Dissertation

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The role of FOXD1 in melanoma dedifferentiation and resistance towards targeted therapy

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Declarations

Declarations according to § 8 (3) b) and c) of the doctoral degree regulations:

b) I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or materials than those expressly indicated,

c) I hereby declare that I have not applied to be examined at any other institution, nor have I used the dissertation in this or any other form at any other institution as an examination paper, nor submitted it to any other faculty as a dissertation.

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Qian Sun 孙倩

This thesis is dedicated to my grandparents, my parents and my sister who always support me, believe in me and inspire me.

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Abstract

Metastatic melanoma is one of the most aggressive skin cancers and is associated with poor prognosis. BRAF and MEK inhibitors are used to treat patients with $BRAF^{V600E}$-mutated advanced melanoma. However, the development of resistances to these treatments compromises therapeutic success. Our lab previously demonstrated that forkhead box D1 (FOXD1) plays a critical role in melanoma migration and invasion. Here, I found that FOXD1 was highly expressed in melanoma cells. Immunohistochemical assessment of 105 samples from patients with metastatic melanoma revealed that high FOXD1 expression in tumors was associated with poor survival and correlated with low MITF, SOX10 and high AXL expression. Upregulation of FOXD1 expression enhanced the resistance of melanoma to vemurafenib (BRAF inhibitor) or combinatorial treatment with vemurafenib and cobimetinib (MEK inhibitor). On the other hand, loss of FOXD1 increased the sensitivity of naïve melanoma cells towards vemurafenib or combinatorial treatment with vemurafenib and cobimetinib. Furthermore, high FOXD1 expression levels were found in BRAF inhibitor (BRAFi)-resistant cells. Downregulation of FOXD1 resulted in a resensitization of BRAFi-resistant cells to vemurafenib. By using microarray analysis, connective tissue growth factor (CTGF) was found to be one of the most downregulated genes in FOXD1 knockdown (KD) cells while its expression was highly increased upon FOXD1 overexpression. Thus, CTGF was identified as a downstream factor of FOXD1. In addition, *in vitro* expression analysis and evaluation of clinical samples demonstrated that CTGF and FOXD1 expression were positively correlated. By using a CHIP assay and a dual reporter luciferase assay, I discovered that FOXD1 could regulate the expression of CTGF by directly binding to the CTGF promoter. This result was confirmed with RT-PCR and western blot. In addition, I found that the protein level of CTGF was highly increased in BRAFi-resistant cells. Similar to FOXD1 knockdown, the knockdown of CTGF resensitized BRAFi-resistant cells to vemurafenib. FOXD1 KD cells treated with recombinant CTGF protein were less sensitive towards vemurafenib compared to untreated FOXD1 KD cells. Based on these findings, I conclude that the transcription factor FOXD1 could promote dedifferentiation and targeted therapy-resistance in melanoma cells by regulating the expression of CTGF. Apart from the results above, I also demonstrated that cytokines such as TGF-β are regulated by FOXD1, and FOXD1 could promote EGFR-RAS-MAPK/AKT pathways activation. Taken these results together, FOXD1 might be a promising new diagnostical marker and a therapeutic target of targeted therapy resistant melanoma.

Zusammenfassung

Das metastasierte Melanom ist eine der aggressivsten Hautkrebsarten und mit einer schlechten Prognose verbunden. BRAF- und MEK-Inhibitoren werden zur Behandlung von Patienten mit $BRAF^{V600E}$-mutiertem, fortgeschrittenem Melanom verwendet. Die Entwicklung von Resistenzen gegen diese Behandlungen beeinträchtigt jedoch den therapeutischen Erfolg. Unser Labor hat zuvor gezeigt, dass Forkhead Box D1 (FOXD1) eine entscheidende Rolle bei der Migration und Invasion von Melanomenzellen spielt. In dieser Arbeit konnte ich zeigen, dass FOXD1 in Melanomzellen stark exprimiert wurde. Die immunhistochemische Untersuchung von 105 Proben von Patienten mit metastasiertem Melanom ergab, dass eine hohe FOXD1-Expression in Tumoren mit einem schlechten Überleben verbunden war und mit einer niedrigen MITF-, SOX10- und hohen AXL-Expression korrelierte. Die Hochregulierung der FOXD1-Expression erhöhte die Resistenz der Melanomzellen gegen die Behandlung mit Vemurafenib (BRAF-Inhibitor) oder die Kombinationstherapie mit Vemurafenib und Cobimetinib (MEK-Inhibitor). Andererseits erhöhte eine Herunterregulierung von FOXD1 die Empfindlichkeit von naiven Melanomzellen gegenüber einer Behandlung mit Vemurafenib oder der Kombinationstheraoie mit Vemurafenib und Cobimetinib. Darüber hinaus wurden hohe FOXD1-Expressionsniveaus in BRAF-Inhibitor (BRAFi) -resistenten Zellen gefunden. Die Herunterregulierung von FOXD1 führte zu einer erhöhten Empfindlichkeit von BRAFi-resistenten Zellen gegenüber Vemurafenib. Unter Verwendung der Microarray-Analyse wurde festgestellt, dass der Bindegewebswachstumsfaktor CTGF eines der am stärksten herunterregulierten Gene nach FOXD1-Knockdown war. Zudem bewirkte die Überexpression von FOXD1 ebenfalls eine starke Erhöhung des CTGF-Expressionsniveaus. Somit wurde CTGF als ein von FOXD1 regulierter Faktor identifiziert. Darüber hinaus konnten sowohl *in vitro*-Expressionsanalysen, als auch die Auswertungen klinischer Proben zeigen, dass die CTGF- und FOXD1-Expression positiv korrelieren. Unter Verwendung eines CHIP-Assays und eines Dual-Reporter-Luciferase-Assays entdeckte ich, dass FOXD1 die Expression von CTGF durch direkte Bindung an den CTGF-Promotor kontrollieren kann. Dieses Ergebnis wurde mit RT-PCR und western blot bestätigt. Zusätzlich fand ich, dass das Proteinlevel von CTGF in BRAFi-resistenten Zellen stark erhöht war. Ähnlich wie beim FOXD1-Knockdown werden BRAFi-resistente Zellen nach Knockdown von CTGF gegenüber Vemurafenib resensibilisiert. Mit rekombinantem CTGF-Protein behandelte FOXD1-KD-Zellen waren gegenüber Vemurafenib weniger empfindlich als unbehandelte FOXD1-KD-Zellen. Basierend auf diesen Befunden schließe ich, dass der Transkriptionsfaktor FOXD1 die Dedifferenzierung und gezielte Therapieresistenz in Melanomzellen fördern könnte, indem er die Expression von CTGF reguliert. Abgesehen von den obigen Ergebnissen konnte ich auch zeigen, dass Zytokine wie TGF-β ebenfalls durch FOXD1 reguliert werden und FOXD1 die Aktivierung der EGFR-

RAS-MAPK / AKT-Signalwege fördern könnte. Zusammengenommen könnte FOXD1 ein vielversprechender neuer diagnostischer Marker und ein therapeutisches Ziel für die Behandlung von therapieresistenten Melanomen sein.

List of abbreviations

18S	18S ribosomal RNA
ANOVA	analysis of variance
AXL	AXL receptor tyrosine kinase
ATCC	American type culture collection
ARAF	serine/threonine-protein kinase A-RAF
DMEM	Dulbecco's modified eagle's medium
BCA	Bicinchoninic Acid Protein Assay
BME	basement membrane extracts
CTLA-4	cytotoxic T lymphocyte antigen 4
BRAF	B-RAF proto-oncogene, serine/threonine kinase
CCLE	complementary deoxyribonucleic acid
CDK4	cyclin-dependent kinase 4
BSA	bovine serum albumin
CDK6	cyclin-dependent kinase 6
CDKN2A	cyclin dependent kinase inhibitor 2
COBI	cobimetinib
CRAF	C-RAF proto-oncogene serine/threonine-protein kinase
AKT	v-akt murine thymoma viral oncogene
CTGF	connective tissue growth factor
FACS	fluorescence activated cell sorting
CTNNB1	catenin beta 1
CXCL5	C-X-C motif chemokine 5
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
Edu	5-ethynyl-2´-deoxyuridine

PVDF	polyvinylidenfluorid
DAPI	4’,6-diamidino-2-phenylindole
DKFZ	German Cancer Research Center
ECM	extracellular matrix
E.coli	Escherichia coli
etc.	et cetera
EMT	epithelial-to-mesenchymal transition
hESCs	human embryonic stem cells
EDN	endothelin
ESCs	embryonic stem cells
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
FCS	fetal calf serum
IL-2	interleukin-2
GTP	guanosine-5’-triphosphate
GNAQ	G protein subunit alpha Q
mRNA	messenger ribonucleic acid
hiPSCs	human induced pluripotent stem cells
IFNα	interferon α
IHC	immunohistochemistry
MDSC	myeloid-derived suppressor cells
IL-6	interleukin 6
NOD/SCID	nonobese diabetic/severe combined immunodeficient
KIT	KIT proto-oncogene, receptor tyrosine kinase
LB	Lysogeny broth
NHM	normal human melanocytes
mTOR	mechanistic target of rapamycin kinase

MAPK	mitogen-activated protein kinase
MDM2	mouse double minute 2
iPSCs	induced pluripotent stem cells
NCCs	neural crest cells
NC	neural crest
NCI	National Cancer Institute
PVDF	polyvinylidenfluorid
NF1	neurofibromin 1
PTEN	phosphatase and tensin homolog
NGFR	nerve growth factor receptor
PBS	phosphate buffered saline
OE	overexpressing
PD-L2	programmed cell death 1 ligand 2
p53	tumor protein P53
PAX3	paired box gene 3
PCR	polymerase chain reaction
PD-L1	programmed cell death 1 ligand 1
PFS	progression-free survival
PD-1	programmed death 1
PVDF	polyvinylidenfluorid
PMEL	premelanosome protein/melanosomal matrix protein 17
SOX2	SRY (sex determine region Y)-BOX2
PFA	paraformaldehyde
siRNA	small interfering RNA
SOB	Super Optimal Broth
PDT	photodynamic therapy
PTT	photothermal therapy

TGF-β	transforming growth factor β
PI	propidum iodide
TERT	telomerase reverse transcriptase
PDGF	platelet-derived growth factor
RAS	rat sarcoma
RTKs	receptor tyrosine kinases
TCR	T-cell receptor gene
TRP1	tyrosinase-related protein 1
VEGF	vascular endothelial growth factor
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
UV	ultraviolet
TILS	tumor-infiltrating lymphocytes
Tregs	regulatory T cells
VEM	vemurafenib
TMA	tissue microarray
vs.	versus
ZEB1	zinc finger E-box binding homeobox 1

1. Introduction

1.1 Melanoma

Increasing incidence rates of cutaneous melanoma have been observed over the last 50 years, especially in places where fair-skinned populations live[1,2], such as Northern America, Australia, New Zealand[3] and Northern Europe[4]. According to a large amount of studies, sun exposure shows a strong positive correlation with melanoma which is considered to be the determinant factor for melanoma development[5]. Besides sun exposure, other important aspects (including white skin, fair hair, the tendency to freckle etc) and immunosuppression are also considered as risk factors[6]. As shown in Figure 1, cutaneous melanoma has been mainly classified into four different subtypes according to the present driver mutation: BRAF-mutant (~50%), NRAS-mutant (~25%), neurofibromin 1 (NF1)-deficient (~10%) and triple wild-type (TWT) harboring other mutations including KIT and CTNNB1 mutations[7,8]. In the following part, I will give an introduction about the four main subtypes of melanoma and the current therapy strategies against melanoma.

