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Menin represses malignant phenotypes of melanoma through regulating multiple pathways

Shu-Bin Gao [a], Zi-Jie Feng [a], Bin Xu [a], Yan Chen [b, c], Hong-Hua Zheng [a], Ping Yin[c], Xianxin Hua [d], Guang-Hui Jin [a, *]

[a] *Department of Basic Medical Sciences, Medical College, Xiamen University, Xiamen, Fujian, China*
[b] *Department of Pathology, Fujian Medical University, Fuzhou, Fujian, China*
[c] *Department of Pathology, Affiliated Zhongshan Hospital, Xiamen University, Xiamen, Fujian, China*
[d] *Department of Cancer Biology, University of Pennsylvania, Philadelphia, PA, USA*

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Abstract

Substantial genetic evidence suggests that chromosome 11q is involved in regulating initiation and progression of malignant melanomas. Mutations of the *MEN1* gene, located in chromosome 11q13, predispose individuals to the multiple endocrine neoplasia type 1 (MEN1) familial syndrome. MEN1 patients develop primary malignant melanoma, suggesting a potential link between MEN1 syndrome and development of melanomas, but the precise molecular mechanism is poorly understood. Here we show that the *MEN1* gene suppresses malignant phenotypes of melanoma cells through multiple signalling pathways. Ectopic expression of menin, the product of *MEN1* gene, significantly inhibited melanoma cell proliferation and migration *in vitro* and *in vivo*. The inhibition was partly achieved through suppressing expression of growth factor pleiotrophin (PTN) and receptor protein tyrosine phosphatase (RPTP) β/ζ, accompanied with the reduced expression of phosphatidylinositol 3-kinase (pI3K) and decreased phosphorylation of focal adhesion kinase (FAK) and extracellular signal regulated kinase (ERK1/2). Interestingly, reduced expression of menin was associated with hypermethylation of the CpG islands of the *MEN1* promoter in melanoma cells. Taken together, these findings suggest a previously unappreciated function for menin in suppressing malignant phenotypes of melanomas and unravel a novel mechanism involving in regulating PTN signalling by menin in development and progression of melanomas.

Keywords: melanoma • menin • pleiotrophin • RPTP β/ζ

Introduction

The *MEN1* gene is located on chromosome 11q13, which is mutated in patients with an inherited tumour syndrome, multiple endocrine neoplasia type 1 (MEN1) [1]. *MEN1* knockout mice develop parathyroid, pancreatic, pituitary and adrenal tumours, mimicking human MEN1 syndrome, indicating that *MEN1* as a bona fide tumour suppressor gene in endocrine tumours [2]. Recently, several reports have demonstrated that *MEN1* is associated with non-endocrine tumours. For instance, menin has been shown to associate with trxG family proteins in a histone methyltransferase complex including trxG proteins MLL (mixed lineage leukaemia), retinoblastoma binding protein 5, WD repeat domain 5 and ASH2 (absent, small or homeotic) and promote histone 3 Lysine 4 (H3K4) methylation at the promoter of target genes [3, 4], and it is required for maintenance of Hox family gene expression, and initiation of MLL-mediated leukemogenesis and myeloid transformation [3, 5, 6]. Recently, we have found that menin represses PTN transcription through Polycomb gene-mediated trimethylation of H3K27 and development of lung adenocarcinoma [7].

Malignant melanoma is the deadliest form of skin cancer, which is an increasing worldwide health problem because of its highly aggressive and drug-resistant nature [8]. Recent advances in understanding development and maintenance of melanoma provide novel insights into the molecular mechanisms. Kit/stem-cell factor (SCF) signalling and Mitf-dependent transcription is essential for melanoma initiation and development [8]. Disruption of Mitf in

*Correspondence to: Guang-Hui JIN,
Department of Basic Medical Sciences, Medical College, Xiamen University, Daxue road 168, Xiamen, Fujian 361005, China.
Tel.: +86-592-218-6173
Fax: +86-592-218-1535
E-mail: ghjin@xmu.edu.cn

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melanocytes or melanoma triggered apoptosis that can be blocked by B cell lymphoma 2 (Bcl-2) overexpression [9]. Genome-wide RNA-interference screening has uncovered 17 genes, including insulin like growth factor binding protein (IGFBP7), which have a central role in activated BRAF oncogene (BRAFV600E)-mediated apoptosis of melanocyte [10]. Selective inhibition of B-Raf drives oncogenic RAS-dependent BRAF binding to C-Raf, CRAF activation and mitogen-activated protein kinase kinase (MEK)-extracellular signal regulated kinase (ERK) signalling, revealing another paradigm of BRAF-mediated signalling that promotes tumour progression [11]. These findings indicate a key role of Mitf, BRAF and BCL2 in promoting progression of melanoma, and partly explained the well-known treatment resistance of melanoma. Pleiotrophin (PTN) is a heparin-binding growth factor that is highly expressed in certain solid cancers, including melanoma [12, 13]. Targeted disruption of PTN decreases melanoma tumour growth, metastasis and angiogenesis [14, 15]. PTN-dependent cell growth required both mitogen-activated protein kinase (MAPK) and pI3-kinase activity [16]. In melanoma, both MAPK and phosphatidylinositol 3-kinase (pI3K)-serine/threonine protein kinase (AKT) signalling pathways are constitutively activated through multiple mechanisms, and they exert a crucial regulating role in malignant phenotype of melanoma [17].These advances highlight the importance of understanding signalling pathways in clinical practice and genotyping of tumours prior to administering gene selective drugs, to identify patients who are likely to respond to the treatment with the drugs.

At present, it is unclear whether menin's function is associated with melanoma. In patients with MEN1 syndrome, various skin tumours of mesenchymal origin, including angiofibromas, collagenomas and lipomas, as well as malignant melanoma had been reported [18, 19]. Nord *et al.* have found that LOH in 11q13 was detected in six tumours of melanoma, and the deletion including the *MEN1* locus in 19 cases of sporadic metastatic melanoma [18]. Previous implications of multiple melanoma tumour suppressors are localized in chromatin 11q, including the *MEN1* region [8], raising the possibility of an association between *MEN1* and melanoma.

