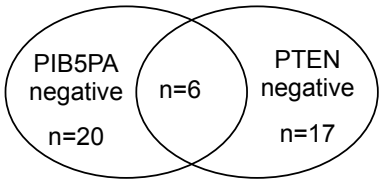
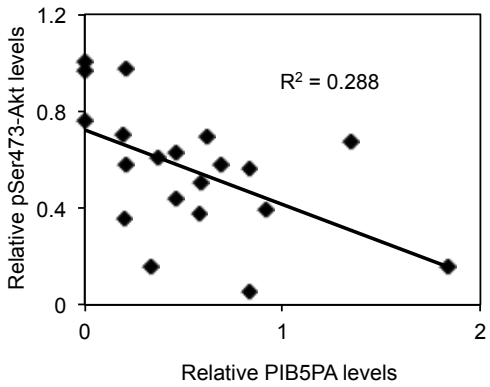
Supplementary Figures

PI(4,5)P2 5-phosphatase A regulates PI3K/Akt signalling and has a tumour suppressive role in human melanoma

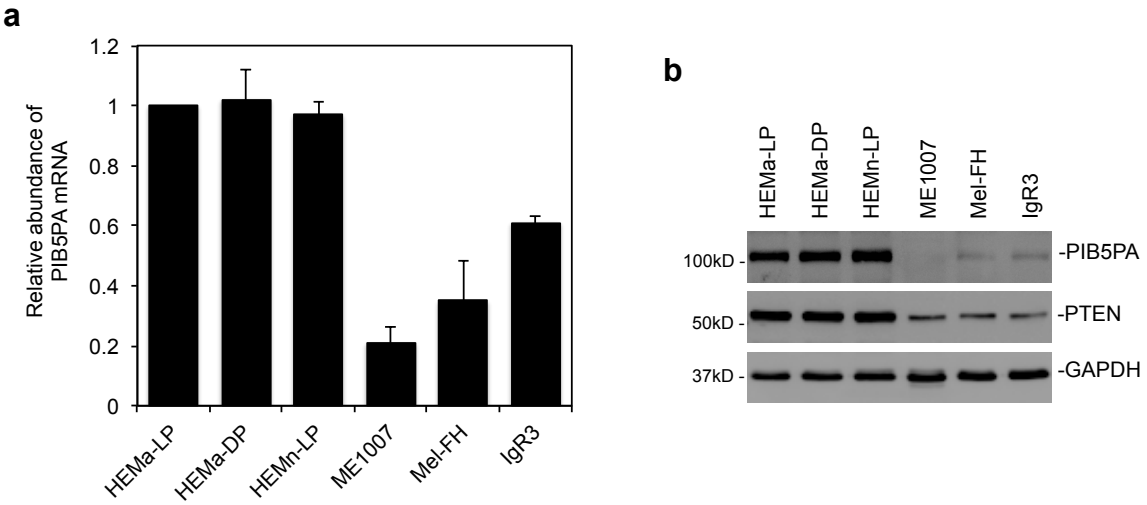
Yan Ye, Lei Jin, James S. Wilmott, Wang Lai Hu, Benafsha Yosufi, Rick F. Thorne, Tao Liu, Helen Rizos, Xu Guang Yan, Li Dong, Kwang Hong Tay, Hsin-Yi Tseng, Su Tang Guo, Charles de Bock, Chen Chen Jiang, Chun Yan Wang, Mian Wu, Lin Jie Zhang, Peter Hersey, Richard A. Scolyer, Xu Dong Zhang



Supplementary Figure S1. Loss of PIB5PA and/or PTEN in melanomas *in vivo*. Results were derived by quantitation of PIB5PA and PTEN positive melanoma cells on tissue sections as shown in Fig. 1a,b and Supplementary Table S1, and are depicted schematically.



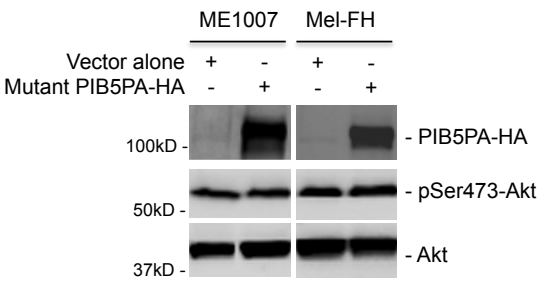
Supplementary Figure S2. There is no overall correlation between PIB5PA and pSer473-Akt levels in fresh melanoma isolates. Regression analysis of relationship between PIB5PA and PTEN expression as shown in Fig. 1c. Levels of PIB5PA and PTEN determined by Western blot were normalized to those of GAPDH. Quantitation of each band was determined using NIH Imager J.



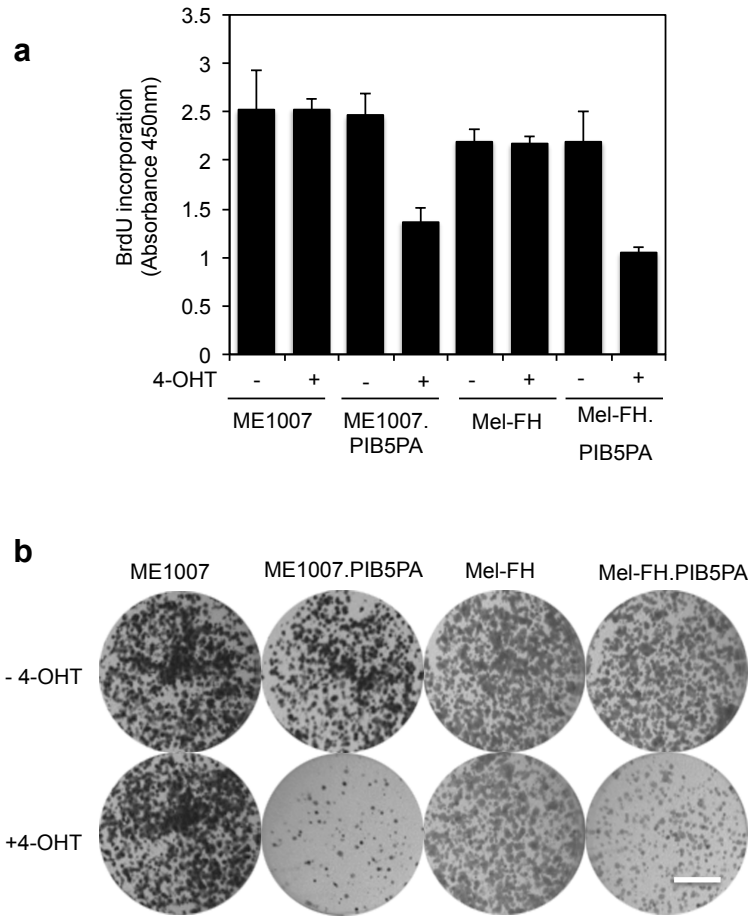
Supplementary Figure S3. Melanocyte lines of different origin (HEMa-LP, HEMa-DP, and HEMn-LP) express similarly higher levels of PIB5PA than melanoma cell lines.

