Supplementary Materials for

Protein Arginine Methyltransferase 5 Regulates ERK1/2 Signal Transduction Amplitude and Cell Fate Through CRAF

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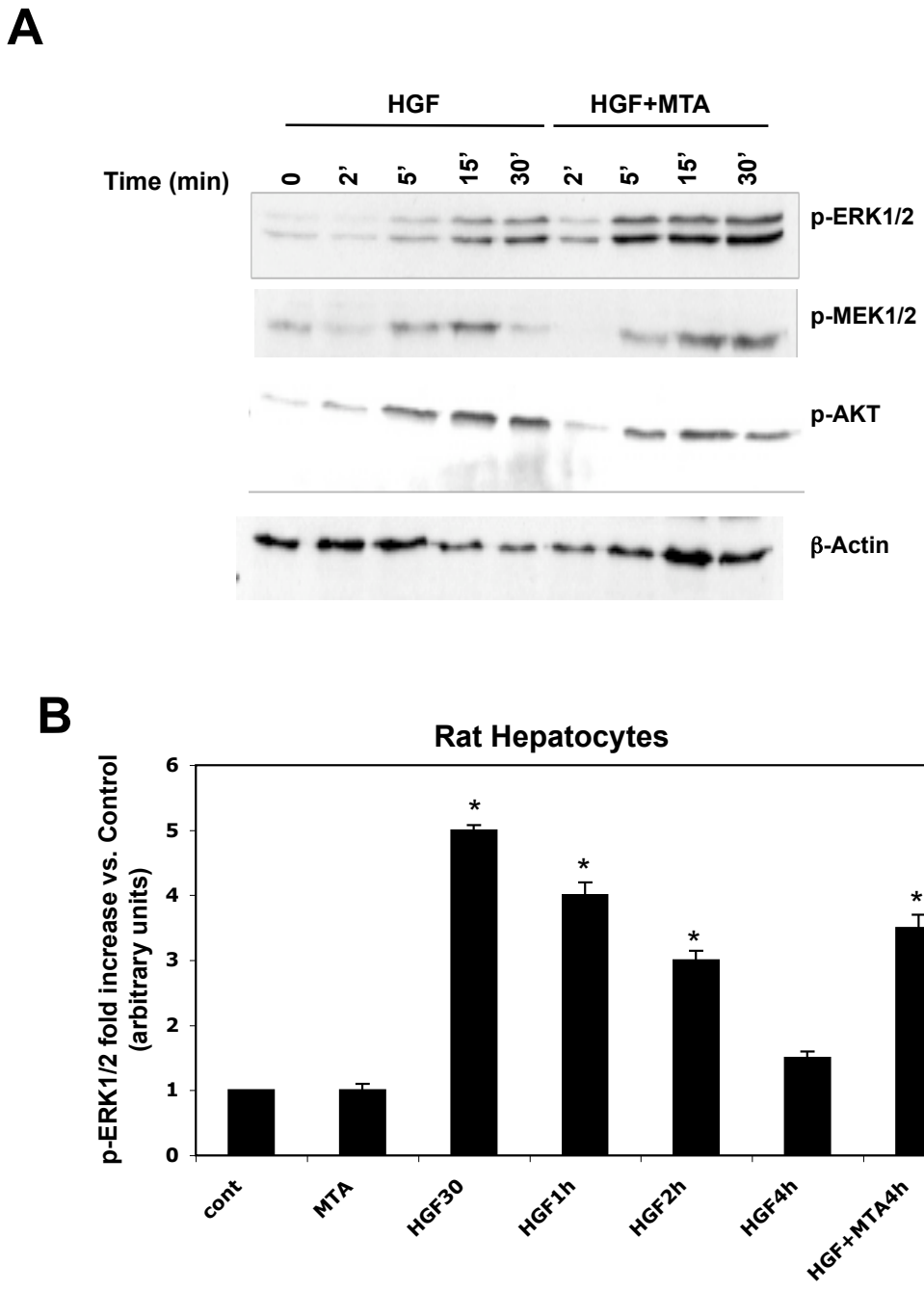
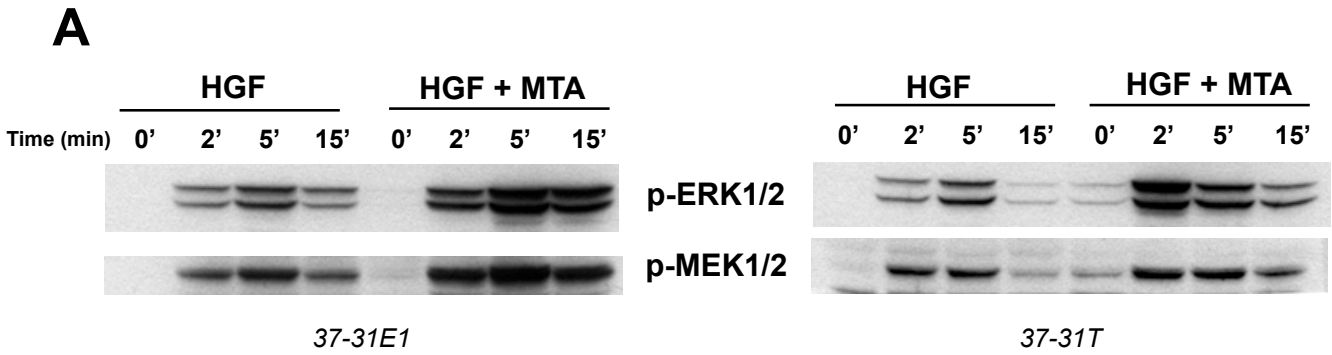


Fig. S1. MTA increases ERK1/2 phosphorylation in response to HGF in primary rat hepatocytes. **(A)** Rat hepatocytes in primary culture were treated with HGF for the times indicated with or without MTA pre-treatment. Western blot shows phosphorylation of ERK1/2, MEK1/2, and AKT. Actin is shown as a loading control. Representative images of three independent experiments are shown. **(B)** Quantification of p-Erk1/2 in response to HGF and MTA in rat primary hepatocytes. Bars indicate the SD. *P-value* was calculated performing a Student's *t*-test (N=3 independent experiments). *=p<0.001.



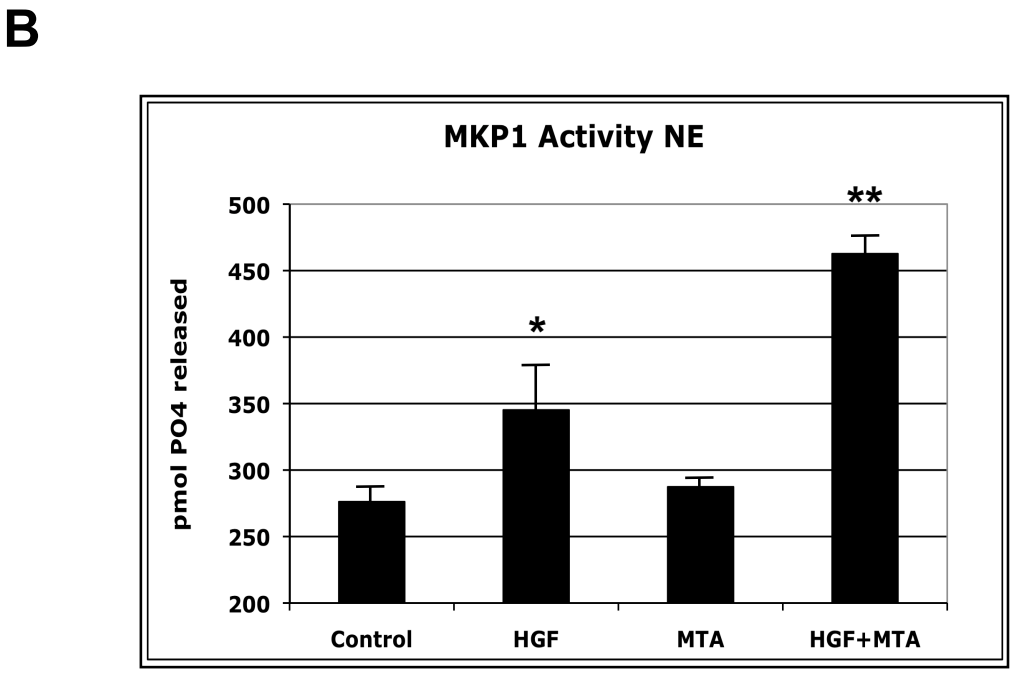


Fig. S2. Modulation of HGF induced MEK1/2-ERK1/2 signaling by MTA in melanoma cells. **(A)** Western-Blot showing the time course of MEK1/2 and ERK1/2 phosphorylation in response to HGF and increased signal seen with MTA pre-treatment in 37-31E and 37-31T melanoma cell lines. **(B)** MKP1 nuclear phosphatase activity**.** MKP1 phosphatase was immunoprecipitated from nuclear extracts after the indicated treatments. Phosphatase activity was measured in the immunoprecipitates. Values represent means ±SD. *P-value* was calculated performing a Student’s *t*-test (N=3 independent experiments). *=p<0.05; **= p<0.001.

