Supplemental Information

4E-BP1 Is A Key Effector of the Oncogenic Activation of the AKT and ERK Signaling Pathways That Integrates Their Function in Tumors

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Inventory of Supplemental Information

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Supplemental Experimental Procedures

References

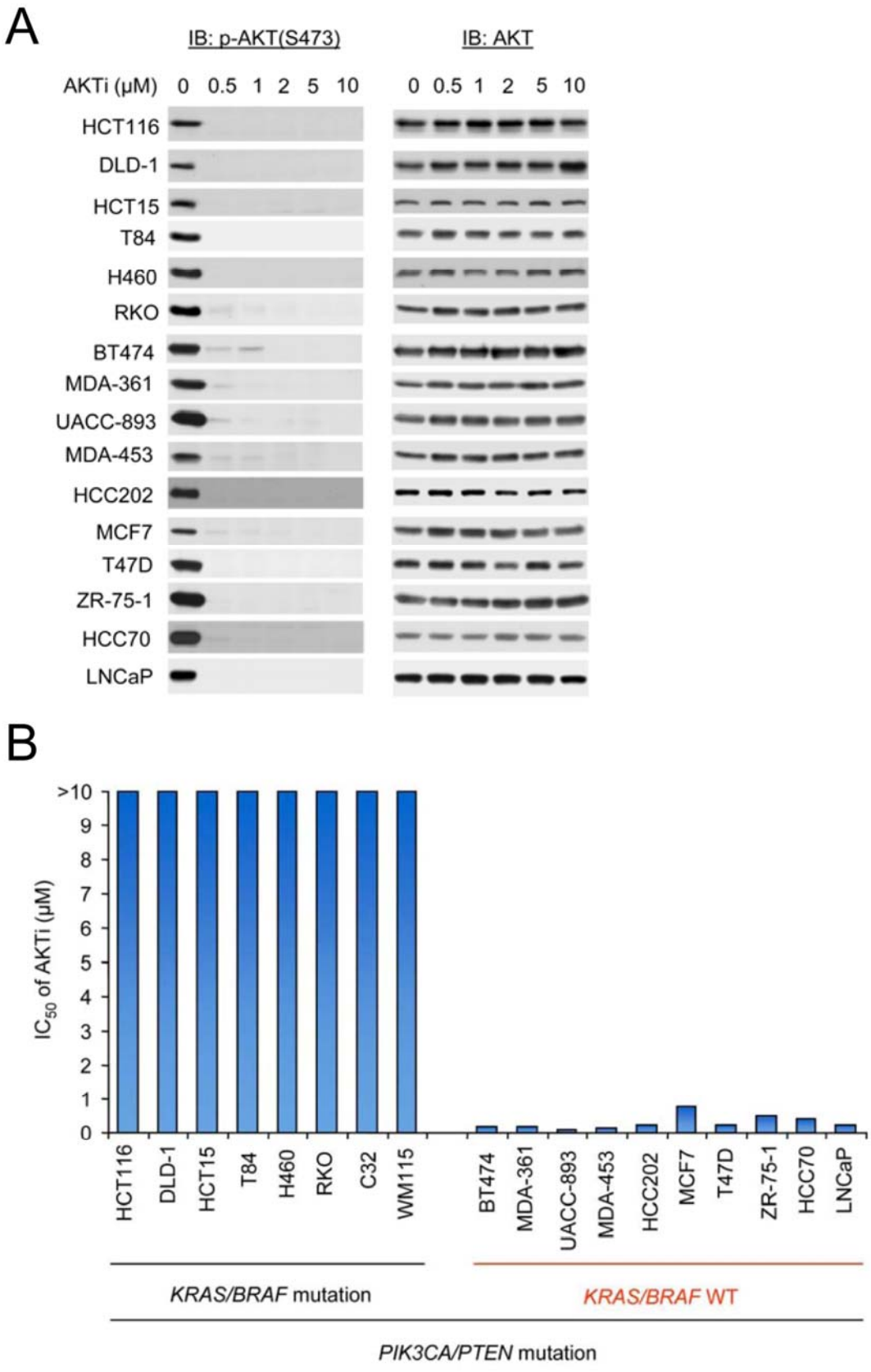

A
IB: p-AKT(S473)
IB: AKT
AKTi (μM) 0 0.5 1 2 5 10
0 0.5 1 2 5 10
HCT116
DLD-1
HCT15
T84
H460
RKO
BT474
MDA-361
UACC-893
MDA-453
HCC202
MCF7
T47D
ZR-75-1
HCC70
LNCaP
B
IC50 of AKTi (μM)
>10
9
8
7
6
5
4
3
2
1
0
HCT116
DLD-1
HCT15
T84
H460
RKO
C32
WM115
BT474
MDA-361
UACC-893
MDA-453
HCC202
MCF7
T47D
ZR-75-1
HCC70
LNCaP
KRAS/BRAF mutation
KRAS/BRAF WT
PIK3CA/PTEN mutation

Figure S1 (related to Figure 1). *PIK3CA* or *PTEN* mutant tumor cells with coexistent *KRAS* or *BRAF* mutations are insensitive to AKT inhibition

(**A**) AKTi inhibits AKT phosphorylation and signaling in tumor cells. Cells were treated with various concentrations of AKTi for 24 h. Cell lysates were subjected to immunoblot analysis of Ser473 phosphorylated AKT and total AKT.

(**B**) Cell proliferation was assessed by a BrdU incorporation assay after 3 days of treatment with AKTi (0-10 μM). The results are expressed as half-maximal growth inhibitory concentration (IC_{50}) of AKTi.

