Supplementary Information

Garcia *et al*.: Regulation of human myoblast differentiation by hPEBP4

Supplementary Materials and Methods

Cell culture and transfections

SkMC cells (Clonetics) were maintained as undifferentiated myoblasts/satellite cells in SkBM medium (Clonetics) supplemented with hEGF (10μg/ml), fetuin (50mg/ml), bovine serum albumin (50mg/ml), insulin (10mg/ml), gentamycin-amphotericin and 2.5% foetal calf serum. HSMM cells (Clonetics) were maintained as undifferentiated myoblasts in SkBM-2 medium supplemented with foetal bovine serum (10%), gentamycin-amphotericin, hEGF (10μg/ml), dexamethasone and L-glutamine. To induce differentiation, cells were allowed to reach confluence then switched to Dulbecco's Minimal Essential Medium (DMEM) + 2% horse serum. COS-1, MCF7 and HEK293 cells were maintained in DMEM + 10% FCS. COS1 cell transfections were done using either Polyfect (Qiagen) or FuGENE (Roche). SkMC and MCF7 cells were transfected using Polyfect. HSMM myoblasts were transfected using the nucleofection kit R from Amaxa and programme T20. IGF-1 was from Sigma.

Plasmids

The CORK-1 cDNA (GenBank accession number AY730275) was subcloned as an EcoRI/NotI fragment from pT7T3 into pcDNA3. The coding region also was PCR-amplified and cloned into pcDNA3 with a Flag tag at the amino terminus. It is called Flag-hPEBP4 in this paper for consistency. pGEX-hPEBP4 and pGEX-hRKIP were cloned in pGEX-KG and pGEX-4T respectively. The baculovirus vector pbGST-MEK was described previously (Yeung et al., 2000).

RNA isolation, Northern blotting and RT-PCR

Total RNA was isolated from SkMC cells (Clonetics) using Trizol (Invitrogen) and checked for integrity by electrophoresis through agarose-formaldehyde gels. For profiling hPEBP4 mRNA tissue distribution, a Multiple Tissue Northern blot (Clontech) was probed with ^{32}P-labelled hPEBP4 or β-actin cDNA in ExpressHyb buffer (Clontech) as per manufacturers' recommendations. RT-PCR was carried out using standard protocols with oligonucleotides corresponding to the N- and C- termini of hPEBP4 or to a segment of the human GAPDH sequence. The forward and reverse primers used were 5'-ATG GGT TGG ACA ATG AGG

CTG-3' and 5'-CTA GCA GGC AGC TAT CTC CGC-3' and 5'-CAA CTA CAT GGT TTA CAT GTT C-3' and 5'-GCC AGT GGA CTC CAC GAC for hPEBP4 and GAPDH, respectively. All PCR reactions were carried out within the exponential phase of amplification as determined in separate experiments.

Reporter gene assays

The Gal-Elk and Gal-luc plasmids (Kortenjann et al., 1994) used for reporter assays were kindly provided by Dr. Peter Shaw. SkMC cells were seeded in 6-well plates at 100,000 cells per well 16 hours before transfection. 50ng of Gal-luc and 1μg of Gal-Elk plasmids were co-transfected together with the appropriate expression plasmids. The RSV-βgal plasmid was also co-transfected to monitor and normalise transfection efficiency. Luciferase activity in lysates was measured using Promega's luciferase assay system I as per manufacturer's recommendations in a TR717 microplate luminometer. All experiments were done in triplicates and in at least three independent experiments.

Antibodies

Anti-Raf C12 polyclonal, anti-MEK1 H-8 monoclonal, anti-HA F-7 monoclonal, anti-HA Y-11 polyclonal, anti-myogenin M-225 polyclonal and F5D monoclonal, anti-MCK N-13 polyclonal, anti-tubulin TU-02 polyclonal and anti-GST B14 monoclonal antibodies were from Santa Cruz Biotechnology. Phospho-p38 MAPK(Tyr180/Tyr182), anti-MEK1/2 polyclonal, anti-phospho-MEK1/2 polyclonal, anti-phospho-ERK1/2 polyclonal and anti-Akt polyclonal antibodies were from Cell Signalling. Anti-Raf monoclonal antibody was from BD Transduction Laboratories, anti-ERK1/2 polyclonal antibody and anti-Flag M2 monoclonal antibodies from Sigma; phospho-c-Jun(Ser 73) and anti-RKIP polyclonal antibody from Upstate and anti-Ras and anti-myc 9E10 from Calbiochem. Anti-mouse horse radish- peroxidase (HRP) and anti-rabbit HRP secondary antibodies were from Cell Signalling. The anti-myosin MF20 monoclonal antibody used for immunocytochemistry was obtained from the Developmental Hybridoma Bank, University of Iowa, Iowa City, IA, USA. The hPEBP4 antiserum was raised against a C-terminal 12mer peptide unique to hPEBP4. The antiserum was affinity purified using a peptide column to select hPEBP4 reactive antibodies.

Immunoprecipitations , immunoblotting, and Raf kinase assays

Cells were lysed in IP buffer containing 50mM TrisHCl pH 7.5, 15mM EGTA, 150mM NaCl, 0.1% (w/v) Triton X-100, 1mM PMSF, 1mM DTT, 10μg/ml leupeptin and 10μg/ml aprotinin.