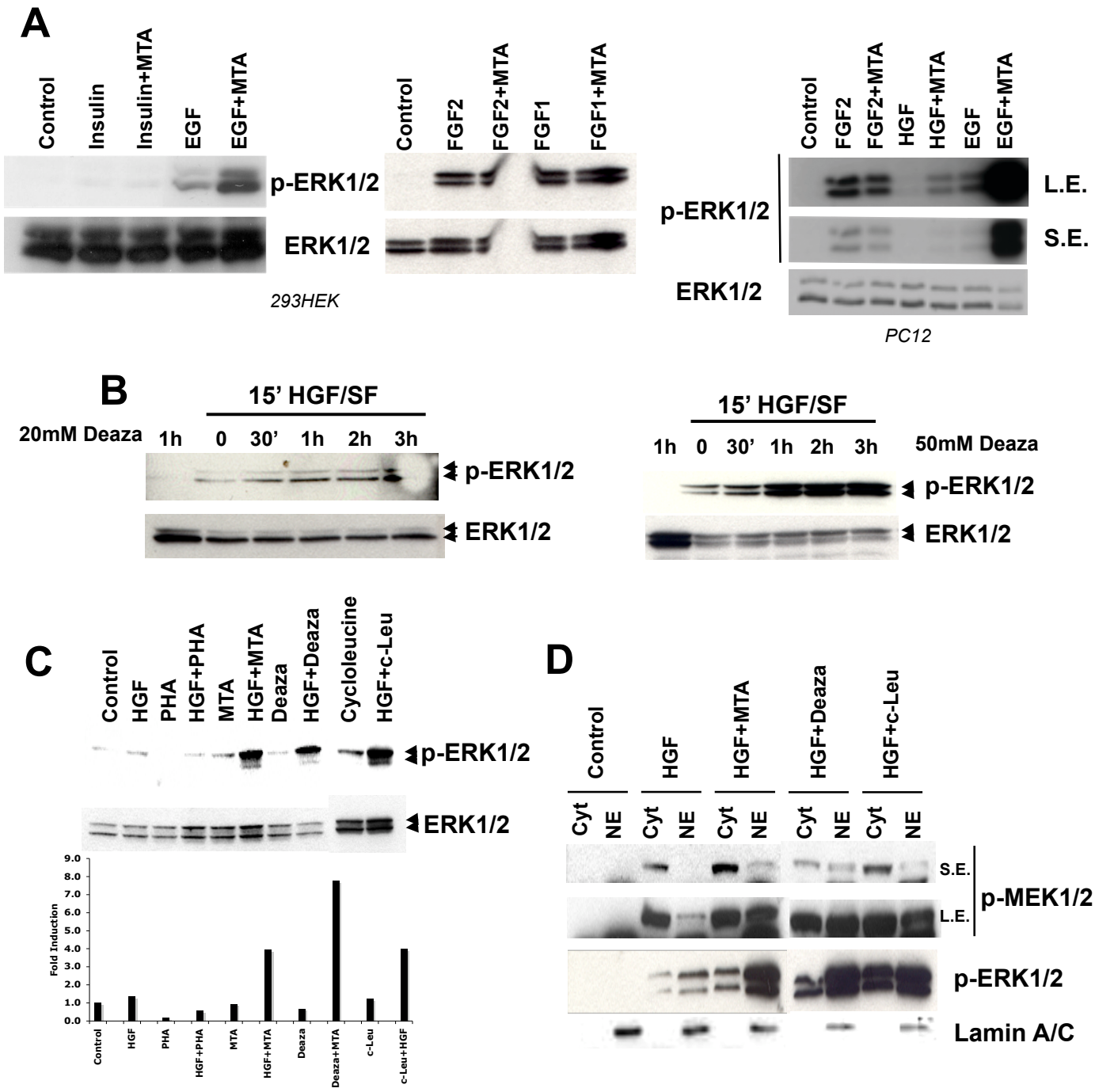


Figure S3. **(A)** MTA modulates ERK1/2 phosphorylation upon specific growth factor treatment in human cells, and protein methylation inhibition modulates ERK1/2 signal amplitude in response to HGF. PC12 cells were treated with FGF2, HGF or EGF with or without MTA. The increase in Erk1/2 phosphorylation is shown. Effect of protein methylation inhibition mediated by Deaza on ERK1/2 phosphorylation. **(B)** 37-31T melanoma cells were exposed to with either 20mM or 50 mM of Deaza for the times indicate and then treated 15 min with HGF. Western–blots show p-ERK1/2 and ERK1/2 at the indicated time points. **(C)** Protein methylation inhibition increases Erk1/2 phosphorylation in response to HGF in 293HEK cells. Cells were incubated either with MTA, PHA, Deaza, or Cycloleucine as indicated and then treated with HGF. p-ERK1/2 and ERK1/2 are shown. Quantification of the bands from different experiments was performed with ImageJ software. **(D)** Nuclear translocation of p-MEK1/2 and p-ERK1/2 in response to HGF in the presence or absence of protein methylation inhibitors in B16F1 melanoma cells. A short exposure (S.E.) and a longer exposure (L.E.) is shown for p-MEK1/2. Lamin A/C is shown as a loading control and as marker for the nuclear fraction. All the experiments were done at least in triplicates. Representative images of each experiment are shown.