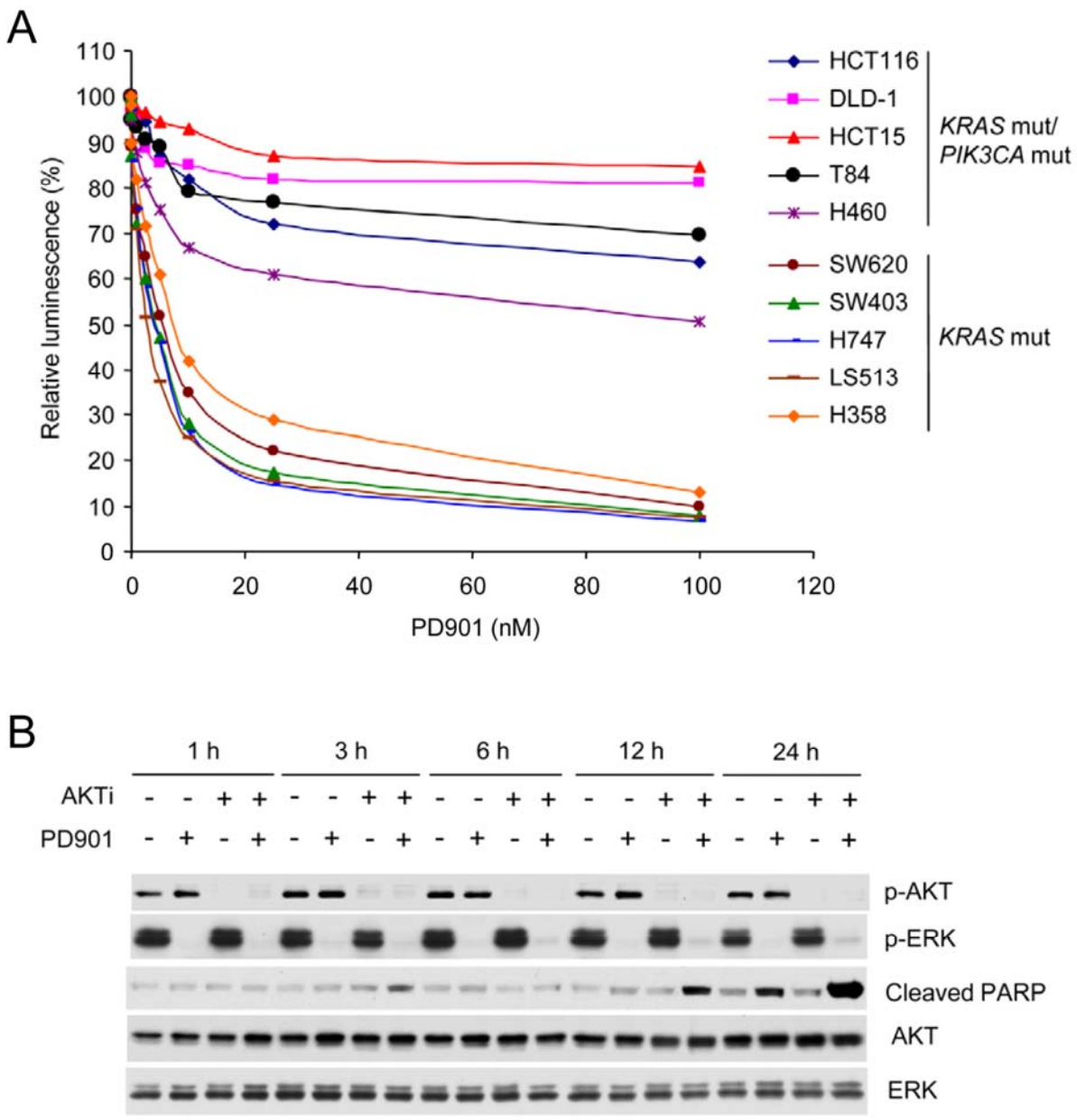