Given these observations, we explored menin's potential role in suppressing malignant melanoma. Our findings suggest a previously unappreciated function for menin in suppressing malignant phenotypes of melanoma. Menin suppresses proliferation and migration of mouse and human melanoma cells *in vitro* and *in vivo*, partly through regulating PTN/RTPT β/ζ signalling. In addition, inactivation of menin was associated with hypermethylation of CpG islands of the *MEN1* promoter region in A375 melanoma cells. These data suggest a novel mechanism involving regulation of PTN signalling by menin in controlling malignant phenotypes of melanoma.

Materials and methods

Cell Culture and gene transfection

The non-pigmented human melanoma A375 cells and pigmented mouse melanoma (B16) cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% foetal bovine serum (Hyclone), 100 U/ml penicillin and 1× Penicillin-Streptomycin (100 U/ml– 100 μg/ml) (Invitrogen, Carlsbad CA, USA). Plasmids were introduced into cells by polyethylenimine-mediated transfection [7] or pLNCX2 retrovirus vector (BD, Franklin Lakes, NJ, USA) system according to the protocol. The transfected cells were selected by either G418 or puromycin, and continuously cultured until harvested for analysis.

RT-PCR and real-time qRT-PCR

Regular RT-PCR and quantitative RT-PCR (qRT-PCR) were performed as previously described [7], using an ABI PRISM 7300 detection system (ABI, Foster, CA, USA) with primers listed in Table S1. The RT-PCR reactions were repeated at least for three times.

Western blotting

The Western blot detections were performed as described [7]. Antibodies are listed in Table S2.

Methylation-specific PCR (MSP)

Genomic DNA from cells was prepared using a DNA Extraction Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The genomic DNA was modified and purified using an CpGenome™ DNA Methylation Kit (Chemicon International, Temecula, CA, USA), following the manufacturer's protocol. MSP was performed with methylation-specific primers, which were designed to recognize bisulphite-induced modifications of unmethylated cytosines. The primer was used to target the CpG islands located in the putative promoter region of *MEN1*.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously described [7]. Briefly, 1×10^6 cells were treated with 1% formaldehyde, followed by pulsed ultrasonication to shear cellular DNA according to the protocol of the ChIP assay kit (Millipore, Billerica, MA, USA). After overnight incubation with the antibodies, protein G-agarose beads were added. The crosslinks between nuclear proteins and genomic DNA were reversed, and the antibody pulled down DNA was purified by phenol/chloroform extraction. The primer pair sequences and antibodies for ChIP assays were shown in Tables S1 and S2.

Xenograft of melanoma cells and tumour development in mice

Tumours were generated in female C57BL/6J mice (vitalriver, Beijing, China) by subcutaneous injection of B16 cells (5×10^5 cells in 100 μl PBS) into the right dorsum of each mouse. Tumour measurements were converted to tumour volume (V) using the formula ($L \times W^2 \times 0.52$), where L and W are the length and width, respectively, and the growth of tumours was measured once every 2 days using vernier callipers. For the pulmonary metastasis model, the B16 cells (5×10^5 cells in 25 μl PBS) were injected into the foot pad of C57BL/6J mice, which were performed as previously described [20]. The mice were killed around day 40. The number and the size of

metastatic foci on the pulmonary surface were macroscopically quantified. Macroscopic lung pictures were acquired with Canon camera and processed with Adobe Photoshop CS Version 8.0. All procedures were performed according to animal welfare and other related ethical regulations approved by the Institutional Animal Care Committee of Medical College at Xiamen University.

5-Aza-2′-deoxycytidine treatment

A375 cells were grown for 7 days in the presence of various concentrations of 5′-aza-dc (0, 3 and 5 μM). Fresh drug was added every 24 hrs. RNA and genomic DNA were separately isolated.

The study of clinical melanoma samples

The study was approved by the Xiamen University Medical Ethics Committee, and written informed consent was obtained from all participants or from patients' representatives if direct consent could not be obtained. We collected 12 malignant melanoma and 6 pigmented nerves samples from Zhongshan Hospital of Xiamen University. The method of immunohistochemistry was as described previously [7].

Data analysis and statistics

Data were presented as the mean ± S.D. or ±S.E. as indicated for each figure. Statistical comparisons between groups were performed with the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Menin inhibits proliferation and migration of melanoma cells

Loss or mutation of *MEN1* acutely promotes pancreatic islet cell proliferation [21, 22]. We have also found that menin suppresses proliferation of lung cancer cells, but the *MEN1* point mutations, A242V and L22R, which were identified from inherited MEN1 patients [23], lost or partially lost ability to repress cell proliferation [7]. Melanomas secrete melanin just like endocrine organs secrete their respective hormones. To explore whether menin affects proliferation of pigmented melanoma cells, we stably transfected B16 cells with either a control vector or a menin-expressing construct. The 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) assay showed that ectopic expression of menin significantly reduced the number of B16 cells on day 4 ($P < 0.05$) (Fig. 1A and B). Furthermore, B16 cells with *Men1* knockdown significantly increased cell proliferation ($P < 0.05$) (Fig. S1a). To further confirm whether menin affects non-pigment melanoma cell phenotype, we generated menin overexpressing A375 cells, a human non-pigmented melanoma cell line, through transduction with either vector or menin-expressing pLNCX2 retroviruses. The BrdU assay clearly showed that overexpression of menin (Fig. 1C) reduced the proliferation of A375 cells on days 2 and 4 (Fig. 1D, $P < 0.05$, respectively). Next, another pair of control and menin overexpressing A375 cell line was established *via* using retrovirus-mediated transduction, and similar results on the role of menin in regulating proliferation of A375 cells were observed by using cell counting assays (Fig. S1b). In malignant melanoma, dysregulation of cell adhesion molecules is associated with tumour progression and metastasis [14]. Menin has been shown to control endocrine cell migration and cell–cell adhesion through interacting with a scaffold protein, IQ motif containing guanosine triphosphatase (GTPase) activating protein 1 [24]. We also found that menin expression was markedly reduced in 23% of certain lung adenocarcinoma, which was correlated with lymph node metastasis [7]. Therefore, we performed a modified transwell chamber assay to evaluate the impact of stably ectopic menin expression on migration of melanoma cells. The results indicated that *MEN1* overexpression significantly decreased migration of B16 cells (Fig. 1E, $P < 0.05$) and A375 cells (Fig. S1c and d). We next used an alternative approach, the scratch wound assay, to compare the motility of mock and menin overexpressing B16 cells. The extent of wound closure achieved by control cells within 48 hrs of wounding was much higher than that menin overexpressed B16 cells (Fig. 1F and G). The dramatic difference in wound healing between these two types of cells reinforces the notion that menin represses migration of melanoma cells. These results reveal a previously unappreciated function for menin in suppressing proliferation and migration of melanoma cells.