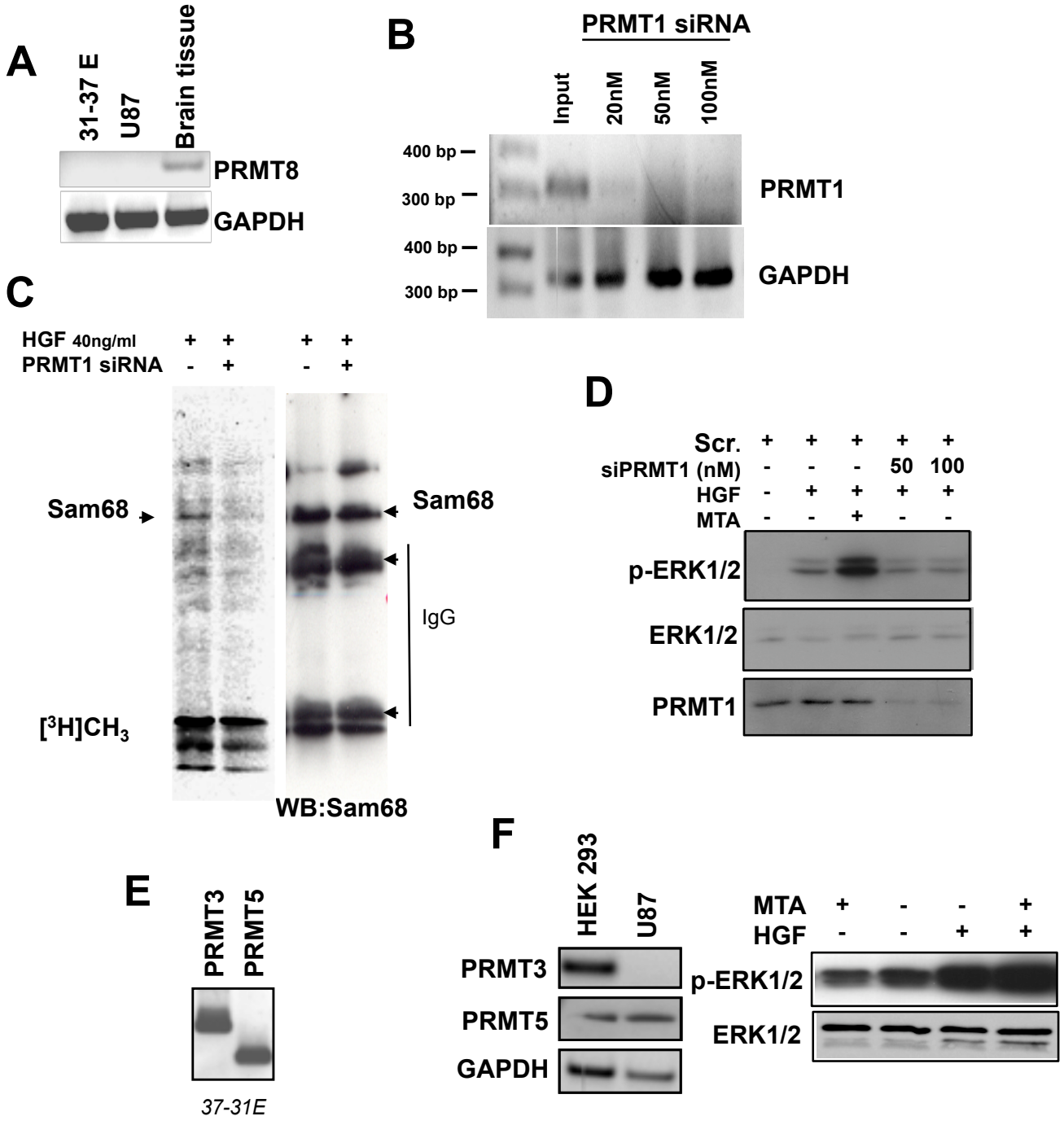


Figure S4. (**A**) PRMT8 arginine protein methyltransferase is not present in 37-31 E mouse melanoma cells and U87 human glioma cells. Human brain tissue was used as positive control. GAPDH was used as a loading control. (**B-D**) Knock-down of *PRMT1* does not reproduce MTA effect on signal amplitude. **(B)** 37-31E melanoma cells were transiently transfected for 60h with increasing concentrations of mouse specific On-Target- PRMT1-siRNA (Dharmacon). PRMT1 RNA abundance was assessed by R-PCR, GAPDH is shown as a loading control. (**C**) To demonstrate the functional inhibition of PRMT1 we used an *in vivo* methylation assay of Sam68, a PRMT1 substrate. After in vivo methylation, Sam68 was immunoprecipitatedthe complexes were resolved by SDS-P and the membrane was exposed to a film for 2 weeks, Sam68 methylation labeling is shown on the left. IP is shownby western analysis after re-blotting with anti-Sam68 antibody on the right. (**D**) 37-31E cells were transiently transfected for 60 hours with 50nM and 100nM of either scrambled siRNA or two different PRMT1 siRNAs (#1 and #2). Then, cells were starved in serum free medium for 3 hours in the presence of 0.4mM MTA . Cells were treated with 50ng/ml of HGF for 10 min. p-Erk1/2, Erk1/2 and PRMT1 are shown. (**E**) RT-PCR showing the expression of *Prmt3* and *Prmt5* in 37-31E cells. (**F**). U87 human glioma cells do not express *PRTM3. PRMT3* expression in 293HEK cells is shown as positive control of the RT-PCR analysis. Right panel, Cells were serum starved overnight and treated with MTA (0.4mM) for 3h followed by 10min with HGF. Western-blot shows the increase in p-ERK1/2 abundance by MTA (0.4mM) upon HGF treatment.

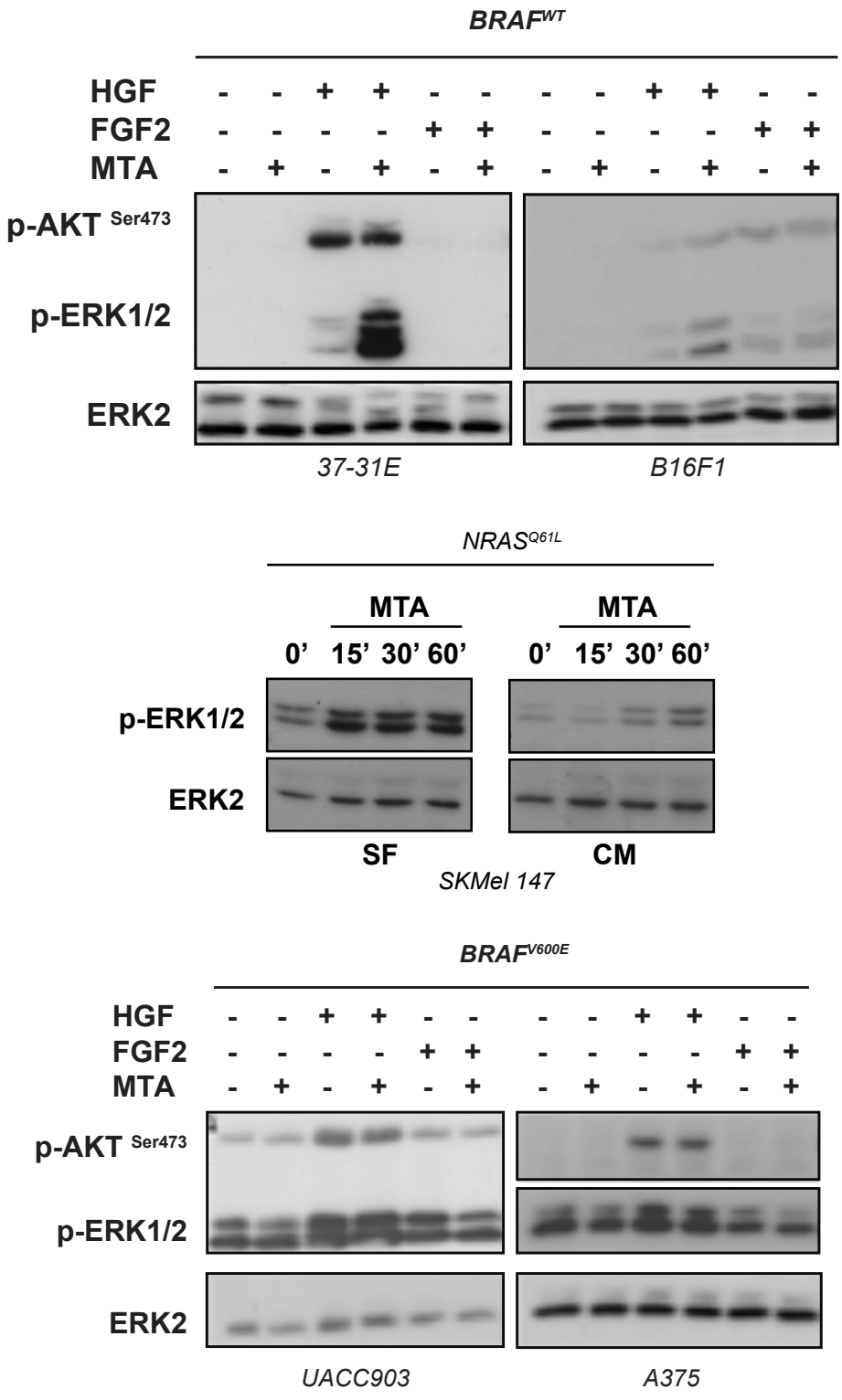


Figure S5: The MTA-dependent increase in Erk1/2 phosphorylation depends on RAS activation. (A) 37-31E and B16F1 cells (harboringwild type RAS and BRAF) were serum-starved and treated with MTA (0.3mM) for 3h. Then cells were treated either with HGF (40ng/ml) or FGF2 (40ng/ml) for 10 minutes. Western blots were probed against p-AKT and p-ERK1/2. ERK1/2 is showed as loading control. (**B**) RAS^{Q61L} mutant melanoma cells SKMel147 were treated for the times indicated with 0.3mM MTA in serum free (SF) or complete medium (CM). Western blot shows the amount of p-ERK and ERK2. (**C**) $BRAF^{V600E}$ mutant melanoma cells (UACC903 and A375)were serum-starved and treated in with MTA (0.3mM) for 3h and then, treated with HGF (40ng/ml) for 15 min. Western-blot shows the amounts of p-ERK, p-AKT and ERK2.