Extracts were either incubated with antibody for 90 minutes on ice followed by addition of protein A or protein G slurry and incubated for a further 60 minutes at 4^{o}C, or simply incubated with antibody and protein A or protein G slurry overnight at 4^{o}C. Immunoprecipitates were collected by centrifugation, washed three times in IP buffer and analyzed by Western blot. For multiple probing Western blots were stripped by incubation in 3%SDS, 100mM glycine pH2.5, 10mM DTT for 3x5 minutes, extensively rinsed with water and re-equilibrated with blocking buffer (20mM TrisHCl pH 7.5, 150mM NaCl, 5% non-fat dry milk) before further use. At least $2x10^{6}$-10^{7} cells were used for endogenous co-immunoprecipitations. For the sequential immunoprecipitations of endogenous proteins, HSMM cell lysates were incubated for 18 hours at 4^{o}C with Raf-1 C12 rabbit antibody covalently conjugated to agarose beads (Santa Cruz, SC-133 AC). Bound Raf-1 was detached by competition with the cognate peptide antigen C12 (20μg/ml, Santa Cruz, SC-133 P) for 2x10 minutes at 21^{o}C. The eluate was immunoprecipitated with rabbit anti-MEK1/2 antibody (Cell Signalling) at 4^{o}C for 3 hours. Control immunoprecipitations used rabbit IgGs instead of Raf-1 or MEK antibodies. Raf-1 kinase activity was assayed using a linked assay as described previously (Dhillon et al., 2002). Briefly, Raf-1 proteins were immunoprecipitated and used in the first step to activate recombinant MEK and ERK. In the second step an aliquot of the reaction was incubated with myelin basic protein (MBP) in the presence of [^{32}P]-γ-ATP to measure the activity of ERK.

In vitro binding assays

FLAG-Raf-1 was immunoprecipitated from transiently transfected HEK293 cells using anti-FLAG M2 agarose beads (Sigma), and washed extensively with IP buffer. GST-MEK was prepared from Sf9 cells as described previously (Dhillon et al., 2002). hPEBP4 and RKIP were expressed as GST fusion products in bacteria, purified using glutathione sepharose (GE Healthcare) and released from their GST component by thrombin cleavage. Flag-Raf-1 beads were incubated with soluble GST-MEK in the presence of hPEBP4 or RKIP for 1 hour at 4^{o}C in IP buffer. After binding the beads were washed three times with IP buffer, and proteins bound to Raf-1 were visualised by Western blotting.

Fluorescence-activated cell sorting and immunocytochemistry

SkMC cells were seeded at low density and co-transfected with the plasmid DsRed mito (Clontech) and either pcDNA3, pcDNA3-hPEBP4, pSUPER or pSUPER hPEBP4 shRNA at a 1:5 ratio. Cells were grown for 36 to 48 hours and trypsinised before they were 80% confluent. DsRed positive cells were then isolated using a FACS Vantage SE cell sorter at an excitation

wavelength of 488nm. Sorted cells were grown to confluence overnight on 8-chamber glass slides then switched to differentiation medium. HSMM myoblasts were transfected with either pcDNA3 or pcDNA3-hPEBP4 using Amaxa nucleofection kit R. Cells were seeded at high density to achieve confluence overnight after which they were switched to differentiation medium. For immunocytochemistry experiments SkMC and HSMM were fixed with 2% formaldehyde for 15 minutes at room temperature, incubated with 100mM glycine/PBS for 15 minutes to quench excess formaldehyde, permeabilised with 0.1% Triton X-100/PBS for 5 minutes and blocked in Roche incubation buffer (PBS containing 0.5% BSA and 0.1% Tween 20) for 1 hour at room temperature. Cells were then incubated with either 1:100 diluted MF-20 antibody (anti-myosin heavy chain) or 1:100 diluted myogenin antibody for 30 minutes, washed in PBS three times for 5 minutes each, incubated with 1:200 diluted FITC-conjugated goat anti-mouse IgG for 30 minutes at 37^{o}C and washed in Roche buffer for 5 minutes, followed by 2x5 minute washes in PBS at room temperature. Nuclei were stained with DAPI and mounted using Vectashield. Cells were photographed using an Axioplan2 fluorescence microscope at 10x magnification.

RNA interference

The target region of shRNA was 5'-AAC AGC CCG TGT GCC CAT GAG GC-3' which corresponds to the sequon NSPCAHEA (amino acids 27-34) of the hPEBP4 sequence. The sense (5'-GATCCCCCAGCCCGTGTGCCCATGAGTTCAAGAGACTCATGGGCACA CGGGCTGTTTTTGGAAA-3') and antisense (5'-AGCTTTTCCAAAAACAGCCCGTGT GCCCATGAGTCTCTTGAACTCATGGGCACACGGGCTGGGG) oligonucleotides were annealed and cloned into the BglII and HindIII sites of pSUPER as described in Brummelkamp et al. (Brummelkamp et al., 2002). Knockdown was monitored by RT-PCR using oligonucleotides corresponding to the N- and C-termini of hPEBP4 (see above).

Bromodeoxyuridine (BrdU) staining

Cells were labelled by adding 10μM BrdU to the growth medium for 8 hours, and then fixed and stained with the anti-BrdU monoclonal antibody (BD Biosciences, Cat. No. 555627) according to the manufacturer's instructions.

Supplementary Figures

Figure S1. Relationship between CORK-1 and hPEBP4. (A) ***Sequence alignment between human hPEBP4 and RKIP.*** The signature sequence indicating the phosphatidylethanolamine binding (PEB) domain of the PEB protein family is underlined. **(B)** ***Sequence alignment between human CORK-1 and hPEBP4.*** Our clone is deposited in Genbank under the name of CORK-1 (Accession Number AY730275.1), but as it differs by only one amino acid from the previously published hPEBP4 sequence (Genbank Accession Number AY037148.1) (Wang et al., 2004) CORK-1 is most likely representing the same gene as hPEBP4. CORK-1 and hPEBP4 were aligned with the single amino acid change at 125 shown in red. Dots mark identical sequence.

Figure S2. hPEBP4 is mainly expressed in muscle cells. Northern blot analysis of hPEBP4 expression in human tissues.