Menin inhibits melanoma cells partly through repressing PTN signalling

To elucidate how menin represses proliferation and migration of melanoma cells, we turned our attention to the impact of menin on expression of certain signalling pathways. Our previous work has shown that menin suppresses lung cancer cell proliferation and migration partly through epigenetically repressing transcription of growth factor PTN [7]. PTN is a heparin-binding growth factor involved in the differentiation and proliferation of neuronal tissue during embryogenesis, and is highly expressed in certain solid tumours including melanoma and breast carcinoma cells [12, 13]. PTN binds to cell surface receptor RPTP β/ζ and exerts multiple functions including cell proliferation, adhesion and migration [25–27]. Thus, we initially evaluated the impact of menin overexpression on expression of PTN and its receptor RPTP β/ζ in melanoma cells. The results indicate that menin overexpression substantially reduced mRNA levels of PTN and RPTP β/ζ, but not other growth factor vascular endothelial growth factor (VEGF), VEGF-C and basic fibroblast growth factor (bFGF) in B16 cells (Fig. 2A). Menin overexpression also reduced protein levels of PTN and RPTP β/ζ, but not VEGF (Fig. 2B). We further evaluated

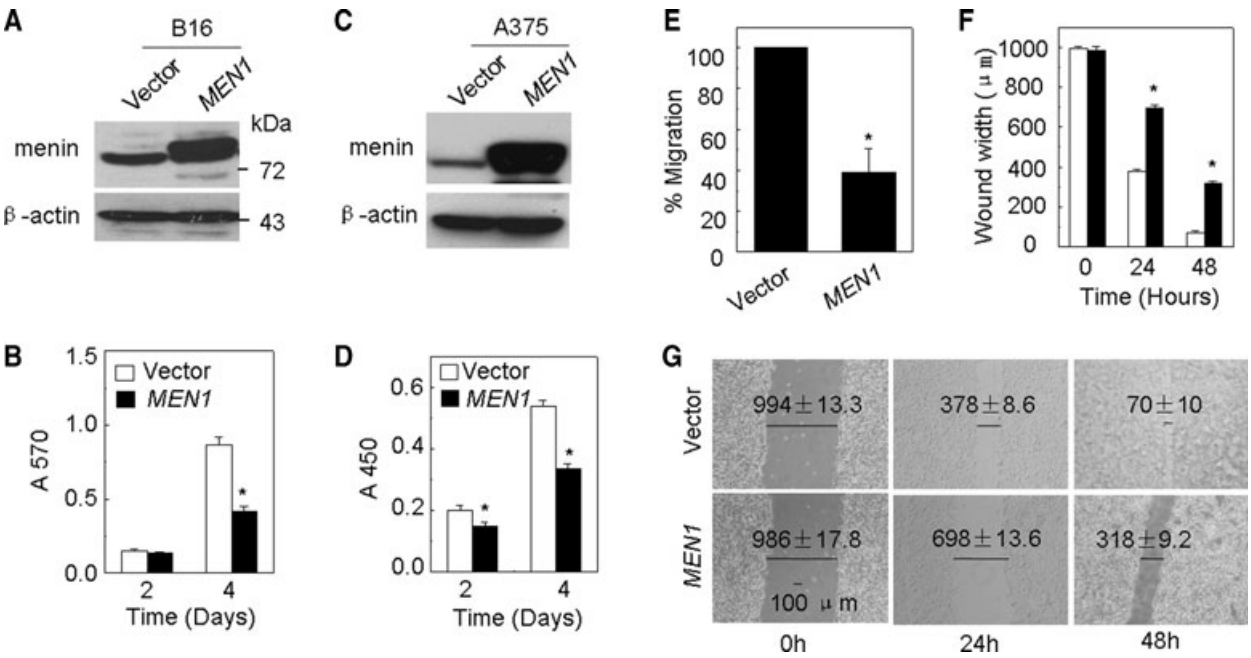

Fig. 1 Menin inhibits proliferation and migration of melanoma cells. (**A**) The efficiency of menin overexpression was detected by Western blot in B16 cells. (**B**) The proliferation of B16 cells which was stably transfected with either pMX-puro or pMX-menin was estimated by MTT assay. (**C**) The efficiency of menin overexpression was detected by Western blot in A375 cells. (**D**) The proliferation of A375 cells, which were stably transfected with either vector or menin, was detected by BrdU assay. (**E**) Stably transfected B16 cells were added to the upper filter, and cell migration was determined. (**F** and **G**) Quantification of the time-dependent effects of menin overexpression on cell motility (wound width). Confluent monolayers of B16 menin overexpression cells were wounded with a pipette tip. Wound closure was monitored by microscopy at the indicated time, $^*P < 0.05$, $N = 3$.

if PTN/RPTP β/ζ signalling is required for menin-mediated repression of migration of melanoma cells. Two distinct PTN shRNAs and a control Luc shRNA vector were stably transfected into B16 cells, and RT-PCR results showed that shRNA1 substantially reduced PTN expression, but shRNA2 failed to knockdown PTN expression (Fig. 2C). Interestingly, correlated with the levels of PTN knockdown by the shRNAs, shRNA1 significantly decreased cell proliferation ($P < 0.05$), but control vector and PTN shRNA2, which were unable to reduce PTN expression, did not significantly decrease proliferation of B16 cells ($P > 0.05$) (Fig. 2D). Notably, PTN knockdown by shRNA1 also reduced migration of B16 cells (Fig. 2E). Furthermore, RPTP β/ζ knockdown effectively reduced intracellular RPTP β/ζ mRNA (Fig. 2F) and protein expression (Fig. 2G), concomitant with reduced migration of B16 cells (Fig. 2H). Together, these data indicate that menin inhibits proliferation and migration of B16 cells at least partly through regulating expression of PTN and RPTP β/ζ.