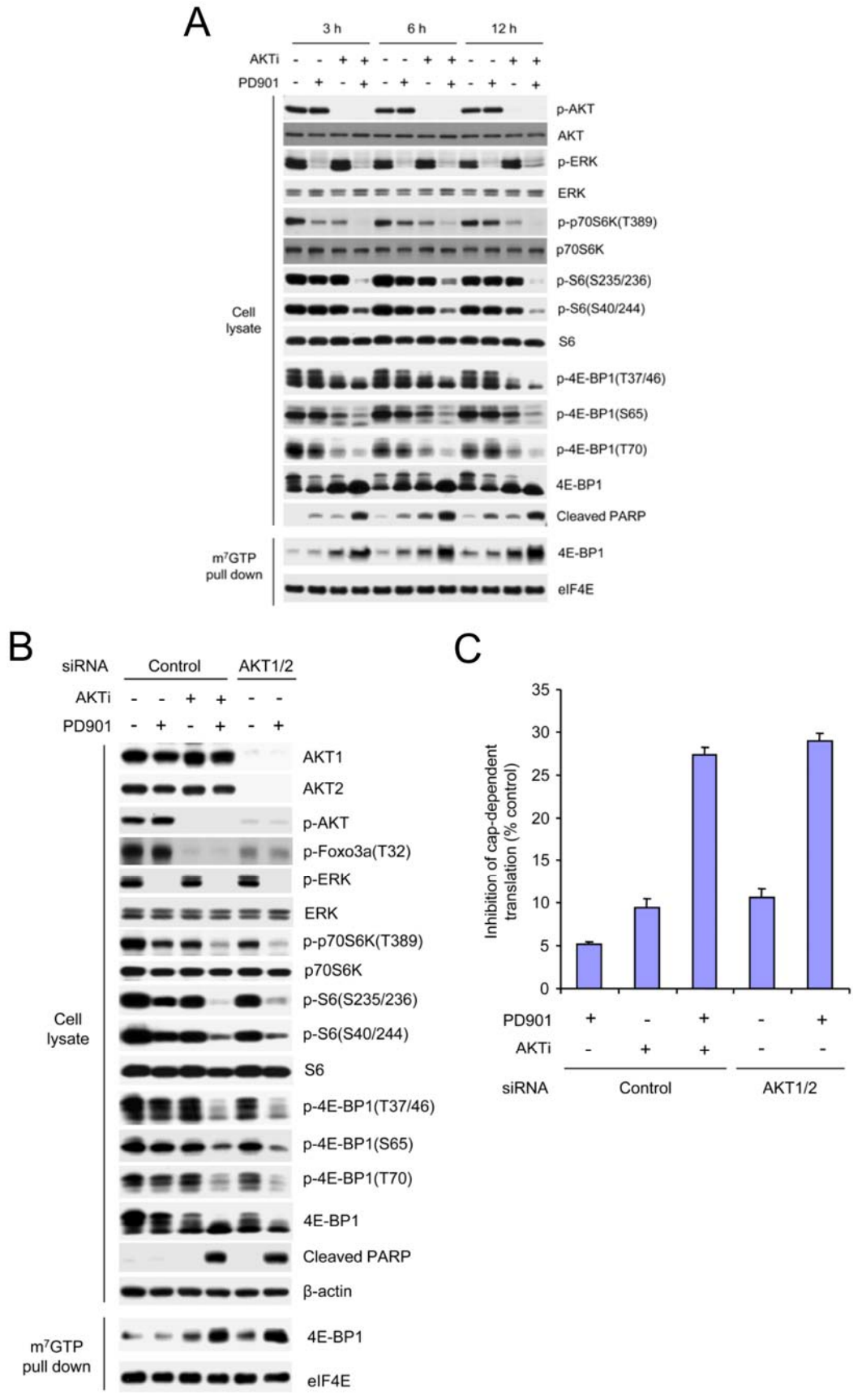