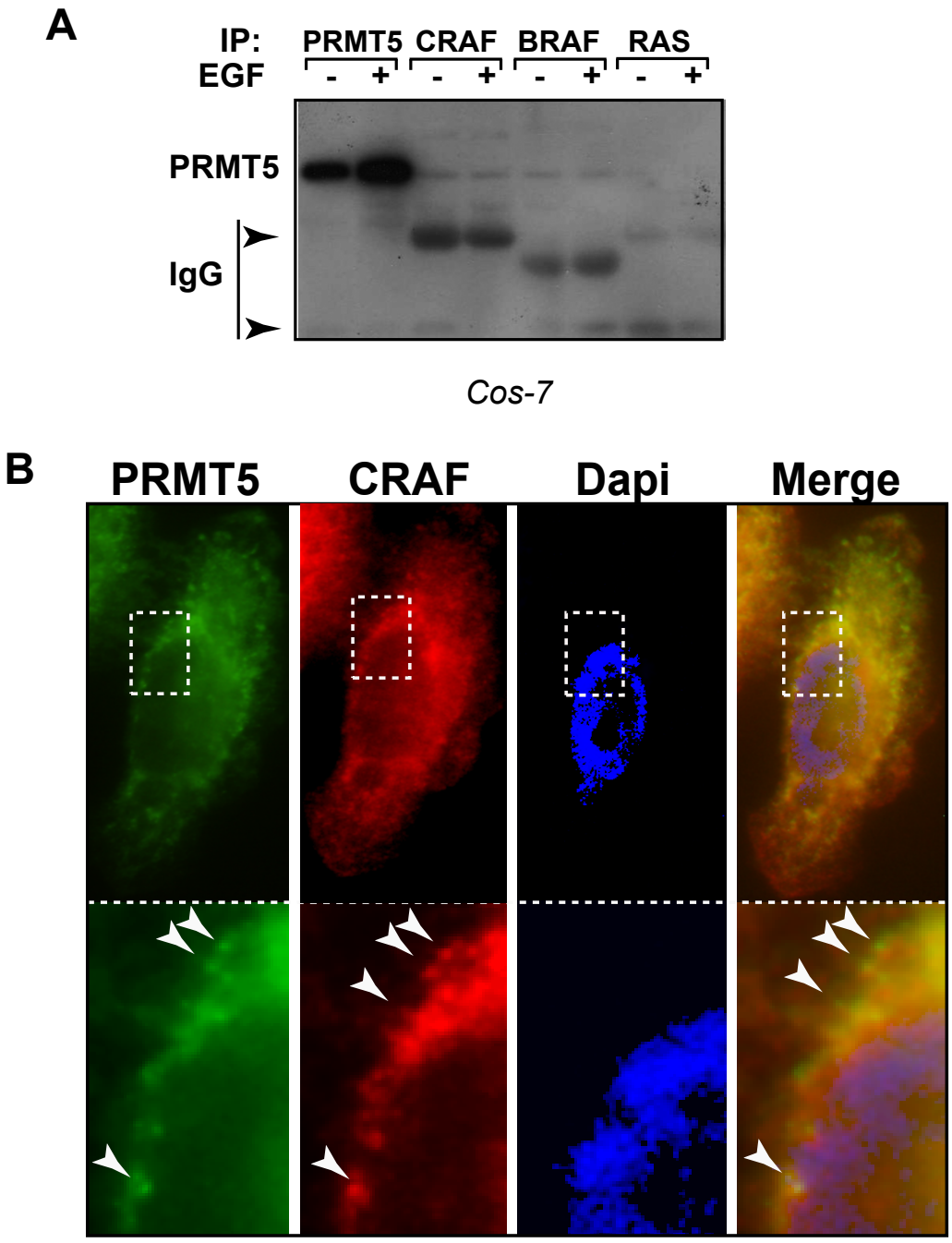
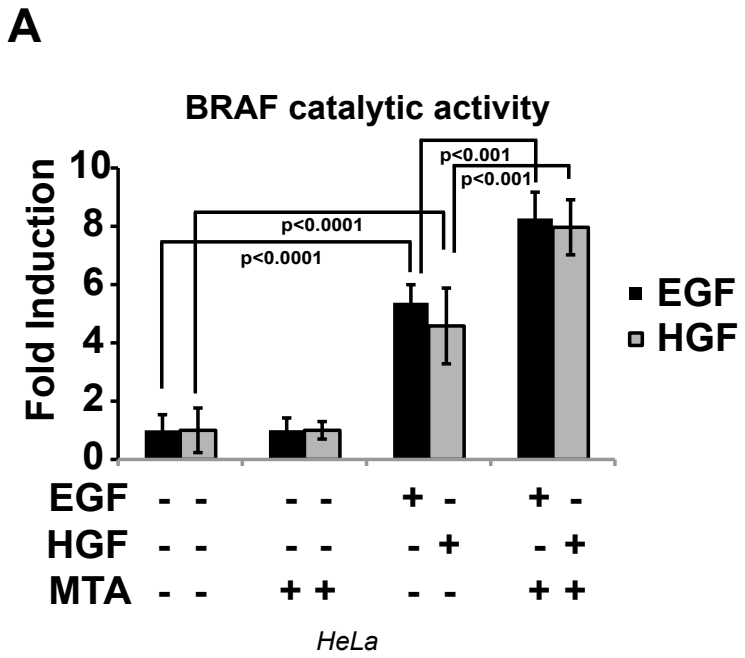


Figure S6: **(A**) Total lysates from Cos7 cells untreated or treated with EGF (50ng/ml) were used to immunoprecipitate endogenous Prmt5, CRaf, Braf, and Ras. Immunocomplexes were analyzed for the presence of Prmt5 by Western -blot. **(B)** Immunofluorescence analysis of HeLa cells transiently transfected with Flag-*CRAF*^{WT} and *PRMT5*. Representative images are shown. Squares indicated by dashed lines are magnified at bottom. Arrows indicate cytoplasmic particles.



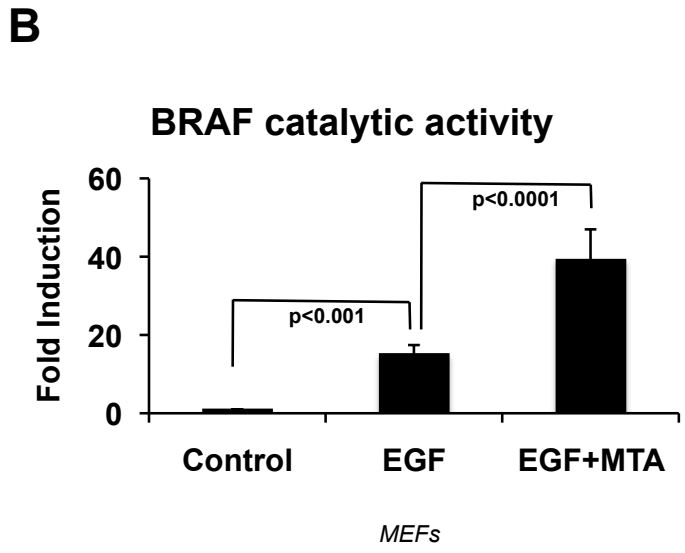


Figure S7: MTA regulates growth factor-induced BRAF kinase activity. **(A)** HeLa cells were transiently transfected with myc -*BRAF*^{WT}, starved, and pre-treated with MTA (0.4mM) for 3h. and then treated with EGF (50ng/ml) or HGF (40ng/ml) for 10 min. After immunoprecipitation of myc-BRAF, immunocomplexes were used to perform an in vitro kinase activity. Graphs show increase of BRAF catalytic activity relative to control under the different conditions. All experiments were done at least in triplicate. Bars indicate the SD. *P-value* was calculated performing a student's *t*-test. **(C)** Endogenous BRAF activity in MEFs. MEFs were starved and pre-treated with MTA (0.4mM) for 3h. Then, cells were treated with EGF (50ng/ml) for 10 min. Graphs show the increase inthe *in vitro* BRAF kinase activity. Bars indicate the SD. *P-value* was calculated performing a student's *t* -test. N=3 independent experiments.