Figure S3. hPEBP4 forms ternary complexes with Raf and MEK in MCF7 cells. Lysates from growing MCF7 cells were immunoprecipitated with Raf-1 C12 antibody (directed against the c-terminal 12 aa of Raf-1) or a control IgG. Both antibodies were covalently crosslinked to beads. Raf-1 protein was eluted from the antibody beads by competition with the synthetic C12 peptide (corresponding to the c-terminal 12 aa of Raf-1). The eluate was immunoprecipitated with a monoclonal MEK1 antibody and blotted with antibodies against Raf-1, MEK and hPEBP4 as indicated. The right panel shows that the MEK1 antibody specifically immunoprecipitates MEK, whereas an unrelated IgG control does not.

Figure S4. hPEP4 scaffolds the MEK-ERK interaction. COS-1 cells were transfected with the indicated plasmids. Co-immunoprecipitation experiments show that hPEBP4 can function as a scaffold for the MEK-ERK interaction.

Figure S5. Elk mediated reporter gene transcription in SkMC is Raf dependent. Gal-Elk luciferase activity was measured in growing SkMC cells transfected with the indicated plasmids. BXB and MEK-DD are constitutively active mutants of Raf-1 (Heidecker et al., 1990) and MEK (Eblen *et al.*, 2001), respectively. As indicated, cells were treated with 10μM U0126 overnight before measuring luciferase activity. The results show that in SkMCs (i) Elk induced

transcriptional activity is due to ERK activation; (ii) hPEBP4 functions upstream of MEK as activated MEK-DD abrogates its effects; and (iii) downregulation of hPEBP4 by siRNA activates Elk transcription in a MEK dependent manner (as U0126 inhibits).

Figure S6. hPEBP4 accelerates differentiation of human HSMM myoblasts. HSMM cells were nucleofected with either vector or pcDNA3-hPEBP4 and examined for extent of myotube formation after 4 days in differentiation medium (DM). **(A)** Cells were stained with an antibody against the myosin heavy chain (MyHC, green) to identify fully differentiated cells as in Fig. 4A. **(B)** ***hPEPB4 increases the number of myosin heavy chain expressing cells.*** Differentiation was quantified by counting the number of nuclei in MyHC positive cells versus the total number of nuclei (differentiation index). **(C)** ***hPEBP4 increases the number of multinucleated myotubes.*** MyHC positive cells were evaluated for the number of nuclei per myotube. Results shown in B) and C) are representative of two independent experiments with a total of >6000 cells counted from randomly chosen sections of the slides.

Figure S7. hPEBP4 overexpression promotes cell cycle arrest. SkMC cells were nucleofected with either vector or pcDNA3-hPEBP4 and examined for DNA synthesis by BrdU incorporation.

Figure S8. hPEBP4 binds but does not regulate Akt. (A) ***hPEBP4 associates with Akt.*** Upper panel: COS-1 cells were transiently transfected with HA-Akt and Flag-hPEBP4. Flag-immunoprecipitates were Western blotted (WB) for associated HA-Akt. Lower panel: Co-immunoprecipitation of endogenous Akt and hPEBP4 from SkMC cells. Association was only detectable in differentiating cells. Note that the expression of both hPEBP4 and Akt is increased during differentiation. **(B)** ***hPEBP4 can form ternary complexes with Raf-1 and Akt.*** COS-1 cells were transfected with GST-Raf-1 or GST (as control) plus Flag-hPEBP4 and HA-Akt. A ternary complex was isolated by sequential immunoprecipitation as in Fig.1C. First, GST-Raf-1 was pulled down with glutathione sepharose beads and eluted from the beads with glutathione. Then, the eluate was immunoprecipitated with Flag antibody and immunoblotted for the presence of Akt with HA antibody. **(C)** ***Akt inhibits Raf-1 kinase activity independent of hPEBP4 and Raf-1 S259 phosphorylation.*** MCF-7 cells were used because the inhibition of Raf-1 by Akt was described in these cells (Zimmermann and Moelling, 1999). MCF-7 cells were transiently transfected with Flag-Raf-1 (left panel), Flag-Raf-1 S259A (right panel), HA-Akt and different amounts of hPEBP4 in the indicated combinations. Cells were serum starved

overnight and treated with 10% foetal calf serum for 10 minutes. Where indicated cells were treated with 20μM inhibitors for PI3 kinase (LY294002; Calbiochem) or Akt (Akt IV; Calbiochem) one hour before stimulation. The activity of the inhibitors was validated by assessing Akt activation (data not shown). Flag-Raf-1 proteins were immunoprecipitated using Flag-agarose beads, and Raf-1 kinase activity was measured in a coupled kinase assay with myelin basic protein (MBP) as substrate. The uppermost panel shows an autoradiogram of MBP phosphorylation with a Western blot of immunoprecipitated Raf-1 proteins below. Both Raf-1 and the Raf-1 S259A mutant are inhibited by Akt co-expression, but this inhibition is not influenced by hPEBP4 or PI3K and Akt inhibitors. **(D)** ***Akt does not induce S259 phosphorylation of Raf-1.*** MCF-7 cells were transfected and treated as above except that IGF-1, a potent Akt activator, was used for stimulation (100ng/ml IGF-1, 5 minutes) instead of serum. Raf-1 immunoprecipitates were Western blotted with a phospho-specific S259 antibody. As previously reported growth factors decreased S259 phosphorylation (Dhillon et al., 2002), and this was not prevented or modulated by co-expression of Akt. hPEBP4 further decreased S259 phosphorylation, while Akt had no effect on that. **(E)** ***Induction of SkMC cell differentiation induces Akt activation but not Raf-1 phosphorylation on S259.*** Differentiation medium (DM) induced a robust activation of Akt, as visualised by staining with a phospho-specific Akt antibody, which was completely blocked by pre-treatment with 10μM LY294002. However, none of these conditions where Akt activity was elevated or blocked changed the phosphorylation of S259 (indicated by an arrowhead).