Menin represses tumour growth and metastasis of melanoma cells *in vivo*

To determine whether menin affects growth of melanoma cell-derived tumours in animal model, we stably transfected B16 cells with either control or menin-expressing construct, and the resulting cells were subcutaneously transplanted into C57BL/6J mice ($n = 8$ per group). Ectopic expression of menin was confirmed by Western blotting (Fig. 3A). The size of the solid tumour was measured after various periods of time following transplantation. Ectopic menin expression in B16 cells significantly reduced the size of B16 cell-derived solid tumour in C57BL/6J mice after transplantation (Fig. 3B, $P < 0.05$). To determine if menin affects the growth of the established tumours in C57BL/6J mice partly through PTN, the PTN knockdown B16 cells were generated and subcutaneously transplanted into C57BL/6J mice ($n = 8$ per group). The efficiency of PTN silencing was determined by Western blotting (Fig. 3C). As expected, reduction in PTN expression also significantly suppressed the growth of B16 cell-derived solid tumours on indicated days (Fig. 3D, $P < 0.05$). These results suggest that menin represses, but PTN promotes, growth of B16 solid tumour in mice, highlighting a crucial role of menin and PTN in controlling growth of melanoma *in vivo*. In the syngeneic murine metastasis models, we also found that either menin overexpression (Fig. 3E and F) or PTN knockdown (Fig. 3G and H) significantly repressed the number of macroscopic pulmonary metastatic foci. Together, these data show that menin suppresses growth and pulmonary metastasis of solid melanomas partly through repressing PTN signalling *in vivo*.

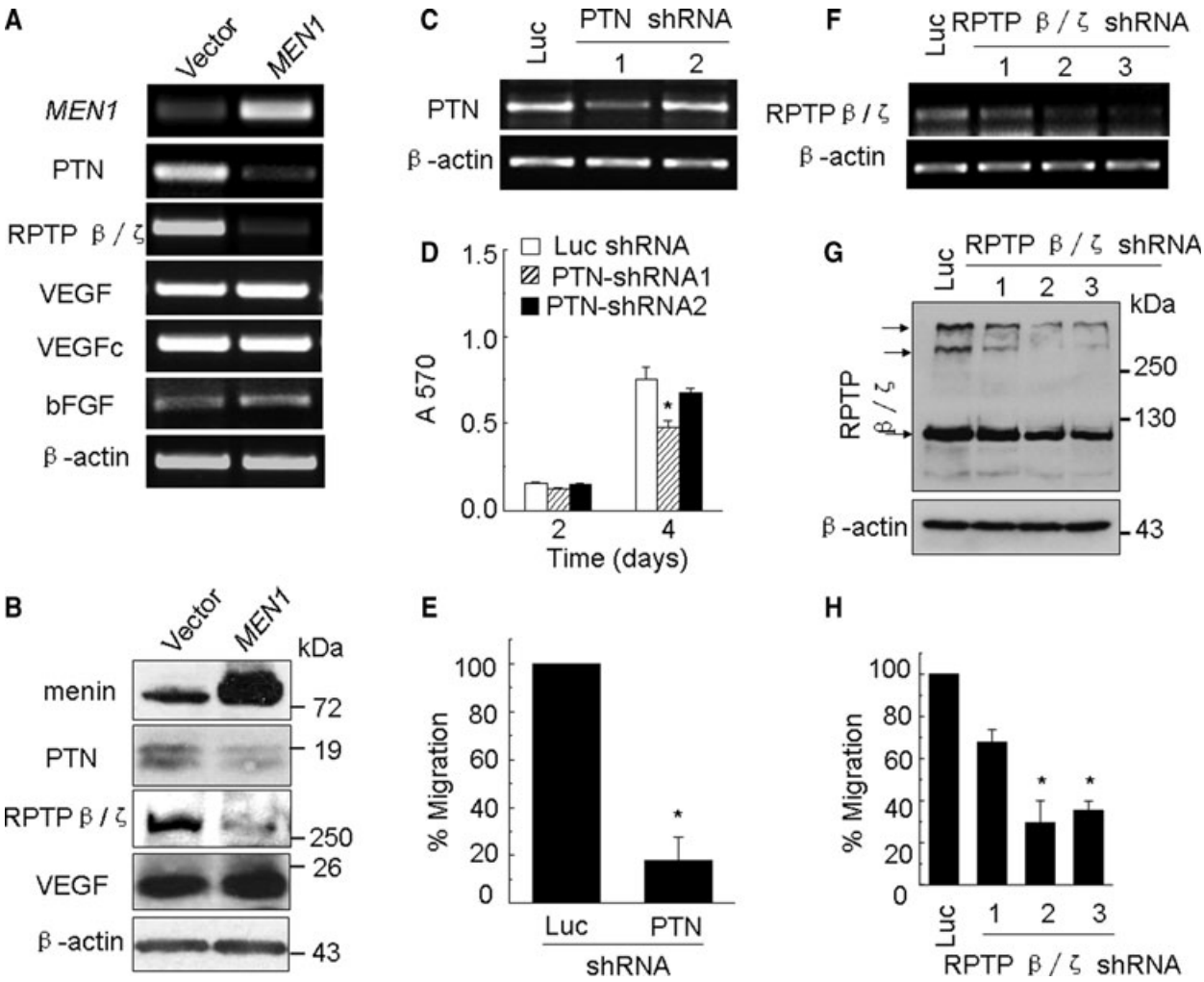

Fig. 2 Menin represses proliferation and migration of melanoma cells partly through PTN signalling. (**A**) *Men1*, PTN, RPTP β/ζ, VEGF, VEGFc and bFGF mRNA levels were detected by RT-PCR. (**B**) The efficiency of menin overexpression and the effect of *Men1* expression on PTN, RPTP β/ζ and VEGF expression were determined by Western blotting and β-actin was used as loading control. (**C**) B16 cells were transfected with either vector expressing shRNAs against Luc or one of the two shRNAs against PTN and selected by G418. The efficiency of PTN silencing was determined by RT-PCR. (**D**) The proliferation of the selected B16 cells was estimated by MTT assay. (**E**) The selected B16 cells were added to upper filter and cell migration was determined. (**F** and **G**) B16 cells were transfected with either vector expressing shRNAs against Luc or one of the there shRNAs against RPTP β/ζ and selected by G418. The efficiency of RPTP β/ζ silencing was determined by RT-PCR and Western blotting. (**H**) The selected B16 cells were added to upper filter and cell migration was determined.

