Supplemental Materials

Research Article

EGFR Signaling Through an Akt-SREBP-1-Dependent, Rapamycin-Resistant Pathway Sensitizes Glioblastomas to Anti-Lipogenic Therapy

Deliang Guo, Robert M. Prins, Julie Dang, Daisuke Kuga, Akio Iwanami, Horacio Soto, Kelly Y. Lin, Tiffany T. Huang, David Akhavan, M. Benjamin Hock, Ava A. Kofman, Steve J. Bensinger, William H. Yong, Harry V. Vinters, Steve Horvath, Andrew D. Watson, John G. Kuhn, H. Ian Robins, Minesh P. Mehta, Patrick Y. Wen, Lisa M. DeAngelis, Michael D. Prados, Ingo K. Mellinghoff, Timothy F. Cloughesy, Paul S. Mischel.

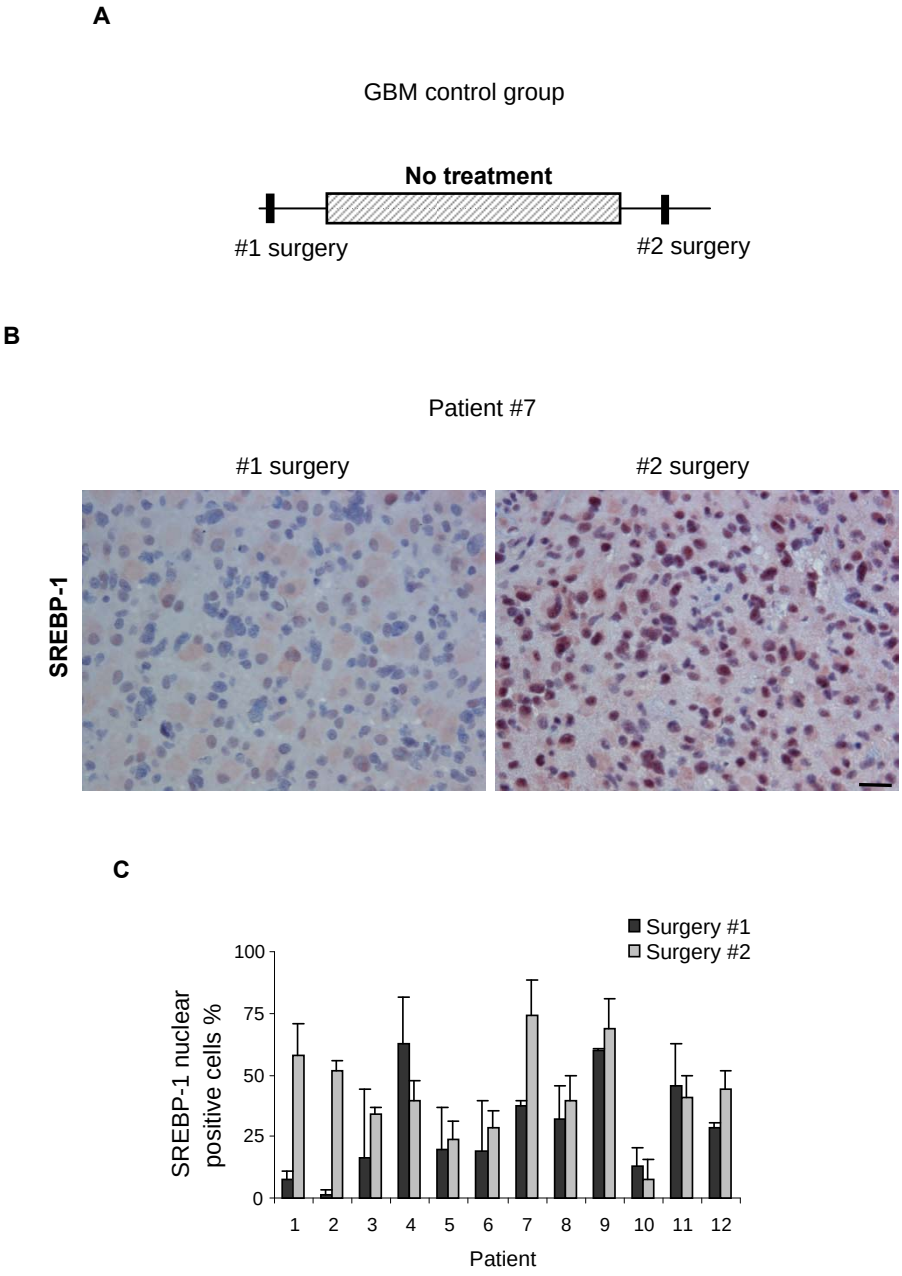


Fig. S1. Nuclear SREBP-1 staining in 12 control GBM patients. (**A**) A set of 12 control GBM patients from whom tumor tissue was available at baseline (Surgery 1) and at recurrence (Surgery 2), but who did not receive lapatinib. (**B**) Representative immunohistochemical image from a control patient. Scale bar = 20 um. (**C**) Nuclear SREBP-1 in tumor samples from Surgery #1 and Surgery #2.