Figure S9. hPEBP does not modulate Akt activity. (A) ***hPEBP does not affect Akt activity in MCF7 cells.*** MCF7 cells were co-transfected with HA-Akt and increasing amounts of hPEBP4. Akt kinase activity was measured using the Akt kinase assay kit from Cell Signaling (Catalogue number #9840) with GSK-3 as substrate. **(B)** ***hPEBP does not affect Akt activity in SkMC cells.*** SkMC cells were transfected with hPEBP4 expression vectors or sRNA and both MEK and Akt activation were measured using phosphospecific antibodies.

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Garcia_ Supplementary Figure S1

A)

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hPEBP4      1  MGWTMRLVTAALLLGLMMVVTGDEDENSPCAHEALLDEDTLFCQGLEVFYPELGNIGCKV
hRKIP       1  -----MPVDLSKWSGPLSLQEVDEQP----------QHPLHVTYAGAAVDELG----KV

hPEBP4     61  VPDCNNYRQKITSWMEPIVKFPGAVDGATYILVMVDPDAPSRAEPRQRFWRHWLVTDIK
hRKIP      41  LTPTQVKNRPTS------ISWDGLDSGKLYTLVLTDPDAPSRKDPKYREWHHFLVVNMK

hPEBP4    120  GADLKEGKIQGQELSAYQAPSPPAHSGFHRYQFFVYLQEGKVIS----LLPKENKTRGS
hRKIP      94  GNDISSG----TVLSDYVGSGPPKGTGLHRYVWLVYEQDRPLKCDEPILSNRSGDHRGK

hPEBP4    175  WKMDRFLNRFHLGEPEASTQFMTQNYQDSPTLQAPRERASEPKHKNQAEIAAC
hRKIP     149  FKVASFRKKYELRAPVAGTCYQAEWDDYVPKLYEQLSGK--------------
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B)

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CORK-1      MGWTMRLVTAALLLGLMMVVTGDEDENSPCAHEALLDEDTLFCQGLEVFYPELGNIGCKV  60
hPEBP4      ............................................................  60

CORK-1      VPDCNNYRQKITSWMEPIVKFPGAVDGATYILVMVDPDAPSRAEPRQRFWRHWLVTDIKG 120
hPEBP4      ............................................................ 120

CORK-1      ADLKKGKIQGQELSAYQAPSPPAHSGFHRYQFFVYLQEGKVISLLPKENKTRGSWKMDRF 180
hPEBP4      ....E....................................................... 180

CORK-1      LNRFHLGEPEASTQFMTQNYQDSPTLQAPRERASEPKHKNQAEIAAC  227
hPEBP4      ...........................................  227
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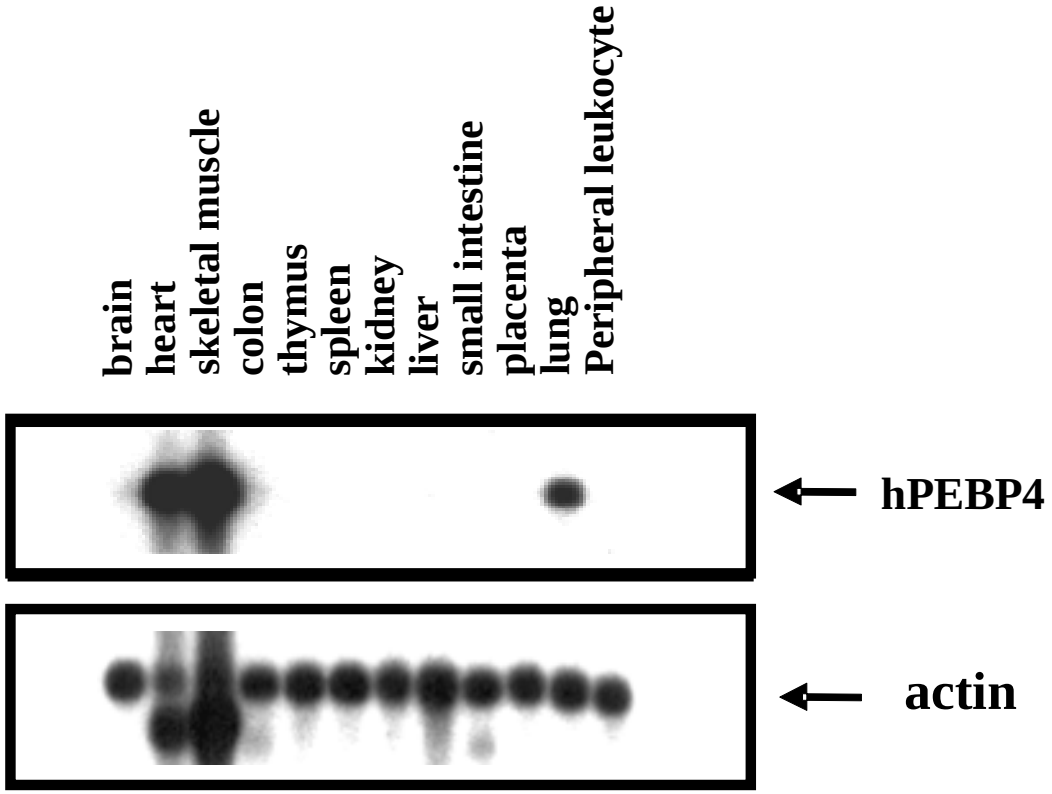
Garcia_Supplementary Figure S2



