

Original Article

Inhibition of peptidyl-prolyl isomerase (PIN1) and BRAF signaling to target melanoma

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Abstract: PIN1 is a phosphorylation-dependent peptidyl-prolyl cis/trans isomerase, overexpressed in many cancers, including melanoma. Our immunohistochemistry data of melanoma patient tissue underline the up-regulation of PIN1 in metastases. Here, we demonstrate important functions of PIN1 and its selective and cell permeable inhibitor 37 for the treatment of melanoma. To analyze its possible role in oncogenesis and as a therapeutic target, we first suppressed PIN1 expression by a siRNA pool. PIN1 knockdown potently inhibited melanoma cell proliferation and vascular mimicry by influencing several cancer-relevant pathways. Furthermore, inhibitor 37 inhibited cell growth in melanoma and induced apoptosis. Normal healthy melanocytes, keratinocytes and fibroblasts are not affected by the PIN1 inhibitor 37. Combinatorial treatment of melanoma cells is with Vemurafenib as a common therapeutic option for BRAF-mutated melanoma and inhibitor 37 resulted in a strong, synergistic effect on apoptosis of melanoma cell lines. In summary, targeting PIN1 offers a promising therapeutic approach to simultaneously downregulate multiple cancer-driving pathways in cancer.

Keywords: BRAF, melanoma, peptide 37, PIN1, proliferation

Introduction

An important oncogenic signaling pathway is the Ras/MEK/ERK pathway. MEK is constitutively activated in the vast majority of melanomas due to activating mutations in the upstream proteins such as NRAS and BRAF, with BRAF mutations being the oncogenic driver of ~50% of all melanomas [1]. Furthermore, both normal and oncogenic BRAF proteins are 'feedback' phosphorylated on four Ser/Thr-Pro sites (S151, T401, S750, and T753) by activated MAP kinase ERK to interrupt the signaling pathway. This feedback phosphorylation prevents the binding of BRAF to activated RAS or between C-RAF and BRAF, which alters the signaling pathway of BRAF. Subsequently, the peptidyl-prolyl cis-trans isomerase PIN1 contributes to the 'recycling' of BRAF proteins which allows again the re-assembling of BRAF to build heterodimers (RAS/BRAF; C-RAF/BRAF) to reactivate the signaling pathway [2]. Here, PIN1 catalyzes conformational changes of the protein which represent a novel and tightly controlled signaling mechanism regulating a spec-

trum of protein activities during physiological and pathological processes [1].

In recent years, a growing number of proteins has been reported as PIN1's substrates, including tumor suppressor p53 [3, 4], proto-oncogene c-MYC [5], cell-cycle proteins CDC25 [6], Cyclin D1 [7], and many others proteins that are generally not related to each other [8].

Activities of multiple transcriptional factors such as c-FOS [9], c-JUN [7], NFκB [10] and STAT3 [11] were influenced by nuclear PIN1 whereas the described BRAF signaling pathway is dependent on cytoplasmic PIN1 [2]. Zhou et al. [12] reported that PIN1 can switch on more than 40 oncogenes and growth promoters and/or switch off at least 20 tumor suppressors and growth inhibitors through positive and negative feedback mechanisms to simultaneously activate and/or amplify numerous cancer-driving pathways.

Of particular interest is the observation that PIN1 is often overexpressed in human cancers including ~44% of melanoma [2, 3] and acti-

vates multiple cancer-driving pathways [13]. PIN1 has been proposed as potential target for the development of anticancer drugs. However, until recently development of PIN1 inhibitors as therapeutic agents has been challenging [13]. Although several families of small-molecule and peptidyl inhibitors have been reported, they often have poor potency or specificity (e.g., EGCG, Juglone), poor solubility (e.g., PiB) or low cell-permeability (e.g., peptide inhibitors) [14]. Recently, Jiang and Pei reported a potent, selective, and cell-permeable nonphosphorylated bicyclic peptidyl inhibitor against PIN1, peptide 37 [15]. Peptide 37 inhibited HeLa cell growth in a concentration-dependent manner (with an IC_{50} value of 1.0 μ M), whereas a control peptide (Peptide 47, which is cell-permeable but defective in PIN1 binding) did not. The improved cell permeability and metabolic stability of peptide 37 makes it an attractive candidate for *in vivo* applications. Here, we show that genetic knockdown of PIN1 via a siRNA pool suppresses melanoma cell proliferation and tube formation by blocking several cancer-driving pathways. Furthermore, peptide 37 induces apoptosis of melanoma cells. These results demonstrate that specific PIN1 inhibitors may provide a novel treatment option for melanomas.

Targeted therapies for melanoma have revolutionized the management of advanced BRAF-mutated melanoma, specifically by using BRAF inhibitors such as vemurafenib and dabrafenib and MEK inhibitors such as cobimetinib and trametinib. Combination regimens of the inhibitors have improved clinical benefit compared with monotherapy [16]. New therapies would either have to enhance initial drug efficacy, repress acquired drug resistance or inhibit downstream targets of MEK in an alternative manner. However, the role of PIN1 for improvement of the efficacy of e.g. vemurafenib against melanoma is unknown. Therefore, we consider a combination of vemurafenib plus PIN1 inhibitory peptide 37 in our analysis.

Material and methods

Cell culture and tissue samples

Mel Im and SK-Mel-28 (derived from human metastases of malignant melanoma) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (400 U/ml), streptomycin (50 μ g/ml) and 10% fetal calf

serum (all from Sigma-Aldrich, Munich, Germany). HaCaT cells [17] and FV fibroblasts were cultivated in DMEM. All cell lines were incubated at 37°C in a humidified atmosphere containing 8% CO₂.

Human tissue samples with clear-cut pathological classification were obtained a tissue collection (Institute of Pathology, University of Regensburg, Germany). Sampling and handling of patient material were carried out in accordance with the ethical principles of the Declaration of Helsinki.

Transfection experiments and luciferase analysis

Cells were plated 2×10^5 cells/well into 6-well plates and transfected with a siPool against PIN1 (siTools Biotech GmbH, München, Germany), 2 nM in a 6-well chamber, using the RNAiMax (Invitrogen/Life Technologies GmbH, Darmstadt, Germany), according to the manufacturer's instructions.

For luciferase analysis 0.5 μ g plasmid DNA using the Lipofectamine Plus method (Invitrogen/Life Technologies GmbH) according to the manufacturer's instructions, was performed. For the transient transfection, the plasmids pAP-1-Luc (TGAATA)₇, pSRE-Luc (TAGTTTCACTTTCCC)₄, pNF κ B-Luc (TGGGGACTTTCCGC)₅ and p21/WAF1-promoter-Luc [18] (Addgene, LGC Standards Teddington, UK) were used. TOPflash/FOPflash reporter constructs for measuring TCF/LEF activity in the beta-catenin signaling pathway, were purchased from Upstate/MERCK Millipore (Billerica, MA, USA). The pRLTK (Renilla) (Promega, Mannheim, Germany) activity was used to standardize the transfection efficiency of the luciferase constructs.

Scratch closure

Migration of cells was assayed by scratch assays. For scratch assays cells were seeded at 5×10^5 per well into 6-well plates and scratched by a pipette tip in a definite array. Migration into this array was recorded by digital photography at the same point in the scratch and measured after 24 and 48 h.

Measurement of proliferation

The xCELLigence System (Roche, Mannheim, Germany) is based on measurement of electri-

cal impedance and permits real-time analysis of proliferation. E-plates were used and basic protocols recommended by the manufacturer were followed. The bottom chambers contained culture supernatant from human fibroblasts as chemo-attractant. Upper chambers contained serum-free DMEM. Cells suspended in serum-free DMEM were added to the upper chambers (2×10^2 /well). Thereafter, impedance can be measured continuously over 72 h or longer.