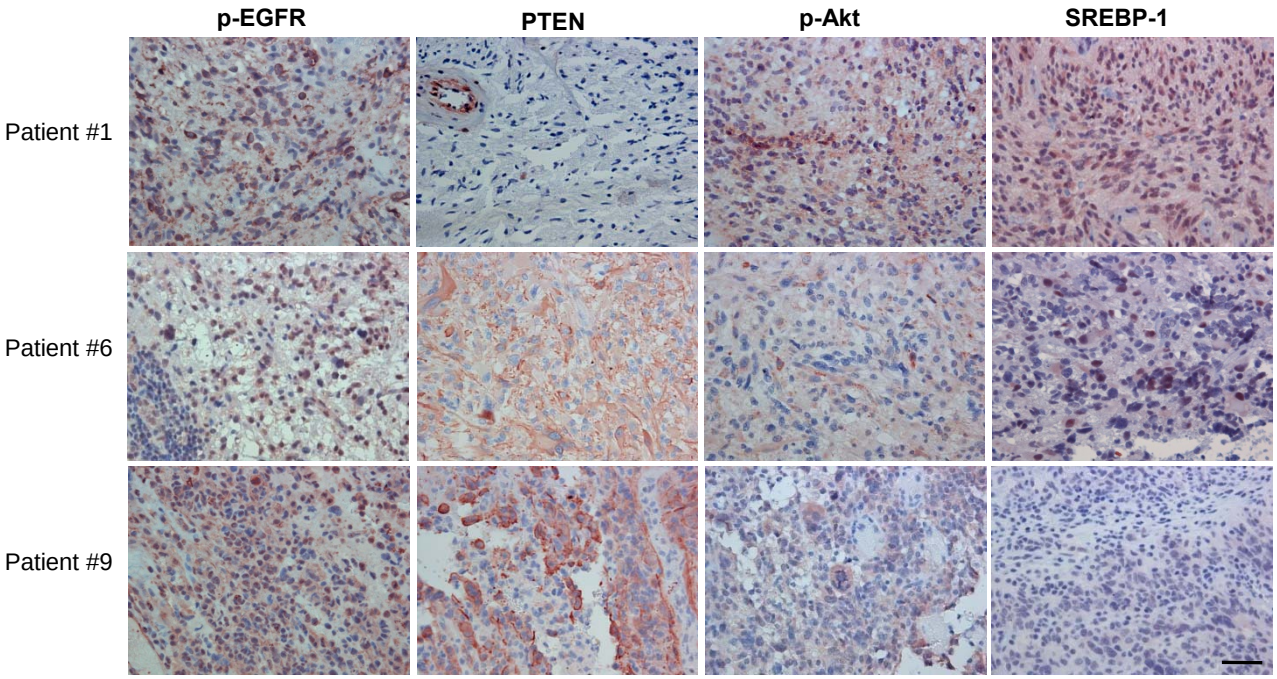


Fig. S2. Effect of PTEN on SREBP-1. Representative immunohistochemical images of p-EGFR, PTEN, p-Akt, and SREBP-1 staining in pre-treatment tissue from 3 lapatinib-treated patients. PTEN is not apparent in patient 1, whose tissue also shows increased nuclear SREBP-1 staining relative to that of patients 6 and 9, whose tumors stained positive for PTEN (reddish brown). Scale bar = 50 um.

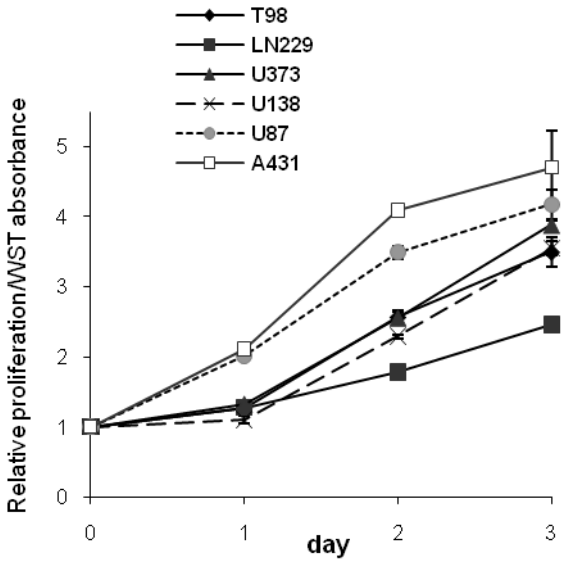


Fig. S3. Proliferation rates of GBMs (T98, LN229, U373, U138, U87) and epidermoid carcinoma cell line (A431). Tumor cell lines were grown in 1% FBS for 3 days and relative growth was measured by WST-1 cell proliferation assay kit (Chemicon).