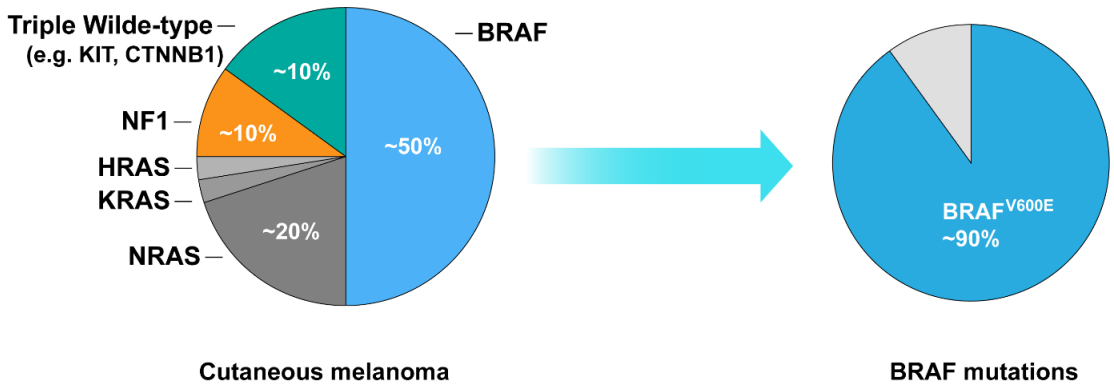


Figure 1. Mutations in cutaneous melanoma

Left: Abundance of driver mutations in melanoma including BRAF mutations (50%), RAS mutations (25%) including the extremely rare KRAS and HRAS mutations, NF1 loss (10%), and infrequent driver mutations affecting genes such as KIT, CTNNB1. Right: Among BRAF mutations, 90% of cases are $BRAF^{V600E}$ point mutations. The figure was adjusted from Wiesner et al[8].

1.1.1 BRAF-mutant melanoma

The serine/threonine protein kinases BRAF, ARAF and CRAF comprise the RAF (rapidly accelerated fibrosarcoma) family. BRAF has stronger elevated basal kinase activity than ARAF and CRAF, which means BRAF is easily activated by RAS[9–11]. This high potential of being activated by RAS accounts for the fact that the BRAF mutation represents the most frequent (over 50%) driver mutation identified in melanoma, whereas ARAF and CRAF mutations are extraordinary rare[12,13]. Based on the report from Aubhishek Zaman[14], about

two hundred different mutations of BRAF have been found and a large amount of them are not well studied. In more detail, BRAF mutations can be classified into 3 variants. RAS-independent monomers with high activity of BRAF such as $BRAF^{V600E}$, $BRAF^{V600K}$ and $BRAF^{V600D}$ belong to variant 1; RAS-independent dimers normally with lower activity of BRAF such as $BRAF^{K601E}$, $BRAF^{K601N}$ belong to variant 2; whereas the BRAF activity of variant 3 such as $BRAF^{D594G}$, $BRAF^{D594N}$,etc. is RAS-dependent and lower than the activity of variant 1 and 2. Briefly, in cells with a variant 1 or 2 BRAF mutation, the BRAF kinase is constitutively activated without being stimulated by RAS. On the other hand, in cells with a variant 3 BRAF mutation, the function of BRAF depends on RAS activation (Table 1). Understanding the potential mechanisms of these BRAF mutations could help us provide more personalized medical treatment[15–17].

Table 1. Variants of BRAF mutations

Variant	RAS Dependent	Mutation
Variant 1	No	V600E, V600K, V600D, etc.
Variant 2	No	G469E, K601E, K601N, etc.
Variant 3	Yes	D594N, D594G, F595H, etc.

As shown in Figure 3, in normal cells, RAS is activated by receptor tyrosine kinases (RTKs) and in turn switches on the RAF-MAPK signaling pathway. Variant 1 BRAF mutants function as monomers with high kinase activity, constitutively activating the MEK/ERK signaling pathway and in this way triggering uncontrolled cell growth. Variant 2 BRAF mutants share some similarities with variant 1, such as high kinase activity and MEK/ERK pathway activation (RAS-independent). The main difference is that variant 2 mutants form dimers in order to activate the downstream signaling pathway. Since BRAF and MEK/ERK are the decisive factors controlling melanoma cell proliferation and survival, inhibitors targeting these kinases are used in clinical treatment, such as vemurafenib (VEM) and dabrafenib for BRAF inhibition as well as cobimetinib (COBI) and trametinib for MEK inhibition. In cells with variant 3 BRAF mutations, the BRAF/MEK/ERK pathway is triggered by activated RAS. Since the BRAF/MEK/ERK pathway is RTK/RAS-dependent, RTK inhibitors can also be used for targeted therapy of tumors with variant 3 BRAF mutations besides BRAF/MEK inhibitors.

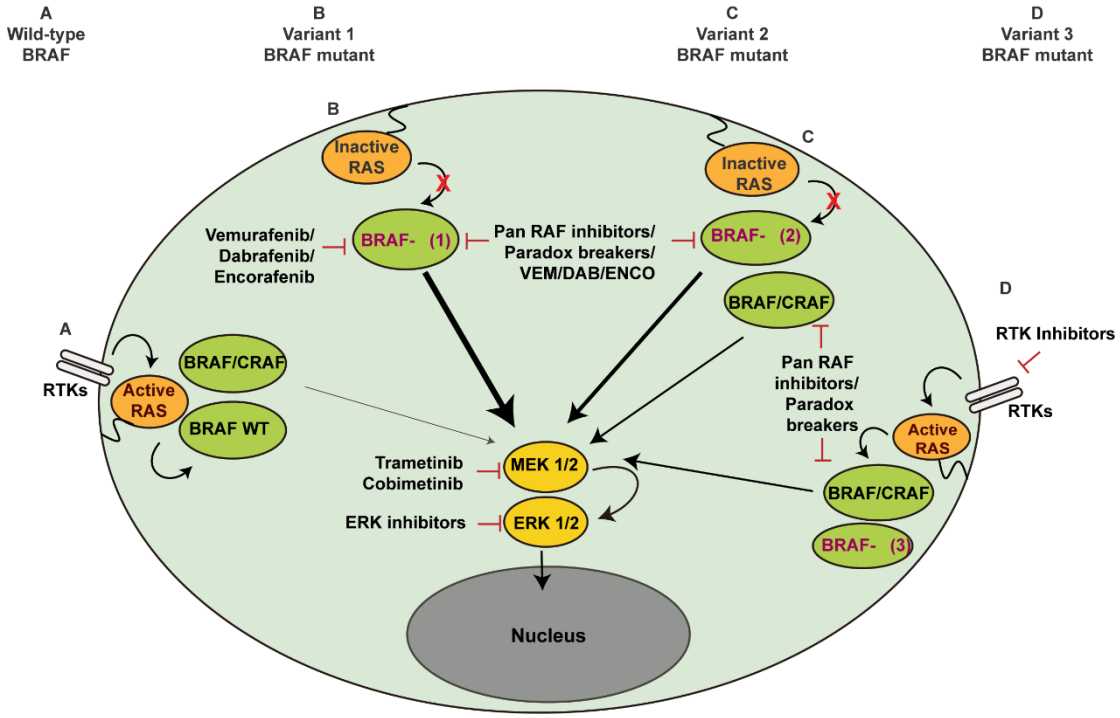


Figure 2. BRAF mutations in cells

Three different variants of BRAF mutations affect the MAPK pathway. **A** In cells with wild-type BRAF, RAS is activated by RTKs and in turn activates the RAF-MAPK signaling pathway. **B** Variant 1 BRAF mutations create highly active BRAF monomers (RTK/RAS-independent) that constitutively trigger the MAPK pathway. BRAF inhibitors (vemurafenib etc.) or MEK inhibitors (cobimetinib etc.) are used to treat melanoma patients with this mutation. **C** Variant 2 BRAF mutants dimerize with CRAF and activate the MAPK pathway in an RTK/RAS-independent fashion. **D** The function of variant 3 BRAF mutants is RTK/RAS-dependent. The figure was modified from Matthew Dankner et al[15].

1.1.2 NRAS-mutant melanoma

The *RAS* gene family comprises three ubiquitously expressed members: NRAS, KRAS and HRAS[18]. NRAS was found to be the most frequently mutated among the three isoforms of melanoma[19]. As shown in Figure 1, approximately 20% of all melanomas harbor NRAS mutations[20]. Wild-type NRAS requires upstream stimuli for activation, whereas mutated NRAS is capable of constitutively activating MEK/ERK and PI3K/AKT pathway without prior extracellular stimulation. Indeed, in NRAS-mutant melanoma cells, NRAS permanently binds GTP, which keeps NRAS in its active conformation. Active NRAS can then provide constant stimulation downstream signaling pathways resulting in cell growth and survival. Melanoma patients with NRAS mutations normally have a poor prognosis. BRAF and MEK inhibitors are widely used for melanoma patients carrying NRAS-mutation since there is still no specific therapeutic agent available that directly targets NRAS in melanoma[21,22].

1.1.3 NF1-mutant melanoma

NF1 function as a negative regulator of RAS and its mutation also belongs to the driver mutation in melanoma. The alterations of NF1 are found in approximately 10 percent of total melanoma cases[23]. According to clinical data, NF1 mutations strongly correlate with UV exposure, age, and specific melanoma subtypes. For example, Michael Krauthammer found that elderly patients with melanoma are more likely to develop NF1 mutations[24] and Thomas Wiesner reported an extremely high frequency (93%) of NF1 mutations in a subtype of melanoma called desmoplastic melanoma[25]. The inactivating mutation of NF1 could trigger the activation of MAPK and PI3K/AKT pathways. Therefore, it is recommended to use relevant inhibitors to block these pathways in NF1 mutant melanoma[24,26].

1.1.4 Triple wild-type melanoma

The term triple wild-type (TWT) melanoma refers to melanomas that do not carry any BRAF, RAS, or NF1 mutation. KIT mutation is one of the most common aberrations in TWT melanomas[27]. KIT belongs to the RTK family and its oncogenic mutation can trigger the activation of several signaling pathways that control cell proliferation and survival[28]. Normally, the mutation rate of KIT varies with different melanoma subtypes. Acral and mucosal melanoma display higher KIT mutation frequency than other subtypes[29–31].

The *CDKN2A* gene encodes multiple proteins, among them the two isoforms $p16^{INK4A}$ and $p14^{ARF}$ function as negative regulators in melanoma. $p16^{INK4A}$ has an essential role in regulating the activity of cyclin-dependent kinases CDK4 and CDK6 (these two factors are important kinases in cell cycle progression)[32]. $p14^{ARF}$ regulates apoptosis by suppressing MDM2, thereby preventing it from blocking the function of p53.

GNAQ and *GNA11* are two genes commonly mutated in uveal melanoma[33]. The oncogenic mutations of these two factors cause abnormal cell proliferation and survival in melanoma[34].

Apart from the somatic mutations mentioned above, mutations of PTEN (phosphatase and tensin homolog), TERT, CNNB1, and EZH2, etc. have also been reported for TWT melanoma. As a tumor suppressor, their inactivating mutations were found in multiple cancer types, including melanoma, head and neck cancers, blood cancer and so on[35–38].

1.2 Strategies of melanoma treatment

The treatment strategy of melanoma mainly depends on the subtype and stage, the location of the tumor, and whether there are factors that affect the prognosis and recurrence. Patients with metastatic melanoma normally already have or will develop metastases in the brain and/or other internal organs and usually barely respond to clinical

treatment and show poor prognosis. For example, of all patients diagnosed with metastatic melanoma, 20% have already had brain metastases[39], which is one of the most severe types of metastases going along with extremely short life expectancy[40]. Therefore, early detection, diagnosis and treatment are essential and able to significantly improve the survival rate of patients. Hitherto numerous treatment methods such as targeted therapy, chemotherapy and immunotherapy have been used in the clinical treatment of advanced melanoma.