(a). Total RNA from HEMa-LP, HEMa-DP, and HEMn-LP melanocytes and ME1007, Mel-FH, and IgR3 melanoma cells were subjected to qPCR analysis. The PIB5PA mRNA levels in HEMa-LP cells were arbitrarily designated as 1.

(b). Whole cell lysates were subjected to Western blot analysis. Data shown are representative of 3 individual experiments.



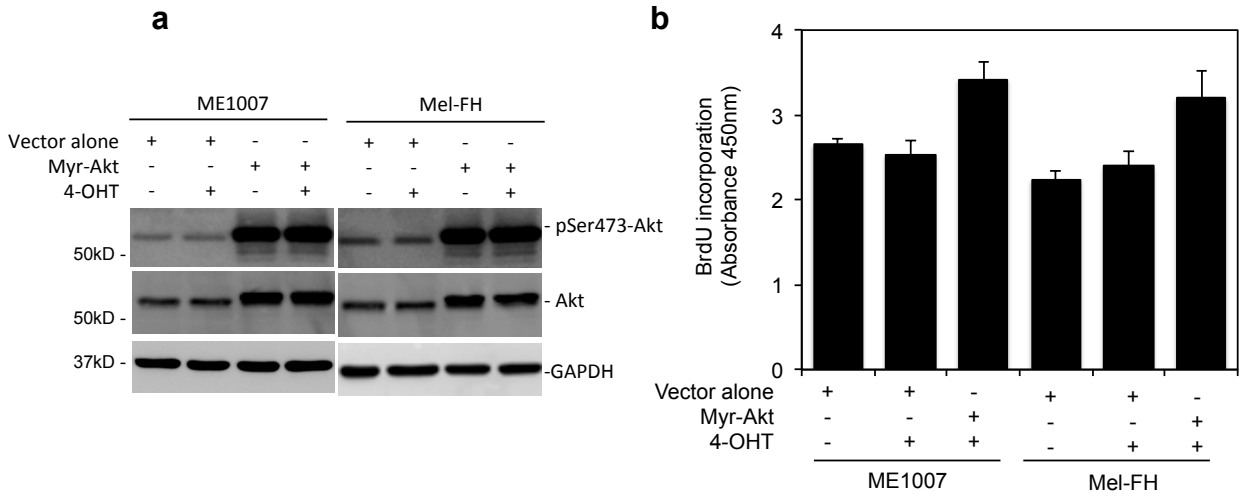
Supplementary Figure S4. Overexpression of a catalytically inactive PIB5PA (PIB5PA^{SKICH}) does not affect activation of Akt. Whole cell lysates from ME1007 and Mel-FH cells transfected with empty HA or mutant PIB5PA constructs were subjected to Western blot analysis of HA, pSer473-Akt, and total Akt. Data shown are representative of 3 individual experiments.



Supplementary Figure S5. Inducible PIB5PA inhibits melanoma cell proliferation.

(a). Mel-FH.PIB5PA, ME1007.PIB5PA, and their corresponding parental cells with or with out treatment with 4-OHT (10nM) for 72 hours were subjected to proliferation assays using the BrdU incorporation method.

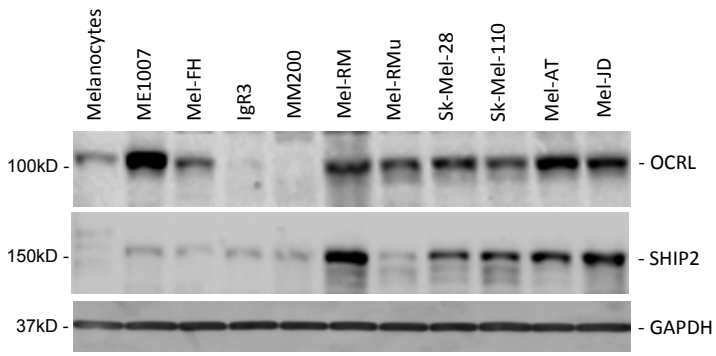
(b). Mel-FH.PIB5PA, ME1007.PIB5PA, and their corresponding parental cells were seeded onto 6-well plates (2000 cells/well) for 24 hours before the addition of 4-OHT (10nM). Twelve days later, cells were fixed with methanol and stained with crystal violet. Data shown are representative of 3 individual experiments. Scale bar, 1cm.



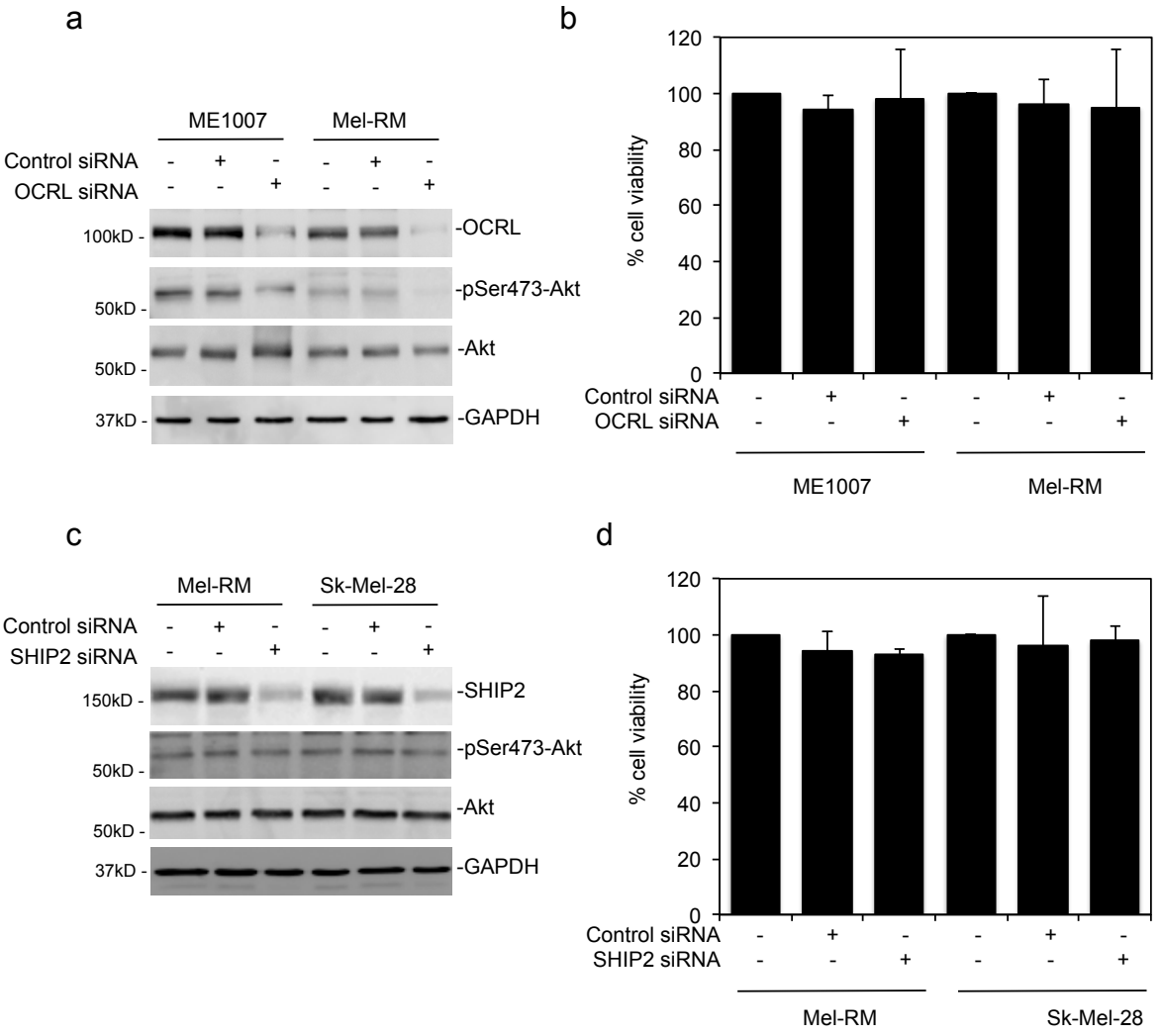
Supplementary Figure S6. Overexpression of myr-Akt moderately promote cell proliferation in Mel-FH and ME1007 cells.