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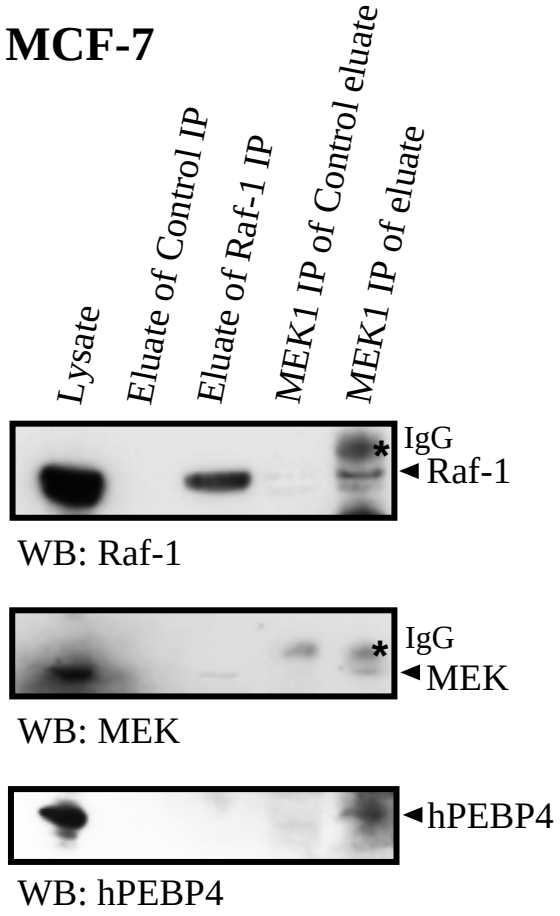

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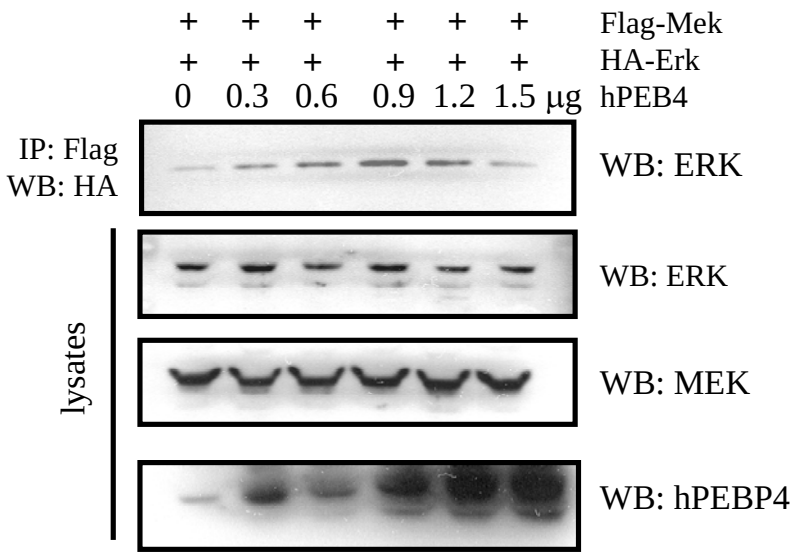

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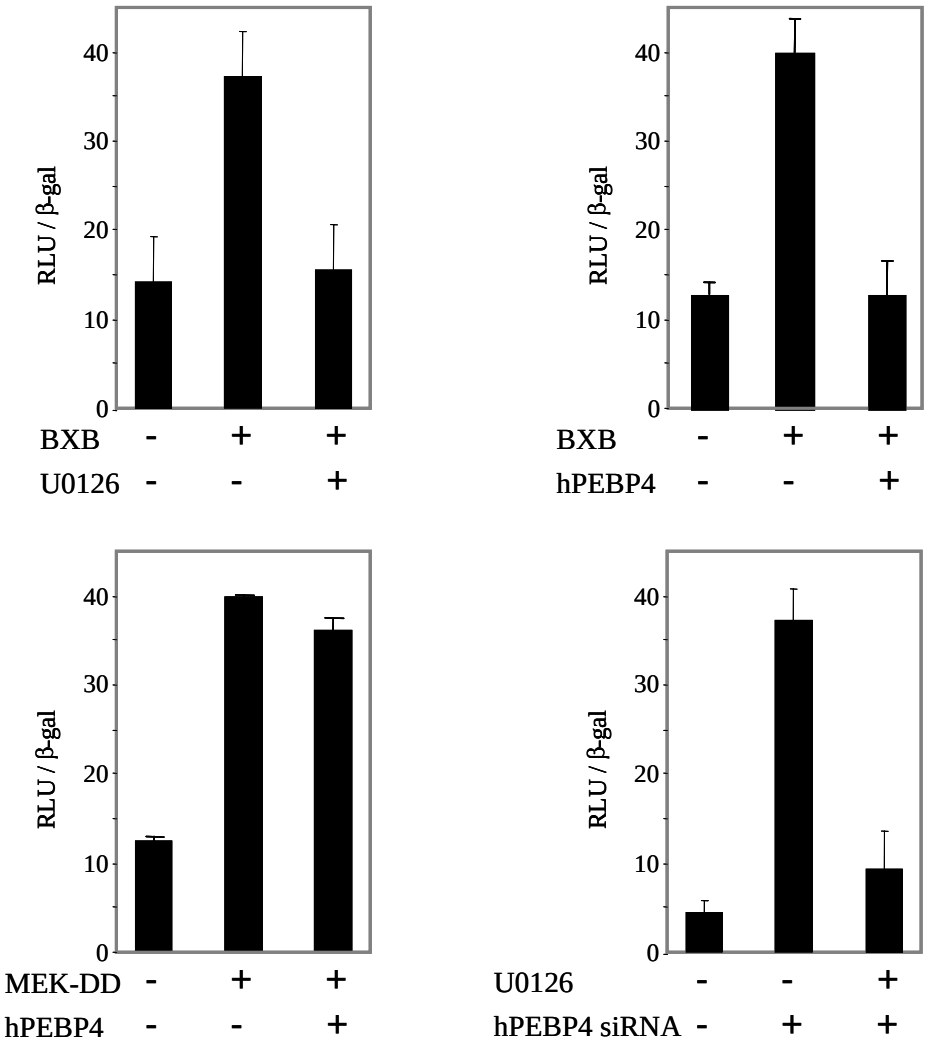