1.2.1 Targeted therapy

In the past decade, more and more studies have pointed out that melanoma exhibits a high heterogeneity[41]. As mentioned previously, the mutations of BRAF, NRAS, KIT, etc. are frequently found in melanoma patients[23]. These specific genetic abnormalities lead to multiple aberrant signaling pathways which promote melanoma proliferation and progression[26,30,42,43]. Based on that, small molecules were designed to block these signaling pathways.

BRAF inhibitors

The BRAF inhibitor vemurafenib (BRAFi, PLX4032) has been used clinically for the treatment of $BRAF^{V600E}$-mutant melanoma since 2011[44]. According to a study from 2012 from Chapman and colleagues, patients who received traditional chemotherapy (dacarbazine) only showed 5% response rate, while exhibiting an extraordinarily high response and progression-free rate (48% and 90% respectively) when treated with vemurafenib[45]. Three years later, a study from the same clinical trial reported that patients who undergoing targeted therapy with vemurafenib had a longer survival time than patients receiving chemotherapy (vemurafenib vs. dacarbazine: 13.6 months vs. 9.7 months)[46]. In 2013, the FDA approved another selective BRAF inhibitor termed dabrafenib. Similar to vemurafenib, clinical studies by Hauschild et al. indicated that the progression-free survival (PFS) of patients receiving dabrafenib treatment was significantly higher than that of patients treated with dacarbazine[46]. Although melanoma patients showed high response rates towards these targeted therapy drugs, the clinical benefits of these therapies are limited since a high proportion of patients develop drug resistance within a few months after BRAF inhibitor monotherapy, leading to relapse and disease progression[47].

MEK inhibitor

Apart from blocking BRAF function, targeting downstream effectors of BRAF (such as MEK) is another effective option in clinic treatment. For example, Trametinib, one of FDA approved MEK inhibitors in 2013. According to a phase 1 monotherapy report from Falchook et al., in patients with BRAF mutant melanoma, 33% showed a confirmed response to trametinib compared to a 5% response rate to dacarbazine. Furthermore, trametinib also increased patients' median PFS more than dacarbazine (trametinib vs.

dacarbazine: 5.7 months vs. 1.6 months)[45,48]. At the same time, Flaherty and his colleagues conducted a clinical trial for trametinib treatment. The results showing that melanoma patients who took 2 mg of trametinib per day showed better drug response in contrast to chemotherapy with dacarbazine (trametinib vs. dacarbazine: 22% vs. 8%) and higher median PFS (trametinib vs. dacarbazine: 4.8 months vs. 1.5 months)[49]. Previous research from Paraiso et al. indicated that although BRAF blockade could effectively inhibit the growth of melanoma cells, the MAPK pathway is reactivated, which triggers cell resistance. Based on this fact, a combination treatment using BRAF and MEK inhibitor provided a more effective therapeutic strategy to overcome the onset of melanoma resistance[50]. In fact, the clinical trial has demonstrated the success of combination therapy for melanoma patients. Flaherty and colleagues conducted a phase 1 and 2 trial and found that the combination of trametinib and dabrafenib could increase the response rate and PFS of BRAF-mutant melanoma patients compared with monotherapy[51]. Furthermore, a phase 3 clinical trial from Larkin and colleagues also found that the combinatorial use of vemurafenib and trametinib is beneficial for the clinical outcome[51].

PI3K/AKT-mTOR pathway inhibitors

Hyperactivation of the PI3K/AKT pathway can be frequently found in melanoma[52]. A clinicopathologic study conducted by Dai et al. showed that the upregulation of AKT is tightly associated with melanoma progression. Buparlisib is a PI3K inhibitor, which effectively prevents melanoma cell growth. When Buparlisib is used in combination with specific MEK and BRAF inhibitors, its inhibitory effect on melanoma cell proliferation can even be enhanced[53]. Yaguchi and colleagues synthesized the novel PI3K inhibitor ZSTK474, which very effectively inhibits melanoma growth *in vivo*[54]. mTOR, which is often activated in melanoma cells, is one of the downstream factors in the PI3K/AKT pathway. Multiple inhibitors have been developed to block the PI3K/AKT/mTOR axis[55]. A study has shown that the dual PI3K/mTOR inhibitors such as NVP-BAG956 have longer lasting effects on preventing melanoma cell proliferation than the PI3K inhibitor LY294002[56]. Interestingly, the antitumor effect of mTOR inhibitors is augmented when a MAPK pathway inhibitor is administered at the same time[57]. So far, research on PI3K inhibitors has produced some promising results: By analyzing the data from 46 randomized control trials Li et al. found that blocking the PI3K and its downstream pathway could increase the treatment efficacy in advanced solid tumors[58].

Cyclin-dependent kinase (CDK) inhibitor

As aforementioned, CDK4 and CDK6 are essential in regulating cell cycle and proliferation, and both of them can be negatively regulated by p16, which is encoded by the *CDKN2A* gene[32]. Ribociclib is a selective inhibitor of CDK4/6 and a study has reported that the combination treatment with ribociclib and binimetinib (MEK inhibitor) could prevent the growth of melanoma xenografts *in vivo*[59,60]. In clinical trials, the CDK4/6 inhibitor in

combination with other therapeutic compounds exhibited promising effects on melanoma treatment. For example, combinatorial treatment with ribociclib and binimetinib was very effective in patients with NRAS-mutant melanomas[60]; while ribociclib plus encorafenib (BRAF inhibitor) improved the treatment of melanoma patients carrying BRAF mutantion[61].

1.2.2 Immunotherapy

After Rudolf Virchow first mentioned that cancer is related to the immune system, it took more than a century for his hypothesis to attract widespread attention[62]. Up to now, researchers have worked on immunotherapy for more than 20 years. Although the results of some clinical trials are not satisfactory, a certain degree of progress has also been made. For example, increasing specific cytokines with antitumor activities such as Interleukin-2 (IL-2) in a better overall clinical response[63]. In addition, lymphokine-activated killer cells (LAK) and tumor infiltrating lymphocytes (TILs) are also being used in clinical practice[64,65]. Increasing evidence indicates that immunotherapy is one of potential treatment strategies for malignant melanoma. Especially, in clinical studies with patients with advanced melanoma, immunotherapy showed long-lasting treatment effects.

Programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1)

A study in 1992 from Ishida and colleagues indicated that PD-1 was an inducer of cell death which could be found on the cell surface of lymphoid cells[66]. Eight years later, the same research group proved that PD-L1 binds to PD-1 and inhibits the proliferation of T cells[67]. Hino et al. collected 59 clinical melanoma samples and analyzed them with regard to PD-L1. They demonstrated that high expression levels of PD-L1 go along with poor prognosis and low overall survival (OS) rate[68]. In 2014, another group conducted a study to evaluate primary lesions and metastases of 81 patients with malignant melanoma with respect to PD-L1 expression coming to a similar conclusion[69]. Besides PD-L1, Latchman et al. discovered PD-L2 sharing properties with PD-L1: it could also prevent T cell proliferation[70]. Nowadays, blocking this pathway has become an important approach in melanoma treatment. For example, nivolumab is an FDA approved monoclonal antibody against PD-1 for melanoma treatment in 2014. By binding to PD-1, nivolumab prevents the interaction between PD-L1/PD-L2 and PD-1, thereby increasing the anti-tumor activity of the immune system and reducing tumor progression[71]. Another study found that blocking PD-1 by using nivolumab is a much more effective treatment for melanoma patients compared to chemotherapy[72]. In addition, combining nivolumab with different checkpoint inhibitors such as the CTLA4 (cytotoxic T lymphocyte-associated protein 4) inhibitor ipilimumab, led to an even better survival outcome among patients[73]. Pembrolizumab is a second PD-1 antibody which also has high treatment efficacy and low toxicity[74].

Interleukin-2 (IL-2)

IL-2 is one of the cytokines with antitumor activity. It is a glycoprotein that binds to the IL-2

receptor which is present on helper T cells (Th cells), effector T cells (Teffs) and regulatory T cells (Tregs) and which can promote cell proliferation and maintain cell survival[63]. IL-2 is the first immunotherapy for melanoma and was approved by FDA in 1988. Data obtained from 270 patients with melanoma showed that 16% of patients receiving high-dose cytokine therapy responded to IL-2 treatment. 6% of patients with a complete response had long-term survival and two-thirds of them were still in complete remission after 10 years of IL-2 treatment[75]. In addition, a clinical trial conducted by the NCI (National Cancer Institute, USA) showed that patients had longer PFS when they received treatment of IL-2 and GP100 peptide vaccine combination treatment (the PFS of the group with the combination therapy and the IL-2 group alone were 2.2 months and 1.6 months, respectively). Moreover, the combination treatment group also showed a longer mean OS in contrast to the monotherapy group (the OS of the group receiving combination therapy and the IL-2 group alone were 17.8 months and 11.1 months, respectively). This study indicates that patients with advanced melanoma who use a vaccine and IL-2 in combination therapy have a higher response rate and longer tumor-free survival compared to patients undergoing IL-2 therapy alone. Such results are very exciting, but more studies are required to determine whether a high-dose IL-2 treatment regimen can be combined with a vaccine for melanoma therapy. In addition, the use of IL-2 can cause side effects such as hypotension, azotemia, oliguria, renal failure, etc., which restrict its potential for clinical application.

Interferon α (IFNα)

IFNα is a cytokine and has anti-angiogenic, anti-proliferative, and anti-tumorigenic properties[76]. It can negatively regulate anti-inflammatory cells, such as Tregs and MDSC and induce the activity of T cells and NK cells among others[77]. Based on its distinctive functional properties, IFNα therapy has been used as adjunctive therapy in clinical melanoma treatment. Recently, a study noted that ancillary IFNα therapy can significantly reduce the chances of cancer recurrence as well as improve the survival of melanoma patients. Unfortunately, only a few patients showed a response to IFNα treatment[78]. In recent years, IFNα combined with additional immunotherapies and other therapies have shown higher effects than monotherapy and has attracted widespread attention[77].

1.2.3 Chemotherapy and other treatment methods

Although melanoma does not have a high sensitivity to many chemotherapeutics, chemotherapy is still one of the main treatments for patients with advanced and relapsed melanomas[79]. To date, several drugs have been used to treat melanoma, including dacarbazine, the first chemical drug approved by the FDA[80]. Patients with melanoma achieved a longer survival time after receiving dacarbazine treatment and it is currently clinically recommended to use dacarbazine in combination with other treatment methods

(such as targeted therapy, immunotherapy) to enhance the therapeutic outcome for melanoma patients[81].

Compared with other treatment strategies, radiation therapy is rarely applied in melanoma treatment because melanoma is not sensitive to this therapy. For patients with larger and thicker facial lesions who refuse surgery, radiotherapy can be considered[74]. Recent studies have shown that hyperfractionated radiotherapy is more effective than traditional segmented radiotherapy. It reduces the radiation response and promotes the regeneration of normal tissues. Cells in healthy organs easily get damaged after chemotherapy and radiotherapy. Photothermal therapy (PTT) is a non-invasive treatment method that could overcome this problem as its shows fewer side effects on the organism[82,83]. As the name suggests, PTT uses certain nanomaterials to convert the energy of light outside the tumor into thermal energy inside the tumor in order to kill tumor cells. Chen et al. performed an *in vivo* study and found that the combination of PTT and immunotherapy could effectively eradicate melanoma in mice[83]. However, it remains to be determined whether PTT would be applicable to the treatment of melanoma patients. Photodynamic therapy (PDT) is another promising adjuvant therapy in cancer treatment. This therapy consists of two major steps: first, the photosensitive drug is delivered to the tumor tissue, followed by activating it using irradiation with visible light and thereby destroying the tumor tissue. Sheleg and colleagues conducted a clinical study on PDT. They found that all of the 14 melanoma patients in this study showed complete tumor regression after PDT treatment[84]. Furthermore, PDT plus dacarbazine could overcome drug resistance in metastatic melanoma[85]. Currently, a new melanoma PDT clinical trial is underway (NCT02685592).