Figure S2 (related to Figure 2). *KRAS* mutant tumor cells with coexistent *PIK3CA* mutation are resistant to MEK inhibition

(**A**) Cell growth was assessed by using the CellTiter-Glo luminescent cell viability assay after 3 days of treatment with PD0325901 (0-100 nM). The results are expressed as the cell numbers relative to those with the DMSO-treated controls.

(**B**) Combined inhibition of AKT and ERK synergistically induces expression of cleaved PARP. HCT116 cells were treated with treated with 50 nM PD901 and 1 μM AKTi, alone or in combination, and the cell lysates were immunoblotted with the indicated antibodies.


A
3 h
6 h
12 h
AKTi
PD901
p-AKT
AKT
p-ERK
ERK
p-p70S6K(T389)
p70S6K
p-S6(S235/236)
p-S6(S40/244)
S6
p-4E-BP1(T37/46)
p-4E-BP1(S65)
p-4E-BP1(T70)
4E-BP1
Cleaved PARP
Cell lysate
m7GTP pull down
4E-BP1
eIF4E
B
siRNA
Control
AKT1/2
AKTi
PD901
AKT1
AKT2
p-AKT
p-Foxo3a(T32)
p-ERK
ERK
p-p70S6K(T389)
p70S6K
p-S6(S235/236)
p-S6(S40/244)
S6
p-4E-BP1(T37/46)
p-4E-BP1(S65)
p-4E-BP1(T70)
4E-BP1
Cleaved PARP
β-actin
Cell lysate
m7GTP pull down
4E-BP1
eIF4E
C
Inhibition of cap-dependent translation (% control)
35
30
25
20
15
10
5
0
PD901
AKTi
siRNA
Control
AKT1/2