pI3K and ERK1/2 were crucial for menin-mediated regulation of melanoma cells

To further elucidate cell signalling underlying menin/PTN regulated cell proliferation and migration, we tested the impact of menin on pI3K and ERK1/2, which is essential for regulating phenotype of melanoma [17]. The results showed that ectopic expression of menin reduced expression of pI3K as well as phosphorylation (Thr202/Tyr204) of ERK1/2 in A375 cells (Fig. 4A). FAK (focal adhesion kinase) is a protein tyrosine kinase that is recruited at an early stage to focal adhesions and mediates many of the downstream responses, including activation of the MAPK and pI3K p85-subunit in epithelial tumour cells and fibroblasts [28, 29]. To further dissect the potential relationship between menin, FAK, ERK1/2 and pI3K, the stable menin-expressing A375 cells were analysed. Our results showed that menin overexpression did not affect the total amount (Fig. 4A) and cell localization (data not shown) of FAK, but reduced the level of its Tyr 397-phosphorylated form (Figs 4A and S2a). Next, serum-starved A375 cells were stimulated by addition of rhPTN and allowed to progress for

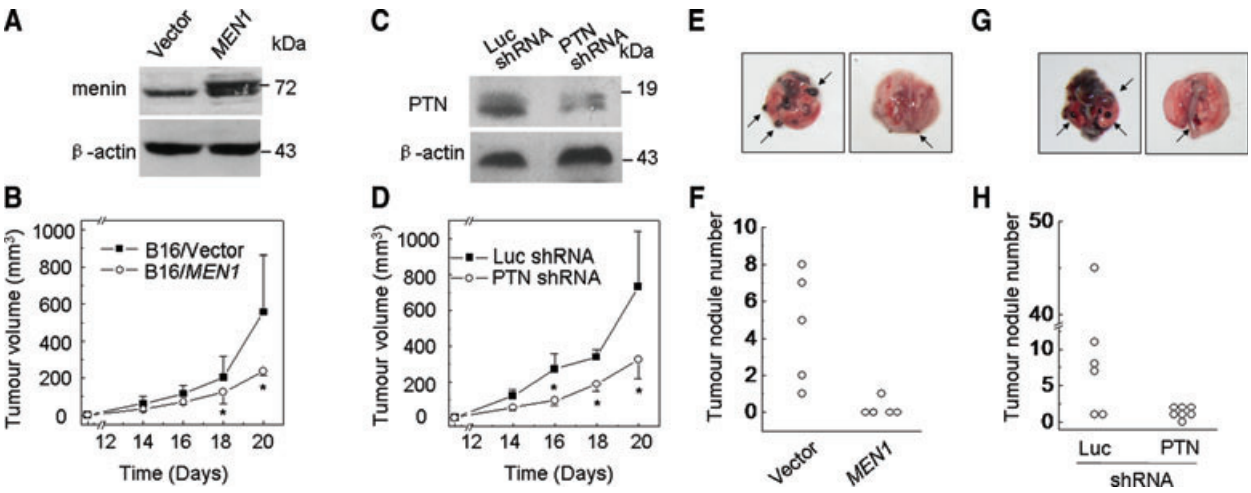

Fig. 3 Menin represses tumour growth and metastasis of melanoma cells *in vivo*. (**A**) The efficiency of menin overexpression was determined by Western blotting. (**B**) Menin overexpressing B16 cells were injected subcutaneously into nude mice and tumour formation was examined day 14 after transplantation. $N = 8$, $^*P < 0.05$. (**C**) The efficiency of PTN silencing was determined by Western blotting. (**D**) The PTN-shRNA expression B16 cells were injected subcutaneously into nude mice, and tumour formation was examined day 14 after transplantation, $N = 8$, $^*P < 0.05$. (**E** and **F**) The number of macroscopic pulmonary metastases from each mouse treated with menin overexpressing B16 cells, $N = 5$. (**G** and **H**) The number of macroscopic pulmonary metastases from each mouse treated with PTN-shRNA B16 cells, $N = 6$ or 7.

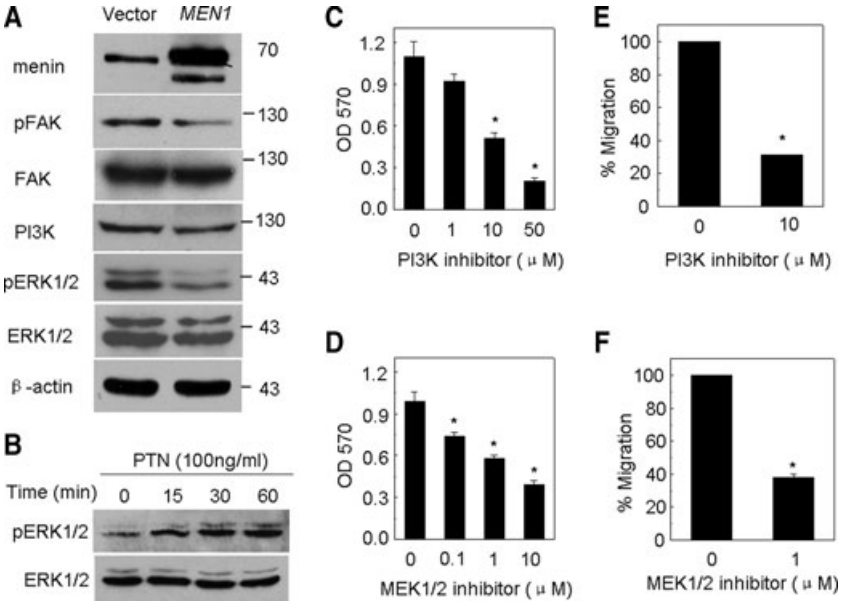

Fig. 4 pI3K and ERK1/2 were crucial for menin-mediated regulation of melanoma cells. (**A**) Menin, pFAK, pI3K and pERK1/2 protein level were detected by Western blot. (**B**) Serum-starved A375 cells were treated with 100 ng/ml rhPTN and harvested at various time-points. The activation of ERK1/2 was detected by Western blotting. (**C**) A375 cell lines were treated using LY294002, a pI3K inhibitor 48 hrs, and cell proliferation was measured by MTT. (**D**) A375 cell lines were treated with U0126 at 0.1, 1 and 10 μM, a MEK1/2 inhibitor 48 hrs and cell proliferation was measured by MTT. (**E**) A375 cells treated with LY294002 were added to upper filter and cell migration was determined. (**F**) A375 cells treated with U0126 were added to upper filter and cell migration was determined.