Garcia_Supplementary Figure S6

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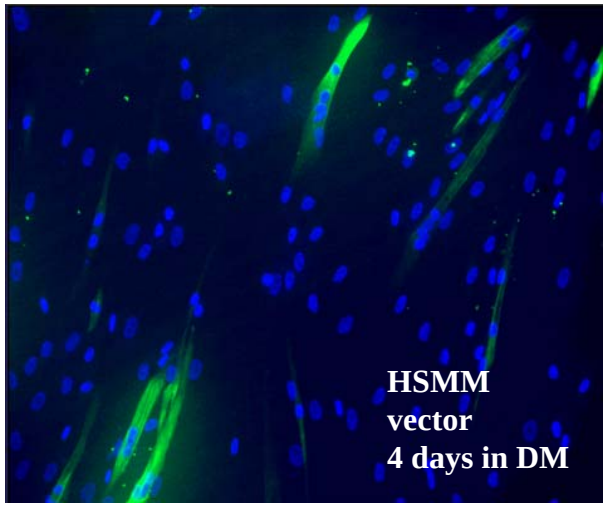

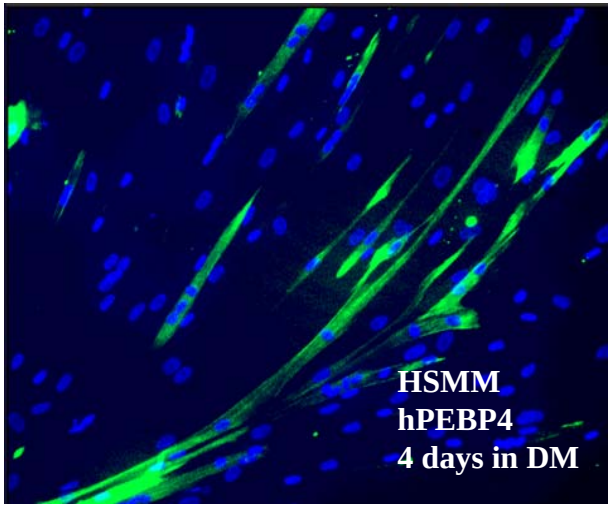

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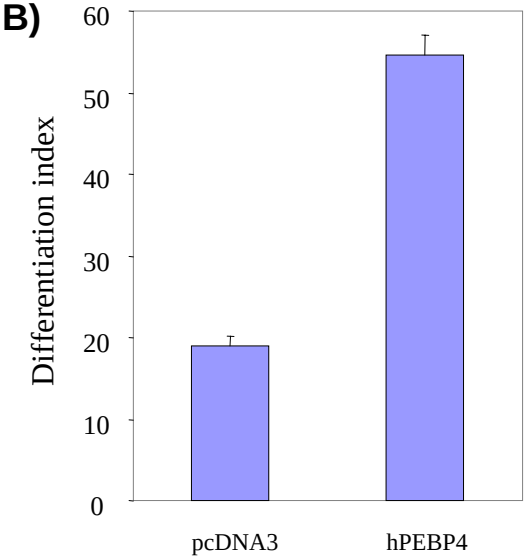