(a). Whole cell lysates from ME1007 and Mel-FH cells transfected with vector alone or myr-Akt cDNA with or without treatment with 4-OHT (10nM) for 24 hours were subjected to Western blot analysis. Data shown are representative of 3 individual experiments.

(b). ME1007 and Mel-FH cells transfected with vector alone or myr-Akt cDNA with or without treatment with 4-OHT (10nM) for 24 hours were subjected to proliferation assays using the BrdU incorporation method. Data shown are mean ± SEM of 3 individual experiments.



Supplementary Figure S7. Western blot analysis showing that the 5-phosphatases OCRL and SHIP2 are generally expressed at higher levels in melanoma cell lines than cultured melanocytes. Data shown are representative of 3 individual experiments.



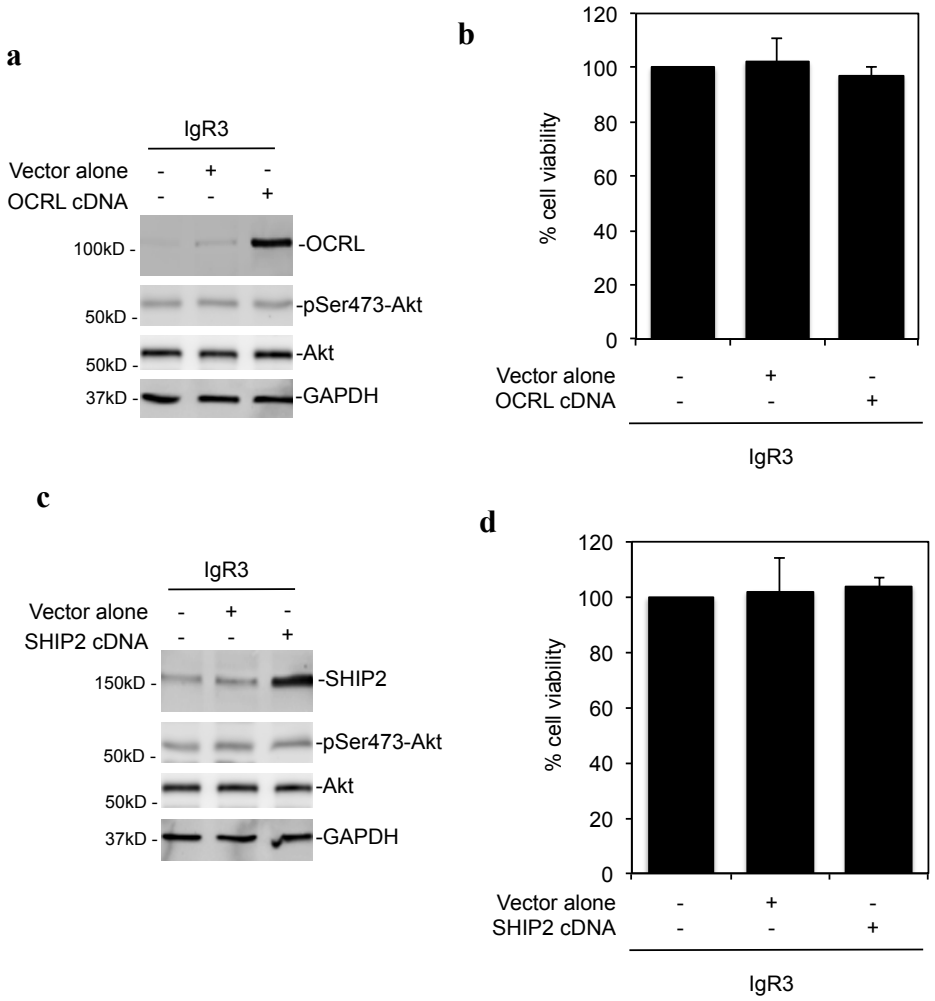
Supplementary Figure S8. Inhibition of OCRL or SHIP2 does not impinge on activation of Akt and cell viability in melanoma cells.

(a). Whole cell lysates from ME1007 and Mel-RM cells transfected with the control or OCRL siRNA were subjected to Western blot analysis. Data shown are representative of 3 individual experiments .

(b). ME1007 and Mel-RM cells were transfected with the control or OCRL siRNA. Seventy-two hours later, cells were subjected to MTS assays. Data shown are mean ± SEM of 3 individual experiments.

(c). Whole cell lysates from Mel-RM and Sk-Mel-28 cells transfected with the control or SHIP2 siRNA were subjected to Western blot analysis. Data shown are representative of 3 individual experiments.

(d). Mel-RM and Sk-Mel-28 cells were transfected with the control or SHIP2 siRNA. Seventy-two hours later, cells were subjected to MTS assays. Data shown are mean ± SEM of 3 individual experiments.