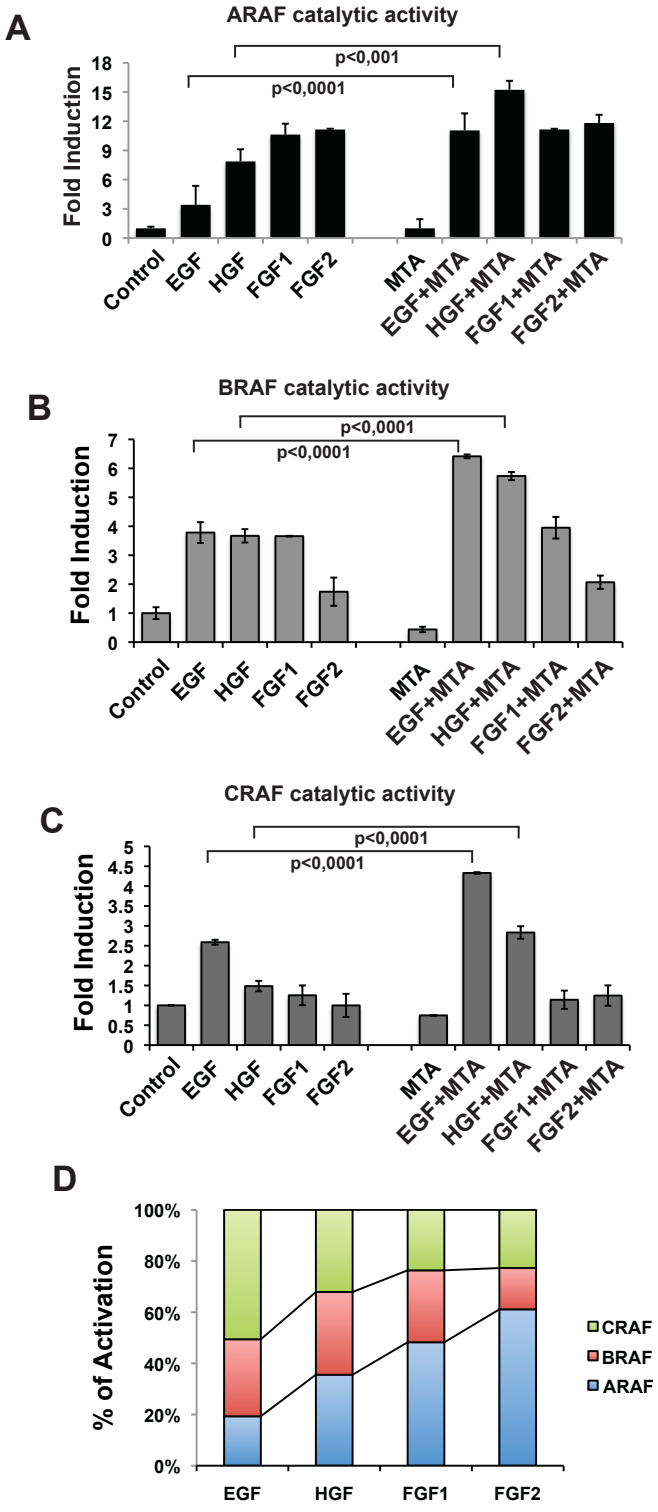


Figure S8. Differential activation of RAF isoforms by growth factors. 293HEK or HeLa cells were serum-starved and pre -treated with MTA (0.4mM) for 3h. Then, cells were treated either with EGF (50ng/ml), HGF (40ng/ml), FGF1 (40ng/ml) or FGF2 (40ng/ml) for 10 min. Alicuots of the same lysates were used to immunoprecipitate either ARAF, BRAF or CRAF. Immunocomplexes were used to perform an *in vitro* kinase activity. All the experiments in the two cell lines were done at least in triplicate (three different experiments per condition, per cell line). Graphs show the average catalytic activity of ARAF(**A**), BRAF (**B**) or CRAF (**C**) in fold induction under the different conditions. Bars indicate the SD. *P-value* was calculated performing a student's *t*-test. (**D**) Graph showing the percentage of the catalytic activation of ARAF, BRAF and CRAF by EGF, HGF, FGF1 and FGF2. Graph shows the combined data obtained inin 293HEK and Hela cells. Where a 100% represent the sum of the catalytic activity relative to Control of ARAF, BRAF and CRAF in response to a specific growth factor.

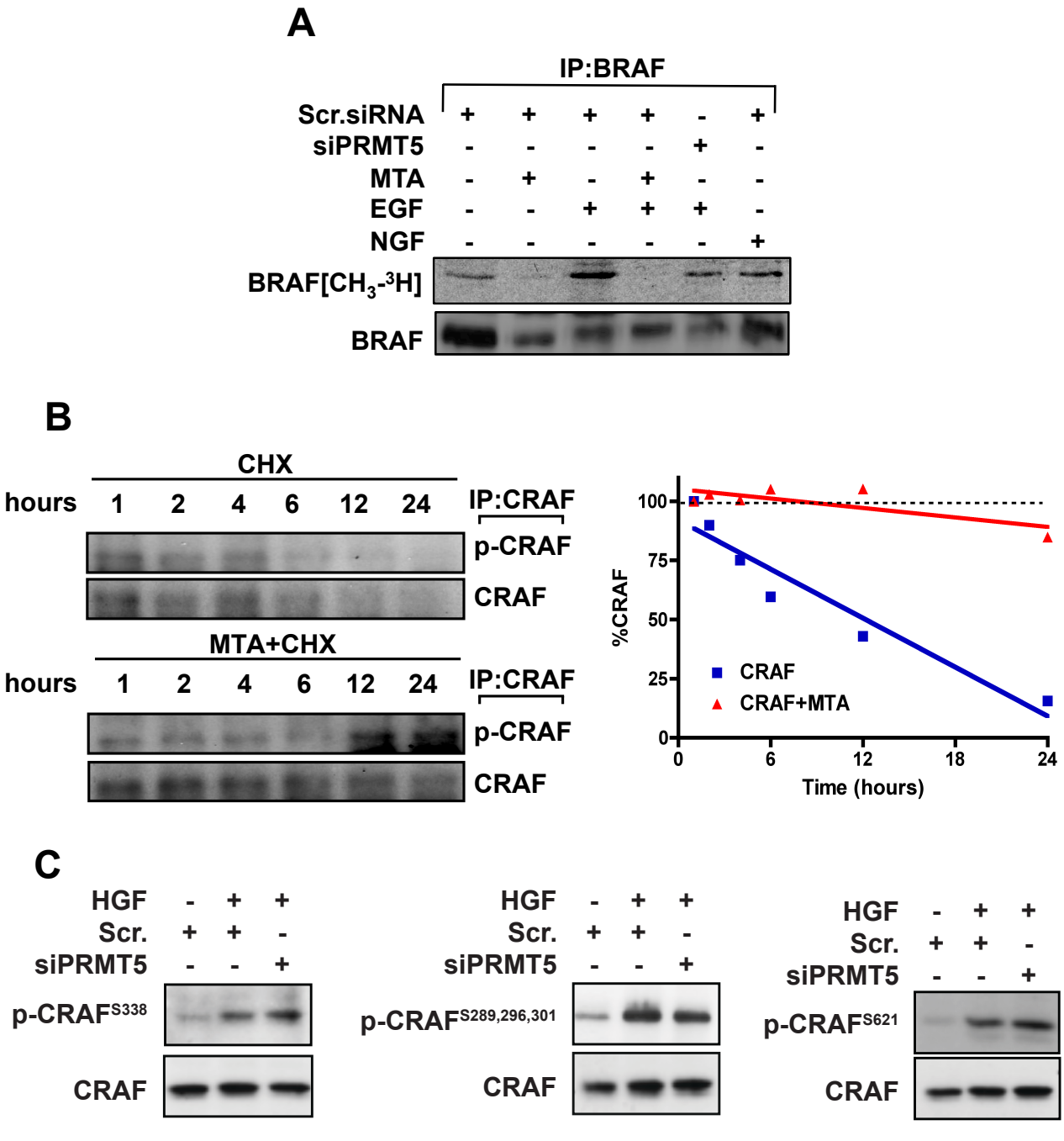


Figure S9.BRAF is in vivo methylated and MTA and PRMT5 Knocked-down increase CRAF stability. **(A)** In vivo methylation assay in PC12 cells. Cells were transiently transfected with siRNA for *Prmt5* for 50-60h. Then, cells were metabolically labelled with [H^3] methionine as previously described (1-2) and treated with EGF (50ng/ml) and MTA (0.4mM). After immunoprecipitation of endogenous BRAF and SDS-PAGE, membranes were exposed to film to capture the [^{3}H] radioactive signal. Immunoprecipitated BRAF is shown as a loading control. Input samples were the same as in Figure 4E. **(B)** SkMel147 $NRAS^{Q61L}$ mutant cells grown in complete medium were treated with cycloheximide (CHX) in the presence or absence of MTA (0.3mM) and chased for up to 24h. Then, CRAF was immunoprecipitated from 500µg of total lysates. Western-blot shows p-CRAF (Ser 621) and total CRAF in total lysates at the indicated time points. Graph shows CRAF quantifications. **(C)** HeLa cells were transfected with ether scrambled (Scr) or *PRMT5* siRNA (siPRMT5) for 0 hours then cells were serum-starved for 3 hours and pre-treated with MTA (0.4mM). After treatment with HGF for ten min cells were lysated and proteins were analyzed by SDS-PAGE. Wester-blots show the amounts of CRAF, p-$CRAF^{S338}$, p-$CRAF^{S289,296,301}$ and p-$CRAF^{S621}$ under the different conditions. Representative images of three independent experiments are shown.