Tube formation (vasculogenic mimicry)

Growth factor reduced Matrigel (BD Biosciences, Heidelberg, Germany) was added to eight-chamber polystyrene vessel tissue culture-treated glass slides (BD Bioscience, Heidelberg, Germany) and allowed to gelatinize for 20 min at 37°C. To assay vasculogenic mimicry, 7×10^4 melanoma cells were seeded onto Matrigel-coated culture slides. Tube formation was assessed by phase contrast microscopy after 16 h and recorded with a digital camera.

Angiogenesis array

The Human Angiogenesis Array Kit (R&D Systems, Proteome Profiler-Antibody Arrays, Wiesbaden-Nordenstadt, Germany) was performed as recommended by the manufacturer's protocol. The pixel density was calculated with ImageJ software.

Clonogenic assay

The *in vitro* cell survival assay was performed as described by Franken et al. [19].

RNA isolation, reverse transcription and quantitative RT-PCR

Total RNA was isolated using the E.Z.N.A. MicroElute Total RNA Kit (Omega Bio-Tek, VWR Darmstadt, Germany) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop spectrophotometer (Peqlab Biotechnology GmbH, Erlangen, Germany) and cDNA was generated by reverse transcription using the Super Script II Reverse Transcriptase Kit (Life Technologies, Carlsbad, USA), with each reaction containing 500 ng of total RNA. Analysis of mRNA expression was performed using quantitative Real-Time PCR on the LightCycler 480 system (Roche, Mannheim, Germany).

Protein isolation and western blot analysis

Cells and tissues were lysed in 150 µl RIPA buffer (Roche, Mannheim, Germany) for 15 min at 4°C and cell debris was separated via centrifugation at 13,000 rpm and 4°C for 10 min. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA). For each sample, 40 µg of total lysate were separated on 10% SDS-PAGE gels and subsequently transferred onto a PVDF membrane. After blocking for 1 h with 5% BSA/PBS the membrane was incubated overnight (4°C) with one of the following antibodies: anti-beta-ACTIN (Sigma-Aldrich, Missouri, USA; 1:5000), anti-c-PARP (1 in 1000 dilution; Cell Signaling Technology, Frankfurt a.M., Germany), anti-cytochrom c (1 in 5000 dilution, Abcam, Cambridge, UK), anti-AKT (1 in 2000 dilution, Cell Signaling Technology), anti-p-AKT (1 in 3000 dilution, Cell Signaling Technology), anti-LaminB2 (1 in 1000 dilution, Cell Signaling Technology), anti-ERK (1 in 1000 dilution, Cell Signaling Technology), anti-p-ERK (1 in 1000 dilution, Cell Signaling Technology), anti-PIN1 (Santa Cruz Biotechnology), anti-PIN1 (1 in 1000 dilution, R&D Systems).

Nuclear and cytoplasmic separation of protein

The cells were lysed by incubating (4°C, 15 min) with Buffer I (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT). After the addition of NP-40 (at a final concentration of 0.1%), the lysates were centrifuged at 10,000 rpm for 1 min at 4°C. The supernatant with cytoplasmic proteins were collected and frozen, the nuclear pellets were agitated (4°C, 15 min) with Buffer II (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) and were centrifuged for 10 min at 13,000 rpm at 4°C. The supernatants with the nuclear extracts were shock-frozen in liquid nitrogen.

Quantification of apoptosis

For quantification of apoptosis, FACS analysis was carried out after PI (propidium iodide, Promokine, PK-CA707-40017, 1 mg/mL) staining versus annexin-V staining (V-FITC, Promokine, PK-CA577-1001-200, 0.15 mg/mL). Flow cytometry was performed as previously described [20].

PIN1 in melanoma

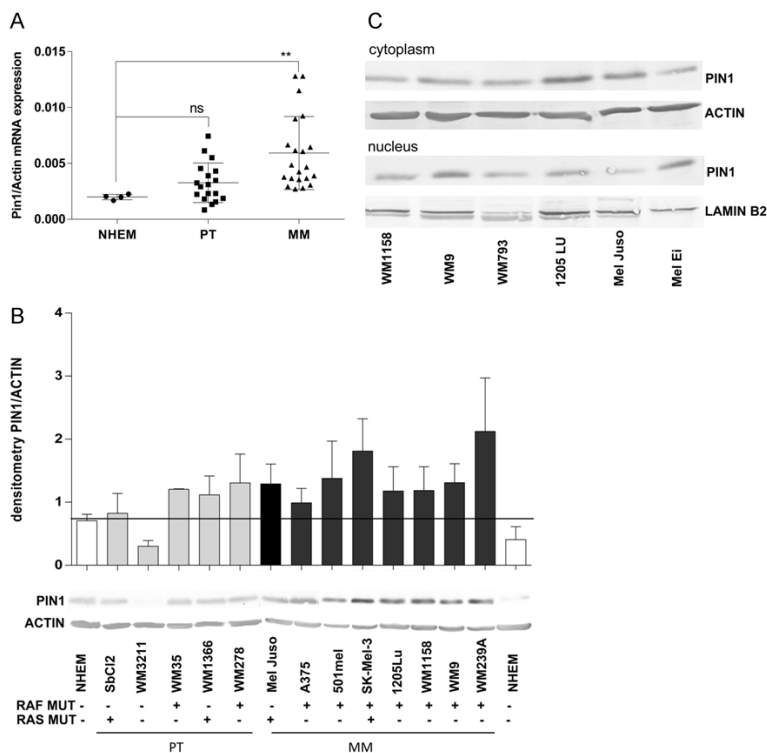


Figure 1. PIN1 expression in melanoma cell lines. A. Quantitative real time PCR analysis of PIN1 expression in melanoma cell lines in comparison to four different donors of normal human epidermal melanocytes isolated from human foreskin (NHEM). B. Representative western blot analysis of different melanoma cell lines which show the PIN1 expression in comparison to NHEM. The densitometry was calculated using the ImageJ software. C. Comparison on PIN1 distribution in the cytoplasmic and nuclear compartments of melanoma cells. β -actin was used as loading control for cytoplasmic proteins and LaminB2 was used as loading control for nuclear proteins.

Statistical analysis

The values for the experiments were presented as the means \pm s.e.m. (standard error of the mean) or percent. Significant differences were determined with a 1-way ANOVA, except for **Figure 3** which was analyzed using the paired t-test. GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA) was used as graphical and statistical tool. A p -value < 0.05 was considered as statistically significant (ns: not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Results

PIN1 expression in melanoma

We examined PIN1 expression in melanoma cell lines available in our laboratory, on both mRNA (**Figure 1A**) and protein levels (**Figure 1B**). Approximately 76% of the melanoma ce-

ll lines showed increased PIN1 expression. Interestingly, PIN1 expression is elevated in cell lines expressing either mutant NRAS (WM1366, SK-Mel-3) or BRAF (e.g. WM239A, 501mel), as compared to cell lines that contain wild-type BRAF and RAS (**Figure 1B**). Western blot analysis showed that PIN1 is localized in both the nucleus and cytoplasm of melanoma cell lines (**Figure 1C**).