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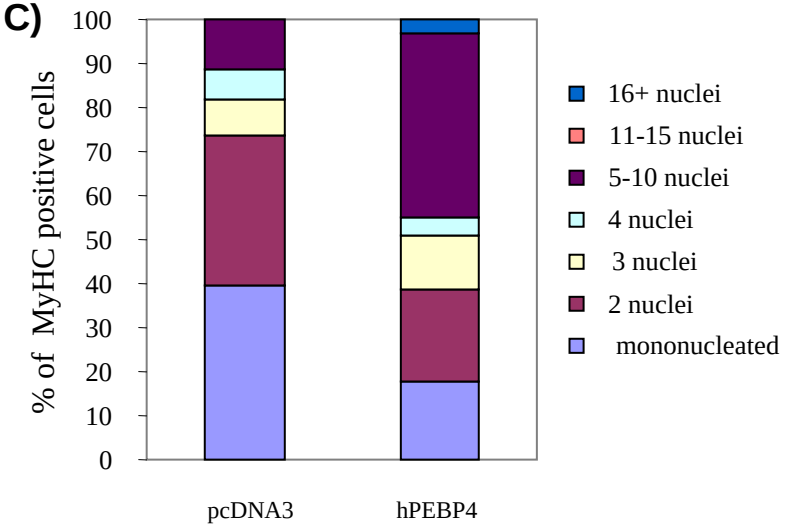
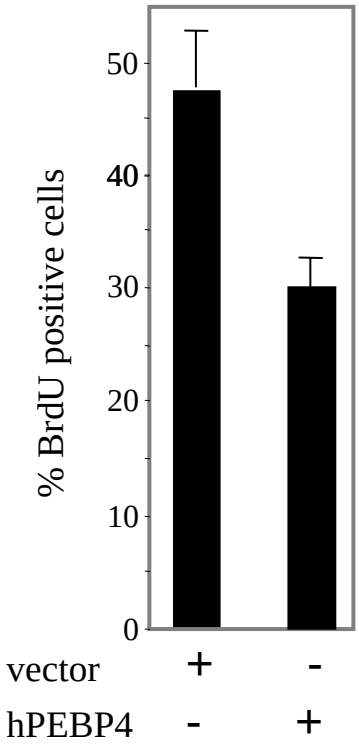
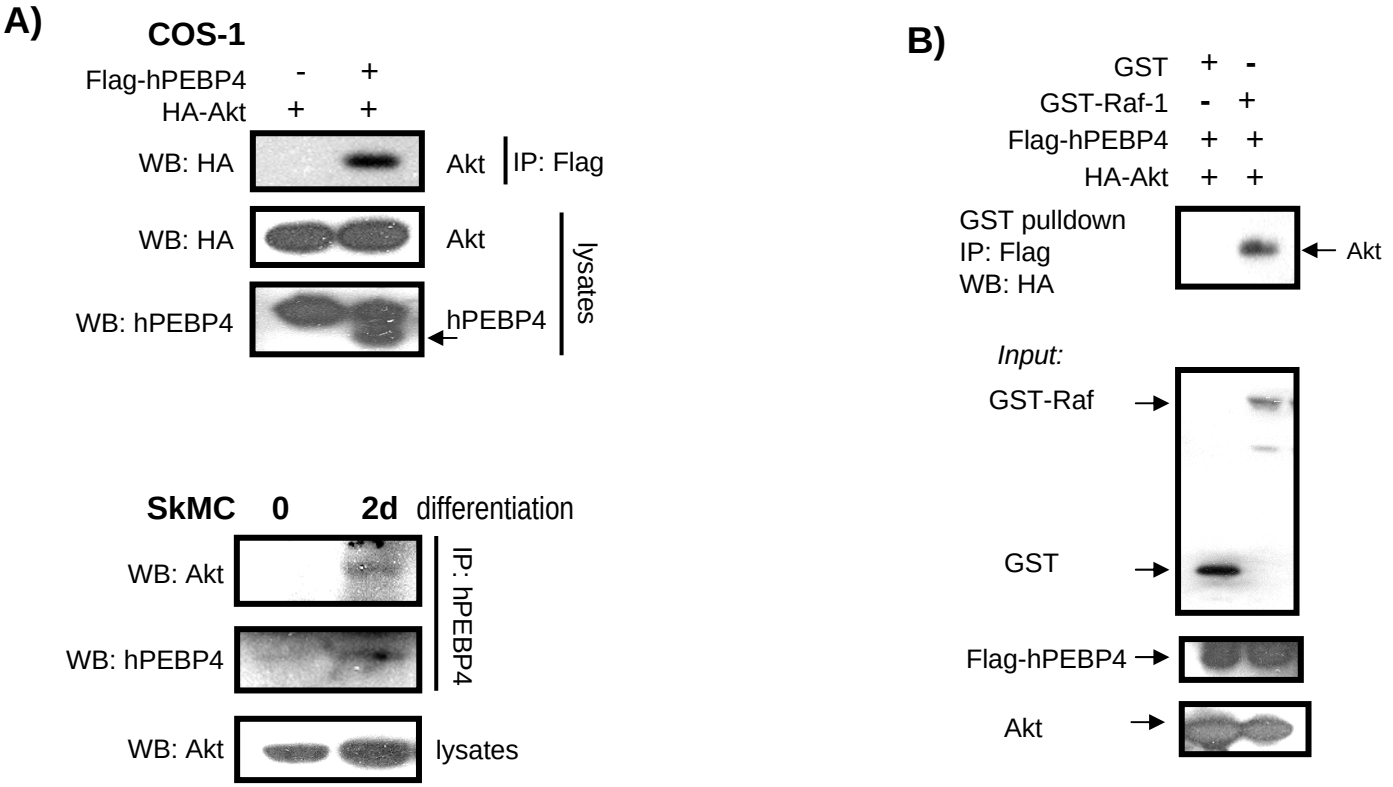


Garcia_Supplementary Figure S7



Garcia_Supplementary Figure S8



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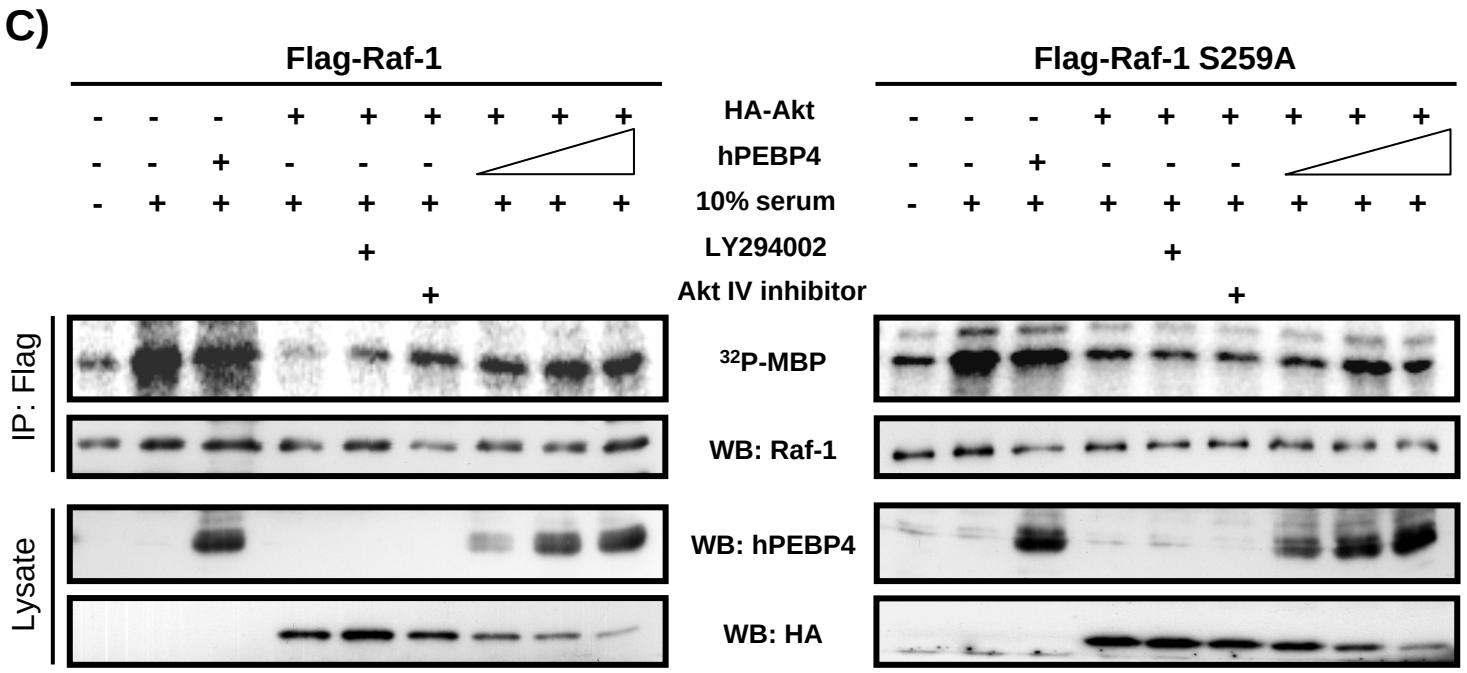