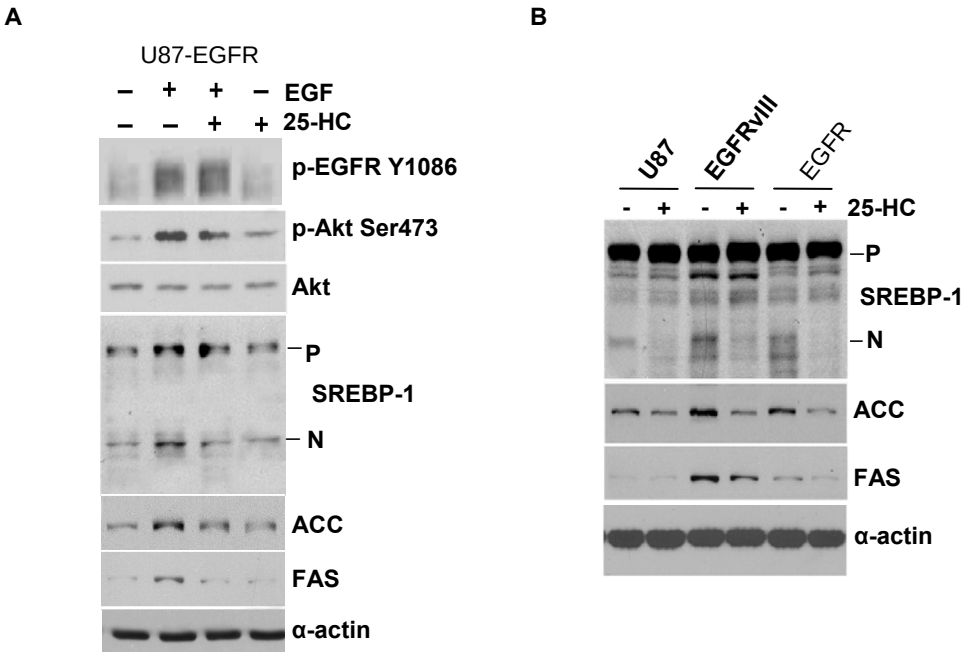


Fig. S4. Effect of 25-hydroxycholesterol (25-HC) on SREBP-1 cleavage and ACC and FAS abundance. (**A**) U87-EGFR bearing tumor cells were grown in serum-free conditions for 24 hours and exposed to 25-HC (1 ug/ml) or vehicle control for 30 mins before treatment with EGF (20 ng/ml) for 6 hours. Lysates were immunoblotted with indicated antibodies. (**B**) Cells were treated with 25-HC (1 ug/ml) or vehicle control for 24 hrs in medium containing 1% serum and lysates were immunoblotted for SREBP-1, ACC, or FAS.

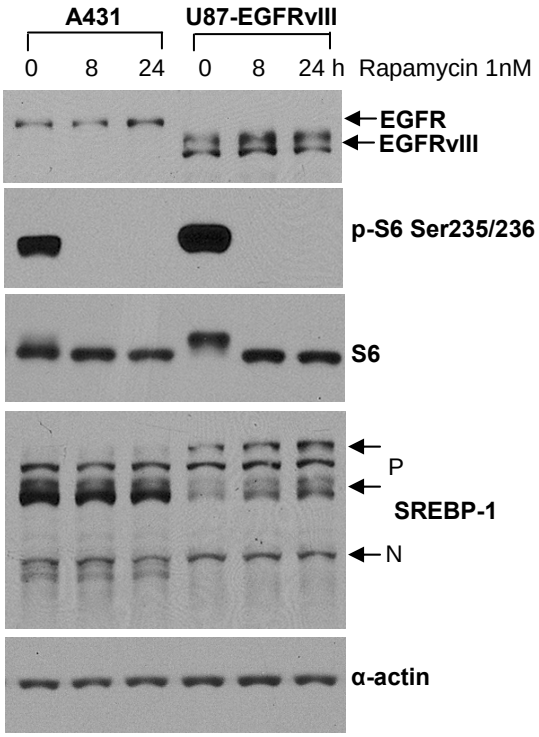


Fig. S5. Inhibition of mTORC1 signaling with rapamycin does not prevent SREBP-1 cleavage in cancer cells with highly abundant EGFR. A431 epidermoid carcinoma and U87-EGFRvIII cells were cultured in 1% FBS media for 24 hrs and treated with rapamycin (1 nM) for the indicated times. Immunoblot analysis of cellular lysates was performed using the indicated antibodies. P designates precursor band of SREBP-1, N designates NH2-terminal band of SREBP-1.