We next analyzed the PIN1 expression levels in tissue samples derived from melanoma patients. Based on the mRNA isolated from patient samples, strongest PIN1 expression was detected in melanoma metastases compared to primary tumors (**Figure 2A**). Using NCBI geo profile data set GDS3966/202927_at, highest expression of PIN1 was detectable in melanoma cases compared to normal skin and nevi (**Figure 2B**). The mRNA databank of Talantov et al. [21] confirmed the induced PIN1 expression in melanoma ($n =$

70 samples) (**Figure 2C**). Elevated PIN1 protein levels were also confirmed by using human protein atlas results (<http://www.proteinatlas.org/>) (**Figure 2D**) and melanoma patient tissues provided by the local Pathology laboratory (**Figure 2E**). Immunohistochemistry showed an induction of PIN1 protein in all eleven melanoma tissues with a cytoplasmic, membranous and nuclear localization (**Figure 2D**). Based on the nuclear localization of PIN1, we detected that ~96% of visceral melanoma metastasis were positive for PIN1 (**Figure 2F**). In summary, PIN1 expression can be observed in all parts of the cell, membranous, cytoplasmic and nuclear.

PIN1 knockdown suppresses cell proliferation and tube formation

To assess the role of PIN1 in melanoma, we transfected two metastatic cell lines (Mel Im, SK-Mel-28) with a siRNA pool against PIN1.

PIN1 in melanoma

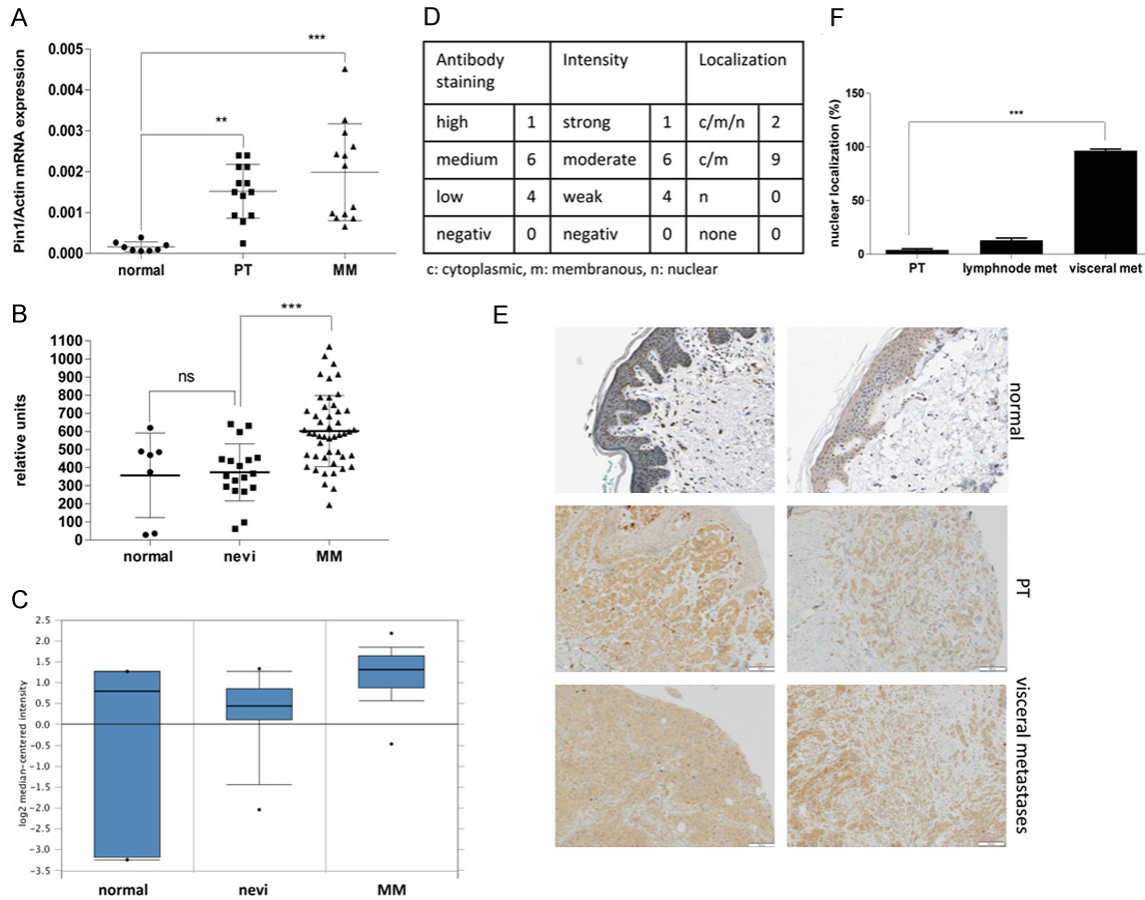


Figure 2. *In vivo* expression of PIN1. A. Quantitative real time PCR of micro-dissected melanoma tissues from different patients. Melanocytes and normal skin are in comparison to primary melanoma tissues (PT) and melanoma metastases (MM). B. Results of the GEOprofile data set (GDS1375/202927_at) comparing normal skin, nevi and melanomas from patients. C. Results of the Talantov dataset including 70 tissues of melanoma patients which was published [21]. D. Immunohistochemistry results of The Human Protein Atlas dataset for eleven different patient tissues. E. Immunohistochemistry of a tissue microarray (TMA) with staining against PIN1 in melanoma samples (10 primary and 10 metastases) versus normal (10) tissues. F. The graph gives an overview of nuclear staining of PIN1.

After 24 to 72 h, successful knockdown of PIN1 was confirmed on both mRNA and protein levels (Figure S1A and S1B). In fact, the PIN1 levels in the melanoma cell lines were reduced to the endogenous protein level of normal human epidermal melanocytes (Figure S1C). We next performed functional assays with the transiently transfected cell lines. The diameter of spheroids formed as 3-D culture and colony formation of the melanoma cell lines were not affected by PIN1 knockdown (Figure S1D and S1E). We also assessed the migratory activity of the transfected cells by using the “cell migration boyden chamber” (data not shown) and the “wound-scratch” assay (Figure 3A). PIN1 knockdown did not significantly affect the migration of melanoma cells (see the scratch closure after 24 hours in Figure 3A).

The scratch assay of Figure 3A hints to a proliferative effect of PIN1 knockdown when we consider the significant PIN1 knockdown effects after 48 hours. Reduced cell proliferation (by ~40%) after PIN1 silencing was confirmed via an XCelligence real time proliferation assay (Figure 3B).

Since vascular mimicry is a characteristic of melanoma cells, we next performed a 3-D tube formation assay. PIN1 knockdown dramatically inhibited tube formation and almost completely prevented vascular mimicry processes (Figure 3C). Consequently, a human angiogenic array was conducted using supernatants of control and siPIN1 transfected Mel Im cells, respectively. Densitometric analysis of the array displayed that pro-MMP-8 was sig-