various periods of time prior to analysis. The results indicated that pERK1/2 was rapidly increased after exposure to rhPTN at 15–60 min. (Fig. 4B). It showed that menin regulated activation of ERK1/2 partly through repressing PTN. These results suggest that FAK signalling may link menin/PTN to cell proliferation and migration partly through regulating pI3K and ERK1/2 pathways. To further confirm this observation, we determined whether pI3K and ERK1/2 signalling were necessary for the menin/PTN regulating phenotypes of melanoma cells. To this end, A375 cells were treated with either LY294002 or U0126, which are specific inhibitors for pI3K and MEK1/2, respectively. As expected, both LY294002 and U0126 decreased proliferation of A375 cells in a dose-dependent manner (Fig. 4C and D). Migration of A375 cells treated with either LY294002 or U0126 was also reduced (Fig. 4E and F). β-catenin acts as a key factor in E-cadherin-mediated cell–cell adhesion [30]. We further determined if menin/PTN regulated cell migration was dependent on β-catenin signalling. However, menin did not effectively suppress expression and phosphorylation (Tyr 142) of β-catenin (Fig. S2b). Cell morphology and migration were regulated by members of the Rho family of small GTPases, including

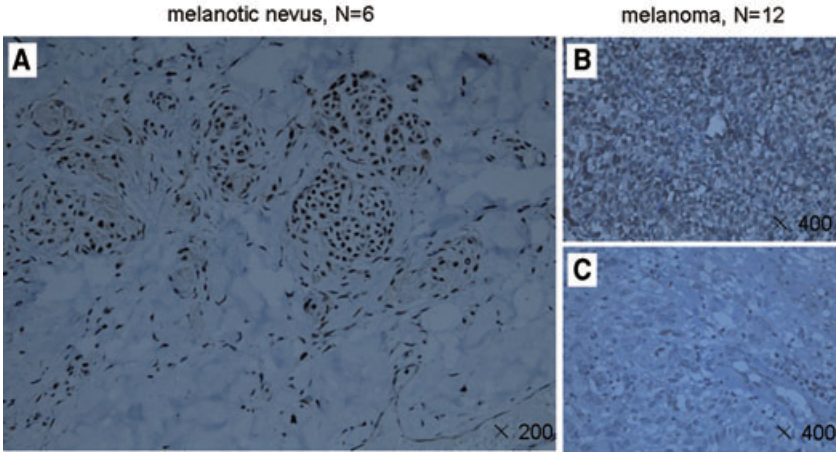

Fig. 5 Menin expression is reduced in certain primary melanoma cells. Sections from paraffin-embedded samples were stained with affinity-purified anti-menin antibody for immunohistochemistry staining. (**A**) Menin was easily detectable in the nucleus of the pigmented nerves (×200). (**B** and **C**) In melanoma, staining for menin was slightly weaker (three cases) or undetectable (nine cases), as compared to that in the pigmented nevus cells (×200).

Rho, Rac1 and Cdc42 [31]. Hence, we further examined if menin controls cell migration partly through Rho family signalling. Ectopic menin expression did not alter the amount of either activated forms (GTP bound) or the total amount of Rho, Rac1 and Cdc42 in A375 cells (Fig. S2b).

Next, we determined whether the level of expression of menin in melanoma cell lines is correlated with cell sensitivity to the cytotoxic effects of cisplatin and dacarbazine, the two most commonly used drugs for treating malignant melanoma. The time-course results indicate that menin was gradually increased after exposure to cisplatin at 0–24 hrs (Fig. S3a). Meanwhile, the dose–response result also indicates that menin was increased after exposure to indicated concentrations of cisplatin at 16 hrs (Fig. S3b). However, there was no significant correlation between menin levels and sensitivity of melanoma cell lines to dacarbazine (Fig. S3c and d). Because it is well known that menin can induce cell apoptosis [32], we determined whether menin could serve as a means to enhance killing of malignant melanoma cells. Overexpression of menin indeed increased cisplatin induced apoptosis of A375 cells (Fig. S3e). Further studies indicated that menin repressed phosphorylation (S139) of γ-H2AX, a marker of DNA damage repair, and cell cycle regulators, such as cyclin B1 and B2 (Fig. S3f). These results raise a possibility that menin also regulates apoptosis of melanoma cells, and this process may be associated with controlling DNA damage response and cell cycle progression. The precise mechanism for menin regulated apoptosis remains to be investigated. Together, our results suggest that menin inhibits ERK1/2 phosphorylation partly through PTN expression, and FAK, pI3K and ERK1/2 signalling might be involved in menin-mediated repression of phenotype of melanoma cells.

DNA Methylation of the MEN1 promoter correlates with menin inactivation in A375 cells

Since we observed a crucial role for menin in repressing phenotype of melanoma cells, we wondered if the menin protein level is altered in patients' primary melanoma. We examined 12 malignant melanoma samples and 6 pigmented nevus. These tumours were from male and female patients with ages ranging from 28 to 88 (Table S3). Sections from paraffin-embedded samples were stained with affinity-purified anti-menin antibody for immunohistochemistry (IHC) staining, and the specificity of the anti-menin antibody was verified in menin-null and menin-expressing cells [7]. Menin was easily detected in the nucleus of the normal six pigmented nevus cells (Fig. 5A). However in melanoma tumours, staining for menin was slightly weaker (three cases) or undetectable (nine cases), as compared to that in the pigmented nevus cells (Fig. 5B, C and Table S3). To determine the cause for inactivation of menin in A375 cells, we designed the primers to determine if *MEN1* was mutated (Fig. S4). Unexpectedly, DNA sequencing data did not reveal any mutation in the sequence of *MEN1*.

Transcriptional silencing of tumour suppressor genes, associated with DNA hypermethylation of CpG islands [33]. Hence we considered if reduced menin expression is related to epigenetic regulation. In MSP analysis, we designed methylation-specific primers and unmethylation-specific primers which were targeted to CpG sites (Fig. 6A), and examined the methylation status of the *MEN1* promoter in A375 cells using real-time qPCR. In order to clarify the functional association between *MEN1* promoter methylation, 5′-aza-dc, an agent reducing DNA methylation, was used to treat A375 cells. The quantitative methylation-specific PCR (qMSP) results showed that the level of DNA hypermethylation *at the MEN1* promoter was reduced by treatment with 5′-aza-dc in A375 cells (Fig. 6B). After 7 days treatment with 5′-aza-dc at 3 μM or 5 μM, the increased *MEN1* mRNA re-expression was detected by real-time qRT-PCR (Fig. 6C).