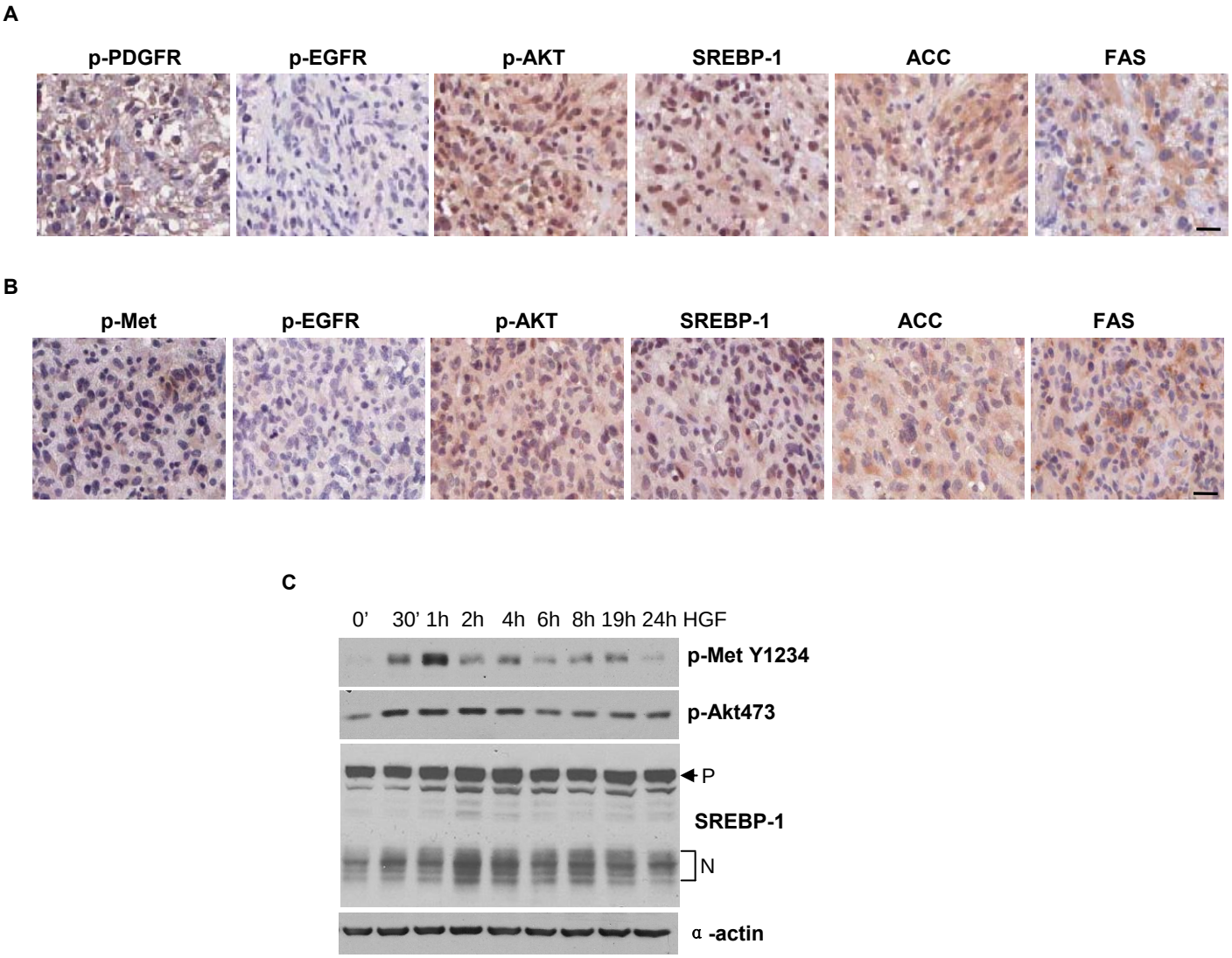
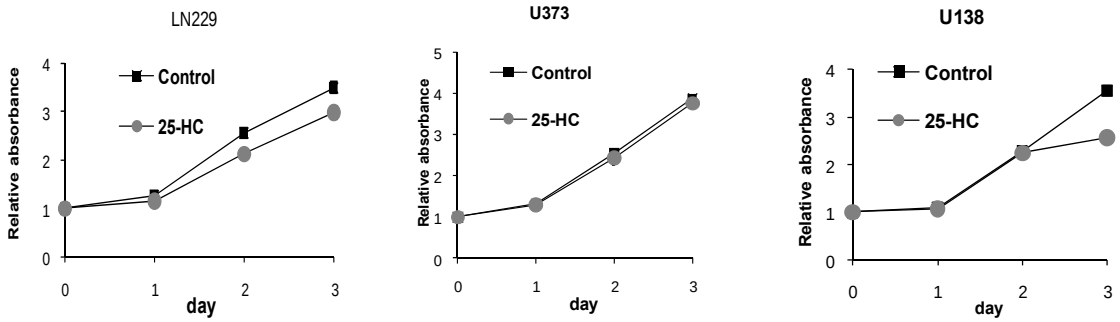


Fig. S6. P-PDGFR and p-Met are also associated with p-Akt and nuclear SREBP-1 staining in glioblastoma tissue microarrays and Met activation can promote SREBP-1 cleavage in glioblastoma cells in vitro. (**A, B**) Representative images from immunohistochemical analysis of two representative patients on the tissue microarray. Scale bar = 20 um. (**C**) Western blot analysis of U251 glioblastoma cells at selected time points following treatment with HGF (60 ng/ml) in serum free medium.

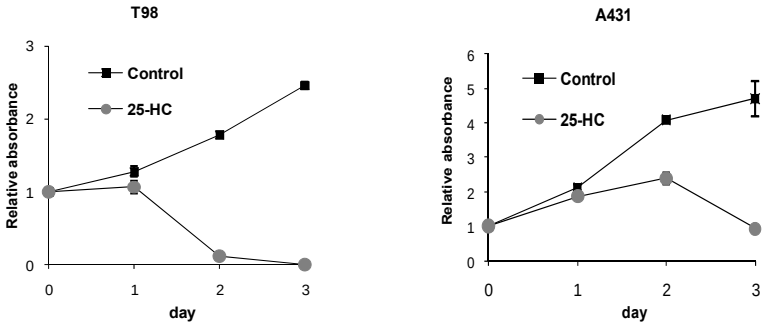
A

Low EGFR



B

High EGFR



C

Modulation of EGFR signaling in isogenic cell context

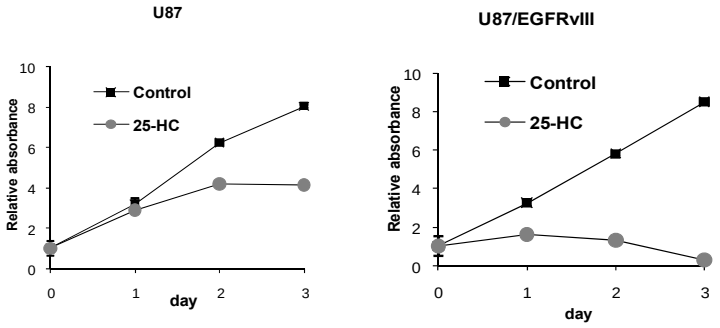


Fig. S7. Effect of 25-hydroxycholesterol (25-HC) on tumor viability. Tumor cell lines with little EGFR (**A**) or with abundant EGFR (**B**) were grown in medium containing 1% serum conditions and treated with 25-HC (1 ug/ml) for 3 days. Tumor viability was measured by WST-1 Assay (Chemicon). (**C**) U87 cells and isogenic U87-EGFRvIII cells were similarly treated and viability was measured by WST-1 assay.