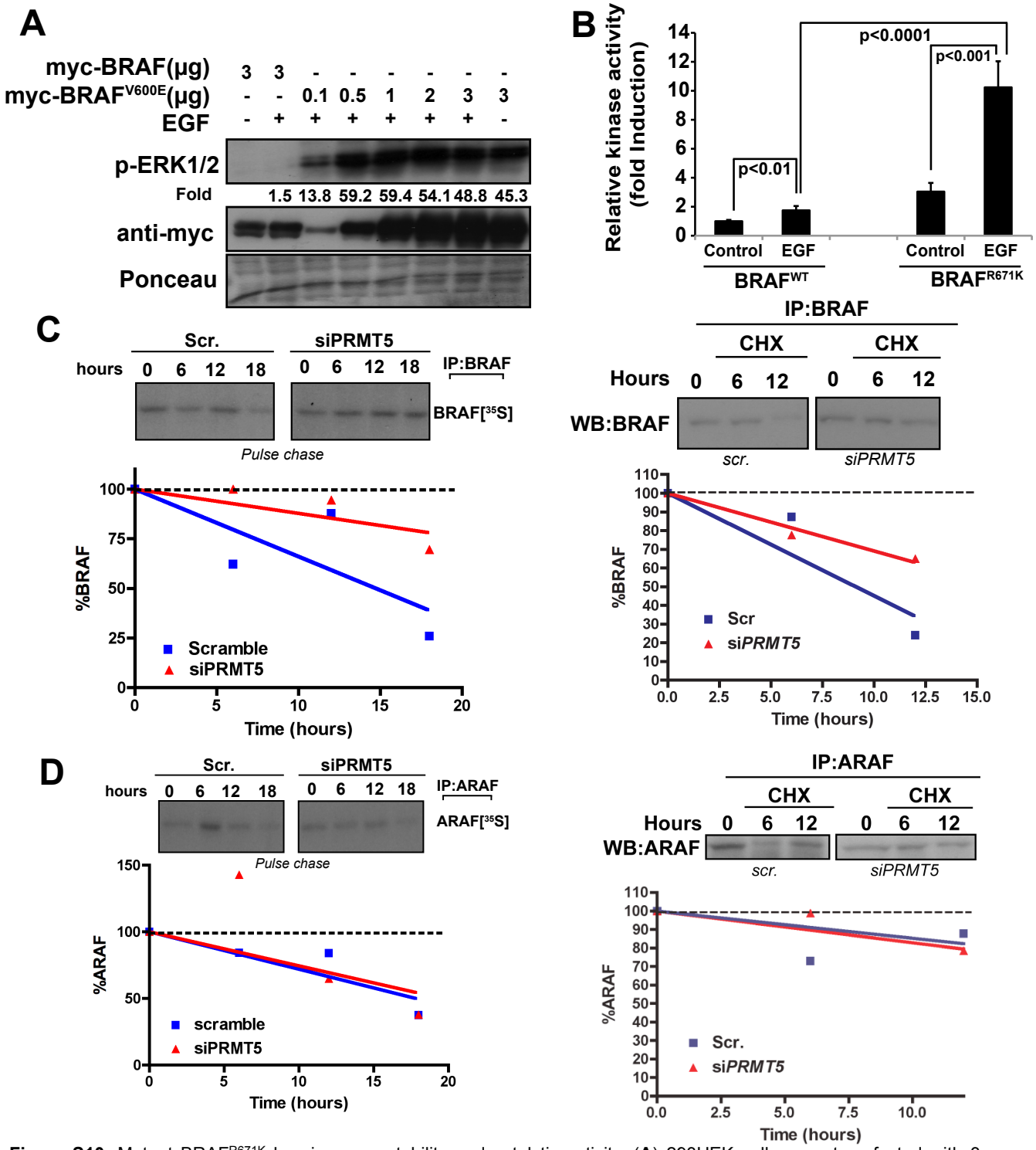
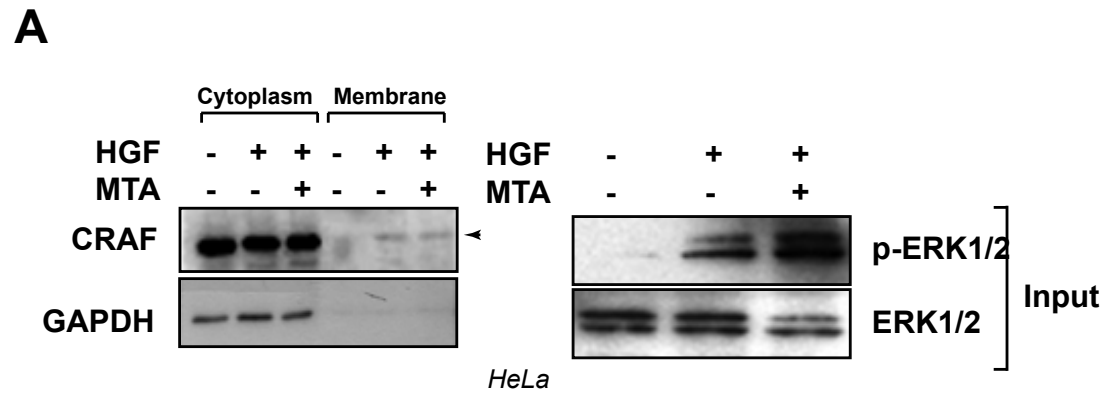


Figure S10. Mutant BRAF^{R671K} has increase stability and catalytic activity. (**A**) 293HEK cells were transfected with 3μg myc -BRAF^{WT} or 0.1 to 3μg of myc-BRAF^{R671K} serum-starved for 3h, and then treated with EGF (50ng/ml) for 10 minutes. Western-blot shows the abundant of transfected BRAF and p-ERK1/2. Ponceau S staining of membrane is shown as loading control. (**B**) HeLa cells were transfected with 3μg of myc-BRAF^{WT} or 0.5μg myc-BRAF^{R671K}. After serum-starvation for 3h, cells were treated with EGF (50ng/ml) for 10 minutes and BRAF was immunoprecipitated with an anti-myc antibody to perform a *in vitro* kinase assay. Graphs show the increase in BRAF catalytic activity in response to EGF for both myc-BRAF^{WT} and myc-BRAF^{R671}. All the experiments were done at least in triplicate. Bars indicate the SD. *P-value* was calculated with student's *t*-test. (**C**) HeLa cells were transiently transfected with scramble-siRNA (Scr.) or *PRMT5*-siRNA (siPMT5) for 24h. Cells were pulse labeled with Met [^{35}S] and and samples were obtained over a time course of up to 18 hours in the presence of EGF (40ng/ml). BRAF was immunoprecipitated from 500μg of total lysate. Samples were separated by SDS-PAGE and dried-gel was exposed to capture the ^{35}S signal. Hela cells grown in serum-containing medium plus EGF were treated with cycloheximide (CHX) in the presence or absence of MTA (0.3mM). Then, samples were taken at the indicated time points.Immunoprecipitaion of BRAF was performed from 500μg of total protein Wester-blot show the abundance of BRAF. Graph shows BRAF quantification (N=3 different experiments). Levels of PRMT5 from input samples are showed in Fig 4E. (**D**) Same experiments as in (C) were performed immunoprecipitating ARAF.