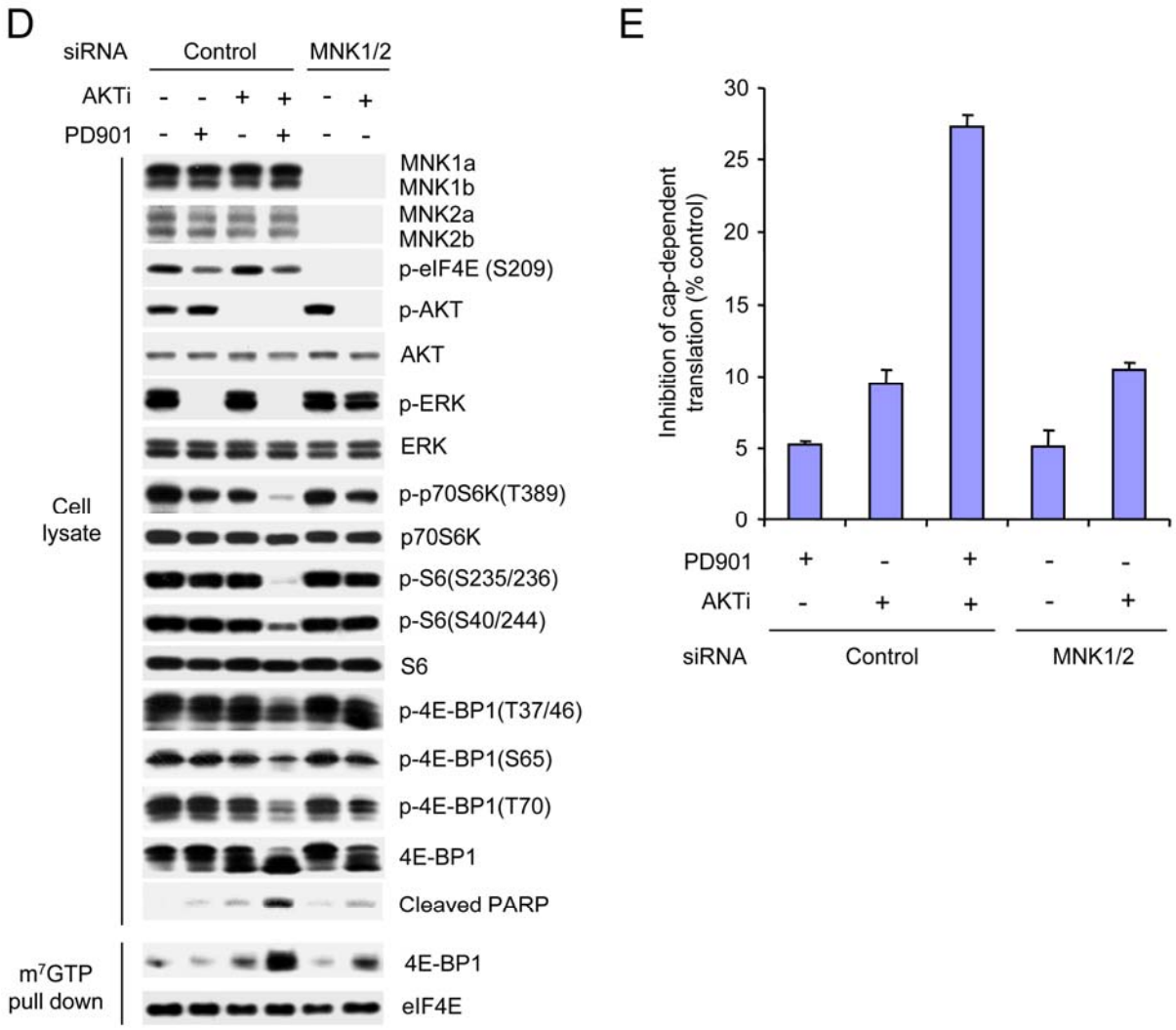

D
siRNA
Control
MNK1/2
AKTi
PD901
MNK1a
MNK1b
MNK2a
MNK2b
p-eIF4E (S209)
p-AKT
AKT
p-ERK
ERK
p-p70S6K(T389)
p70S6K
p-S6(S235/236)
p-S6(S40/244)
S6
p-4E-BP1(T37/46)
p-4E-BP1(S65)
p-4E-BP1(T70)
4E-BP1
Cleaved PARP
Cell lysate
m7GTP pull down
4E-BP1
eIF4E
E
Inhibition of cap-dependent translation (% control)
30
25
20
15
10
5
0
PD901
AKTi
siRNA
Control
MNK1/2