PIN1 in melanoma

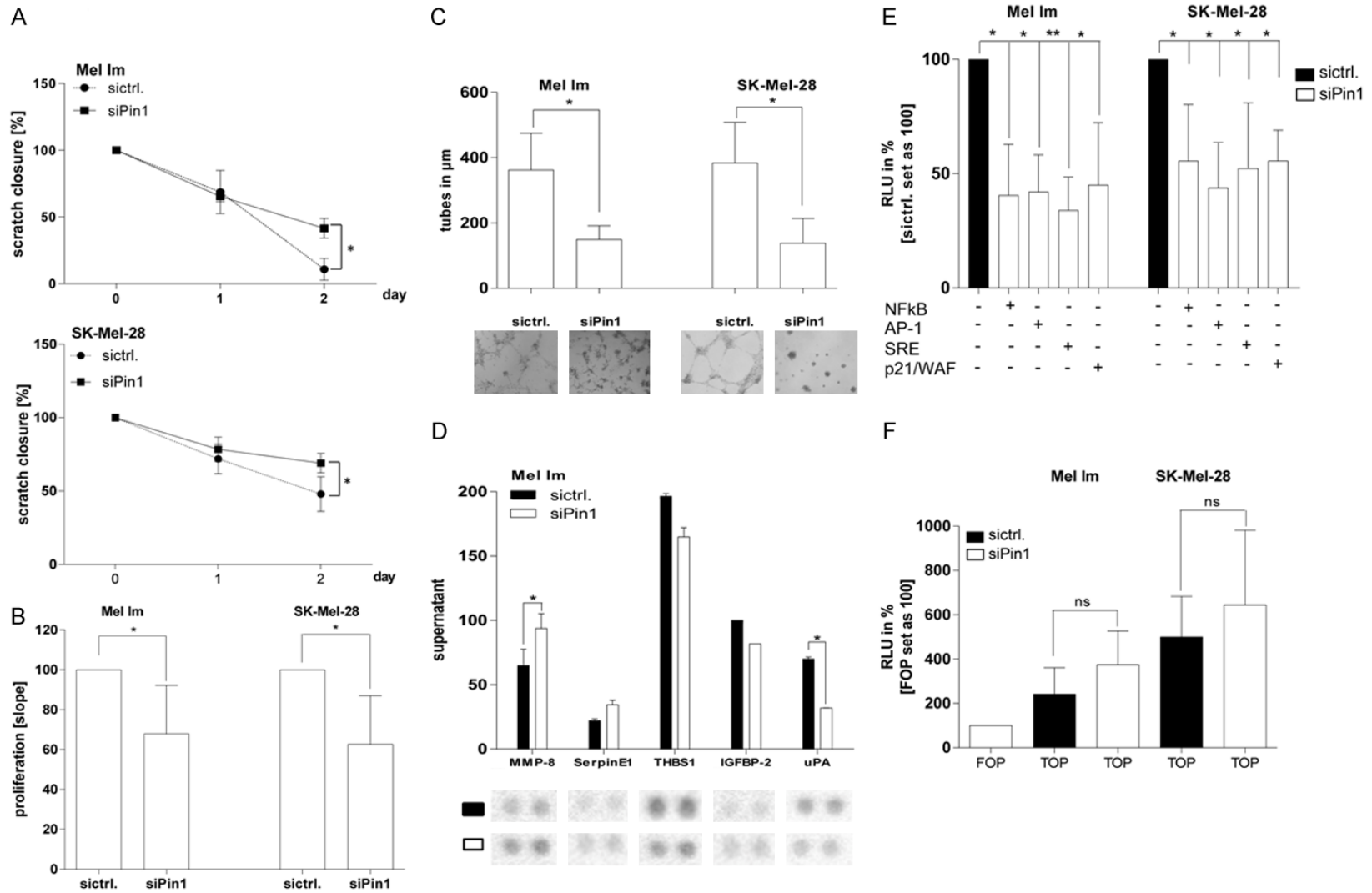
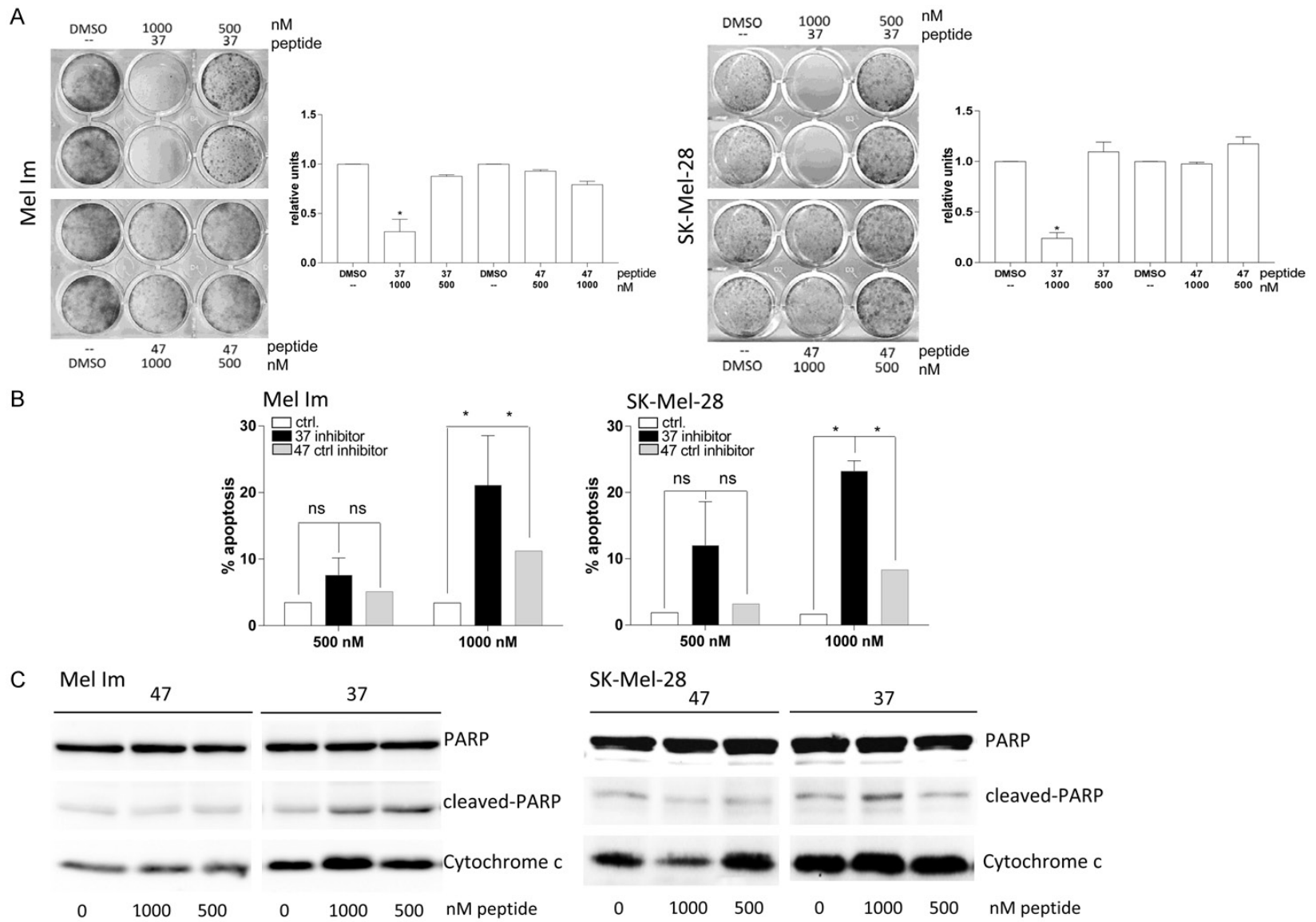


Figure 3. Analysis of the transient knockdown of PIN1. A. Analysis of migration of Mel Im and SK-Mel-28 cell lines in a two dimensional “scratch assay” where the closure of a scratched “wound” was measured in percentage of closure after 48 h. B. xCELLigence system for analyzing the migration of Mel Im and SK-Mel-28 cells after knockdown of PIN1 compared to control siRNA transfected cells (siCtrl). C. 3-D assay: tube formation after knockdown of PIN1 compared to control siRNA transfected cells (siCtrl). D. Supernatant of Mel Im cells after knockdown of PIN1 was analyzed using The Human Angiogenesis Array Kit. Proteins which were affected by PIN1 were shown in the graph and by immunospots. E. Luciferase assays were performed after co-transfection of Mel Im and SK-Mel-28 with siCtrl. or siPIN1 and with various reporter constructs for transcription factor binding cloned into pGL3basic. pRLTK transfection was used as internal control. pGL3basic/siCtrl. were set as 100%. F. The TOPflash luciferase assay was used to measure TCF/LEF transcription factor activity in PIN1 dependency. FOPflash has the TCF/LEF binding sites mutated and therefore was used as control and was set as 100%.