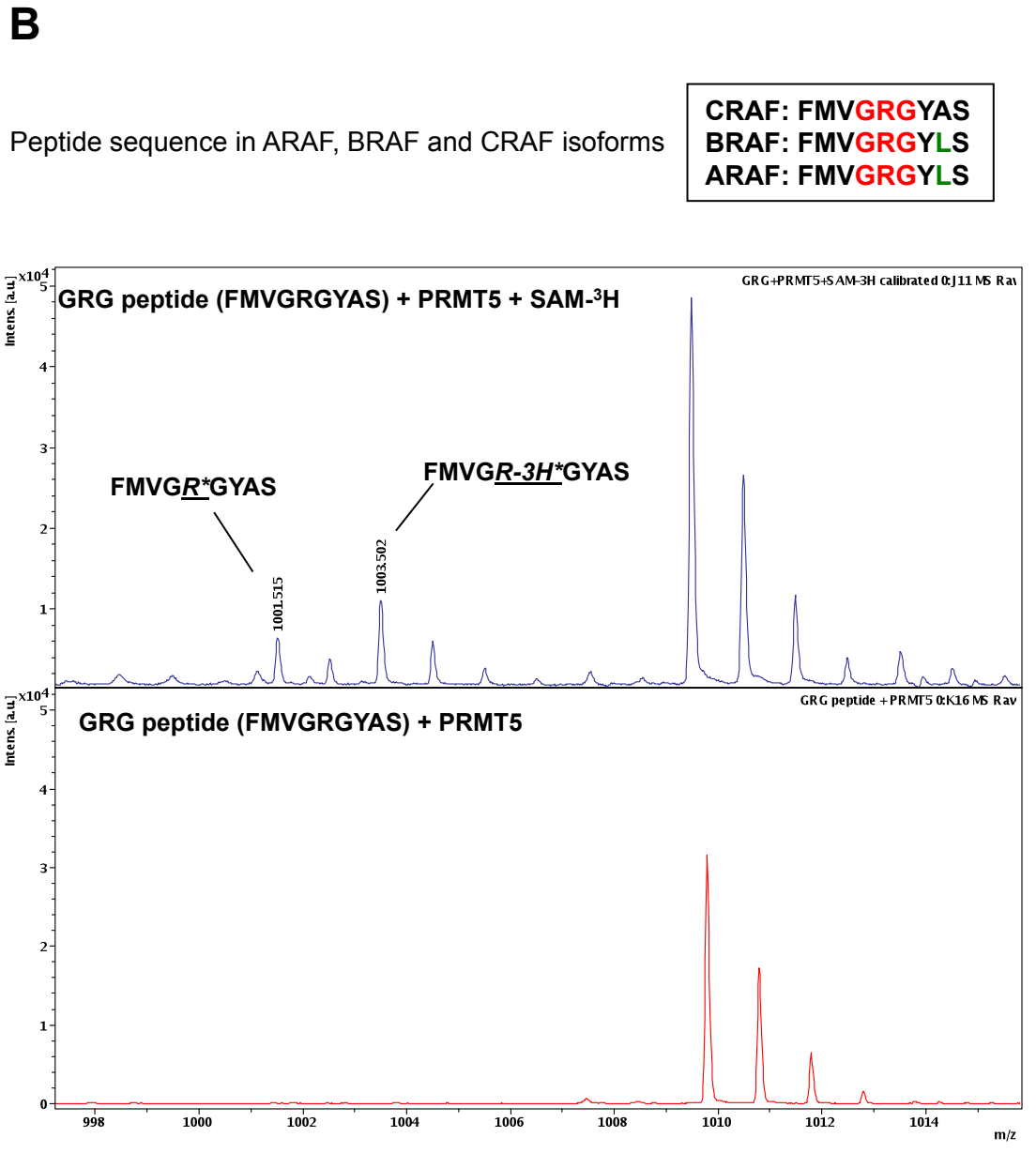


Figure S11. A small fraction of total CRAF is recruited to the membrane in response to growth factor signaling and GRG containing peptide of CRAF is methylated by PRMT5. **(A)** HeLa cells were serum-starved and then treated with HGF (40ng/ml) and MTA (0.4mM) for 2h. Western blot shows the amount of CRAF in membrane an cytosolic fractions. GAPDH shows the purity of fractions. Input samples are shown on the right. Representative images of three independent experiments are shown. **(B)** Alignment of the assayed peptide sequence among the RAF isoforms. Purified peptides after the in vitro methylation assay were analyzed by mass spectrometry. Graphs show the MALDI spectrum of both the unmethylated and methylated CRAF peptide (FMVGRGYAS) with cold CH_3 or labeled [^{3}H]-CH_3.

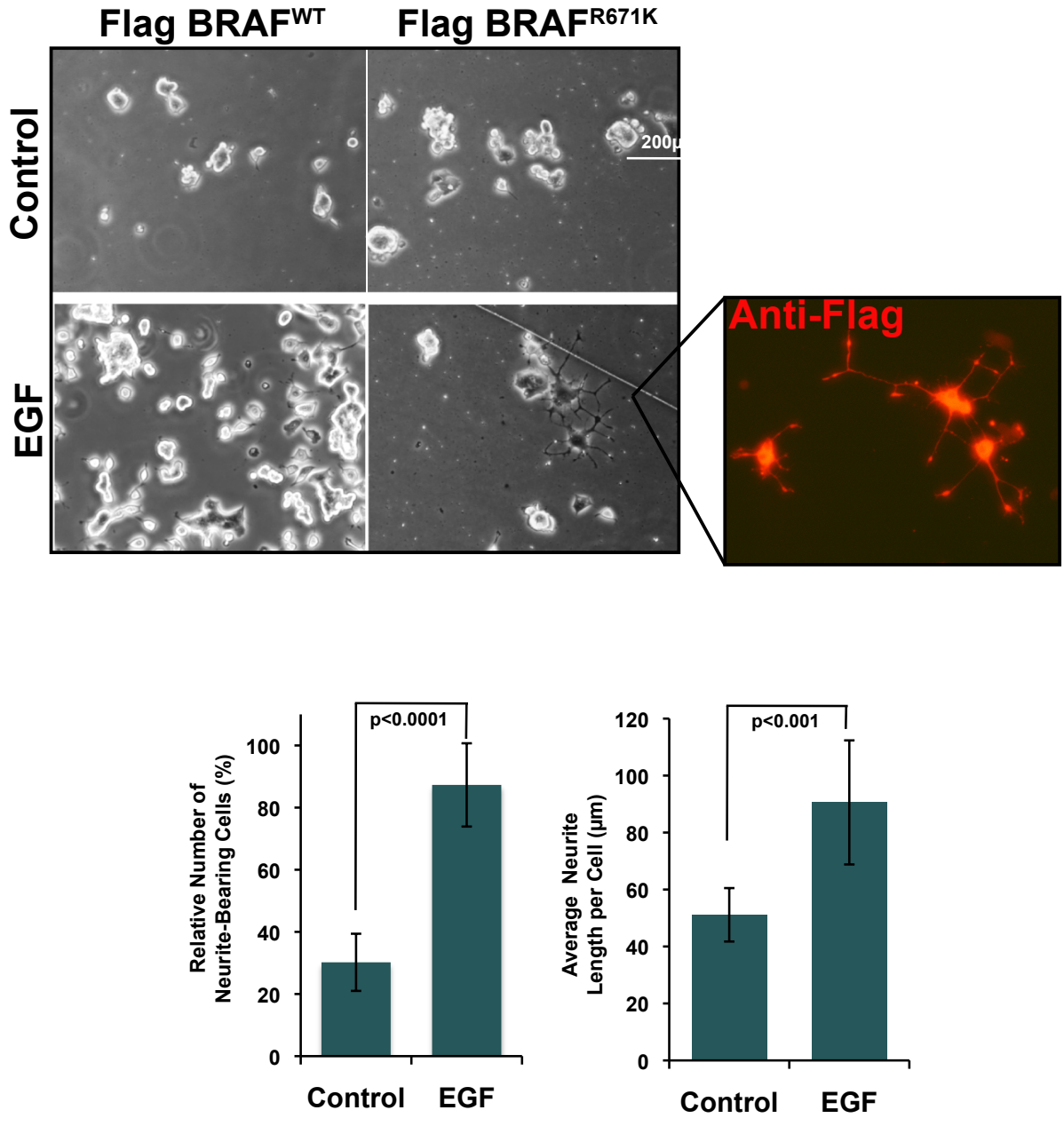


Figure S12. BRAF is in vivo methylated. BRAF^{R671K} mutant mimics the MTA-induced effects on PC12 cells. **(A)** PC12 cells transiently transfected with myc-*BRAF*^{R671K} mutant differentiate in response to EGF. Cells were transiently transfected with either myc-*BRAF*^{WT} (1.2µg) or myc-*BRAF*^{R671K} (0.2µg) as indicated. Then cells were treated with EGF (50ng/ml) for up to 96h. Representative pictures of untreated and treated cells are shown. Transfected cells were identified with an anti-myc antibody to detect myc-CRAF^{R671K} (red staining). Graphs show, quantification of neurite outgrowth in 100 transfected cells per treatment. Bars indicate the SD. *P*-values were calculated by a two-tailed Mann-Whitney test.