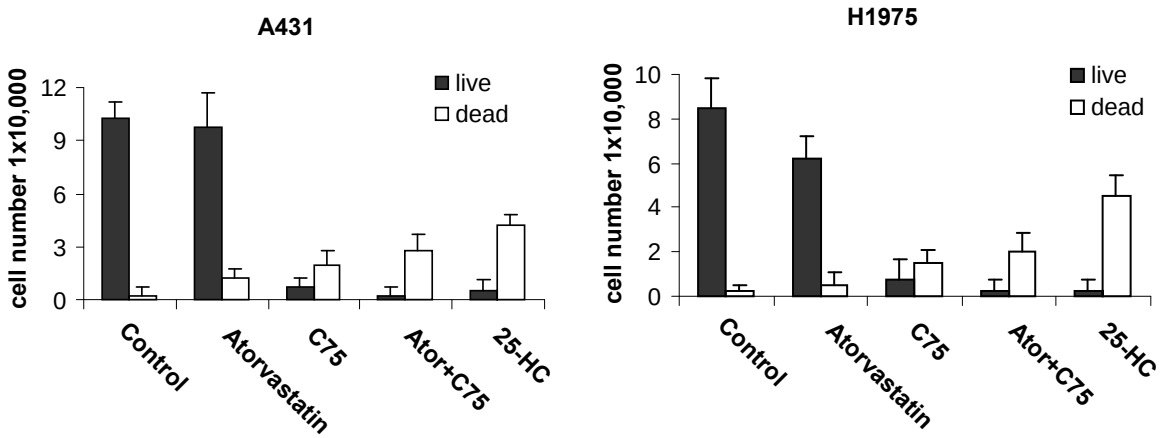


Fig. S8. Lipogenesis inhibitors induce cell death in other cancer types with abundant EGFR. A431 (epidermoid cancerinoma cells bearing EGFR amplification) and H1975 (non-small cell lung cancer, EGFR T795M mutation) cells were seeded in 12 well plates for 24 hrs, then treated with atorvastatin (1 uM), C75 (10 ug/ml) alone or in combination, or 25-hydroxycholesterol (25-HC 1 ug/ml) in 1% FBS medium for 3 days. Cell death was detected using trypan blue exclusion.