1.3 Molecular mechanisms of melanoma resistance

In BRAF-mutated melanoma, the BRAF-MAPK pathway is consistently activated which triggers melanoma progression. The BRAF inhibitor vemurafenib (BRAFi, PLX4032) is considered the first-line drug to treat patients with melanoma carrying a $BRAF^{V600E}$ mutation[44]. Despite the high response rate to vemurafenib, patients with melanoma still easily acquire drug resistance[47]. Melanoma cells could establish either intrinsic or acquired drug resistance to a range of anticancer agents. Several potential resistance mechanisms against BRAFi monotherapy and BRAFi/MEK inhibitor combination therapy have been proposed including MAPK and PI3K/AKT signaling pathways reactivation, constant activation of RTKs, drug-resistance related transcription factors regulation and changes in the tumor microenvironment (Figure 3)[86–90].

As a key kinase in MAPK pathway, RAS can control the progression of melanoma. Vemurafenib selectively inhibits $BRAF^{V600E}$ and disrupts the MAPK signaling pathway, leading to apoptosis of tumor cells. In response to BRAF inhibition, the expression and activity of RTK can be increased and thereby reverse the anti-tumorigenic effect of the

inhibitor. In addition, the overexpression of upstream factors such as NRAS[44], alternative splicing variants of $BRAF^{V600E}$ [91], or the interaction between COT (MAP3K8, a kinase which can activate ERK) and MAPK protein kinases can also lead to the activation of the MAPK kinase, which increases the resistance of tumor cells to vemurafenib[92–94]. According to a study by Nazarian et al., the upregulation of NRAS and PDGFRβ (a member of the RTK family) were both found in vemurafenib-resistant cells *in vitro*. Moreover, they found that upregulation of NRAS reactivates the MAPK pathway, which in turn results in resistance to vemurafenib in melanoma. Furthermore, by analyzing patient-derived samples, they also demonstrated that the overexpression of PDGFRβ as well as EGFR is highly related to tumor resistance to vemurafenib[93].

As a protein phosphatase, PTEN (a tumor suppressor) can regulate cell adhesion and migration by suppressing the activation of MAPK signaling pathway. The loss of the *PTEN* gene neutralizes the inhibition of the PI3K/AKT signaling pathway and results in the occurrence of tumors[82]. Loss or inactivation of PTEN is found in 10 to 30% of melanoma patients[95]. In clinical studies, it was found that PTEN mutations were not related to the patients' overall response to BRAF inhibitors, but to a shorter PFS[96]. PTEN deletion was reported to inhibit BIM-mediated apoptosis by PI3K activation, leading to a resistance of melanoma to BRAF inhibitors. Shi et al. reported that PI3K-PTEN-AKT signaling activation is crucial to the acquisition of resistance to BRAF inhibitors[97].

CDK4 is essential for regulating cell cycle and proliferation[32]. Dysregulation of CDK4 can have different causes. First, CDK4 can be negatively regulated by p16, which is encoded by CDKN2A gene[32]. Mutations of CDKN2A in melanoma can cause the loss of p16 function, thereby losing the restriction on CDK4 and increasing cell proliferation and resistance to inhibitors[98]. Second, the mutation of CDK4 itself can also promote melanoma drug resistance by eliminating the interaction between p16 and CDK4[99]. Furthermore, cyclin D1 is another key regulator of CDK4. Smalley et al. indicated that the upregulation of cyclin D1 could increase the tolerance of melanoma cells to BRAF inhibitor. In addition, when cyclin D1 and CDK4 are both ectopically expressed in the cells, the resistance of melanoma is particularly enhanced[98].

The tumor microenvironment is made up of tumor cells and a variety of stromal cells, cytokines, chemokines, etc., which can promote metastasis as well as sensitivity to drugs in tumor cells. HGF (Hepatocyte Growth Factor) is a well-studied microenvironmental factor that is secreted by stromal fibroblasts and can promote epithelial-to-mesenchymal transition (EMT) of tumor cells[100]. Straussman et al. confirmed that HGF renders patients resistant to multiple targeted drugs by activating PI3K/AKT and MAPK signaling pathways through its receptor c-Met[101].

NF1 is a classic tumor suppressor that can negatively regulate the signaling downstream of RAS by inhibiting the activity of RAS[102]. According to the study of Maertens and

colleagues, the resistance of melanoma patients to BRAF inhibitors correlates positively with the loss of NF1 protein expression and the loss of NF1 function[103]. In addition, studies have shown that NF1 is the main factor that sensitizes melanoma cells to BRAF inhibitors. When NF1 is knocked out, melanomas cells are resistant to the anti-proliferative effect of the BRAF inhibitor vemurafenib[104,105].

RAC1 is GTPase and belongs to the RAS superfamily. RAC has been considered as a melanoma-related biomarker since a lot of evidence shows that oncogenic mutation of RAC is critical for the occurrence of melanoma[106,107]. Watson et al. have emphasized the integral function of Rac1 in promoting melanoma resistance to targeted therapy compounds[108]. Our previous study also showed that the expression of RAC1b (an isoform of RAC1) is related to melanoma migration and invasion[109].

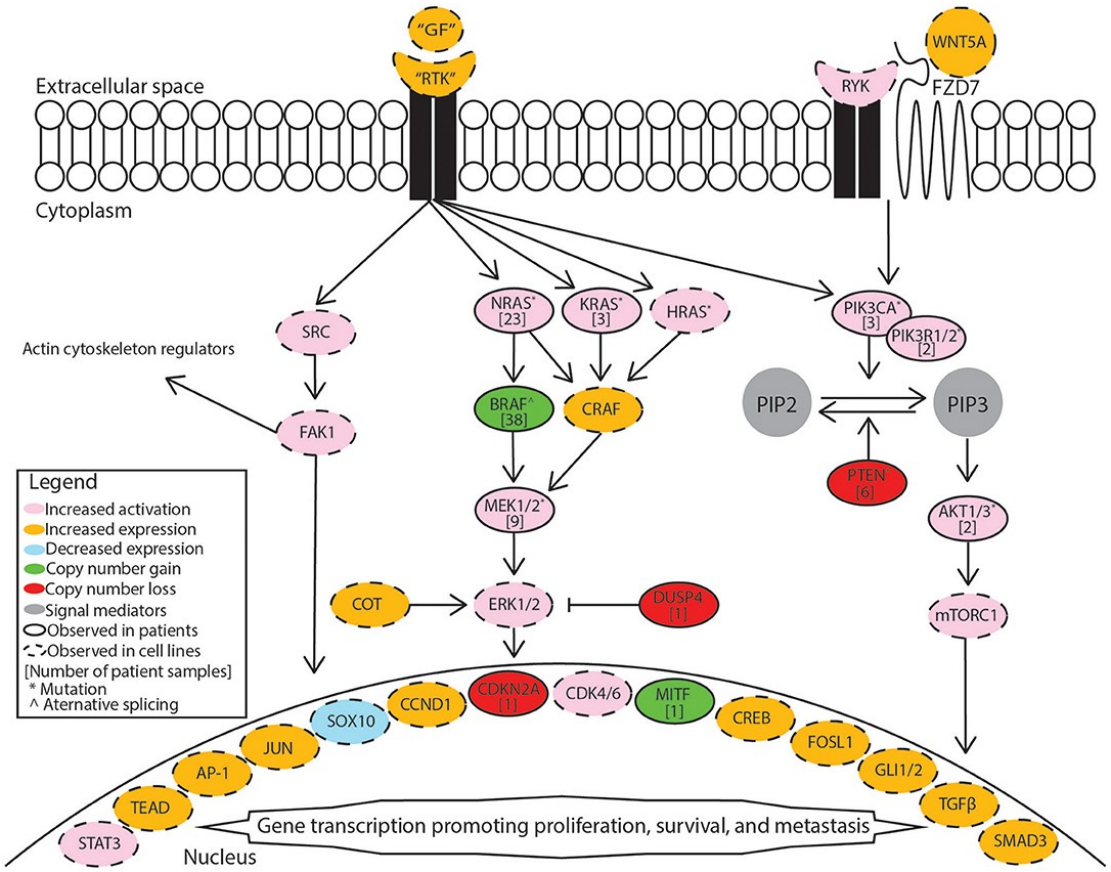


Figure 3. Mechanisms of BRAF inhibitor resistance

Potential resistance mechanisms against BRAFi: 1. Increased expression of growth factors (GF) and their corresponding RTKs, including AXL, EGFR, FGFR1, etc.; 2. Reactivation of MAPK pathway including the Ras mutation, BRAF amplification, MEK upregulation, COT overexpression, DUSP4 inhibition, etc.; 3. PI3K/Akt pathway activation, including the loss of PTEN, activation of PIK3CA, AKT and mTORC1, etc.; 4. SRC pathway activation, including SRC and FAK1 activation; 5. Multiple transcription factors regulation, such as MITF, SOX10, FOSL1, etc. Figure adopted from Stephen A. Luebker et al[110].

1.4 FOXD1

The *FOXD1* gene is a transcription factor of the forkhead box family. The expression of FOXD1 varies in different tissues and the protein is widely involved in physiological processes and biological functions such as cell differentiation, tissue repair and inflammation and normal embryonal development. FOXD1 has a well-defined role in kidney development[111] and is required for cell reprogramming by mediating self-renewal and differentiation[112]. According to a study from Fetting and colleagues, FOXD1 is essential for kidney development. It can promote the differentiation of nephron progenitor cells by inhibiting the protein decorin in the embryonic kidney. The inactivation of FOXD1 leads to failure of nephron progenitor cell development and no maturation of the mouse kidney[113]. In addition, FOXD1 participates in and promotes tissue repair and cellular inflammatory response of renal perivascular cells and myofibroblasts. In addition, the interaction between FOXD1 and RAS is critical during metanephric development[114,115]. Polevoy and colleagues found that FOXD1 is highly related to mesoderm and neural development through the interaction of Wnt and BMP signals[116]. Upregulation of FOXD1 can promote cell metabolism and mitigate osteoarthritis[117]. Shanmughapriya and colleagues reported that FOXD1 can mediate cell differentiation and control the activity of mitochondria by directly regulating MICU1 expression[118].

According to a large number of studies, it has been found that FOXD1 is an imperative factor for the development of various cancers. Nemlich and colleagues found that FOXD1 can promote melanoma cell proliferation and invasion by inhibiting ADAR1 (a tumor suppressor gene) expression[119]. Our previous study showed that FOXD1 is a neural crest-related gene whose deletion leads to reduced invasiveness and migration of melanoma cells[109]. Li et al. pointed out that FZD3 enhances MAPK-mediated signaling indirectly by interfering with the transcriptional network of FOXD1, resulting in increased tumor growth and metastasis of melanoma[120].