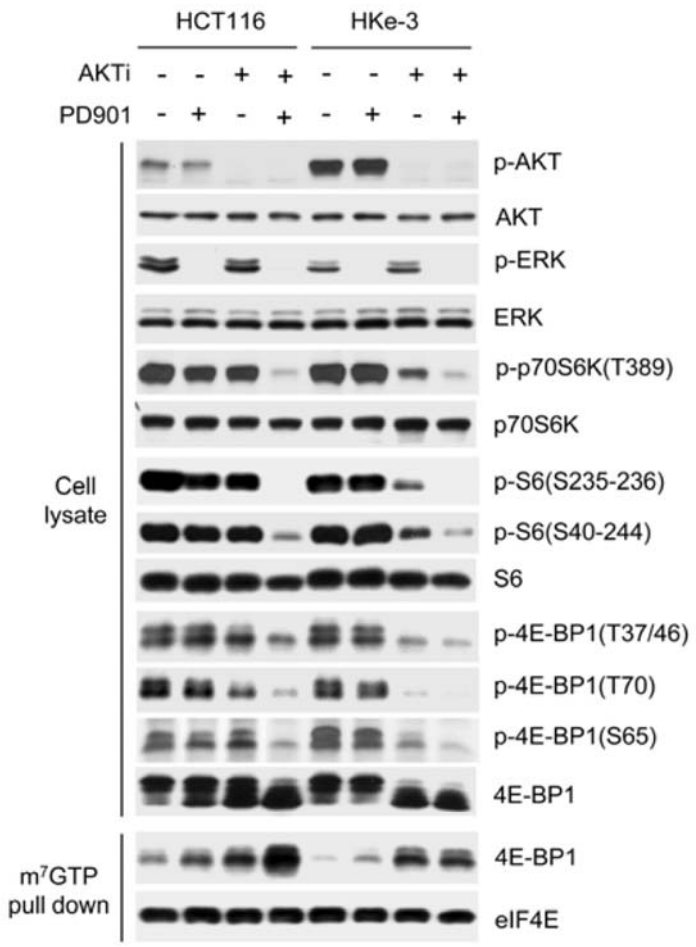
Figure S3 (related to Figure 3).

(**A**) **Combined inhibition of AKT and MEK is required to maximally dephosphorylate 4E-BP1 and induce its binding to eIF4E.** T84 cells were treated with 100 nM PD901 and 1 μM AKTi, alone or in combination. Cell lysates were immunoblotted with the indicated antibodies or precipitated with m^7GTP sepharose beads followed by immunoblotting of 4E-BP1 and eIF4E.

(**B** and **C**) **Knockdown of AKT1 and AKT2 expression and pharmacologic inhibition of AKT activity have similar effects on cap-dependent translation.** (B) siRNAs against the indicated genes or control siRNAs were transfected into HCT116 cells and incubated for 54 h. The cells were then treated with 50 nM PD0325901 and 1 μM AKTi, alone or in combination for 6 h. Cell lysates were immunoblotted with the indicated antibodies or precipitated with m^7GTP sepharose beads followed by immunoblotting of 4E-BP1 and eIF4E. (C) siRNAs against the indicated genes or control siRNAs were transfected into HCT116 cells and incubated for 30 h. The cells were then transfected with a bicistronic luciferase reporter plasmid for 24 h, and then treated with 50 nM PD0325901 and 1 μM AKTi, alone or in combination for an additional 12 h. The inhibition of cap-dependent translation was determined as in Figure 3C. Values represent means ± SEM (n=3).

(**D** and **E**) **MNK1/2 do not mediate the effects of MEK/ERK signaling on Cap-Dependent Translation.** (D) siRNAs against the indicated genes or control siRNAs were transfected into HCT116 cells and incubated for 54 h. The cells were then treated with 50 nM PD0325901 and 1 μM AKTi, alone or in combination for 6 h. Cell lysates were immunoblotted with the indicated antibodies or precipitated with m^7GTP sepharose beads followed by immunoblotting of 4E-BP1 and eIF4E. (E) siRNAs against the indicated genes or control siRNAs were transfected into HCT116 cells and incubated for 30 h. The cells were then transfected with a bicistronic luciferase reporter plasmid for 24 h, and then treated with 50 nM PD0325901 and 1 μM AKTi, alone or in combination for an additional 12 h. The inhibition of cap-dependent translation was determined as in Figure 3C. Values represent means ± SEM (n=3).

A


HCT116
HKe-3
AKTi - - + + - - + +
PD901 - + - + - + - +
Cell lysate
p-AKT
AKT
p-ERK
ERK
p-p70S6K(T389)
p70S6K
p-S6(S235-236)
p-S6(S40-244)
S6
p-4E-BP1(T37/46)
p-4E-BP1(T70)
p-4E-BP1(S65)
4E-BP1
m7GTP pull down
4E-BP1
eIF4E