PIN1 in melanoma



PIN1 in melanoma

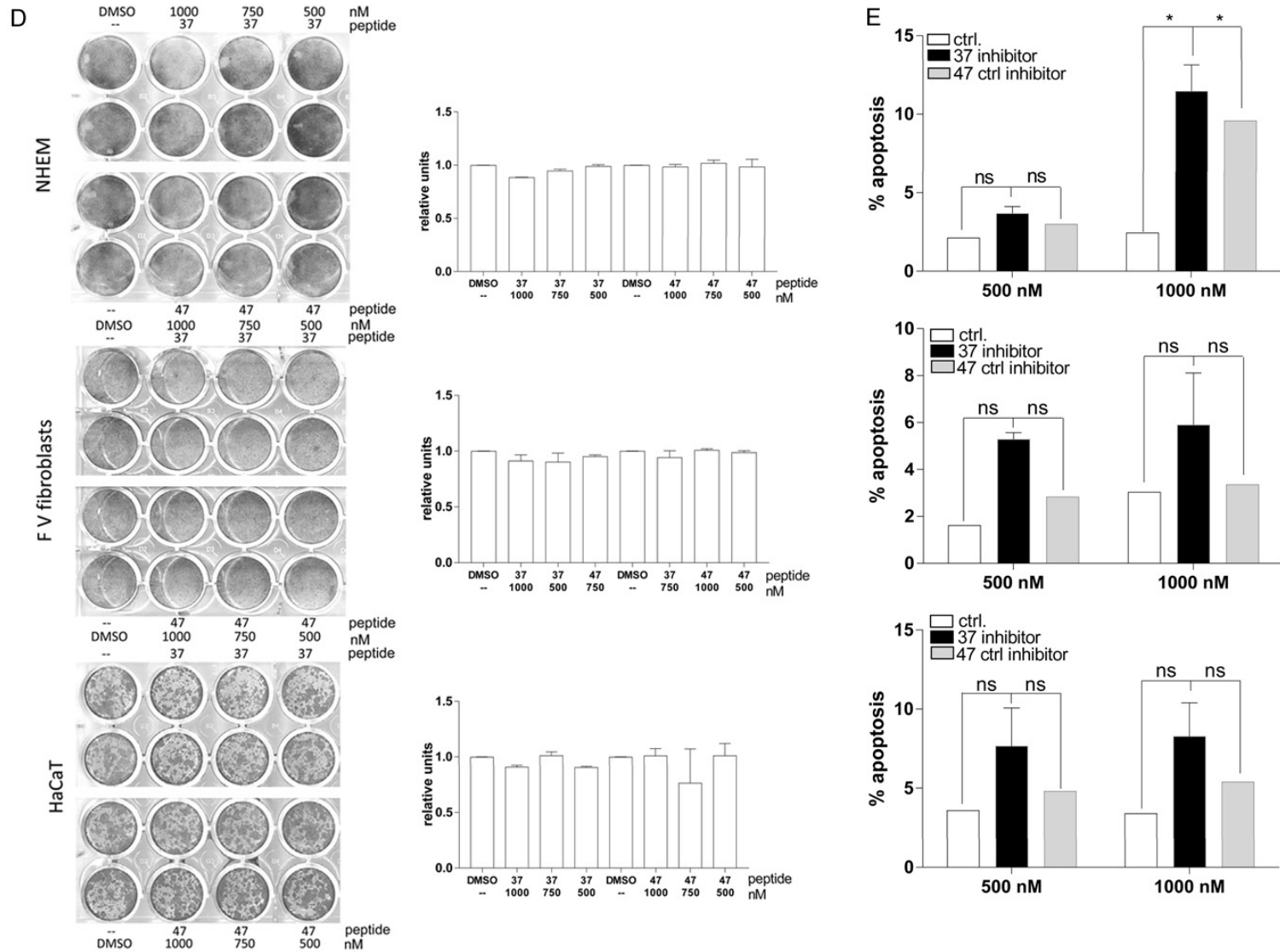


Figure 4. Chemical PIN1 inhibition. A. Mel Im and SK-Mel-28 cells were treated with PIN1 inhibitor 37 and the control inhibitor 47 in a concentration of 500 nM and 1000 nM. DMSO was used as control. Viability of cells was visualized in 6-well chambers and in a graphical overview. B. FACS analysis of apoptosis in melanoma cells in response to treatment with inhibitor 37 compared to 47 (500 and 1000 nM, respectively) is shown. The DMSO control was set as 1. C. Western blot analysis of the apoptotic relevant proteins PARP/cleaved-PARP and Cytochrome C. D. Healthy cells (melanocytes, NHEM; fibroblasts, FV; keratinocytes, HaCaT) were treated with PIN1 inhibitor 37 and the control inhibitor 47 in a concentration of 500, 750, and 1000 nM. DMSO was used as control. Viability of cells was visualized in 6-well chambers and in a graphical overview. (The results were not significant). E. FACS analysis of apoptosis in normal skin cells in response to treatment with inhibitor 37 compared to 47.

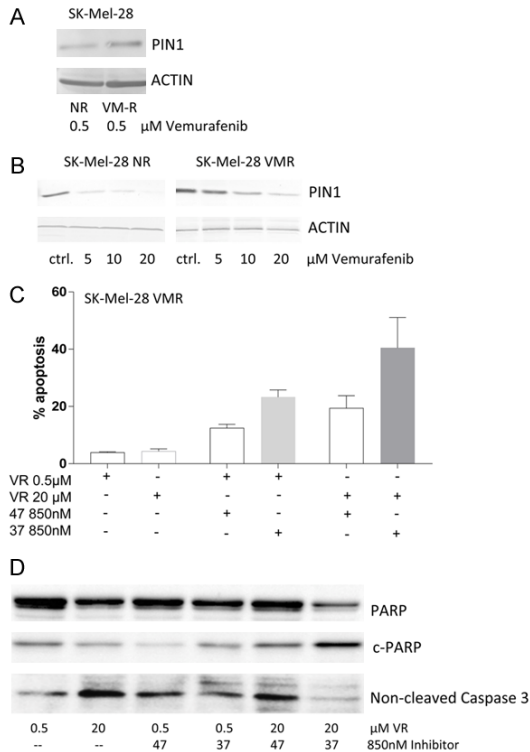


Figure 5. Chemical PIN1 and BRAF inhibition. A and B. PIN1 protein expression of SK-Mel-28 with acquired resistance (VM-R) to BRAF-inhibition (incubated with 0.5 μM to 20 μM Vemurafenib in cell culture), as compared to according non-resistant (NR) cells. C. FACS analysis of apoptosis for SK-Mel-28 VM-R cells treated with Vemurafenib (0.5 μM and 20 μM, respectively) and PIN1 inhibitor 37 and 47, respectively. (Percentage values depict apoptotic cells). D. Western blot analysis of the apoptotic relevant proteins PARP/cleaved-PARP and Caspase-3 after treatment of SK-Mel-28 VM-R cells with Vemurafenib and PIN1 inhibitor 37 and the control inhibitor 47.

nificantly up-regulated and uPA was significantly downregulated after siRNA against PIN1 expression. SERPINE1, THBS1, and IGF-BP2 were not significantly regulated but showed tendencies to be regulated in PIN1 dependency (Figure 3D).

