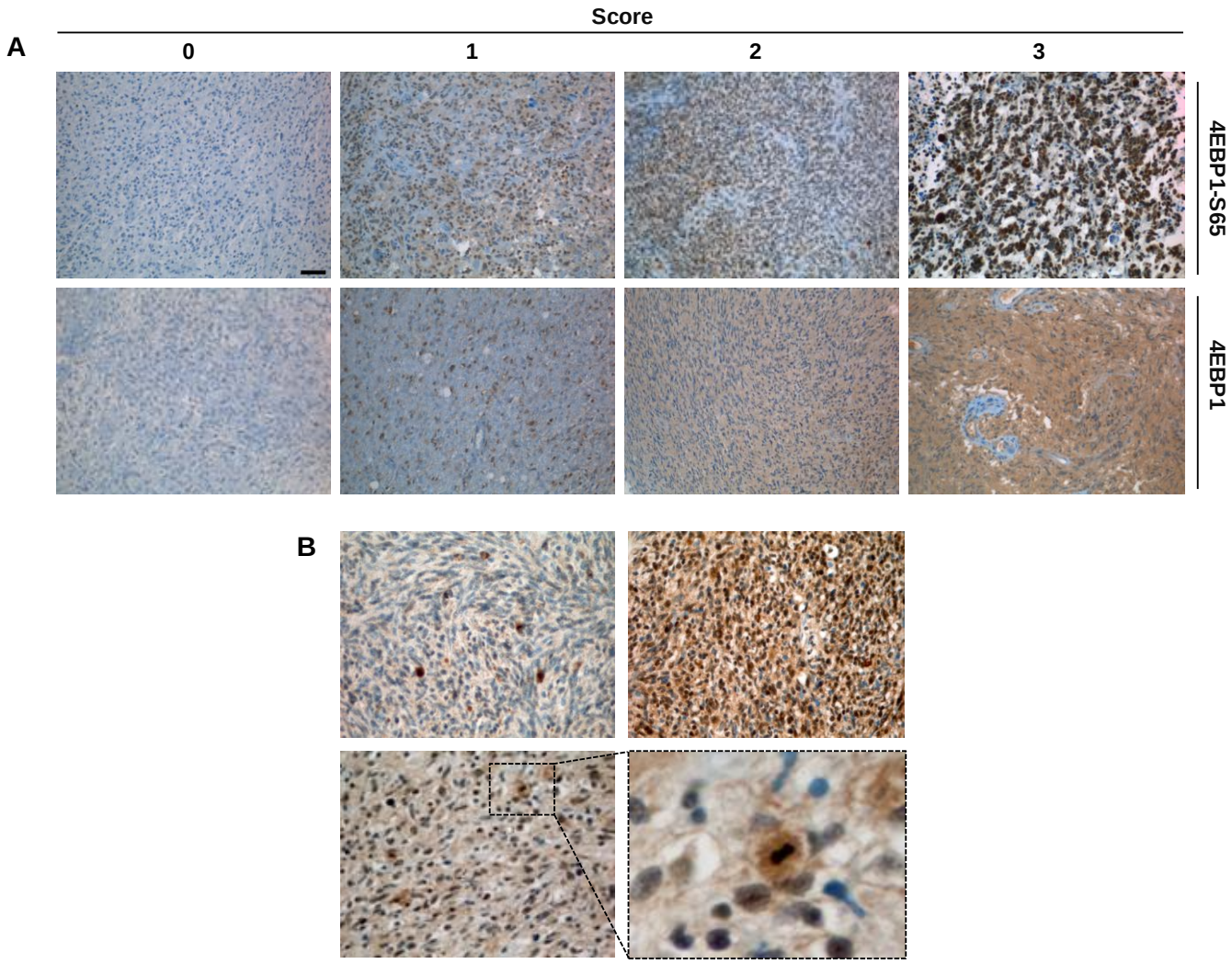
Supplemental Figure 5

Concomitant treatment with U0126 and RAD001 inhibits glioma cell proliferation. (**A**) An MTT-based assay for U373 cell proliferation 3 days after treatment with 10 μM U0126 and/or incubation with 10 nM RAD001. Results were assayed in triplicate and are shown as % proliferation compared with control cells. Data represent mean ± SD. **P* <0.05. (**B**) Phosphorylation of ERK and S6 was monitored by WB analysis. Tubulin was used as a loading control.



Supplemental Figure 6

Overexpression of 4EBP1 mutant inhibits glioma cell proliferation. Stable transfected U373 or LN229 cells, that express 4EBP1 T37A/T46A/S65A/T70A mutant in tetracyclin-inducible manner, were transfected with a plasmid for eIF4E S209A mutant or were treated with 10 μM CGP5730, respectively. (**A**) Construct for GFP expression was used as a transfection efficiency control in U373 cells. (**B, C**) An MTT-based proliferation assay 3 days after transfection or treatment as indicated. Results were assayed in triplicate and are shown as % proliferation compared with control cells. Data represent mean ± SD. (**D**, **E**) Expression levels of 4EBP1, eIF4E as well as level of eIF4E phosphorylation was monitored by WB analysis. Tubulin was used as a loading control. Tet; tetracyclin, ns; non-significant . *P<0.05, **P<0.01.



Supplemental Figure 7

Phosphorylation of 4EBP1 and MNK1 in glioblastoma samples. (**A**) Set of representative images of primary GBM samples used for immunohistological scoring analysis (0-3) of phosphorylated 4EBP1 at Ser65 and total 4EBP1 expression. (**B**) Immunostaining for phosphorylated MNK1 at Thr197/202 in 3 GBM patients. IHC for phosphorylated 4EBP1, MNK1 and total 4EBP1 was accomplished using monoclonal antibodies (*brown*) and counterstained with hematoxylin (*blue*). Scale bar 100 µm.