B

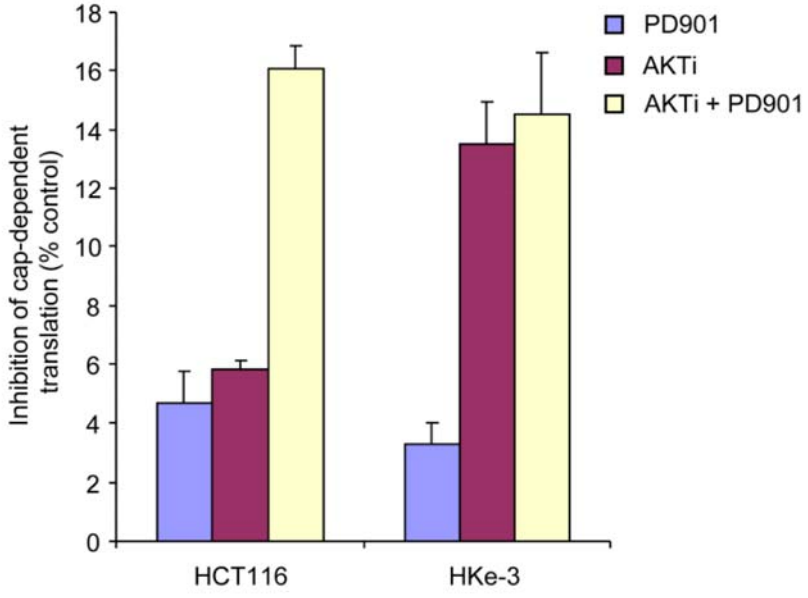

Inhibition of cap-dependent translation (% control)
0
2
4
6
8
10
12
14
16
18
HCT116
HKe-3
PD901
AKTi
AKTi + PD901

Figure S4 (related to Figure 4). Deletion of mutant *KRAS* restores AKT dependence on cap-dependent translation

(**A**) Cells were treated with 50 nM PD901 and 1 μM AKTi, alone or in combination for 6 h. Cell lysates were immunoblotted with the indicated antibodies or precipitated with m^7GTP sepharose beads followed by immunoblotting of 4E-BP1 and eIF4E.

(**B**) Cells were transfected with a bicistronic luciferase reporter plasmid and then treated with 50 nM PD901 and 1 μM AKTi, alone or in combination for 12 h. The inhibition of cap-dependent translation was determined as in Figure 3C, and the results represent mean ± SE of triplicate samples.

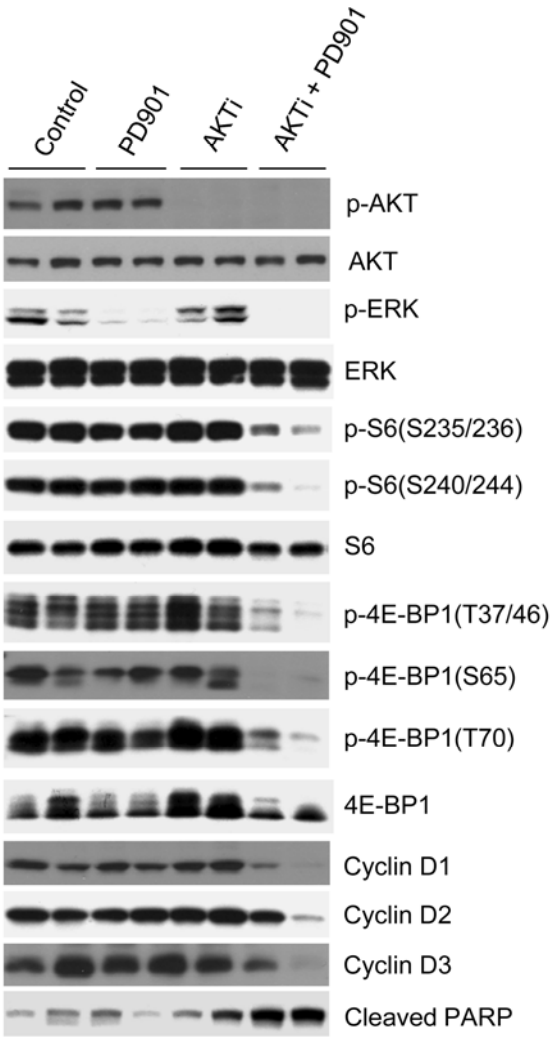


Figure S5 (related to Figure 6). 4E-BP1 phosphorylation is repressed after combined inhibition of AKT and MEK in vivo.