To evaluate regulation of several previously published target molecules of PIN1, we performed western blot analysis (Figure S2). Neither cell cycle proteins Cyclin D1, Cyclin D2, transcription factors c-JUN, FRA-2 (AP-1 family) nor the kinases ERK and p38 were regulated or inactivated/dephosphorylated after successful PIN1 knockdown. A slight reduction of FRA-1 protein amount was detected only in the cell line SK-Mel-28.

As the regulatory influence of PIN1 is difficult to evaluate on the level of one specific target molecule, we conducted a broader approach to proof the participation of PIN1 in generally relevant signaling pathways of melanoma (Figure 3E and 3F). In reporter gene assays PIN1 protein targets were analyzed. Downregulation of PIN1 by siRNA transfection inhibits NFκB, AP-1, SRE, and p53 dependent p21 signaling and has no impact on beta-catenin (TOP/FOP assay) (Figure 3F). In summary, genetic inhibition of PIN1 blocks multiple cancer-driving pathways simultaneously in melanoma.

PIN1 inhibition promotes apoptosis in melanoma cell lines

The strong effects of PIN1 knockdown on melanoma cells suggest that pharmacologic inhibition of PIN1 provide an effective approach for treating melanomas. We tested this by treating melanoma cells with peptide 37, a bicyclic peptidyl inhibitor which demonstrated remarkable potency and selectivity for Pin1, as opposed to other peptidyl-prolyl cis-trans isomerases [15]. Peptide 37 inhibits PIN1 via binding to its active site. It is cell-permeable and metabolically stable and inhibited the proliferation of HeLa cells with an IC₅₀ value of 1.0 μM [15]. We tested the effect of peptide 37 and the control peptide 47 (which has greatly reduced PIN1 binding affinity) on the viability of Mel 1m and SK-Mel-28 melanoma cells (Figure 4A). Treatment of the cells with 1000 nM peptide 37 resulted in floating cells within five hours (the cells did not recover after seven days of incubation), whereas peptide 47 had no effects. At 500 nM, neither peptide had significant visible impact on the cell lines. Flow cytometry analysis of the cells revealed that both peptides caused apoptosis at a concentration of 1000 nM but the effect was much greater for peptide 37 (Figure 4B). Incubation of the cells with peptide 37 also resulted in greater amounts of cleaved PARP, an apoptotic substrate of Caspase-3, as compared to peptide 47 (Figure 4C). Mel 1m cells appear to be particularly sensitive to peptide 37, since significant PARP cleavage was observed already at 500 nM peptide 37 compared to 1000 nM peptide 37 for SK-Mel-28 cells. Peptide 37 also caused cytochrome c release from mitochondria into the cytosol and again the effect of peptide 47 was less strong. We additionally treated norm-

al human epidermal melanocytes (NHEM), immortalized fibroblasts (FV) and keratinocytes (HaCaT) with peptides 37 and 47. After treatment for 1 day at 500-1000 nM peptide, no significant floating cell population was observed for all three “normal” cell types (**Figure 4D**). Only NHEM failed to reach confluency after treatment with 1000 nM peptide 37. NHEMs were also the only cell line that underwent significant apoptosis after 1-day treatment with 1000 nM peptide 37 or 47 (**Figure 4E**). These results demonstrate that melanoma cells are more sensitive to the PIN1 inhibitor peptide 37 than healthy control cells.

PIN1 inhibition sensitizes melanoma cells to chemotherapeutic agents

RAF, MEK, extracellular signal-regulated kinase (ERK) proteins are core components of de-regulated signaling pathways in melanoma leading to abnormal cell proliferation [1]. Phosphorylation/de-phosphorylation recycling processes of molecules of this signaling cascade are dependent on protein phosphatase 2A (PP2A) and PIN1 [2, 22]. We analyzed the RAF-MEK-ERK-dependent PIN1 regulation also in melanoma after inhibition of this important pathway. For BRAF inhibition, we used the clinically approved drug PLX-4032 (Vemurafenib) to treat BRAF inhibitor-sensitive and BRAF inhibitor-resistant sub-populations of SK-Mel-28 cells [23]. The resistant cells reveal acquired drug resistance to BRAF inhibition and are constantly under Vemurafenib selection (5 μ M) in cell culture medium. The cells do not have secondary BRAF mutation beyond V600E. For the next experiment resistant SK-Mel-28 cells (VM-R) were compared to non-resistant SK-Mel-28 (NR) cells which were treated with 0.5 μ M Vemurafenib for 48 hours. The PIN1 protein level was elevated in VM-R cells compared to NR cells (**Figure 5A**). Afterwards we repeated the experiment comparing the PIN1 protein level in VM-R cells (5 μ M Vemurafenib) directly with that of untreated control cells (**Figure 5B**). VM-R SK-Mel-28 cells (ctrl.) have a higher PIN1 protein amount than NR SK-Mel-28 cells (ctrl.). Then we used different concentrations of Vemurafenib and compared the PIN1 protein level in VM-R cells with that of NR cells. A decline of PIN1 protein was visible which was slower in VM-R cells compared to NR cells in Vemurafenib-concentration dependency

(**Figure 5B**). We speculated that PIN1 induction could support resistance of VM-R and that a combination treatment with PIN1 and BRAF inhibitors may overcome the resistance against Vemurafenib. We analyzed the effects of peptide 37 and Vemurafenib on resistant SK-Mel-28 and revealed that peptide 37 was more effective than Vemurafenib alone in inducing apoptosis (**Figure 5C**). PIN1 inhibition resulted in a significant decline in cell viability when adding 20 μ M Vemurafenib and peptide 37 in combination to resistant cells. Western blot analysis of cleaved PARP and Caspase 3 confirmed the induction of apoptosis in resistant melanoma cell line SK-Mel-28 by Vemurafenib and peptide 37. Significant accumulation of cleaved PARP and reduction in the amount of non-cleaved Caspase 3 were observed (**Figure 5D**).

Discussion

There is substantial evidence that two of the major pathways for melanoma development are the Mitogen-activated protein kinase (MAPK) and Phosphatidylinositol-3-kinase (PI3K) pathway. It is known that PIN1 isomerizes specific phosphorylated Ser/Thr-Pro bonds in key kinases of these pathways and therefore is a major player for controlling the fates of phospho-proteins in these cancer-relevant signaling pathways. Thus, the role of PIN1 in enhancing the oncogenic potential of these proteins via phosphorylation-dependent prolyl isomerization is important during cancer development and progression.

Our data showed that PIN1 is up-regulated in melanoma cell lines and in melanoma tissue of patients. Consistently, several studies have confirmed that PIN1 mRNA and protein are over-expressed in cancer, as compared with non-cancer tissues [7, 24-26]. We evaluated the importance of PIN1 in melanoma using genetic PIN1 knockdown by a siRNA pool and chemical inhibition of PIN1 activity. Functionally, we show that PIN1 regulates proliferation and tube formation. PIN1 inhibition by the inhibitor 37 induces apoptosis of melanoma cell line but not of healthy cells. Both, genetically and chemically inhibition of PIN1 regulates cancer relevant pathways.