Gao and colleagues examined the biological functions of FOXD1 in glioma cells and found that an increased expression of FOXD1 is directly related to the glioma histology grade[121]. Ma et al. also confirmed that FOXD1 has a promoting effect on gliomas[121]. Based on microarray analysis, Li et al. found that abnormal expression of FOXD1 can activate the downstream target Gal-3, which in turn facilitates the growth and motility of lung cancer cells[122]. In addition, a study from Li and colleagues noted that FOXD1 could trigger the progression of non-small cell lung cancer by regulating vimentin. They found patients with high FOXD1 expression showed significantly unfavorable prognosis[123]. Another clinical study from Nakayama et al. has also confirmed a negative correlation between FOXD1 level and OS time[124]. Regarding colorectal cancer, Chen et al. found that CXCL5 promotes angiogenesis in colorectal cancer by upregulating FOXD1[125]. In addition, Han et al. further

confirmed that high expression of FOXD1 can be found in colorectal cancer tissues, and FOXD1 expression is connected to the colorectal cancer stage, which revealed that FOXD1 could function as a prognostic factor for colorectal cancer[126]. Concerning breast cancer, a study by Zhao proved that FOXD1 is a vital oncoprotein regulating breast cancer cell proliferation and chemotherapy resistance[127]. Zhou et al. noted that FOXD1 induce the resistance of pancreatic cancer cells to gemcitabine[128]. In addition, Chen and colleagues integrated an analysis of microarray data and revealed the positive regulation of FOXD1 for liver cancer pathogenesis[129].

Overall, different studies have proved abnormally increased level of FOXD1 could be found in different tumors and its expression negatively correlates with prognosis. Cancer patients with tumors with higher expression of FOXD1 have shorter DFS and OS than those with lower expression.

In terms of cell function, FOXD1 has a strong influence on the modulation of tumor growth, progression and can lead to tumor chemotherapy resistance as shown for prostate cancer, cervical cancer and breast cancer. In terms of mechanisms, studies have found that FOXD1 can activate AKT/NF-κB and MAPK pathway. By activating these pathways, FOXD1 can exert pathological effects such as regulating invasion and metastasis of malignant tumors.

In summary, the transcription factor FOXD1 can modulate several important physiological processes, its expression is also elevated in a variety of malignant tumors and is usually related to poor patient prognosis. Based on these findings, one can conclude that FOXD1 might be a novel target of different cancerous diseases. So far, not much has been reported about the involvement of FOXD1 in providing resistance to melanoma cells towards therapies. Further studies are needed to unravel these mechanisms.

2. Aims of the study

FOXD1 accumulation in tumor cells can influence biological functions, including cell stemness, metastasis and drug resistance. However, the role of FOXD1 in melanoma and its contribution to establishing resistance remains largely unknown and hence require further investigation.

The specifics aims of this study are:

1. To investigate if FOXD1 could regulate melanoma cell resistance towards targeted therapy.
2. To uncover the mechanisms behind FOXD1-mediated melanoma cell resistance to targeted therapy.

3. Materials and methods

3.1 Materials

3.1.1 Reagents and kits

Product	Company	Catalog#
AlamarBlue®	Invitrogen	DAL1100
Albumin fraction V	Carl Roth	8076
Cultrex® BME cell invasion assay	TREVIGEN	3455096K
CellEvent™ Caspase-3/7 Kit	Thermo Fisher Scientific	C10423
Cultrex® Cell Invasion Assays	R&D Systems	3455-024-K
HumanHT-12 v4 Expression BeadChip Kit	Illumina	BD-103-0204
Miniprep Kit	Qiagen	27106
NuPAGE™ Novex™ 4-12% Tris-Acetate Protein Gels	Thermo Fisher Scientific	NP0335BOX
SYBR Green Mix	Applied Biosystems	4309155
RNeasy Plus Mini Kit	Qiagen	74136
EZ-ChIP™	Merck Millipore	17-371
Protein Ladder	Life Technologies	26619
DNA transfection Reagent	Roche Diagnostics	06365787001
Tween® 20	Applichem	A13890500
0,45 µm Syringe Filters	Carl Roth	P667.1
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Stealth RNAi™ siRNA negative control	Life Technologies	12935-300

Ibidi Culture-Insert 500 µm	ibidi	80209
PhosSTOP™ phosphatase Inhibitor Cocktail	Roche Diagnostics	04906845001
TEMED	Carl Roth	2367,3
Click-iT Plus EdU Proliferation	Thermo Fisher Scientific	C10635
PVDF membrane	Merck Millipore	IPVH00010
SB431542	Selleck Chemicals	S1067
Recombinant CTGF	Peprotec	120-19
Cobimetinib	Selleckchem	S8041
Vemurafenib (PLX4032)	Selleckchem	S1267

3.1.2 Cell culture reagents

Product	**Company**	**Catalog#**
FCS	Biochrom	S0115
Non-essential amino acids	Sigma-Aldrich	M7145
Medium 254	Gibco®Life Technologies	M254500
RNAiMAX transfection reagent	Life Technologies	13778075
Puromycin	Carl Roth	240.1
Opti-MEM® I reduced serum medium	Gibco®Life Technologies	31985062
Penicillin/Streptomycin	Sigma-Aldrich	P4333
Trypsin	Sigma-Aldrich	T3924
Human melanocyte growth supplement (HMGS) 100x	Gibco®Life Technologies	S002-5
2-Mercaptoethanol	Gibco® Life Technologies	31350010

3.1.3 Antibodies

Product	Company	Catalog#
α-Actinin	Santa Cruz	sc-17829
FOXD1	LifeSpan bioscience	LSB9155
FOXD1	Abcam	Ab179940
P21		ab38898
CTGF		Ab125943
Anti-rabbit IgG, HRP-coupled	Cell signaling	7074S
ERK		4695
p-ERK		9106S
p-AKT		CST#4058
PAN-AKT		CST#2920
Caspase 3		9662
GAPDH		CTS#2118
Anti-mouse IgG, HRP-coupled		7076
β-Actin		CTS#5125

3.1.4 siRNA/esiRNA

Product	Company	Catalog#
si-FOXD1-1	Invitrogen	HSS142039
si-FOXD1-2	Invitrogen	HSS142041
MISSION® esiRNA CTGF	Merck	EHU016751-50UG

3.1.5 Plasmids

Product	Company	Catalog#
EX-Q0599-Lv122-human FOXD1 (expression plasmid)	Genecopoeia	TRCN0000013970
EX-Q0599-Lv122-empty (control plasmid)	Generated by removing the FOXD1 ORF from the expression plasmid	-
pGL4.10-ctgf prom long (promoter reporter plasmid)	Kindly provided by Prof. Thomas Brabletz, University of Erlangen[130]	-
pGL4.10 (control reporter plasmid)	Kindly provided by Holger Sültmann (NCT, Heidelberg, Germany)	-

3.1.6 Primers

Target	Forward Sequence	Reverse Sequence
18s	GAGGATGAGGTGGAACGTGT	TCTTCAGTCGCTCCAGGTCT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
FOXD1	TGAGCACTGAGATGTCCGATG	CACCACGTCGATGTCTGTTTC
CTGF	CCAGACCCAACTATGATTAGAGC	GAGGCGTTGTCATTGGTAAC
MITF	GCTCACAGCGTGTATTTTTCC	TCTCTTTGGCCAGTGCTCTT
SOX10	GGCTTTCTGTCTGGCTCACT	TAGAGGGTCATTCCTGGGGG
PAX3	TGCCCTCAGTGAGTTCTATCAGC	GCTAAACCAGACCTGCACTCGGGC
AXL	CCGTGGACCTACTCTGGCT	CCTTGGCGTTATGGGCTTC
NGFR/(CD271)	CGACAACCTCATCCCTGTCT	GCTGTTCCACCTCTTGAAGG
SNAI1(SNAIL)	GAGGCGGTGGCAGACTAG	GACACATCGGTCAGACCAG

3.1.7 Buffers and solutions

Transfer buffer (pH 8.3)
20% SDS
25mM glycine
20% methanol
25mM Tris
dH2O

Tris-buffered saline (TBS) (pH 7.6)
137mM NaCl
20mM Tris
dH2O

Western blot washing buffer (TBST) (pH 7.6)
TBS supplemented with:
0.1% Tween® 20
dH2O

Blocking buffer (milk/BSA)
5% Skim milk or BSA powder
1x TBST

3.1.8 Devices

Device	Company
ABI 7500 Real Time PCR machine	Applied Biosystems
BD FACs conto II	BD
NanoDrop ND-1000	Peqlab Biotechnologie
TECAN infinite F200 pro microplate reader	TECAN

3.1.9 Software

Analysis software	Source
GraphPad Prism 5	GraphPad
ImageJ	National Institute of Health
ABI 7500 Software v2.0.5	Applied Biosystems
Leica Application Suite v4.0	Leica
iControl 1.10	TECAN
NIS-Elements Viewer	Nikon
T-scratch	CSElab
Flowjio	7.10

3.1.10 Online databases

Database	Source
Cbioportal	Whitehead Institute Open sourse
R2: Genomics Analysis and Visualization Platform	The department of Oncogenomics in the Academic Medical Center (AMC) Amsterdam
TCGA	National Institute of Health

NCBI	National Center for Biotechnology Information
CCLE	Cancer Cell Line Encyclopedia

3.2 Methods

3.2.1 Cancer cell lines and cell culture

Cell lines[1]	BRAF/NRAS mutation status	Culture medium
A375	$BRAF^{V600E}$	MEF medium[2]
SK-MEL-28	$BRAF^{V600E}$	MEF medium
HT144	$BRAF^{V600E}$	MEF medium
MeWo	Wild-type	MEF medium
WM266-4	$BRAF^{V600E/D}$	MEF medium
SK-MEL-23	Wild-type	MEF medium
SK-MEL-103	$NRAS^{Q61R}$	MEF medium
SK-MEL-173	$NRAS^{Q61R}$	MEF medium
C32	Wild-type	MEF medium
NHM[3]	-	Medium 254 growth medium[4]

[1] Human melanoma cells with BRAF or NRAS mutation or neither of them (wild-type) were selected for current study.

[2] MEF medium comprised of DMEM, 10% FCS, 10mM non-essential amino acid solution (1%), 0.1mM β-mercaptoethanol (0.75%) and penicillin/streptomycin (1%)

[3] NHM (Normal human melanocytes) were collected from donor foreskins according to the ethical regulation (Ethics committee II, University Medical Centre Mannheim, Germany)

[4] Medium 254 growth medium comprised of medium 254, 1x HMGS

All cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C. Cell medium was changed every second day and cell lines were split every 2-6 days when they reached about 80% confluency.

3.2.2 Vemurafenib-resistant cell lines

The vemurafenib-resistant cells were established as described in Hüser et al.[131]. In brief, to generate vemurafenib-resistant cell lines, A375 and HT144 cells were exposed to the BRAFi vemurafenib with gradually increasing the concentration for about 6 months. Two resistant cell lines were generated and termed A375-R and HT144-R. These resistant cell lines were exposed to a maximum of 7 and 2.5 µM vemurafenib, respectively. The resistant SK-MEL-28 cell line was kindly provided by Prof. Joon Kim[132]. This SK-MEL-28-R cell line was exposed to 2 µM vemurafenib.