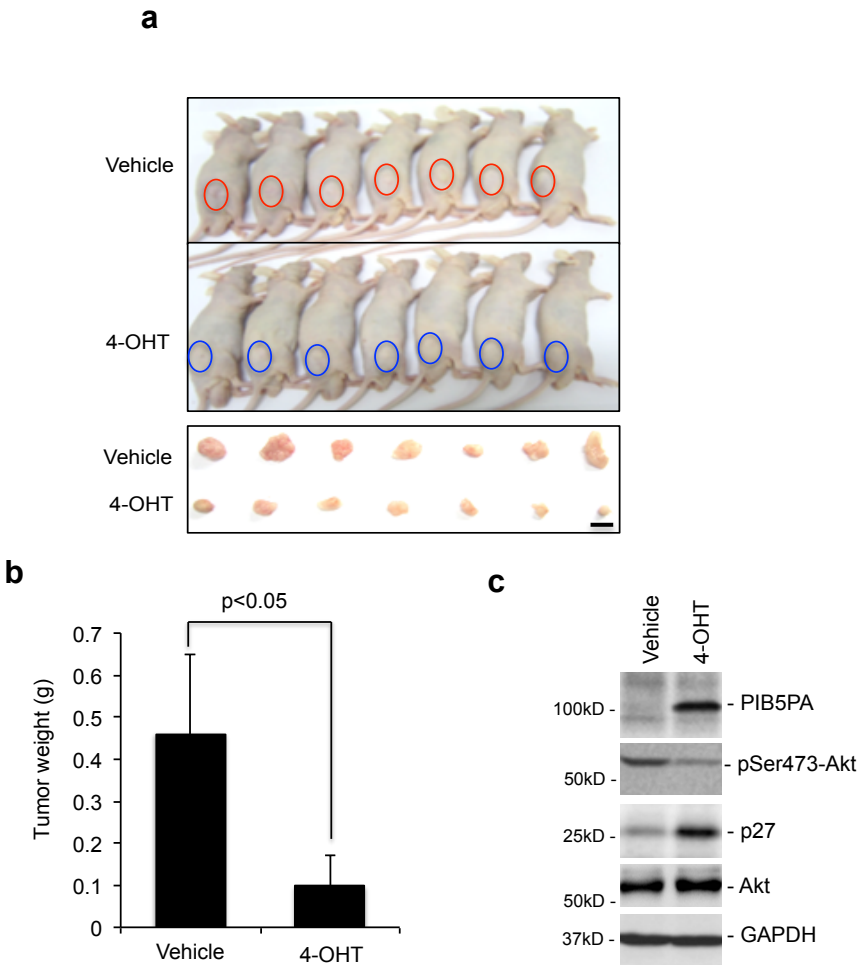
Supplementary Figure S9. Overexpression OCRL or SHIP2 does not impinge on activation of Akt and cell viability in melanoma cells.

(a). Whole cell lysates from IgR3 cells transiently transfected with vector alone or OCRL cDNA were subjected to Western blot analysis. Data shown are representative of 3 individual experiments .

(b). IgR3 cells were transiently transfected with vector alone or OCRL cDNA. Seventy-two hours later, cells were subjected to MTS assays. Data shown are mean ± SEM of 3 individual experiments.

(c). Whole cell lysates from IgR3 cells transiently transfected with vector alone or SHIP2 cDNA were subjected to Western blot analysis. Data shown are representative of 3 individual experiments .

(d). IgR3 cells were transiently transfected with vector alone or SHIP2 cDNA. Seventy-two hours later, cells were subjected to MTS assays. Data shown are mean ± SEM of 3 individual experiments.

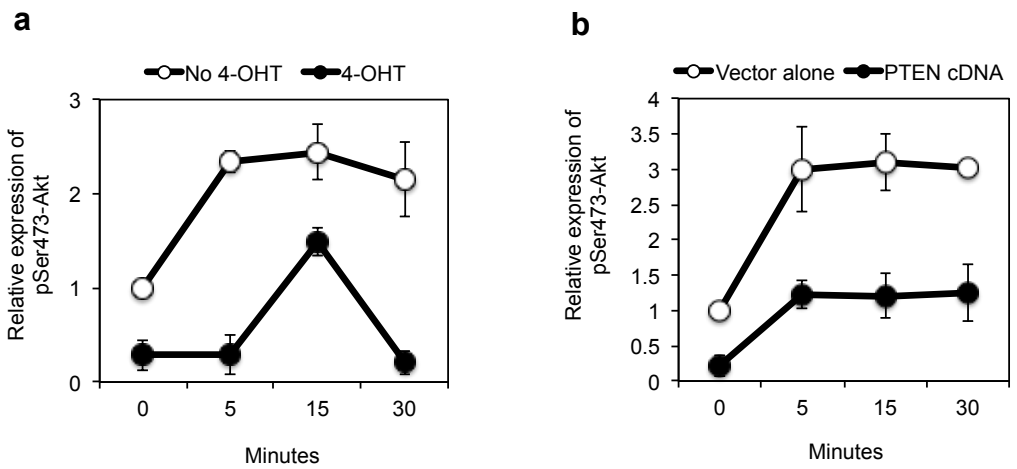


Supplementary Figure S10. Overexpression of PIB5PA inhibits melanoma tumour growth in a xenograft mouse model.

(a). Viable Mel-FH.PIB5PA cells (1×10^7) with or without pretreatment with 4-OHT (10nM) for 36 hours were subcutaneously injected into flanks of nu/nu mice. Two days later, mice were administered with either vehicle (DMSO) (n=7) or 4-OHT (10nM/g) (n=7) via i.p. injections every 3 days. Mice were euthanased and tumor harvested at 36 days after melanoma cell injection. Scale bar, 5mm.

(b). Comparison of weight of tumours from animals treated with 4-OHT and those treated vehicle control. Data shown are mean ± s.e.m. of 3 individual experiments.

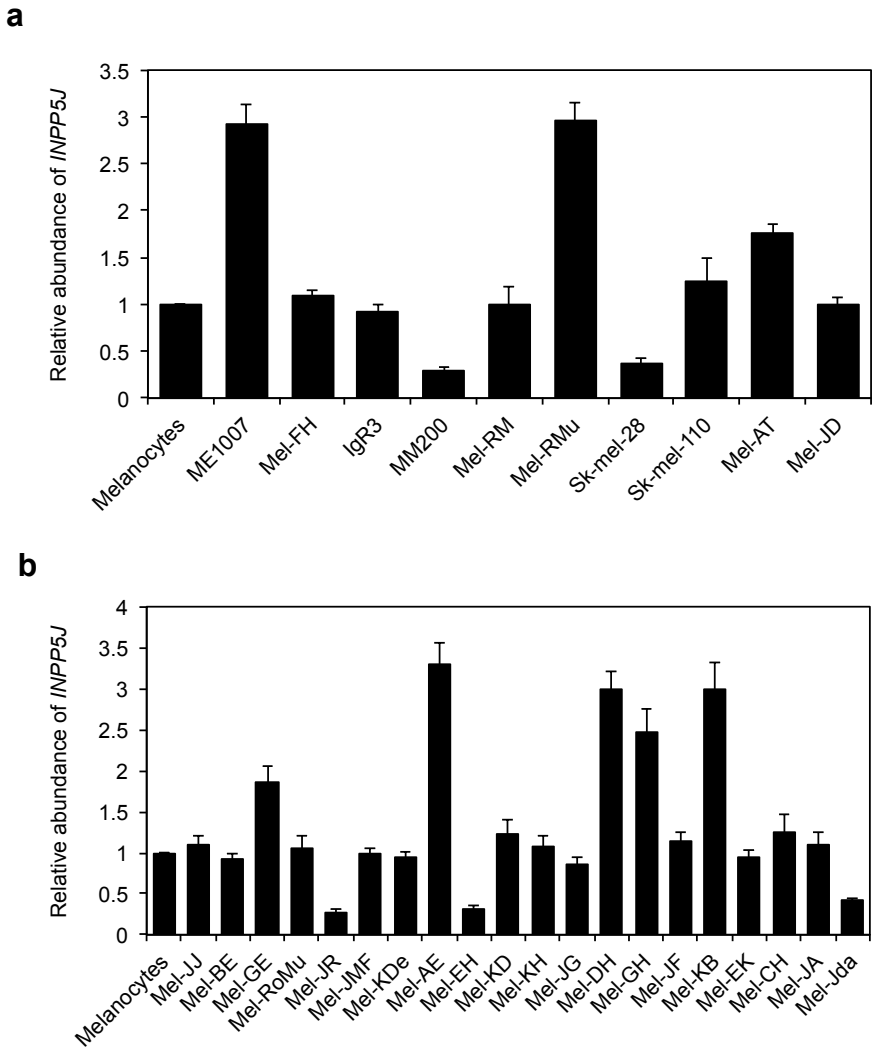
(c). Whole cell lysates of crude tumour tissues were subjected to Western blot analysis. Data shown are representative of 3 individual Western blot analyses.