Indeed, many specific PIN1-interacting partners, such as β -catenin, c-Jun, cyclin D1, Myc, p65, to mention only a few, are known so far

and are implicated in cancer development and progression [9, 12, 27]. It was previously shown by Kruiswijk et al. [26] that PIN1 contributes to FOXM1 signaling in melanoma. Our data approved several PIN1 target molecules also in melanoma, e.g. the transcription factor family NF κ B, AP1, the serum response element, and p53 dependent regulation of p21. Our experiments excluded the transcription factor family TCF/LEF as molecular target of PIN1, although TCF/LEF were described to be specific targets of PIN1 in alternative cancer entities [27].

Obviously, several signaling pathways are regulated by PIN1 in melanoma. However, it was shown previously for the melanoma cell line A375 that phosphorylation of AKT and the protein amount of JNK-1, -2, -3 are PIN1-dependently regulated [28]. Our data using different cell lines (Mel 1m and SK-Mel-28) did not confirm this finding. Phosphorylation of AKT and the protein amount of JNK-1 was not significantly regulated by PIN1. Furthermore, we analyzed the phosphorylation status of p38 and ERK in PIN1 dependency and could not find any regulation of these MAP kinases. It is possible that PIN1 is involved in relevant cancer pathways; still, specific PIN1 target molecules remain to be determined.

PIN1 is also able to bind to the phospho-Thr254-Pro motif of NF κ B/p65 (p65), thereby inhibiting its interaction with I κ B α , contributing to constitutive activation of NF κ B in a variety of human malignancies [10]. Knockdown of PIN1 by siRNA significantly inhibited the expression of p-NF κ B-p65 (Thr254) and p-NF κ B-p65 (Ser276), thereby reducing the activity of NF κ B in HepG2 cells [29]. We revealed that the luciferase reporter activity of NF κ B was reduced using siRNA against PIN1, which hints to an important role of PIN1 in regulation of this relevant pathway also in melanoma cells. Because PIN1 regulates NF κ B, the PIN1 inhibitor may sensitize further cancer cell types also to conventional chemotherapeutic agents such as etoposide and cisplatin.

To target metastatic melanoma, inhibitors of the RAF/MEK pathway, like Vemurafenib have shown partial clinical success, but their effectiveness is hampered due to development of resistance [30]. The development of resistance to previously effective treatments has been a challenge for health care providers and a fear

for patients undergoing cancer therapy. This is an unfortunately frequent occurrence for patients undergoing targeted therapy for tumors harboring the activating V600E mutation of the BRAF gene. Combination of therapies received much attention in clinical trials and therefore we tested the combination of the peptide 37, a PIN1 inhibitor and Vemurafenib, a RAF kinase inhibitor, in melanoma cell lines which were resistant against Vemurafenib [23]. The peptide 37 alone led to growth retardation and apoptosis. Combination of both, peptide 37 and Vemurafenib overcame the resistance of the SK-Mel-28 cells significantly and led to re-induction of apoptosis.

All-trans retinoic acid (ATRA) was recently identified as potent PIN1 inhibitor in hepatocellular carcinoma (HCC) [27] and breast cancer cells [31]. ATRA was also already used as treatment against melanoma [32]. At that time mainly the induction of cell differentiation in the melanoma cells was seen as molecular mode of action. Given the new knowledge about ATRA inhibition of PIN1 and the effects of PIN1 inhibition revealed in this study, potentially also inhibition of PIN1 by ATRA was a component of treatment success. The data underline our idea to consider PIN1 inhibition in clinical trials and indicate that PIN1 inhibition can also be a successful approach for melanoma therapy, eventually in combinatorial trials.

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Disclosure of conflict of interest

None.

Abbreviations

MMP-8, matrix-metalloproteinase 8; uPA, Plasminogen Activator; THBS1, Thrombospondin 1; IGF-BP2, Insulin-like growth factor binding protein 2.

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References

- [1] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR and Futreal PA. Mutations of the BRAF gene in human cancer. *Nature* 2002; 417: 949-954.
- [2] Ritt DA, Monson DM, Specht SI and Morrison DK. Impact of feedback phosphorylation and Raf heterodimerization on normal and mutant B-Raf signaling. *Mol Cell Biol* 2010; 30: 806-819.
- [3] Zheng H, You H, Zhou XZ, Murray SA, Uchida T, Wulf G, Gu L, Tang X, Lu KP and Xiao ZX. The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. *Nature* 2002; 419: 849-853.
- [4] Zacchi P, Gostissa M, Uchida T, Salvagno C, Avolio F, Volinia S, Ronai Z, Blandino G, Schneider C and Del Sal G. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature* 2002; 419: 853-857.
- [5] Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR and Sears R. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol* 2004; 6: 308-318.
- [6] Crenshaw DG, Yang J, Means AR and Kornbluth S. The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. *EMBO J* 1998; 17: 1315-1327.
- [7] Wulf GM, Ryo A, Wulf GG, Lee SW, Niu T, Petkova V and Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. *EMBO J* 2001; 20: 3459-3472.
- [8] Liou YC, Zhou XZ and Lu KP. Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. *Trends Biochem Sci* 2011; 36: 501-514.
- [9] Monje P, Hernandez-Losa J, Lyons RJ, Castellone MD and Gutkind JS. Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. *J Biol Chem* 2005; 280: 35081-35084.
- [10] Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S and Lu KP. Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol Cell* 2003; 12: 1413-1426.
- [11] Lufe C, Koh TH, Uchida T and Cao X. Pin1 is required for the Ser727 phosphorylation-dependent Stat3 activity. *Oncogene* 2007; 26: 7656-7664.
- [12] Zhou XZ and Lu KP. The isomerase PIN1 controls numerous cancer-driving pathways and is a unique drug target. *Nat Rev Cancer* 2016; 16: 463-478.
- [13] Rustighi A, Zannini A, Campaner E, Ciani Y, Piazza S and Del Sal G. PIN1 in breast development and cancer: a clinical perspective. *Cell Death Differ* 2017; 24: 200-211.
- [14] Moore JD and Potter A. Pin1 inhibitors: pitfalls, progress and cellular pharmacology. *Bioorg Med Chem Lett* 2013; 23: 4283-4291.
- [15] Jiang B and Pei D. A Selective, cell-permeable nonphosphorylated bicyclic peptidyl inhibitor against peptidyl-prolyl isomerase Pin1. *J Med Chem* 2015; 58: 6306-6312.
- [16] Dreno B, Ribas A, Larkin J, Ascierto PA, Hauschild A, Thomas L, Grob JJ, Koralek DO, Rooney I, Hsu JJ, McKenna EF and McArthur GA. Incidence, course, and management of toxicities associated with cobimetinib in combination with vemurafenib in the coBRIM study. *Ann Oncol* 2017; 28: 1137-1144.
- [17] Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A and Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 1988; 106: 761-771.
- [18] el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993; 75: 817-825.
- [19] Franken NA, Rodermond HM, Stap J, Haveman J and van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc* 2006; 1: 2315-2319.
- [20] Riccardi C and Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 2006; 1: 1458-1461.
- [21] Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, Atkins D and Wang Y. Novel genes associated with malignant melanoma but not