Furthermore, we also determined if DNA methytransferase 1 (DNMT1) binds to the *MEN1* promoter using ChIP assay. We designed two primers used for ChIP assays at *Men1* promoter loci (Fig. 6D). In A375 cells, an interaction between DNMT1 and the promoter of *MEN1* could be detected (Fig. 6E, lane 3). Following exposure to 5′-aza-dc, the interaction between the DNMT1 and the promoter of *MEN1* was reduced (Fig. 6E, lane 6). To explore whether treatment with 5′-aza-dc affects proliferation and migration

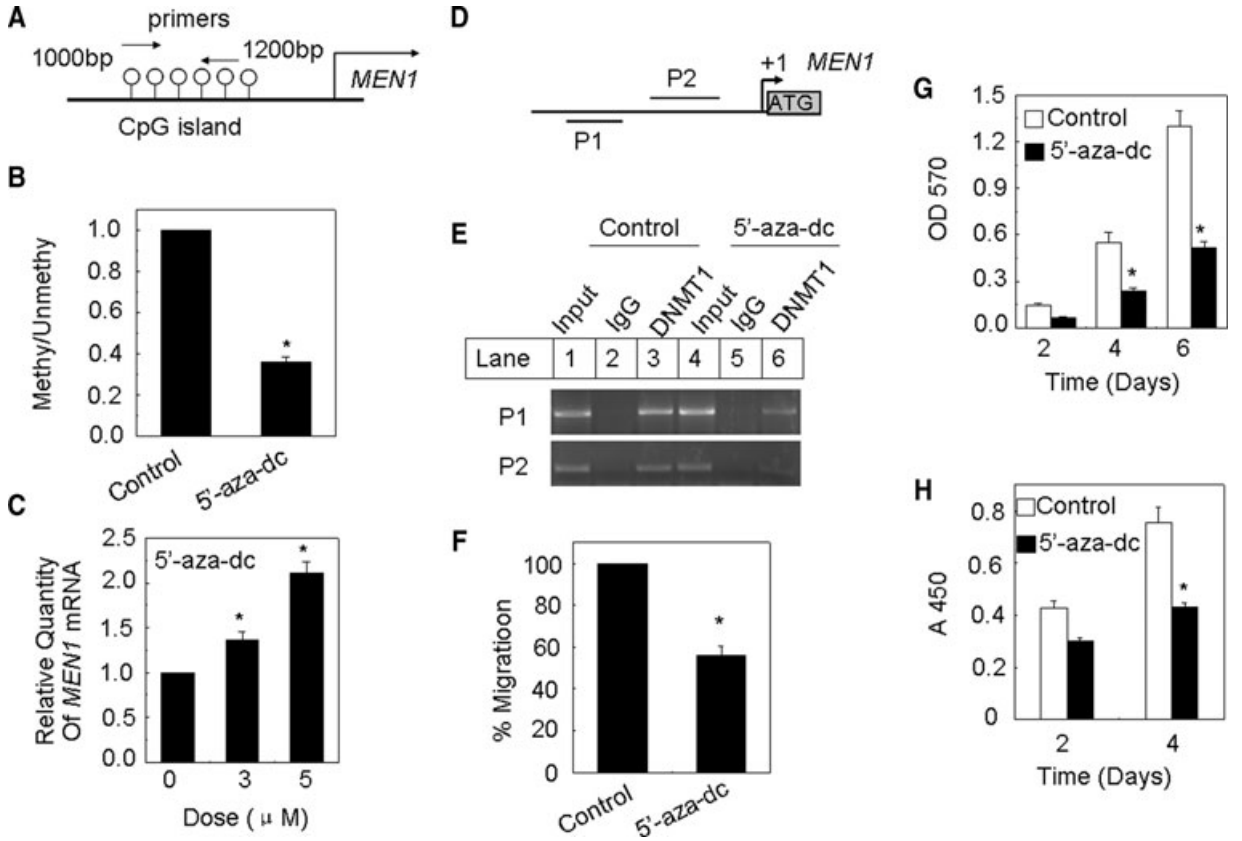

Fig. 6 Methylation of the menin promoter correlates with menin expression in A375 cell. (**A**) Primers for unmethylated and methylated DNA of corresponding CpG islands were used. (**B**) qMSP assay of *MEN1* gene in A375 cells. (**C**) **A375** Cells were treated with 5′-aza-dc at 3 or 5 μM for 7 days with medium changed each day, and *MEN1* mRNA level was determined by real-time qPCR. (**D** and **E**) ChIP assay to demonstrate the association of DNMT with the *MEN1* genes. (**F**) A375 cells treated with 5′-aza-dc at 5 μM for 7 days were added to the upper filter, and cell migration was determined. (**G** and **H**) The proliferation of A375 cells treated with 5′-aza-dc at 5 μM for 7 days was estimated by MTT assay and BrdU cell proliferation assay, respectively.

of melanoma cells, we treated A375 cells with 5 μM 5′-aza-dc for 7 days. The transwell assay showed that treatment with 5′-aza-dc significantly reduced the number of migrated A375 cells on days 4 and 6 ($P < 0.05$, respectively) (Fig. 6F).

In addition, MTT assay confirmed that treatment with 5′-aza-dc reduced the number of A375 cells (Fig. 6G). A similar result was obtained using the BrdU incorporation assay (Fig. 6H). Exposure of A375 cells to 5′-aza-dc effectively demethylated the CpG regions within the *MEN1* promoter, leading to *MEN1* gene expression and suppressed malignant phenotypes of melanoma, including proliferation and migration. Together, these data indicate that *MEN1* silencing was associated with promoter CpG region hypermethylation in melanoma, and suggest a key role for menin in repressing melanomas.