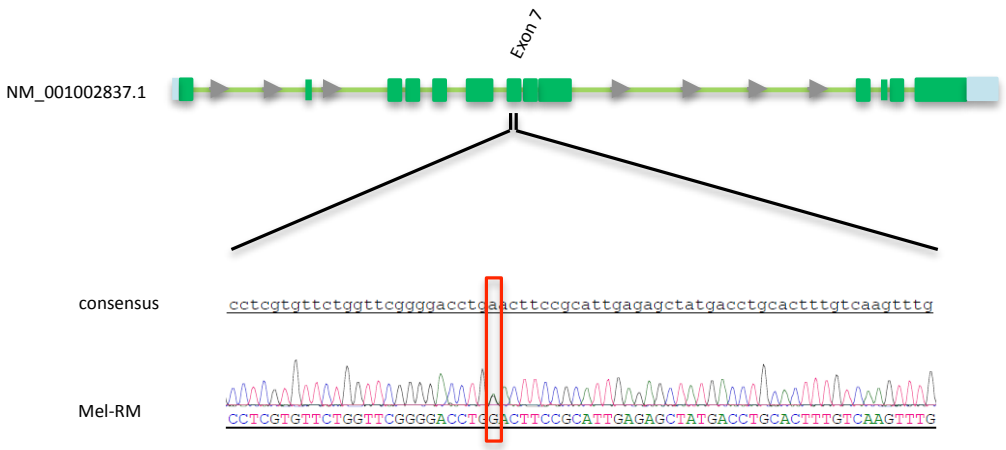
Supplementary Figure S11. Inhibition of Akt activation in response to EGF stimulation in ME1007 cells.

(a). ME1007.PIB5PA cells were serum-starved for 16 hours in the presence or absence of 4-OHT (10nM). Cells were then stimulated with EGF (100nM) for indicated periods. Whole cell lysates were subjected to Western blot analysis as shown in Fig. 6a. Levels of pSer473-Akt were quantitated and normalized to those of total Akt. Data shown are mean ± s.e.m. of 3 individual experiments.

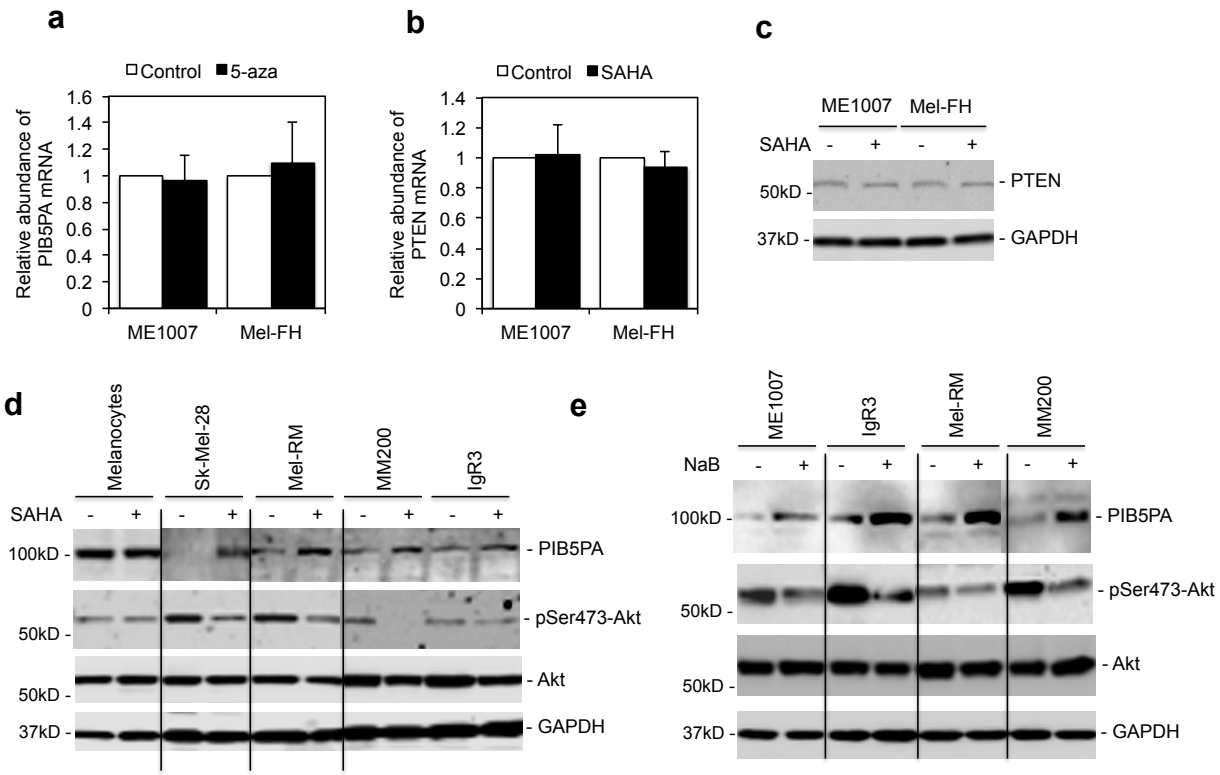
(b). ME1007 cells were transiently transfected with a PTEN construct. Twenty-four hours later, cells were serum starved for 16 hours followed by stimulation with EGF (100nM) for indicated periods. Whole cell lysates were subjected to Western blot analysis as shown in Fig. 6c. Levels of pSer473-Akt were quantitated and normalized to those of total Akt. Data shown are mean ± s.e.m. of 3 individual experiments. Quantitation of each band was determined using NIH Imager J.



Supplementary Figure S12 . Copy number variation of INPP5J in melanoma cell lines (**a**) and fresh melanoma isolates (**b**) as quantitated by qPCR analysis of genomic DNA.



Supplementary Figure S13. A schematic illustration of the PIB5PA gene INPP5J. G for A substation at exon 7 was found in 1 of 10 melanoma cell line (Mel-RM) by exon sequencing.



Supplementary Figure S14. The methylation inhibitor 5-aza does not impinge on the expression of PIB5PA, whereas inhibition of HDACs does not affect the expression of PTEN but up-regulates PIB5PA and decreases Akt activation.