3.2.3 Plasmid preparation

First, 100 ng of plasmid DNA (2 - 10 µL) of interest were added to the 100 µL DH5α E. coli cells and shortly vortexed. After that, the bacteria were incubated on ice for 15 min and were subjected to heat shock for 3 min at 42°C followed by incubation on ice for 10 minutes. Then, 500 µL super optimal broth with catabolite repression (SOC) medium was added and the cells were incubated for 1 h at 37°C with constant shaking. Subsequently, for selection of transformed bacteria were plated on an LB (Lysogeny broth) agar plate containing 100 µg/mL ampicillin by incubation overnight at 37°C. Random colonies were picked separately and grown in 3 mL LB-medium supplemented with ampicillin under constant agitation overnight at 37°C. 12 h later, bacteria were collected and centrifuged. Miniprep Kit (Qiagen) was used for plasmids isolation according to the manufacturer's guideline. Restriction enzyme digestion as well as gel electrophoresis were used to validate the plasmid identity. Thereafter, the bacteria harboring interested plasmids were inoculated into 200 mL LB medium supplemented with ampicillin and incubated overnight at 37°C with constant shaking. Bacteria were collected and lysed, Plasmid Maxiprep Kit (Thermo Fisher Scientific) was used for plasmid DNA isolation according to the manufacturer's instruction. Finally, quality and quantity of DNA were determinated by using the NanoDrop ND-1000 spectrophotometer. The sequence of the plasmid was analyzed for further validation (LGC genomics, Berlin).

3.2.4 Lentiviral particle production

HEK293T cells were used for the production of lentiviral particles harboring the genes of interest. Briefly, HEK293T cells were cultured in MEF medium until they reached approximately 70% confluency. Then, the lentiviral constructs pCMV-dR8.91, pCMV-VSV-G and the plasmid of interest were added to the cells by using the X-tremeGENE®

transfection reagent (Roche). 12 h after transduction, the supernatant was replaced with fresh MEF medium and harvested after 24, 36 and 48 h. The collected supernatants were filtrated and were used for transducing directly.

3.2.5 Lentiviral transduction and antibiotic selection

Fresh transduction mixture (0.5 mL MEF medium plus 1.5 mL lentivirus supernatant 1.5 mL) was added to cells of interest. One day after, Repeat the transduction again. Two days later, cells were washed with PBS three times, transferred to biosafety level I laboratory and incubated with fresh MEF medium in a humidified incubator. The following day, the antibiotic puromycin (1-2 µg/mL, Carl Roth) was added for selection. The selection was performed for 3-5 days. The transduction procedure was conducted in a biosafety level II ("S2") laboratory.

3.2.6 RNA isolation and cDNA synthesis

RNeasy Mini Kit (QIAGEN) was used for the extraction of total cell RNA following the manufacturer's instruction. In brief, cell pellet was lysed with cell lysis buffer. The lysate was transferred to a column and washed three times using the washing buffer. DNase (QIAGEN) treatment was used to avoid genomic DNA contamination. The RNA bound to the column was eluted in RNase-free water. Quality and quantity of RNA were determined with a NanoDrop ND-1000 spectrophotometer. 500 ng of RNA were used for cDNA synthesis using cDNA Reverse Transcription kit (Thermo Fisher Scientific).

3.2.7 Microarray expression analysis

Microarray expression was analysis by the DKFZ Genomics and Proteomics Core Facility. HumanHT-12 v4 expression bead chips (Santa Clara) were utilized for the analysis. Raw data from each condition were normalized, Bayes test was used to compare the differentially expressed genes from two groups (each group consists of at least two biological replicates). Microarray results are uploaded into GEO database (GSE162973). Furthermore, Ingenuity Pathway Analysis (IPA) was used for enrichment analyses.

3.2.8 Quantitative real-time polymerase chain reaction

Gene expression was assessed by RT-PCR using ABI® 7500 Real-Time PCR System (Applied Biosystems) and the SYBR Green PCR master mix (Thermo Fisher Scientific). Briefly, specific primers as well as cDNA were mixed with SYBR Green. 18S and GAPDH were used as endogenous control. The NCBI primer designing tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/ was used for primer design. In this study, the efficiencies of all primers were within the range of 100% ± 10%. The interested gene

expression was quantified by calculating (ΔΔCt).

3.2.9 Protein extraction

Cells used for protein extraction were collected when reaching approximately 80% confluency. Cells were lysed in RIPA buffer (Invitrogen) and incubated on ice for 30 min and centrifuged at 14000 rpm for 30 minutes at 4°C. Protein was then collected and the concentration were analyzed by using the Pierce bicinchoninic acid (BCA) protein assay kit (ThermoScientific).

3.2.10 Immunoblotting

Step 1, loading and electrophoresis: 20 – 40 µg of protein were used and separated with Bis-Tris Protein Gels (Thermo Fisher Scientific) at 180 V for 1 h. Step 2, transfer: proteins were blotted to methanol-activated PVDF membranes with 100 V for 1 h on ice. Step 3, blocking: Once transfer was completed, the PVDF membrane was blocked with 5% BSA in TBST at RT for 1 h. Step 4, primary antibody incubation: after blockage, antibody of interest were added to the blocking buffer and store the membrane overnight at 4°C. Step 5, secondary antibody incubation: membranes were washed three times with TBST, secondary antibodies were added and incubated at RT for 2 h. Step 6, imaging and analysis: signals were visualized using Luminata Forte western HRP substrate (Millipore) and ChemiDoc™ MP Imager (Bio-Rad). Target proteins were then quantified using ImageJ software (Fiji).

3.2.11 Immunohistochemistry of tissue microarrays (TMA)

TMA samples were prepared as previously described[133]. For immunohistochemistry, 4µm thick slices of TMA samples were stained against target antibody. TMA scanning was conducted by the NCT Gewebebank facility, Pathology unit, University of Heidelberg. TMAs were analyzed according to IHC scoring system as previously described[134]. All analyses were performed in accordance with the principles of the Declaration of Helsinki and were approved by the medical Ethics Committee of the Medical Faculty Mannheim, University of Heidelberg.

3.2.12 siRNA transfection

The cells were transfected using 40 pmol target siRNA or negative control using the Lipofectamine® RNAiMAX reagent. After 48h incubation, qPCR or western blot was used for validation. FOXD1-specific siRNAs were purchased from Invitrogen (FOXD1 HSS142039, HSS142041). served as a negative control. For CTGF knockdown, MISSION® esiRNA (heterogeneous mixture of siRNAs, EHU016751) was purchased from

Merck. Stealth RNAiTM siRNA Negative Control Hi GC (catalog number: 12935400) from Thermo Fisher Scientific served as a negative control.

3.2.13 Luciferase assay

The CTGF promoter construct was kindly provided by Prof. Thomas Brabletz. A375 CT or A375 FOXD1 OE cells were transfected with the pcDNA4.10-CTGF promoter luciferase construct or pcDNA4.10 empty plasmid. The next day, the cells were subjected to lysis. The luciferase of each sample was then measured in accordance with the instructions (Promega).

3.2.14 Chromatin immunoprecipitation (ChIP)

First, formaldehyde cross-linking: 3.6% formaldehyde was used for cells (1 - $2x10^6$ cells of each sample) cross-linking for 10min at RT. Then SDS lysis buffer was added for cell lysis; Second, ultrasonic crushing: samples were subjected to ultrasonic crushing using covaris S220 ultrasonicator followed by incubation with agarose bead-coupled antibodies overnight at 4°C; Third, precipitation and cleaning: on the following day, the precipitated complex was washed and the DNA fragments were released and qPCR was used for next step measurement. In this experiment, EZ ChIP kit was used for Chip experiments.

3.2.15 Cell viability assay

$3×10^3$ cells were seeded in per well of a 96-well plate at least in triplicates. After 24-48 h incubation, an increased concentration from 0,0001 µM to 50 µM of vemurafenib was used for cell treatment for one to three days. At determined time points, 20 uL of Alamar Blue solution (10% of final concentration) was added and plates were incubated for 4 h in cell incubator. Subsequently, the final measurement was conducted with a plate reader (Tecan). The GraphPad Prism 5 was used for analyzing.

3.2.16 Analysis of apoptosis with annexin V and PI staining

Cells were seeded one day before treatment. The following day, determined concentrations (based on IC50 values calculated above) of target compounds were added to the cells, followed by 24, 48 or 72 h of incubation. After that, cells in the supernatants as well as attached cells were collected and washed with cold PBS twice followed by Annexin V and PI staining. Then, 100 uL Annexin V Binding buffer were added to the cells and incubated at RT avoiding light exposure around 20 min. For analyzing, 100 µL of FACS buffer was then added to each cell solution, then the samples were tested by FACS machine.

3.2.17 Analysis of apoptosis with caspase 3/7

Cells of interest were cultured in 6-well plates one day before treatment. The following day, determined concentrations (10 uL) of target compounds were added to the cells, followed by 72 h of incubation. After that, cells in the supernatants as well as attached cells were collected in tubes followed by washing with ice-cold PBS twice. Next, 100 µL cold PBS and 4 µL caspase 3/7 were added to the cell solution and incubated at RT for 30 min avoiding light exposure. For analyzing, 200 µL of PBS was added to each cell solution, then the samples were tested using FACS machine.

3.2.18 Proliferation assay

The cell proliferation assay was conducted using Click-iT EdU kit (Invitrogen). Briefly, cells of interest ($1x10^4$ – $5x10^4$/well) were cultured in a 6-well plate one day before measurement. After 24 h, cells were incubated with EdU compound (10 µM) for 2 h and were subjected to fixation. Then, the Click-iT™ Plus reaction probe solution were added to the samples and incubated avoiding light exposure. 30 min later, RNase A as well as PI (50 µg/mL) were added to the cell solutions and tested using FACS machine.

3.2.19 Clonogenic assay

100-500 /well of cells were seeded and incubated for 24 h. Compounds were then added to the wells (24 h incubating). The fresh medium was replaced twice a week. 5 - 10 days later, crystal violet solution (0.5%) was used for cell colonies fixation and staining. ImageJ software was used for colony area calculating.

3.2.20 Scratch migration assay

$2x10^4$ cells of interest were cultures with ibidi 2-well chamber. 12 h later, the insert was removed, and the cell migration was monitored with a light microscope every 4 – 8 h by measuring the gap between the 2 wells.

3.2.21 BME invasion assay

First, cells were starved with serum-free medium for 24 h if necessary, in the meantime, 0.3X BME coating solution (R&D systems) was added to the trans-well chamber and incubated for 24 h in the incubator. On the following day, $1x10^5$ cells with 100uL serum-free medium were added into each of coated trans-well chamber. After 6 - 24 h incubation, the invasive cells were dissociated and stained with calcein. Subsequently, the final measurement was conducted with a plate reader (Tecan). The GraphPad Prism 5 was used for analyzing.

3.2.22 TGF-β ELISA

$1x10^5$ cells /well of interest were cultured in a 6-well plate. After 24 h, cell supernatants were collected and used to perform the Elisa according to the protocol provided by the ELISA kit.

3.2.23 Data set analyses

FOXD1 expression values in melanocytes and melanoma cells were collected from three different GSE databases (GSE130244, GSE35389, GSE4570) and analyzed. Patient survival data were obtained from TCGA Pancancer ATLAS database (www.cbioportal.org) and R2 Genomics analysis platform (http://hgserver1.amc.nl). Furthermore, the expression levels of MITF, SOX10, AXL, CTGF and FOXD1 from GSE7127, GSE10196, GSE68599 and the Cancer Cell Line Encyclopedia (CCLE).

3.2.24 Statistical analyses

Experiments were conducted at least three times if not indicated differently. Data were displayed as mean ± SD and student's two-sided t-test or one sample t-test were applied to compare data between two conditions. One-way ANOVA test was used to compare data among multiple groups. Pearson analysis was used to define correlation between two factors. $P < 0.05$ was considered statistically significant.