Discussion

MEN1 knockout mice develop parathyroid, pancreatic, pituitary and adrenal tumours [2]. Menin interacted with MLL and promoted the development of leukaemia through binding to the locus of Hox family genes and highlight the level of H3K4me3 [3–6]. Recently, we have found that menin inhibits lung cancer cell proliferation and migration *via* epigenetic repression of PTN signalling [7]. Various skin tumours of mesenchymal origin, including angiofibromas, collagenomas and lipomas, as well as malignant melanoma, were detected in MEN1 syndrome patients [18, 19]. However, until recently, little has been known about the precise role and regulatory mechanism of menin in melanoma.

In present study, we have shown that menin inhibits proliferation, migration and metastasis of melanoma cells partly through repressing PTN and its receptor, RPTP β/ζ expression. Further investigation revealed that menin regulates cell phenotype of melanoma *via* PTN/RPTP β/ζ, in conjunction with FAK, pI3K and ERK1/2 signalling. Our previous results show that menin not only inhibits expression of PTN and RPTP β/ζ, but also represses the activation (phosphorylation) of FAK, pI3K and ERK1/2 in lung cancer cells [34]. Therefore, the similar mechanism underlying menin-mediated tumour suppression may exist in lung cancer cells and melanoma cells. However how menin regulates FAK,

pI3K and ERK1/2 signalling through PTN and RPTP β/ζ remains unclear. PTN binds to its receptor, RPTP β/ζ and increases tyrosine phosphorylation of many downstream genes including β-catenin, ALK and integrin β_3 [35–37]. In the present melanoma model, we did not find that menin affects the expression and phosphorylation of β-catenin. Cell morphology and migration were regulated by members of the Rho family of small GTPases [31]. Our results indicate that extopic expression of menin did not alter the amount of either activated forms (GTP bound) or the total amount of Rho, Rac1 and Cdc42 in A375 cells. FAK interacts with integrin β_3 and promotes cell migration and invasion [34]. It has been reported that integrin–FAK interaction may serve as a downstream effector of PTN [37], thus PTN may increase activation of FAK by binding to RPTP β/ζ and increasing tyrosine phosphorylation of integrin β_3. Then activated FAK promotes pI3K-ERK1/2 signalling. Collectively, menin may inhibit FAK, pI3K and ERK1/2 signalling through repressing the ability of menin to repress phosphorylation of the crucial signalling proteins downstream of PTN. During the progression of cutaneous melanomas, matrix metalloproteinases (MMPs) facilitate the tumour cells to traverse the basement membrane and invade the dermis. Melanoma cell lines with low expression of MMP19 exhibited increased adhesion to various substrates and lower migration in comparison with the cell line with higher expression of MMP19 [38]. Dimethylfumarate inhibits tumour cell invasion and metastasis by suppressing the expression and activities of MMPs in melanoma cells [39]. Whether the menin affects melanoma motility *via* one of the multiple MMPs will be determined. In addition, menin also promotes the cisplatin-induced apoptosis of A375 cells, and represses expression of phosphor-γ-H2AX, a DNA damage repair marker. However, how menin may regulate apoptosis of melanoma cell and DNA damage response remains to be further determined.

The function of menin in regulating tumorigenesis is opposite between endocrine tumour and leukaemia. What decides the role of menin in different tissues and cell lines? In the present study, we chose the A375 and B16 cell lines, a non-pigmented and pigmented cell lines, respectively, to demonstrate that the impact of menin on non-pigmented and pigmented melanoma cell lines. Our results reveal that menin has similar effects on both pigmented and non-pigmented melanoma cells. These results indicate that menin has broad-spectrum suppressing effect on melanoma. Melanomas secrete melanin just as endocrine organs secrete their respective hormones. A possible link between melanoma and endocrine is that both of them are secretary tissues. Nonetheless, the precise mechanism remains to be determined. Menin expression was significantly reduced in primary melanoma cells from clinical samples. What is the cause for decreased expression of menin in melanoma cells? Our DNA sequencing data did not reveal any mutation in the sequence of *MEN1*. Treatment of A375 cell with the demethylating agent 5′-aza-dc reactivated menin expression and then repressed proliferation and migration of A375 cells. Based on these results, DNA methylation appears to play a major role in silencing menin expression in A375 cells. And another possibility is that menin has different epigenetic modifications in different tissues and the modifications may determine the various status of menin expression.

Collectively, our findings unravel a previously unrecognized function of menin in controlling melanoma cell proliferation, migration, metastasis and apoptosis. Menin inhibits FAK, pI3K and ERK1/2 signalling through repressing PTN and its receptor, RPTP β/ζ. And this mechanism is similar to what is in lung cancer cells. These findings may suggest that the similar function and regulatory mechanism for menin may exist among lung cancer, melanoma and endocrine tumours, and provide a new insight into further understanding the function of menin in a broader spectrum of tumours.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primer sequences and target sequences of shRNA

Table S2 Antibody and reagent

Table S3 Summarize of IHC results from certain primary melanoma samples

Fig. S1 (**a**) The proliferation of B16 with *MEN1* knockdown. (**b**) The proliferation of A375 cells stably transfected with either empty vector or menin. (**c**) Migrated to lower side of the filter A375 cells were stained with 0.1% crystal violet. (**d**) Stably transfected A375 cells were added to the upper filter, and cell migration was determined, $*P < 0.05$, $N = 3$.

Fig. S2 (**a**) IF detection of menin (green), pFAK (green), DAPI (blue) and merge in the A375 cells. (**b**) PAK1-PBD agarose and Rhotekin RBD agarose were used to isolate GTP-Cdc42, GTP-Rac1 and GTP-RhoA from whole cell lysates from menin-overexpressing A375 cells. The Cdc42-GTP, Rac1-GTP and RhoA-GTP

were detected using Western blotting and normalized by the total input protein. The pβ-catenin protein level was detected by Western blot in menin-overexpressing A375 cells.

Fig. S3 (**a**, **c**) Melanoma cells were treated with 1 μg/ml cisplatin or 250 μg/ml dacarbazine and harvested at various time-points. And the menin expression was determined with Western blotting. (**b**, **d**) Melanoma cells were treated with the indicated concentrations of cisplatin or dacarbazine, and the menin expression was detected by Western blotting. (**e**) A375 cells were treated for 24 hrs with various doses of Cisplatin and then analysed for apoptosis *via* Annexin V-PI staining. (**f**) menin, γ-H2A.X, cyclinB1 and cyclinB2 protein level were detected by Western blot.

Fig. S4 Primers for determining whether menin mutated were used.

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