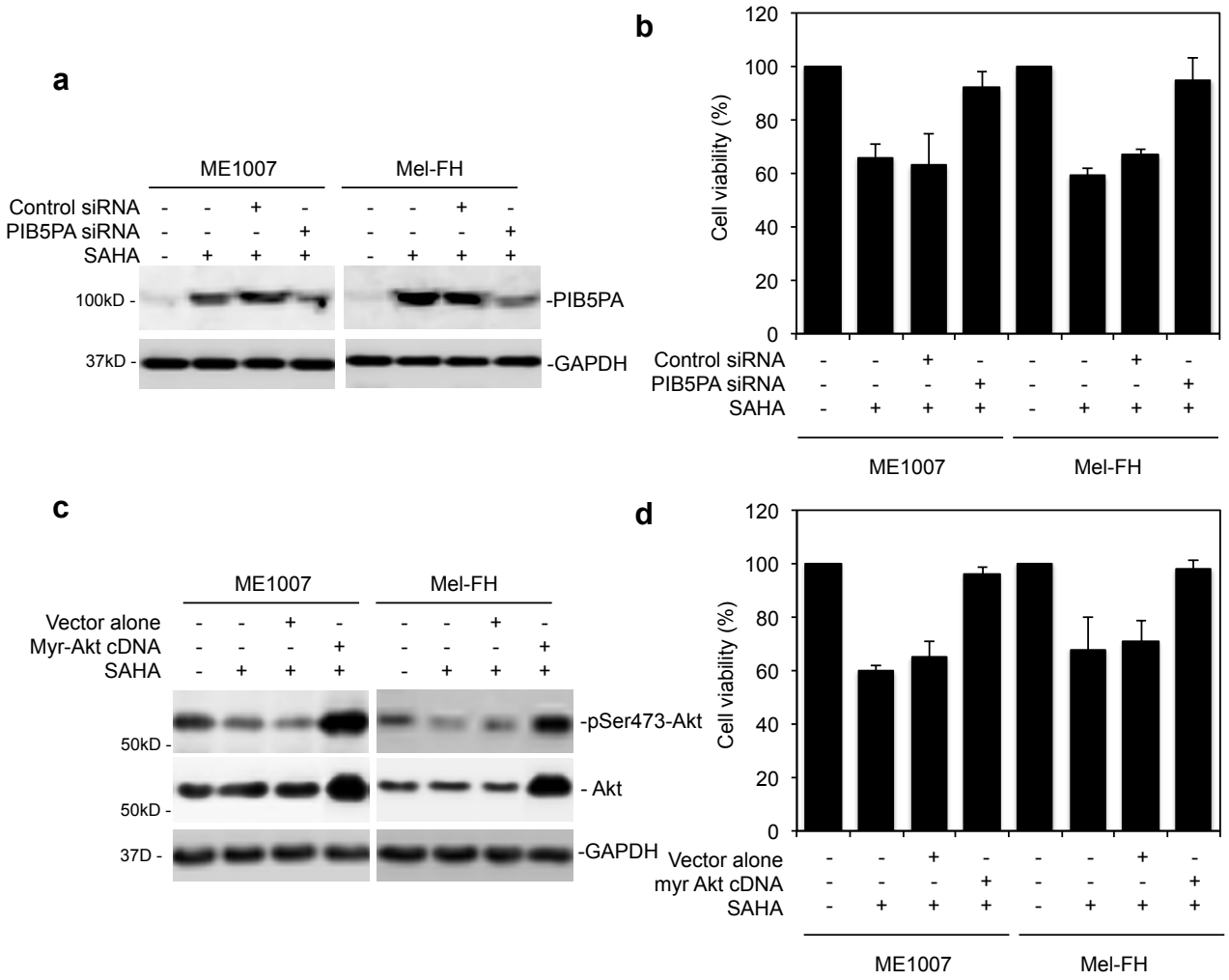
(**a**). Total RNA from ME1007 and Mel-FH cells treated with 5-aza (10μM) for 96 hours were subjected to qPCR analysis of PIB5PA mRNA expression. The relative abundance of PIB5PA mRNA in cells treated with 5-aza was normalized to that in cells treated with vehicle (DMSO). Data shown are mean ± s.e.m. of quantitation of 3 individual experiments.

(**b**). Total RNA from ME1007 and Mel-FH cells treated with SAHA (4μM) for 24 hours were subjected to qPCR analysis of PTEN mRNA expression. The relative abundance of PIB5PA mRNA in cells treated with 5-aza was normalized to that in cells treated with vehicle (DMSO). Data shown are mean ± s.e.m. of quantitation of 3 individual experiments.

(c). Whole cell lysates from ME1007 and Mel-FH cells treated with SAHA (4μM) for indicated periods were subjected to Western blot analysis of PTEN, and GAPDH (as a loading control). Data shown are representative of 3 individual experiments.

(d). Whole cell lysates from HEMa-LP melanocytes and melanoma cells treated with SAHA (4μM) for 24 hours were subjected to Western blot analysis of PIB5PA, pSer473-Akt, total Akt, GAPDH (as a loading control). Data shown are representative of 3 individual experiments.

(e). Whole cell lysates from melanoma cells treated with NaB (5mM) for 24 hours were subjected to Western blot analysis of PIB5PA, pSer473-Akt, total Akt, GAPDH (as a loading control). Data shown are representative of 3 individual experiments.



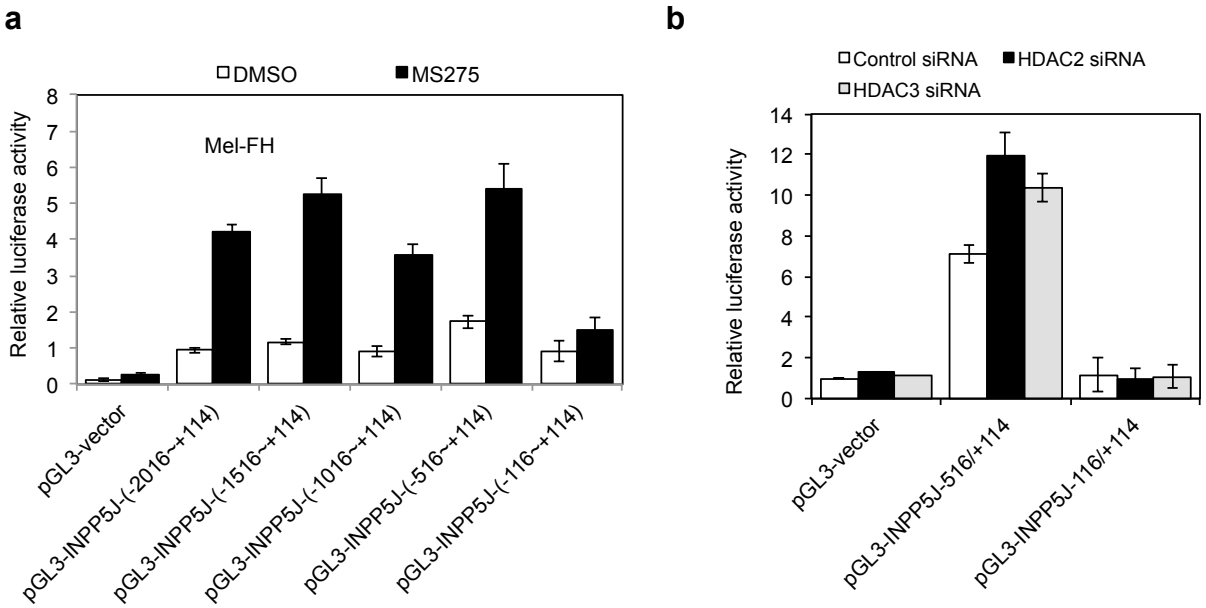
Supplementary Figure S15. Knockdown of PIB5PA or overexpression of myr-Akt reversed the inhibitory effect of SAHA on melanoma cell viability.