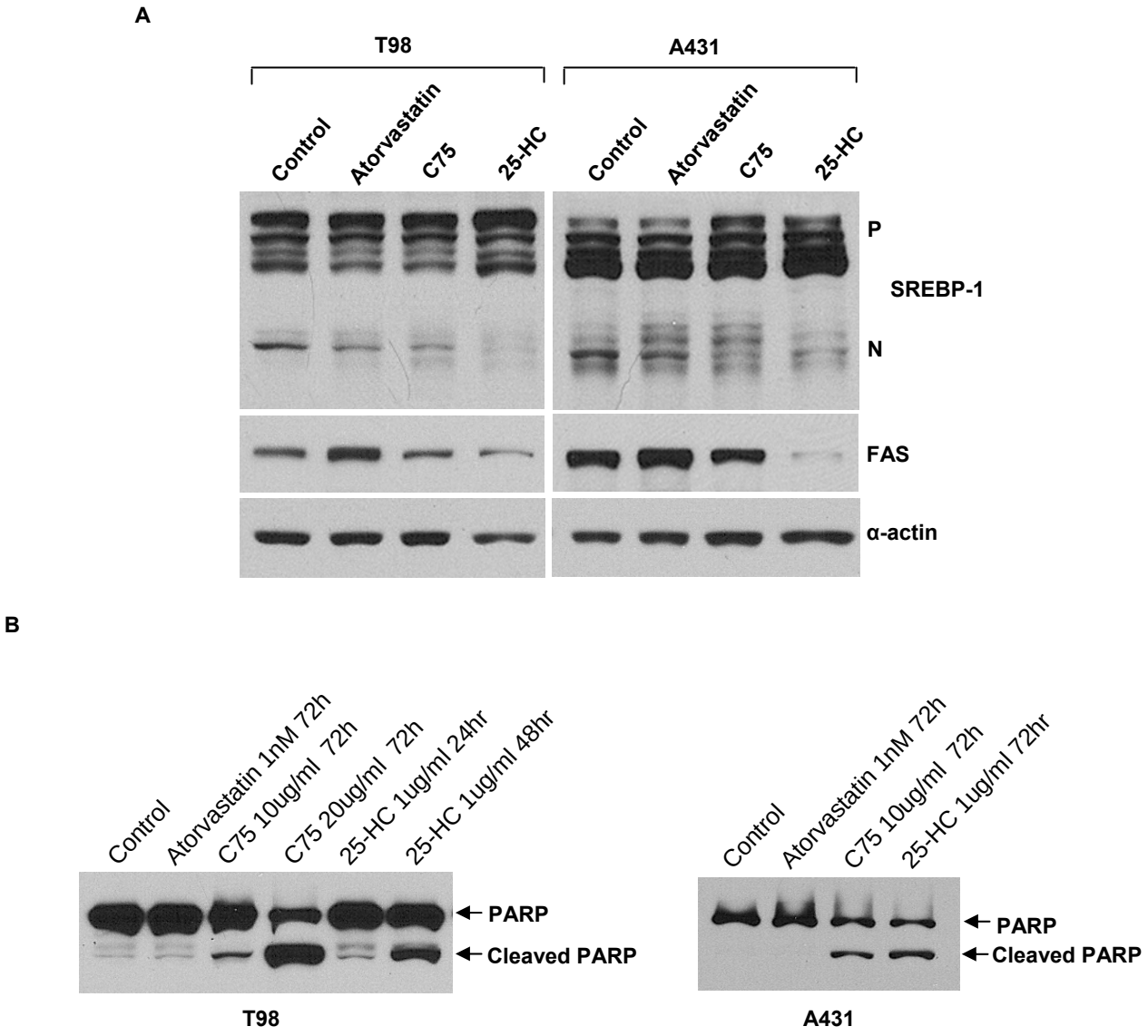


Fig. S9. Analysis of Poly(ADP-ribose) Polymerase (PARP) cleavage confirms that inhibitors of lipogenesis promote apoptotic cell death. (**A**) T98 glioblastoma cells and A431 epidermoid carcinoma cells were treated with atorvastatin (1 nM); C75 (10 ug/ml) or 25-HC (1 ug/ml) for 24 hrs. Immunoblot analysis of cellular lysates was performed using indicated antibodies. P designates precursor band of SREBP-1, and N designates NH2-terminal band of SREBP-1. (**B, C**) T98 or A431 cells were treated with atorvastatin, C75 or 25-HC for the indicated times and immunoblot analysis of cellular lysates for PARP cleavage were performed.

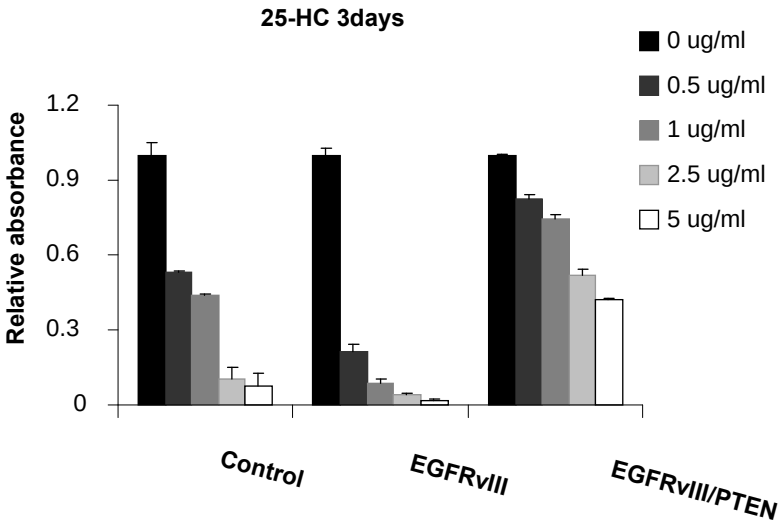


Fig. S10. Effect of PTEN reconstitution on cellular response to 25-HC. U87 cells (control), isogenic U87-EGFRvIII transfected cells, and U87-EGFRvIII-PTEN cells, in which PTEN was transfected into U87-EGFRvIII cells were grown in medium containing 1% serum and treated with 25-hydroxycholesterol (25-HC 1 ug/ml) for 3 days. Cell viability was measured by WST kit (Chemicon).

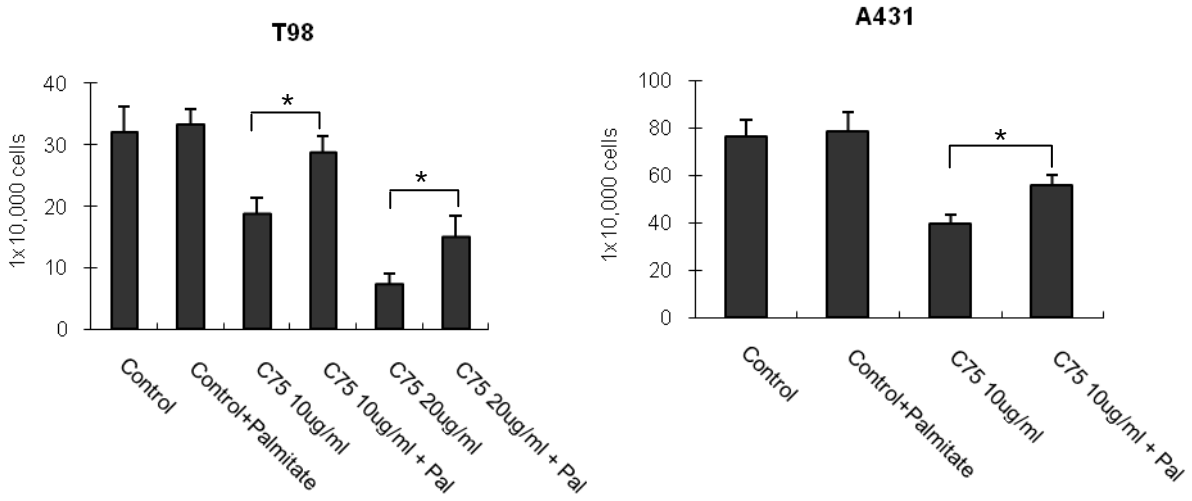


Fig. S11. Palmitate, a product of FAS, significantly rescues C75-mediated cell death. T98 and A431 cells were cultured in 12-well plates for 24 hrs, then medium was replaced with 1% FBS media containing palmitate (Pal 5 uM) for 2 hrs, and then C75 was added into medium at the indicated doses, cells were counted after 3 days treatment. * $P<0.05$.