PIN1 in melanoma

- benign melanocytic lesions. *Clin Cancer Res* 2005; 11: 7234-7242.
- [22] Dougherty MK, Muller J, Ritt DA, Zhou M, Zhou XZ, Copeland TD, Conrads TP, Veenstra TD, Lu KP and Morrison DK. Regulation of Raf-1 by direct feedback phosphorylation. *Mol Cell* 2005; 17: 215-224.
- [23] Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, Wubbenhorst B, Xu X, Gimotty PA, Kee D, Santiago-Walker AE, Letrero R, D'Andrea K, Pushparajan A, Hayden JE, Brown KD, Laquerre S, McArthur GA, Sosman JA, Nathanson KL and Herlyn M. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* 2010; 18: 683-695.
- [24] Ayala G, Wang D, Wulf G, Frolov A, Li R, Sowadski J, Wheeler TM, Lu KP and Bao L. The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer. *Cancer Res* 2003; 63: 6244-6251.
- [25] Bao L, Kimzey A, Sauter G, Sowadski JM, Lu KP and Wang DG. Prevalent overexpression of prolyl isomerase Pin1 in human cancers. *Am J Pathol* 2004; 164: 1727-1737.
- [26] Kruiswijk F, Hasenfuss SC, Sivapatham R, Baar MP, Putavet D, Naipal KA, van den Broek NJ, Kruit W, van der Spek PJ, van Gent DC, Brenkman AB, Campisi J, Burgering BM, Hoeijmakers JH and de Keizer PL. Targeted inhibition of metastatic melanoma through interference with Pin1-FOXO1 signaling. *Oncogene* 2016; 35: 2166-2177.
- [27] Liao XH, Zhang AL, Zheng M, Li MQ, Chen CP, Xu H, Chu QS, Yang D, Lu W, Tsai TF, Liu H, Zhou XZ and Lu KP. Chemical or genetic Pin1 inhibition exerts potent anticancer activity against hepatocellular carcinoma by blocking multiple cancer-driving pathways. *Sci Rep* 2017; 7: 43639.
- [28] Jin J, Zhang Y, Li Y, Zhang H, Li H, Yuan X, Li X, Zhou W, Xu B, Zhang C, Zhang Z, Zhu L and Chen X. RNA-interference-mediated downregulation of Pin1 suppresses tumorigenicity of malignant melanoma A375 cells. *Neoplasma* 2013; 60: 92-100.
- [29] Shinoda K, Kuboki S, Shimizu H, Ohtsuka M, Kato A, Yoshitomi H, Furukawa K and Miyazaki M. Pin1 facilitates NF-kappaB activation and promotes tumour progression in human hepatocellular carcinoma. *Br J Cancer* 2015; 113: 1323-1331.
- [30] Alcalá AM and Flaherty KT. BRAF inhibitors for the treatment of metastatic melanoma: clinical trials and mechanisms of resistance. *Clin Cancer Res* 2012; 18: 33-39.
- [31] Wei S, Kozono S, Kats L, Nechama M, Li W, Guarnerio J, Luo M, You MH, Yao Y, Kondo A, Hu H, Bozkurt G, Moerke NJ, Cao S, Reschke M, Chen CH, Rego EM, Lo-Coco F, Cantley LC, Lee TH, Wu H, Zhang Y, Pandolfi PP, Zhou XZ and Lu KP. Active Pin1 is a key target of all-trans retinoic acid in acute promyelocytic leukemia and breast cancer. *Nat Med* 2015; 21: 457-466.
- [32] Asgari MM, Brasky TM and White E. Association of vitamin A and carotenoid intake with melanoma risk in a large prospective cohort. *J Invest Dermatol* 2012; 132: 1573-1582.

PIN1 in melanoma

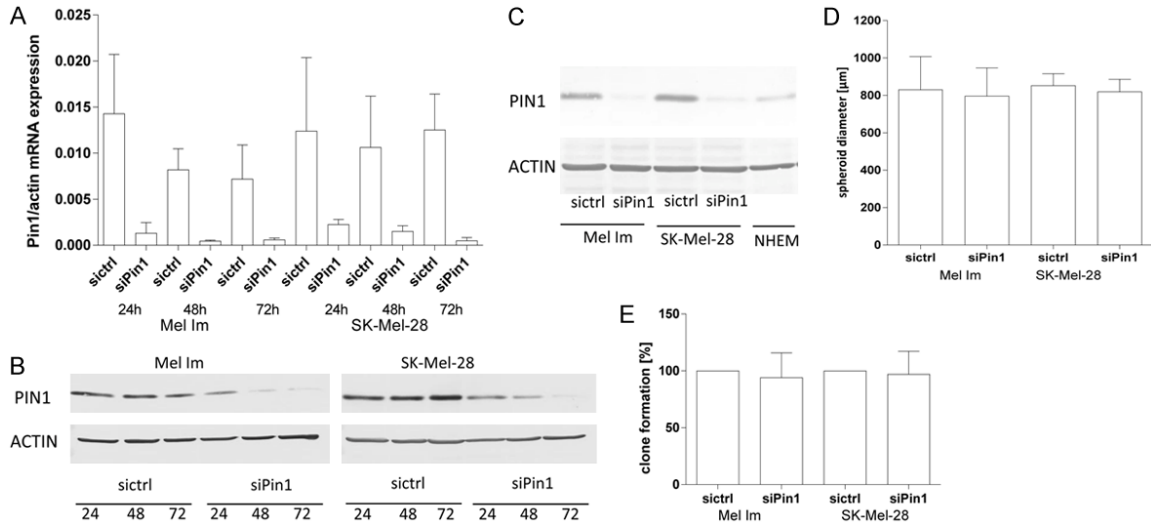


Figure S1. A and B. q-PCR analysis for PIN1 mRNA expression and western blot analysis of PIN1 protein amount after using siRNA against PIN1 (24 to 72 hours). C. PIN1 protein amount was compared after transfection of siRNA against PIN1 in melanoma cell lines compared to the endogenous PIN1 status of NHEM. D. Spheroid assay after knockdown of PIN1 compared to control siRNA transfected cells (sictrl). (Results were not significant). E. Clonogenic assay after knockdown of PIN1 compared to sictrl. (Results were not significant).

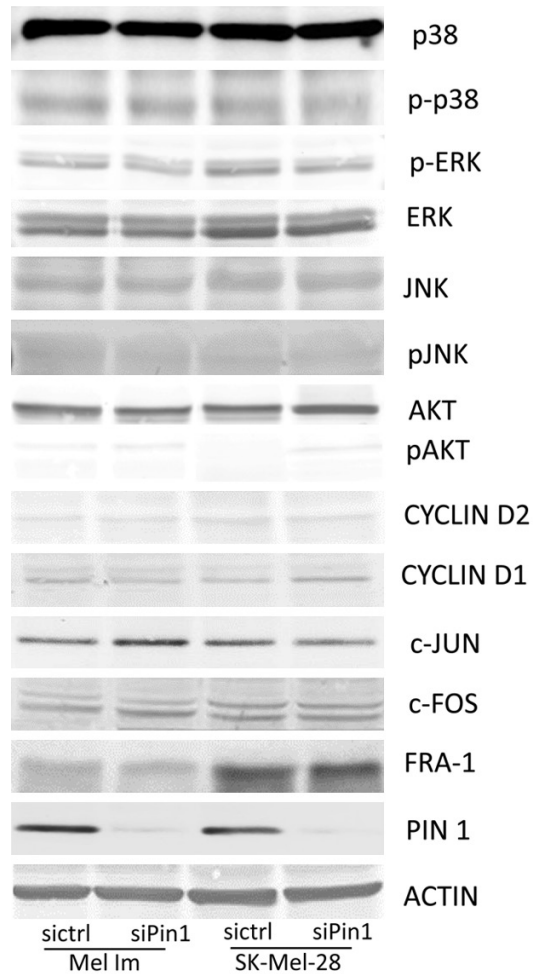


Figure S2. Western blot analysis of MAP kinases/phosphorylated MAP kinases and of proliferative relevant proteins after knockdown of PIN1.