(a). Whole cell lysates from ME1007 and Mel-FH cells transfected with the control or PIB5PA siRNA with or without treatment with SAHA (4μM) for 24 hours were subjected to Western blot analysis. Data shown are representative of 3 individual experiments.

(b). ME1007 and Mel-FH cells transfected with the control or PIB5PA siRNA with or without treatment with SAHA (4μM) for 72 hours were subjected to MTS assays. Data shown are mean ± s.e.m. of quantitation of 3 individual experiments.

(c). Whole cell lysates from ME1007 and Mel-FH cells transiently transfected with vector alone of myr-Akt cDNA with or without treatment with SAHA (4μM) for 24 hours were subjected to Western blot analysis. Data shown are representative of 3 individual experiments.

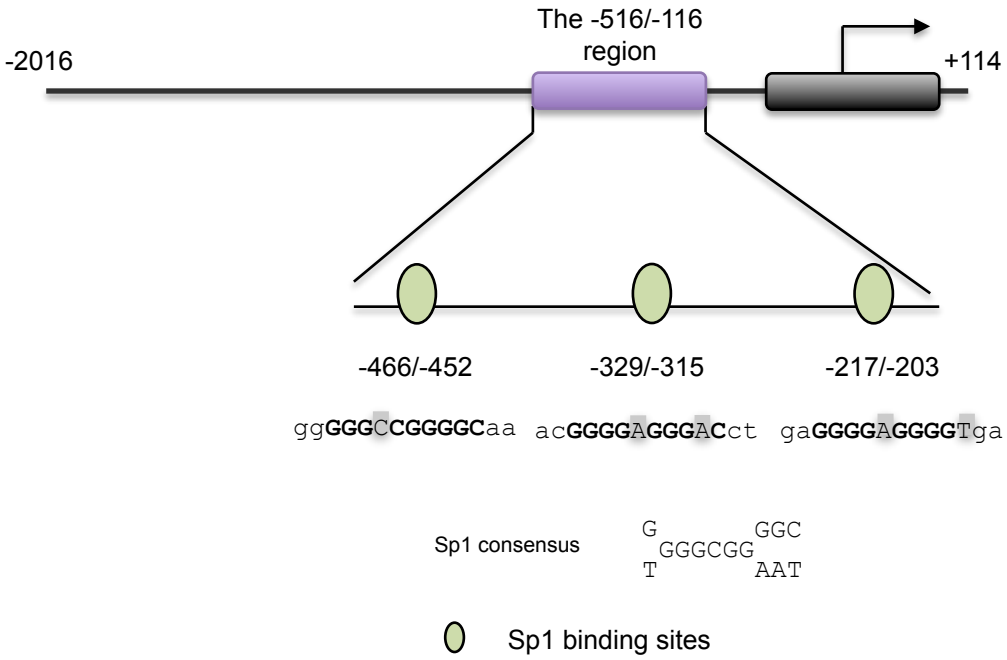
(d). ME1007 and Mel-FH cells transfected with vector alone or myr-Akt cDNA with or without treatment with SAHA (4μM) for 72 hours were subjected to MTS assays. Data shown are mean ± s.e.m. of quantitation of 3 individual experiments.



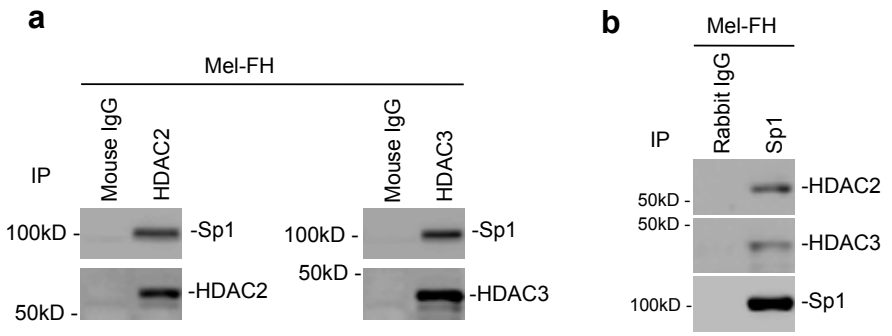
Supplementary Figure S16. HDAC2 and HDAC3 repress *INPP5J* transcription.

(**a).** Mel-FH cells were transiently transfected with indicated pGL3-basic based reporter constructs. Twenty-four hours later, cells were treated with MS275 (5μM) for a further 24 hours followed by measurement of the luciferase activity. Data shown are mean ± SEM of quantitation of 3 individual experiments.

(**b**). Mel-FH cells were co-transfected with the control, HDAC2, or HDAC3 siRNA and indicated pGL3-basic based reporter constructs. Luciferase activity was measured 24 hours after transfection. Data shown are mean ± s.e.m. of quantitation of 3 individual experiments.



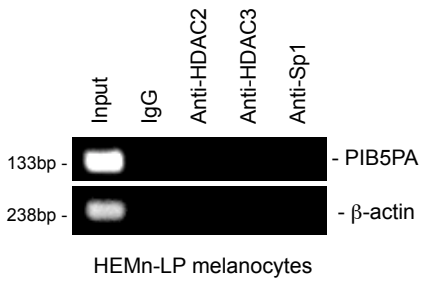
Supplementary Figure S17. A schematic illustration of the Sp1 binding site-enriched fragment(-466/-203) at the -516/-116 region of the *INPP5J* promoter.