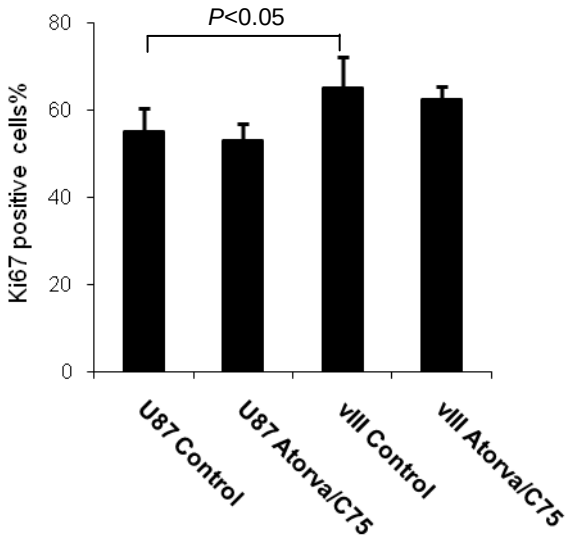


Fig. S12. Effects of atorvastatin and C75 on Ki67 proliferation index in vivo. Immunohistochemical analysis of Ki67 staining of paraffin-embedded tissue sections from U87 or isogenic U87-EGFRvIII tumors implanted in SCID mice before and after treatment with atorvastatin (10 mg/kg daily) and C75 (30 mg/kg weekly) for 15 days.

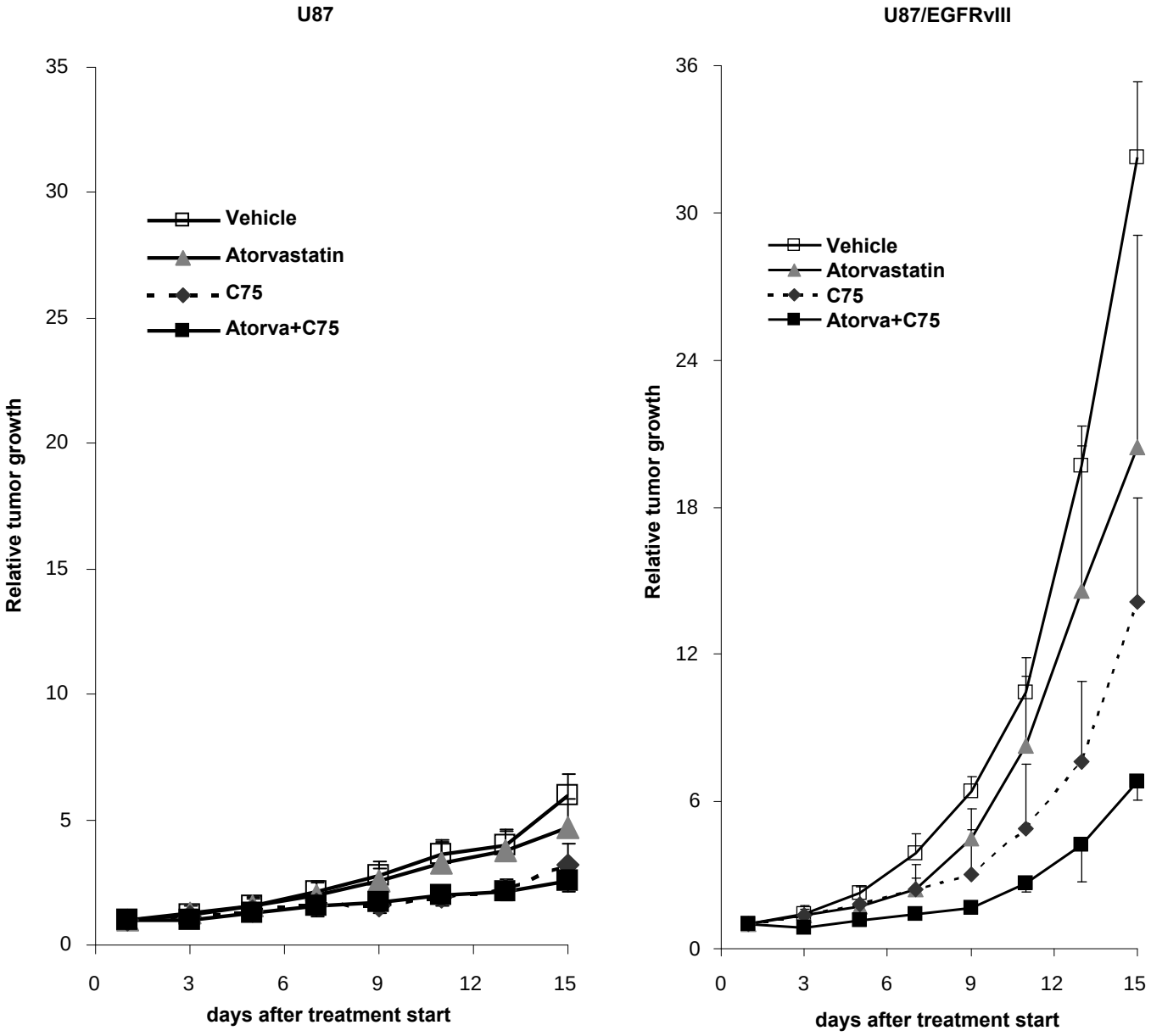


Fig. S13. Effects of atorvastatin or C75 alone or in combination, on the growth of U87 or isogenic U87-EGFRvIII tumors in vivo. Plot of tumor volume over time showing the effect of atorvastatin (10 mg/kg daily) or C75 (30 mg/kg weekly), alone or in combination, on the growth of U87 (**A**) or U87-EGFRvIII tumors (**B**) implanted in the contralateral flank. Tumor volume at multiple time points (days) was normalized to tumor volume at the first day treatment.