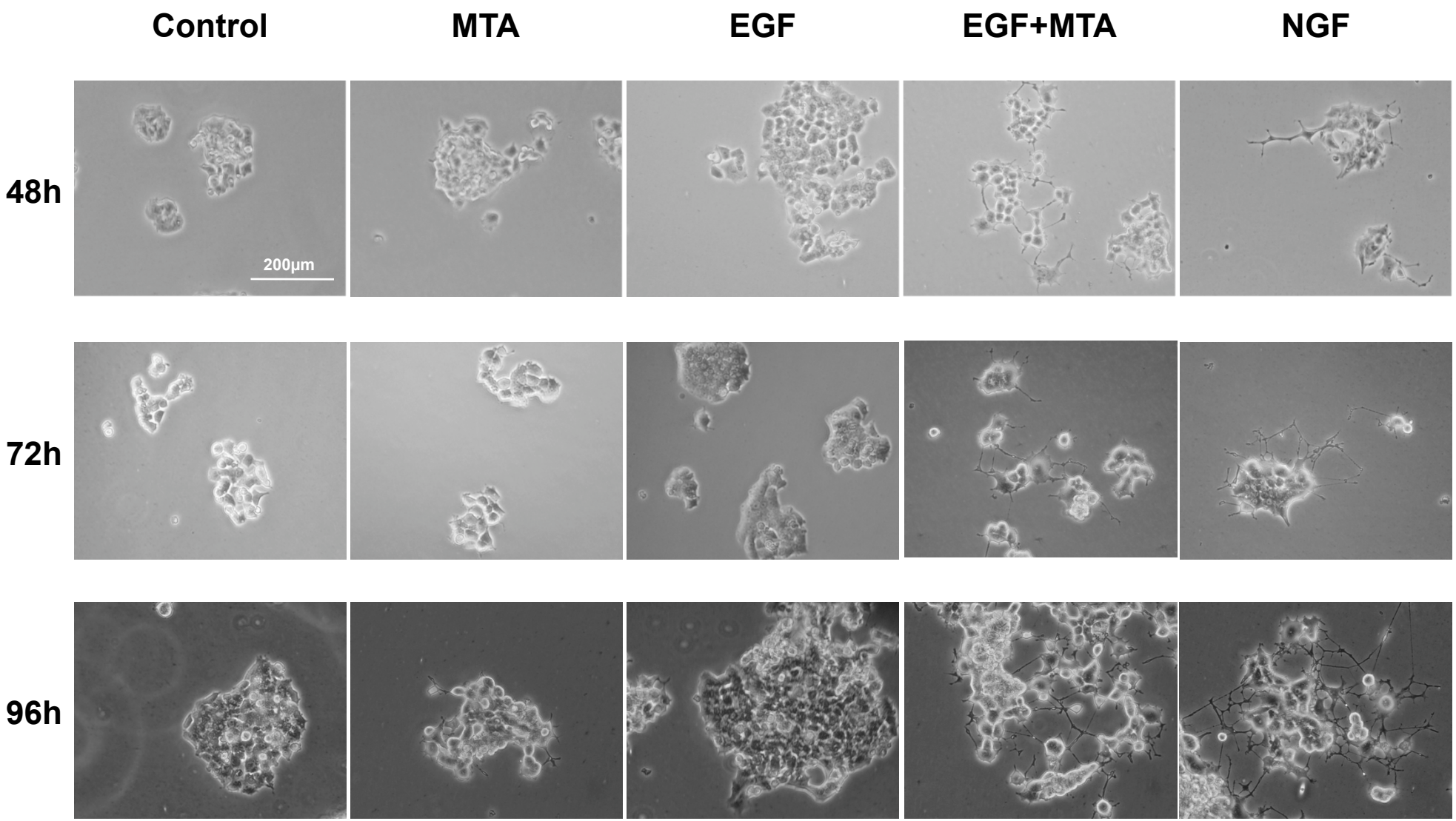


Figure S13. PC12 differentiation assay. Representative pictures of three different experiments performed in triplicates are shown at different time points with the different treatments: Control cells (serum-starved in 1% horse serum); 0.5mM MTA, 200ng/ml EGF; 200ng/ml EGF+0.5mM MTA; 100ng/ml NGF. Pictures were taken after 48, 72, or 96 hours treatment. Treatments were applied every other day.

Supplemental Methods:

Primers and siRNAs sequences:

Name	*Primer*	*Fragment*	*Reference*
PMRT1 f	GAGGCCGCGAACTGCATCAT		Scorilas, A et al. (2000) [3]
PMRT1 r	TGGCTTTGACGATCTTCACC	385 bp	Scorilas, A et al. (2000) [3]
PMRT3 f	GAACTGTCGGACAGCGGAGAC		From mouse sequence
PMRT3 r	GGTCATCTTCCAACACTGGCTT	322 bp	From mouse sequence
PMRT5 f	GCTGACATCATTGTCAGTGAGC		From mouse sequence
PMRT5 r	GGACCTCATTGTACAGCTTGGAG	169 bp	Richard, S et al. (2005) [4]
PMRT8 f	ATGGGCATGAAACACTCC		Lee, J et al. (2005) [5]
PMRT8 r	CTAACGCATTTTGTAGTC	1185 bp	From mouse sequence
GAPDH f	AGAAGACTGTGGATGGCCCC		Lau, A et al. (2004) [6]
GAPDH r	GCAACAGGGTGGTGGACCT	427 bp	Lau, A et al. (2004) [6]

Rat siRNAs PRMT5 (Dharmacon): siRNA1 Sense: CCAGCAGGCCAUCUAUAAAUU; Antisense: UUUAUAGAUGGCCUGCUGGUU
siRNA2 Sense: CUAGAGCGCUACAGAUGGAUU; Antisense: UCCAUCUGUAGCGCUCUAGUU
RT-PCR conditions: Conditions for GADPH and PRMT3: 95ºC for 45 sec; 95ºC for 45 sec; 67ºC for 60 sec; 72ºC for 60 sec (38 cycles). For PRMT8: 95ºC for 45 sec; 95ºC for 45sec; 55ºC for 60 sec; 72ºC for 60 sec (38 cycles). For PRMT1 and PRMT5: 95ºC for 45 sec; 95ºC for 45sec; 62ºC for 60 sec; 72ºC for 60 sec (35 cycles).

Supplemental references:

1. C. Abramovich, B. Yakobson, J. Chebath, M. Revel, A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *Embo J* **16**, 260 (1997).
2. Q. Liu, G. Dreyfuss, In vivo and in vitro arginine methylation of RNA-binding proteins. *Mol Cell Biol* **15**, 2800 (1995).
3. A. Scorilas, M. H. Black, M. Talieri, E. P. Diamandis, Genomic organization, physical mapping, and expression analysis of the human protein arginine methyltransferase 1 gene. *Biochem Biophys Res Commun* **278**, 349 (2000).
4. S. Richard, M. Morel, P. Cleroux, Arginine methylation regulates IL-2 gene expression: a role for protein arginine methyltransferase 5 (PRMT5). *Biochem J* **388**, 379 (2005).
5. A. Lau, R. Kanaar, S. P. Jackson, M. J. O'Connor, Suppression of retroviral infection by the RAD52 DNA repair protein. *Embo J* **23**, 3421 (2004).
6. J. Lee, J. Sayegh, J. Daniel, S. Clarke, M. T. Bedford, PRMT8, a new membrane-bound tissue -specific member of the protein arginine methyltransferase family. *J Biol Chem* **280**, 32890 (2005).