4. Results

4.1 FOXD1 is upregulated in human melanoma cells and negatively associated with OS of melanoma patients

First, the FOXD1 expression levels between normal (N) and tumor (T) samples from three skin cancer databases (GSE130244, GSE35389 and GSE4570) were analyzed and compared. Higher FOXD1 mRNA levels were found in tumor samples compared to normal skin samples (Figure 4A). To answer the question if FOXD1 may be clinically relevant for melanoma progression, I analyzed a cohort of 105 melanoma specimens from patients using tissue microarray analysis (TMA) with a FOXD1-specific antibody and the result showed that FOXD1 expression significantly negatively correlated with overall survival of melanoma patients (Figure 4B). Next, I assessed data about the survival of melanoma patients from two different databases (TCGA database and R2: Genomics Analysis and Visualization Platform). Consistently, melanoma patients with high FOXD1 levels displayed a much lower overall survival (Figure 4C, 4D). These data together imply a critical role of FOXD1 in melanoma progression.

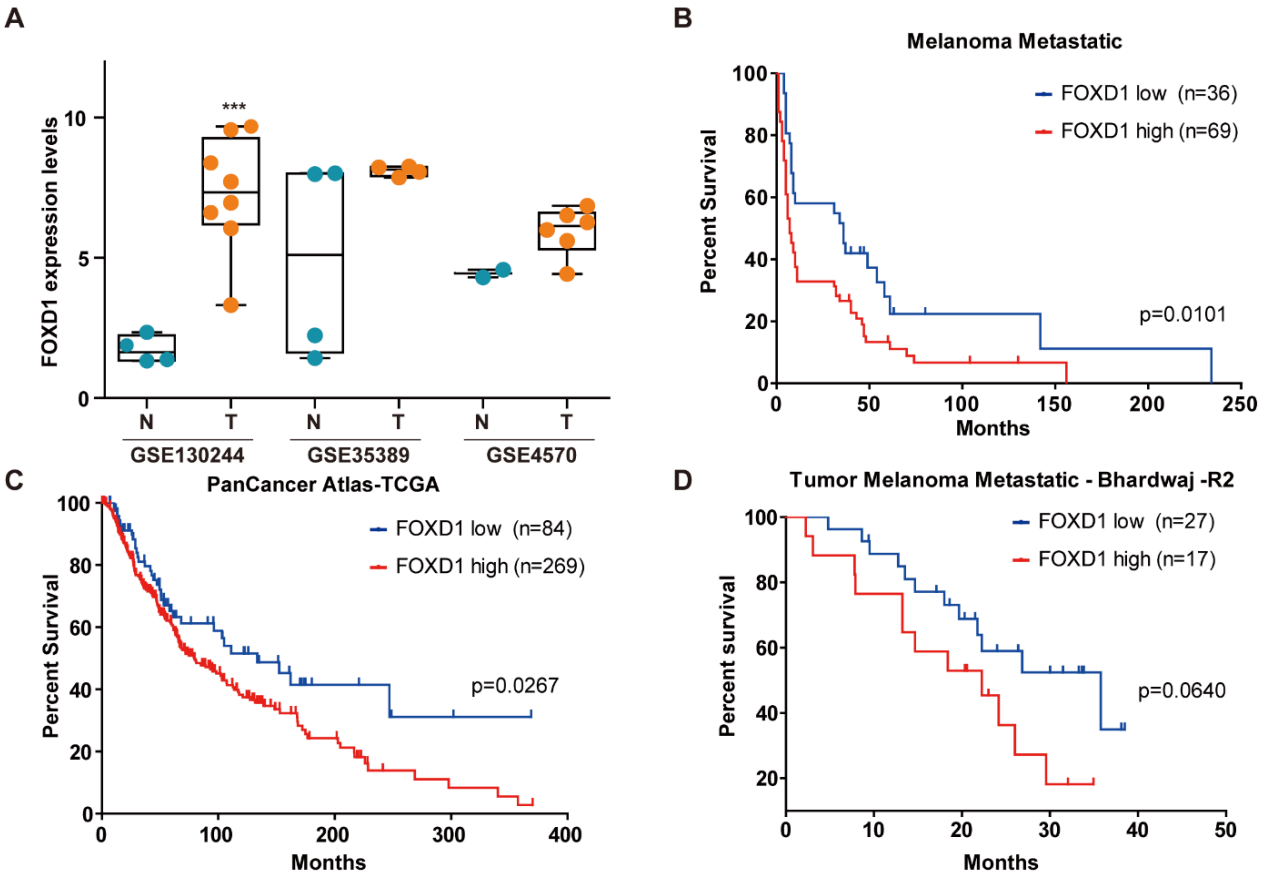


Figure 4. High FOXD1 expression is associated with reduced OS

A FOXD1 expression data from the GSE130244, GSE35389 and GSE4570 databases. (N: non-tumor

cells, T: tumor cells). **B** OS in patients with metastatic melanoma. 105 TMA samples were stained for FOXD1. Kaplan-Meier curve represents high (red) or low (blue) FOXD1 levels. **C**, **D** Kaplan-Meier curve illustrating OS in melanoma patients with high (red) or low (blue) FOXD1 levels. Kaplan-Meier plots were generated using the data from two different databases (PanCancer Atlas-TCGA and Tumor Melanoma Metastatic-Bhardwaj-R2) with the scan method as described previously[135].

4.2 High levels of FOXD1 expression correlate with low MITF, SOX10 and high AXL expression

Previous studies reported that the expression of AXL and MITF involve in melanoma progression as well as drug resistance[136]. AXL (belong to RTKs) that is accumulated in melanoma. The increased expression and activity of AXL is one reason for acquired resistance of melanoma cells[136]. MITF is a key factor in regulating melanoma cells phenotype switch: cells with high expression level of MITF show a differentiated, non-proliferative phenotype, whereas cells with low MITF expression tend to display a quiescent, dedifferentiated phenotype[137–139]. Similar to MITF, SOX10 is another important factor for melanocyte differentiation. Cells with low expression of SOX10 show a more stem-like phenotype and usually associated with drug resistance[140]. By comparing the expression levels of FOXD1, MITF, AXL and SOX10 in human melanoma cells using data from the GSE7127 and GSE10196 databases, I discovered that FOXD1 expression positively correlated with AXL expression and negatively with MITF and SOX10 expression (Figure 5). These results suggest that FOXD1 is an oncogenic factor and might have effects on the differentiation status and resistance of melanoma cells.

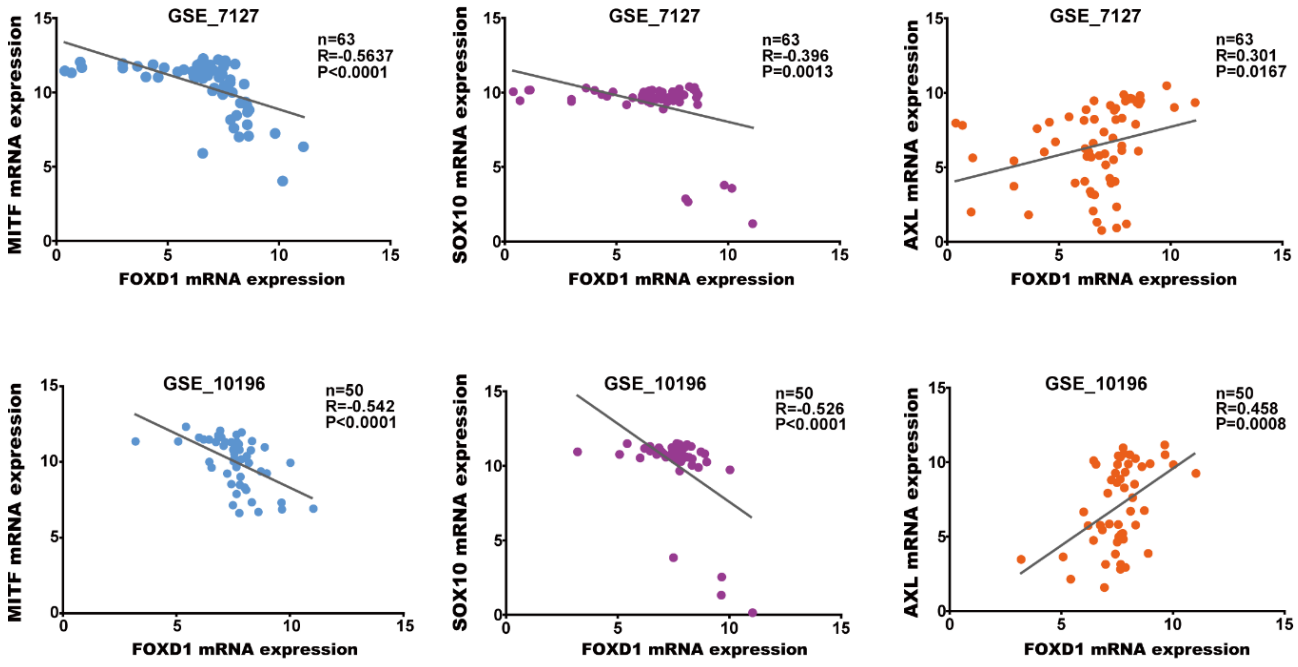


Figure 5. Correlation between FOXD1 and MITF, SOX10 and AXL expression

FOXD1 expression negatively correlated with the expression of MITF, SOX10, and positively correlated with AXL expression according to the data from the two GSE files (GSE7127 and GSE10196).

4.3 FOXD1 expression is highly increased in FOXD1 OE cells

Next, in order to examine the influence of FOXD1 on the differentiation status in melanoma cells and the resistance to BRAFi, I ectopically expressed FOXD1 in three BRAF mutated human melanoma cell lines. By comparing the expression level of FOXD1 between FOXD1 OE cells and control group, I found that the ectopic expression of FOXD1 could significantly increase the FOXD1 level above the natural endogenous level (Figure 6A, 6B).

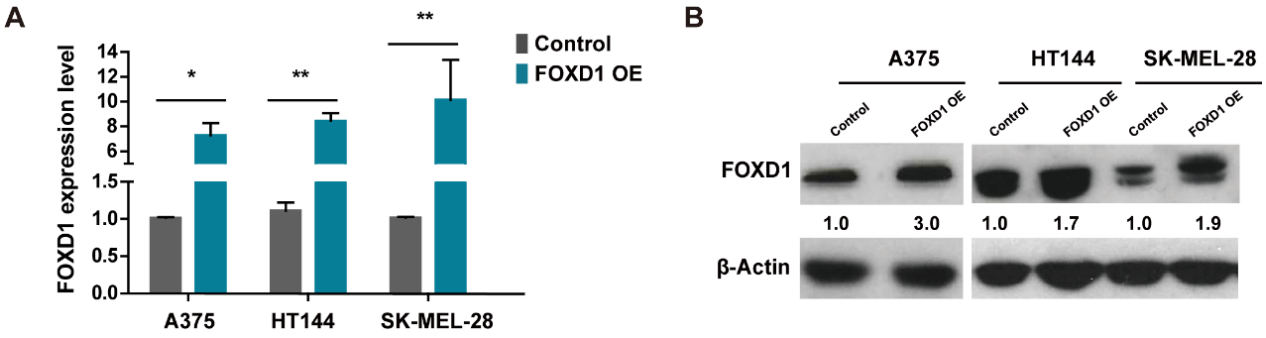


Figure 6. FOXD1 expression level is highly increased in FOXD1 OE cells

A Quantification of the FOXD1 expression by RT-qPCR in three melanoma cell lines transduced with empty vector control (Control) or a FOXD1-overexpressing (OE) plasmid. **B** Expression of FOXD1 in protein level. Densitometric values were normalized to the loading controls β-Actin.