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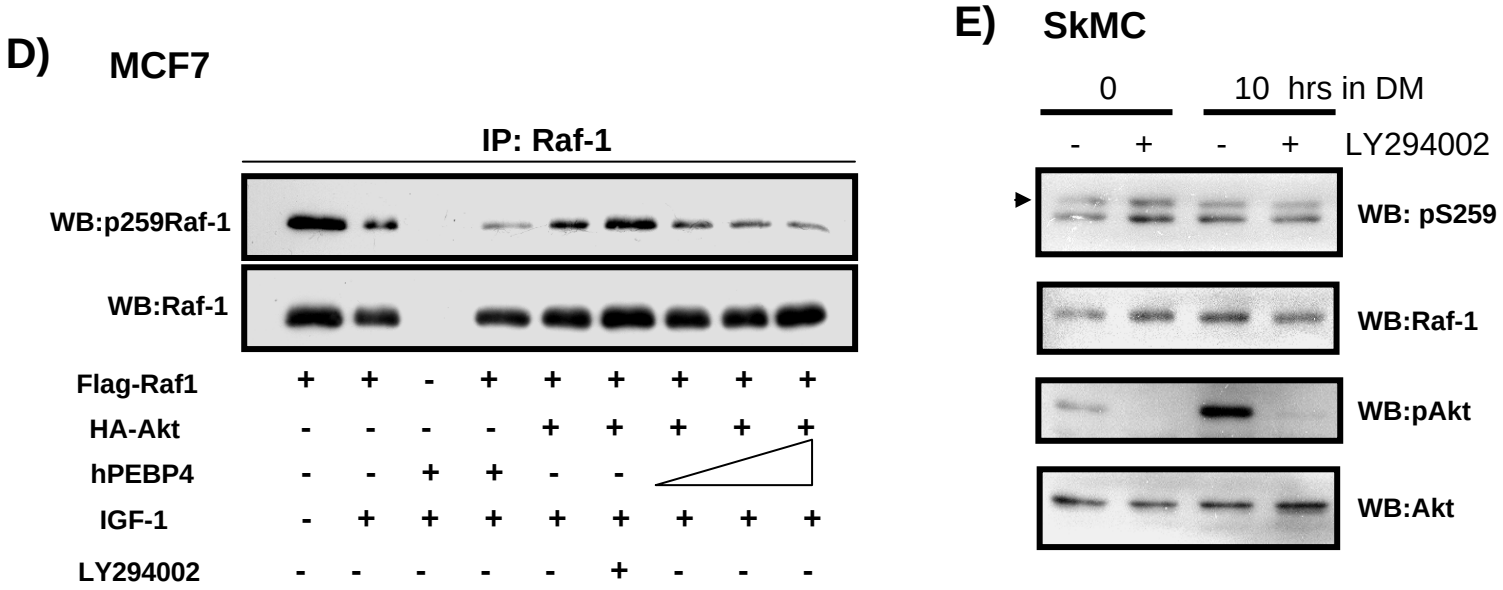
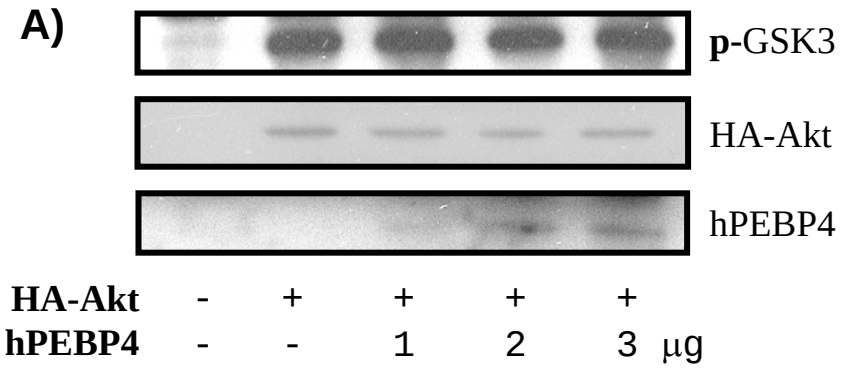


Garcia_Supplementary Figure S9



Garcia_Supplementary Figure S9

B) **SkMC**