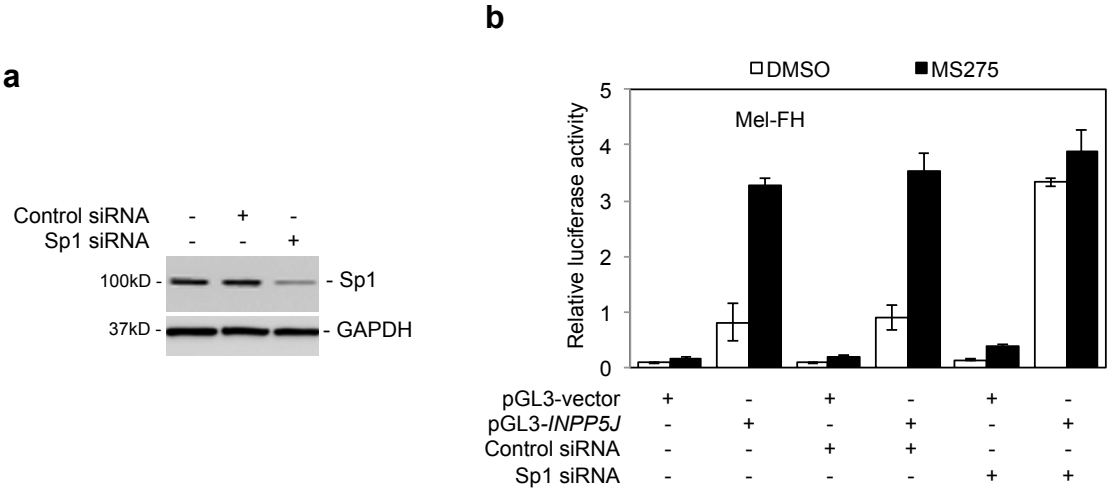
Supplementary Figure S18. HDAC2 and 3 are physically associated with Sp1 in Mel-FH cells.

(**a).** Whole cell lysates from Mel-FH cells were subjected to immunoprecipitation with an antibody against HDAC2 or HDAC3. The resulting precipitates were then subjected to Western blot analysis. Data shown are representative of 3 individual experiments.

(**b**). Whole cell lysates from Mel-FH cells were subjected to immunoprecipitation with an antibody against Sp1. The resulting precipitates were then subjected to Western blot analysis. Data shown are representative of 3 individual experiments.



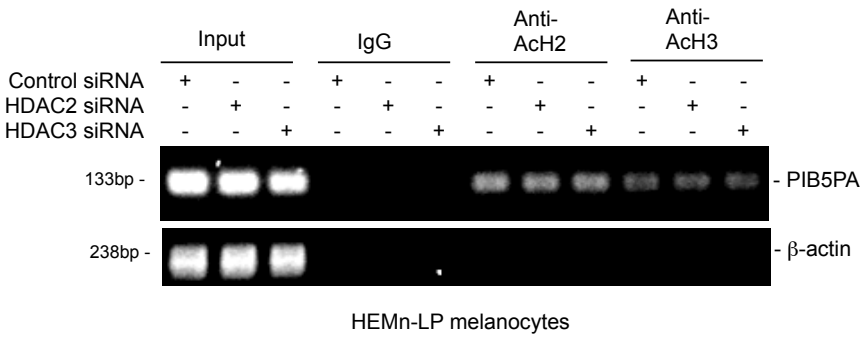
Supplementary Figure S19. ChIP assays demonstrated that there is no association of HDAC2 and 3 and Sp1 with the *INPP5J* promoter in HEMn-LP melanocytes. Formaldehyde-cross-linked chromatin of MEMn-LP melanocytes was subjected to immunoprecipitation with antibodies against HDAC2, HDAC3, or Sp1. The precipitates were subjected to PCR amplification using primers directed to the -516/-383 (133bps) fragment of the *INPP5J* promoter. Data shown are representative of 3 individual experiments.



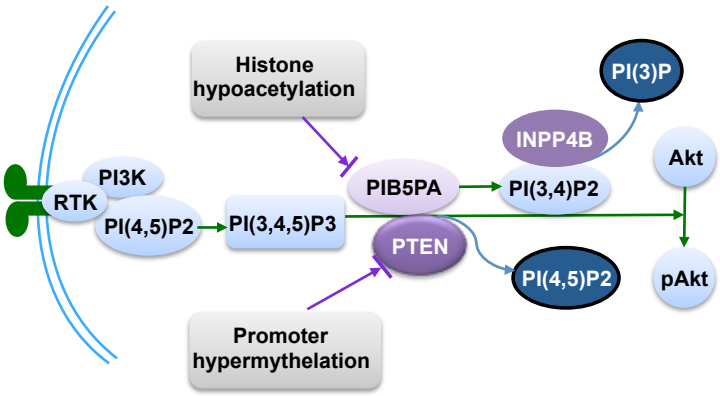
Supplementary Figure S20. Knockdown of Sp1 increases the INPP5J promoter activity that could not be further elevated by MS275.

(a). Western blotting showing siRNA knockdown of Sp1 in Mel-FH cells. Data shown are representative of 3 individual experiments.

(b). Mel-FH cells were co-transfected with the control or Sp1 siRNA and pGL3-vector or pGL3-*INPP5J*-2016/+144 (pGL3-*INPP5J*). Twenty-four hours later, cells were treated with MS275 (5μM) for a further 24 hours followed by measurement of the luciferase activity. Data shown are mean ± s.e.m. of quantitation of 3 individual experiments.



Supplementary Figure S21. ChIP assays show that knockdown of HDAC2 or 3 by siRNA does not alter acetylation of the H3 and H4 histone subunits associated with INPP5J promoter (-516/-383) in HEMn-LP melanocytes. Data shown are representative of 3 individual experiments.



Supplementary Figure S22. A schematic illustration of regulation of PI3K/Akt signaling by PIB5PA (and likely other 5-phosphatases). While PTEN that is supressed by its promoter hypermethylation at the epigenetic level terminates signaling downstream PI3K by hydrolysing PI(3,4,5)P3 to form PI(4,5)P2, PIB5PA that is suppressed by histone hypoacetylation hydrolyses PI(3,4,5)P3 to form PI(3,4)P2. The latter can also activates Akt, but is subjected to dephosphorylation by the 4-phosphatases, in particular, INPP4B.

Supplementary Tables

PI(4,5)P2 5-phosphatase A regulates PI3K/Akt signalling and has a tumour suppressive role in human melanoma

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Supplementary Table S1. Summary of melanocytic tumors and their positivity for PIB5PA, PTEN, and pSer473-Akt

Melanocytic tumors	Numbers	% PIB5PA positive melanoma cells	% PTEN positive melanoma cells	% pSer473-Akt positive melanoma cells
Compound nevus	10	97* (95-100)#	97 (70-100)	93 (60-100)
Dysplastic Naevus	10	98 (95-100)	98 (80-100)	95 (85-99)
Thin Primary (<1mm Breslow depth)	20	37 (0-95)	85 (0-100)	90 (40-100)
Thick Primary (>1mm Breslow depth)	20	43 (0-98)	64 (0-100)	94 (50-100)
Lymph Node Metastases	20	33 (0-100)	47 (0-100)	84 (0-100)
Distant Metastases	20	38 (0-100)	39 (0-100)	80 (5-100)

*Numbers stand for the mean of percentage positive cells.
♯Numbers in brackets represent the range of positive cells.

Supplementary Table S2. Summary of Spearman's Rho coefficient analysis of relationship between expression of PIB5PA, PTEN, and pSer473-Akt

	PIB5PA (IRS)	PTEN (IRS)	pS473-Akt (IRS)
PIB5PA (IRS)	1.000♯	0.373**	0.160
PTEN (IRS)	0.373**	1.000	0.209*
pS473-Akt (IRS	0.160	0.209*	1.000

♯Numbers represent correlation efficiency.
*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
IRS: immunoreactive score.