Representative tumors from mice bearing DLD-1 xenografts in Figure 6D were lysed 6 h after the final treatment with PD0325901 (5 mg/kg), AKTi (100 mg/kg), combination of both drugs, or vehicle control . Tumor lysates were immunoblotted with the indicated antibodies.

Table S1 (related to Figure 1A and Figure S2A). Human tumor cell lines with oncogene mutations in PI3K/AKT and RAS/RAF/MEK/ERK pathways

Cell Line *	Cancer type	PI3K pathway defects	RAS pathway defects
HCT116 †	Colon	*PIK3CA* H1047R	*KRAS* G13D
DLD-1 †	Colon	*PIK3CA* E545K	*KRAS* G13D
HCT15	Colon	*PIK3CA* E545K	*KRAS* G13D
T84	Colon	*PIK3CA* E542K	*KRAS* G13D
RKO	Colon	*PIK3CA* H1047R	*BRAF* V600E
SW620	Colon		*KRAS* G12V
SW403	Colon		*KRAS* G12V
H747	Colon		*KRAS* G13D
LS513	Colon		*KRAS* G12D
C32	Melanoma	*PTEN* null	*BRAF* V600E
WM115	Melanoma	*PTEN* null	*BRAF* V600E
H460	Lung	*PIK3CA* E545K	*KRAS* Q61K
H358	Lung		*KRAS* G12C
BT474 ‡	Breast	*PIK3CA* K111N	
MDA-361 ‡	Breast	*PIK3CA* E545K	
UACC-893 ‡	Breast	*PIK3CA* H1047R	
MDA-453 ‡	Breast	*PIK3CA* H1047R	
HCC202 ‡	Breast	*PIK3CA* E545K	
MCF7 ‡	Breast	*PIK3CA* E545K	
T47D ‡	Breast	*PIK3CA* H1047R	
ZR-75-1 ‡	Breast	*PTEN* null	
HCC70 ‡	Breast	*PTEN* null	
LNCaP	Prostate	*PTEN* null	

* Mutations in *PIK3CA*, *PTEN* , *KRAS* and *BRAF* are from cosmic database, http://www.sanger.ac.uk/genetics/CGP/cosmic/.

† Mutations in *PIK3CA* (Samuels et al., 2005) and *KRAS* (Shirasawa et al., 1993) have been reported in these cell lines.

‡ Mutations in *PIK3CA* and *PTEN* have been reported in these cell lines (Hollestelle et al., 2007; Saal et al., 2005)

Supplemental Experimental Procedures

Immunoblot Analysis

Cells were lysed in NP-40 lysis buffer as described (She et al., 2008) and incubated for 30 min at 4°C. Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4°C and the protein concentration of the supernatant was determined by BCA assay (Pierce). Equal amounts of total protein were resolved by SDS-PAGE, transferred to membranes, immunoblotted with specific primary and secondary antibodies and detected using chemiluminescence (GE Healthcare). Antibodies for p-AKT(S473), p-ERK, p-FOXO1(T24)/FOXO3(T32), p-p70S6K(T389), p-p70S6K(T421/S424), p-S6(S235/236), p-S6(S240/244), p-4E-BP1(T37/46), p-4E-BP1(S65), p-4E-BP1(T70), p-eIF4E(S209), AKT1, AKT2, pan-AKT, ERK, MNK1, 4E-BP1, 4E-BP2, eIF4E and cleaved PARP were from Cell Signaling Technology. Cyclin D1, KRAS, MNK2 and HA antibodies were from Santa Cruz Biotechnology and β-actin antibody was from Sigma.

References

Hollestelle, A., Elstrodt, F., Nagel, J. H., Kallemeijn, W. W., and Schutte, M. (2007). Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. Mol. Cancer Res. *5*, 195-201.

Saal, L. H., Holm, K., Maurer, M., Memeo, L., Su, T., Wang, X., Yu, J. S., Malmstrom, P. O., Mansukhani, M., Enoksson, J., *et al.* (2005). PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res. *65*, 2554-2559.