Table S1. EGFR inhibition and lapatinib drug concentration in tumor.

Patient #	Percent EGFR inhibtion %	Plasma @Resection (ng/ml)	Tumor Tissue Conc. (ug/g)
1	40.8 ± 2.6	948	12.64
2	28.1 ± 1.7	2319	11.74
3	18 ± 3.4	1241	17.4
4	14.3 ± 7.3	1925	9.52
8	-7.8 ± 11.8	1356	3.58
9	-8 ± 9	1340	8.19

Percent EGFR inhibition % - post-lapatinib p-EGFR was compared to pre-lapatinib p-EGFR to determine percent EGFR inhibition % in tumor tissue.

Plasma@Resection - refers to plasma concentration of lapatinib at the time of tumor resection.

Tumor Tissue Conc. - concentration of lapatinib in tumor tissue

Table S2. Immunohistochemical staining of proteins in tissue microarrays of GBM samples and adjacent normal brain.

		Tumor	Normal
p-EGFR	**+**	96	3
	-	121	75
Total number		217	78
Positive percentage		**44.2%**	**3.8%**
P value		*P* <0.0001	
p-PDGFR	**+**	39	0
	-	94	41
Total number		133	41
Positive percentage		**29.3%**	**0.0%**
P value		*P* <0.0001	
p-Met	**+**	30	1
	-	109	44
Total number		139	45
Positive percentage		**21.6%**	**2.2%**
P value		*P* <0.0001	
p-Akt	**+**	171	16
	-	51	63
Total number		222	79
Positive percentage		**77%**	**20.2%**
P value		*P* <0.0001	
SREBP-1	**+**	185	30
	-	47	56
Total number		232	86
Positive percentage		**79.8%**	**34.9%**
P value		*P* <0.0001	
ACC	**+**	198	14
	-	46	71
Total number		244	85
Positive percentage		**81.1%**	**16.5%**
P value		*P* <0.0001	
FAS	**+**	134	13
	-	34	52
Total number		168	65
Positive percentage		**79.8%**	**20%**
P value		*P* <0.0001	

* 252 tumor cores and 91 normal cores from 140 patients on two tissue microarrays.

** Numbers may not add up to 252 or 91 because of missing cores.

P-value were determined by Fisher test.

Table S3. Co-expression of immunohistochemical markers in GBM samples on tissue microarry

		p-Akt		SREBP-1		ACC		FAS	
		+	-	+	-	+	-	+	-
p-EGFR	**+**	92	5	90	6	93	4	62	5
	-	71	43	70	34	78	34	65	25
Percentage in p-EGFR tumor		**94.8%**		**93.8%**		**95.9%**		**92.5%**	
P value		*P* <0.0001		*P* <0.0001		*P* <0.0001		*P* =0.0122	
p-Akt	**+**			151	9	157	11	112	9
	-			**18**	**29**	**18**	**29**	**15**	23
Percentage in p-Akt tumor				**94.4%**		**93.5%**		**92.6%**	
P value				*P* <0.0001		*P* <0.0001		*P* <0.0001	
SREBP-1	**+**					175	10	114	12
	-					13	32	10	16
Percentage in SREBP1 tumor						**94.6%**		**90.5%**	
P value						*P* <0.0001		*P* <0.0001	
ACC	**+**							121	10
	-							7	19
Percentage in ACC tumor								**92.40%**	
P value								*P* <0.0001	

* 252 tumor cores and 91 normal cores from 140 patients on two tissue microarrays.
** Numbers may not add up to 252 or 91 because of missing cores.
P -value were determined by Fisher test.

Table S4. Signaling pathway marker correlations from GBM samples on tissue microarray.

		ACC/FAS	
		+	-
SREBP-1	+	107	11
	-	6	20
Percentage in SREBP1 tumor		**90.7%**	
P value		*P* <0.0001	

		SREBP-1/ACC/FAS	
		+	-
p-Akt	+	99	9
	-	9	16
Percentage in p-Akt tumor		**91.7%**	
P value		*P* <0.0001	

		p-Akt/SREBP-1/ACC/FAS	
		+	-
p-EGFR	+	61	5
	-	30	13
Percentage in p-EGFR tumor		**92.4%**	
P value		*P* =0.003	

		p-Akt/SREBP-1/ACC/FAS	
		+	-
p-PDGFR	+	34	1
	-	67	52
Percentage in p-PDGFR tumor		**97.1%**	
P value		*P* <0.0001	

		p-Akt/SREBP-1/ACC/FAS	
		+	-
p-Met	+	24	4
	-	68	55
Percentage in p-Met tumor		**85.7%**	
P value		*P* =0.002	

* 252 tumor cores and 91 normal cores from 140 patients on two tissue microarrays.
** Numbers may not add up to 252 or 91 because of missing cores.
P -value were determined by Fisher test.