4.4 Overexpression of FOXD1 promotes melanoma cell dedifferentiation

As shown in Figures 7A-C, ectopic FOXD1 expression triggered the downregulation of MITF and SOX10 expression as well as the upregulation of AXL expression in all FOXD1 OE cells in comparison with their control counterparts. As mentioned before, MITF and SOX10 are closely associated with differentiation status and drug resistance. Cells with high level of FOXD1 expressed little MITF and SOX10 indicating the important role of FOXD1 in mediating melanoma cell dedifferentiation and drug tolerance. Consistently, increased AXL expression in combination with decreased MITF expression also provides evidence that FOXD1 OE cells might be more resistant to inhibitor treatment compared with control cells. Furthermore, cell morphology is usually an important parameter that affects and predicts cell function. Cells with different physiological properties may also have differences in cell morphology. In this context, I studied the morphology of control cells and FOXD1 OE cells under an inverted light microscope and compared whether there are differences between the two conditions. I observed that the morphology of SK-MEL-28 cells changes after experimentally increasing FOXD1 expression (Figure 7D). Cells that

overexpressed FOXD1 showed fewer dendritic processes and less differentiated spindle morphology, both of which suggest that ectopic FOXD1 could induce melanoma cell dedifferentiation. Collectively, these results showed above indicate that FOXD1 could stimulate the dedifferentiation of melanoma cells.

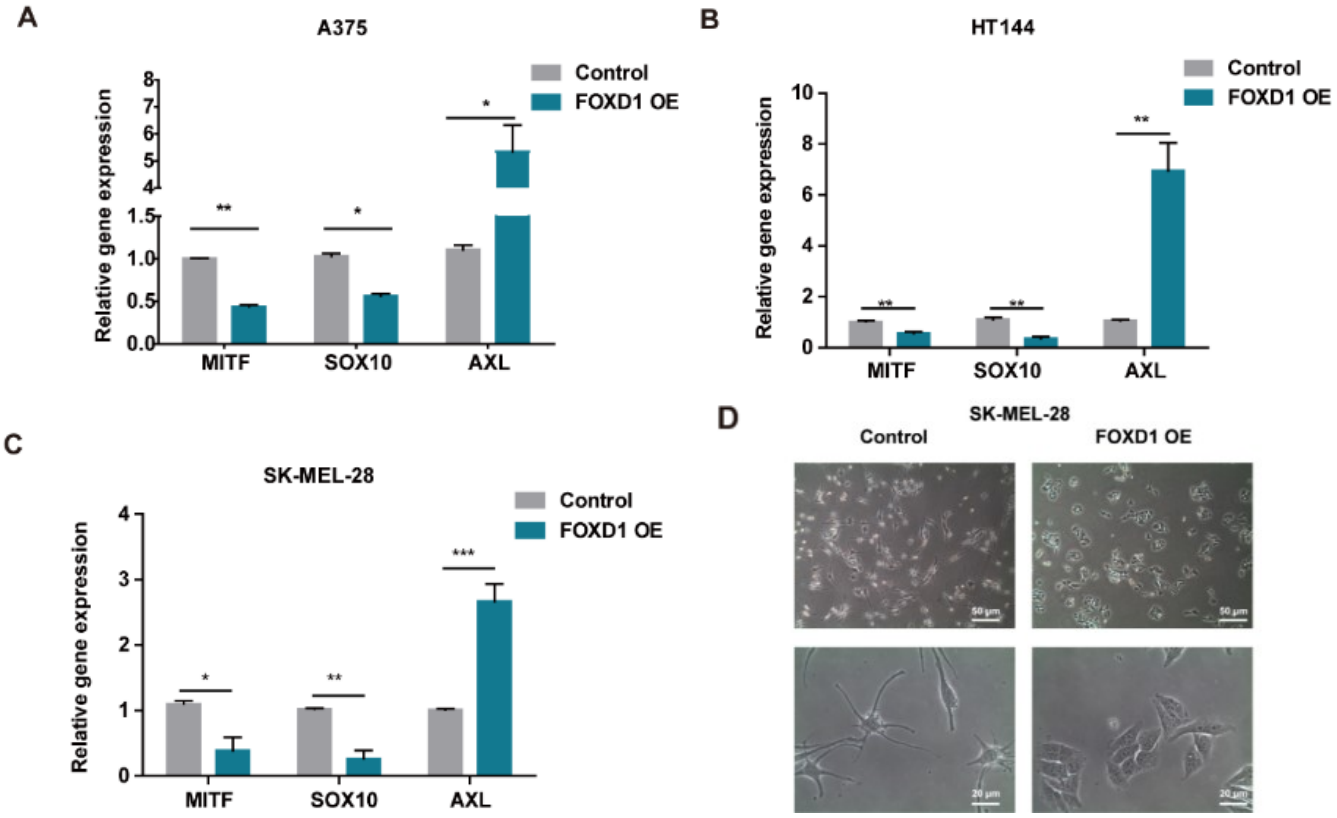


Figure 7. FOXD1 promotes dedifferentiation of melanoma cell

A, B, C mRNA level of MITF, SOX10 and AXL upon ectopic FOXD1 expression in three melanoma cell lines. **D** SK-MEL-28 cells changed their morphology upon ectopic expression of FOXD1.

4.5 Effect of FOXD1 overexpression on cell proliferation

To examine the connection between the aberrant expression of FOXD1 and melanoma pathogenesis, I investigated whether the elevated FOXD1 expression level affects melanoma cell proliferation. To do this, I collected FOXD1 OE and control cells and conducted cell proliferation assays with the Click-iT® EdU kit and flow cytometry analysis. As shown in Figure 8A and 8B, FOXD1 overexpression could only slightly increase the proliferation rate of A375 and HT144 cells since the percentage of cells in S phase was only moderately increased. 43.7% of A375 and 28.1% of HT144 control cells were in the S phase, whereas 48.4% of A375 and 31.3% of HT144 FOXD1 OE cells were found to be in the S phase. However, when I compared the proliferation profiles of SK-MEL-28 control and FOXD1 OE cells, I saw that the ectopic expression of FOXD1 drastically increased the proliferation of SK-MEL-28 cells (Figure 8C). In addition, similar to the results measured

using EdU proliferation kit, cell viability assay using alamar blue also showed no obvious difference between control and FOXD1 OE A375 or HT144 cells (Figure 8D, E). In contrast, the alamar blue assay with SK-MEL-28 cells confirmed the result obtained with the EdU proliferation kit, showing that increased FOXD1 expression could promote cell proliferation (Figure 8F).

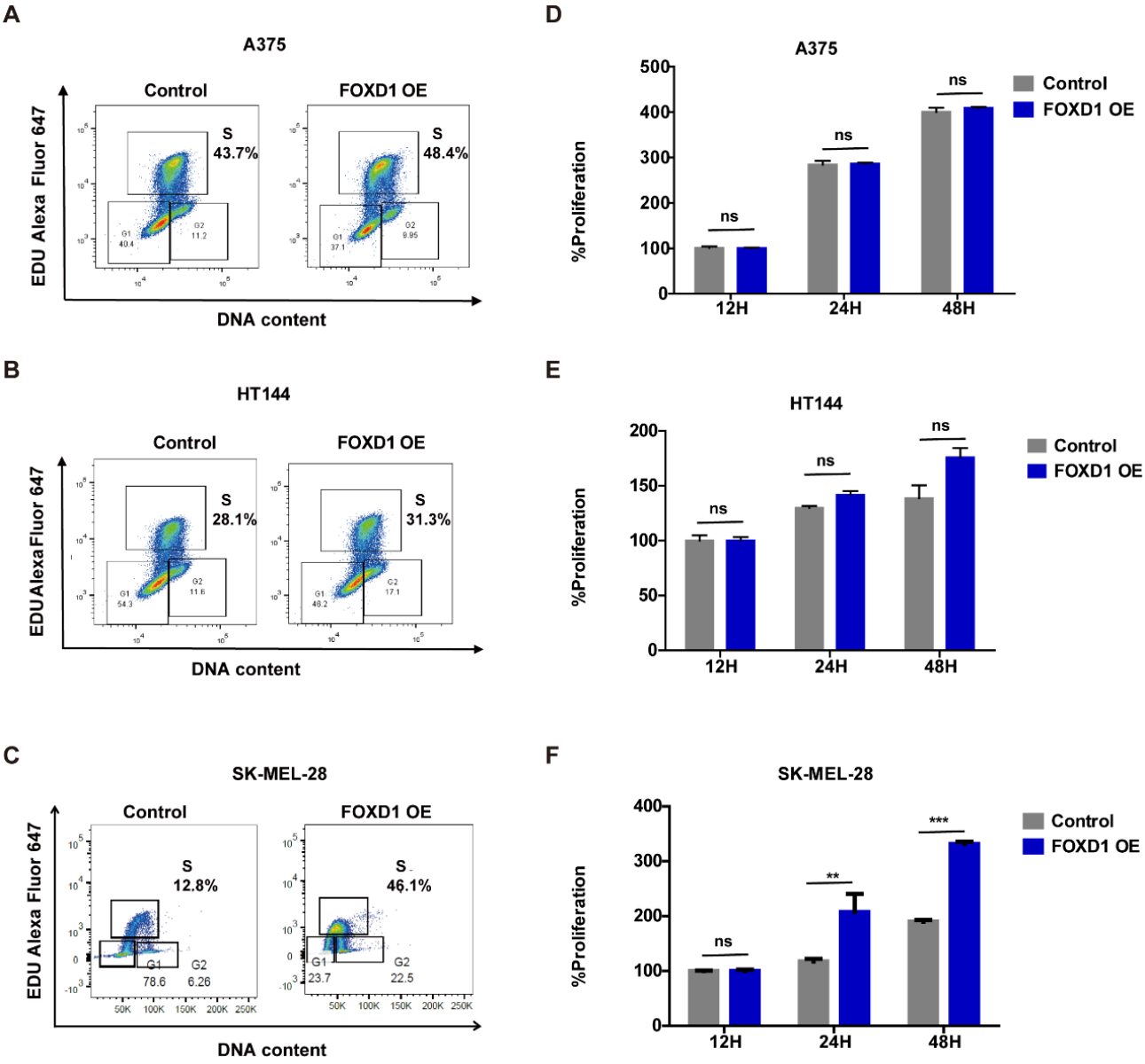


Figure 8. Effect of FOXD1 overexpression on cell proliferation

A, B, C Quantification of the cell proliferation of three melanoma cell lines control and FOXD1 OE groups measured by EdU incorporation and DNA content determination (propidium iodide staining). **D, E, F** Cell proliferation of control and FOXD1 OE cells was analyzed using the alamar blue assay.

4.6 FOXD1 overexpression activates p-ERK and p-AKT and increases the cell tolerance towards BRAFi treatment

The resistance mechanisms of melanoma to targeted therapy are complex and heterogeneous, and the reactivation of p-ERK and p-AKT is the principal mechanism for acquired resistance to targeted therapy in melanoma[141]. Based on these facts, I measured the activation levels of p-ERK and p-AKT in FOXD1 OE and control groups by using western blot. As shown in Figure 9A, increased expression of p-ERK and p-AKT were detected among the three FOXD1 OE cell lines in contrast to the control groups, indicating that the MAPK and AKT pathway were reactivated upon FOXD1 overexpression.

The close connection between high levels of FOXD1 and poor patient survival prompted me to do further study to confirm if FOXD1 overexpression affects tumorigenic properties of melanoma cells. To achieve a particular aim, I performed cell colony formation assay. I did not observe any difference of cell colonies in all three melanoma cell lines upon FOXD1 overexpression compared with their control counterparts. Vemurafenib only slightly influences FOXD1 OE cells to form colonies, but it can greatly weaken colony formation abilities in control cells. These results demonstrate that FOXD1 overexpression promoted resistance of melanoma cells to BRAFi *in vitro* (Figure 9B, 9C).

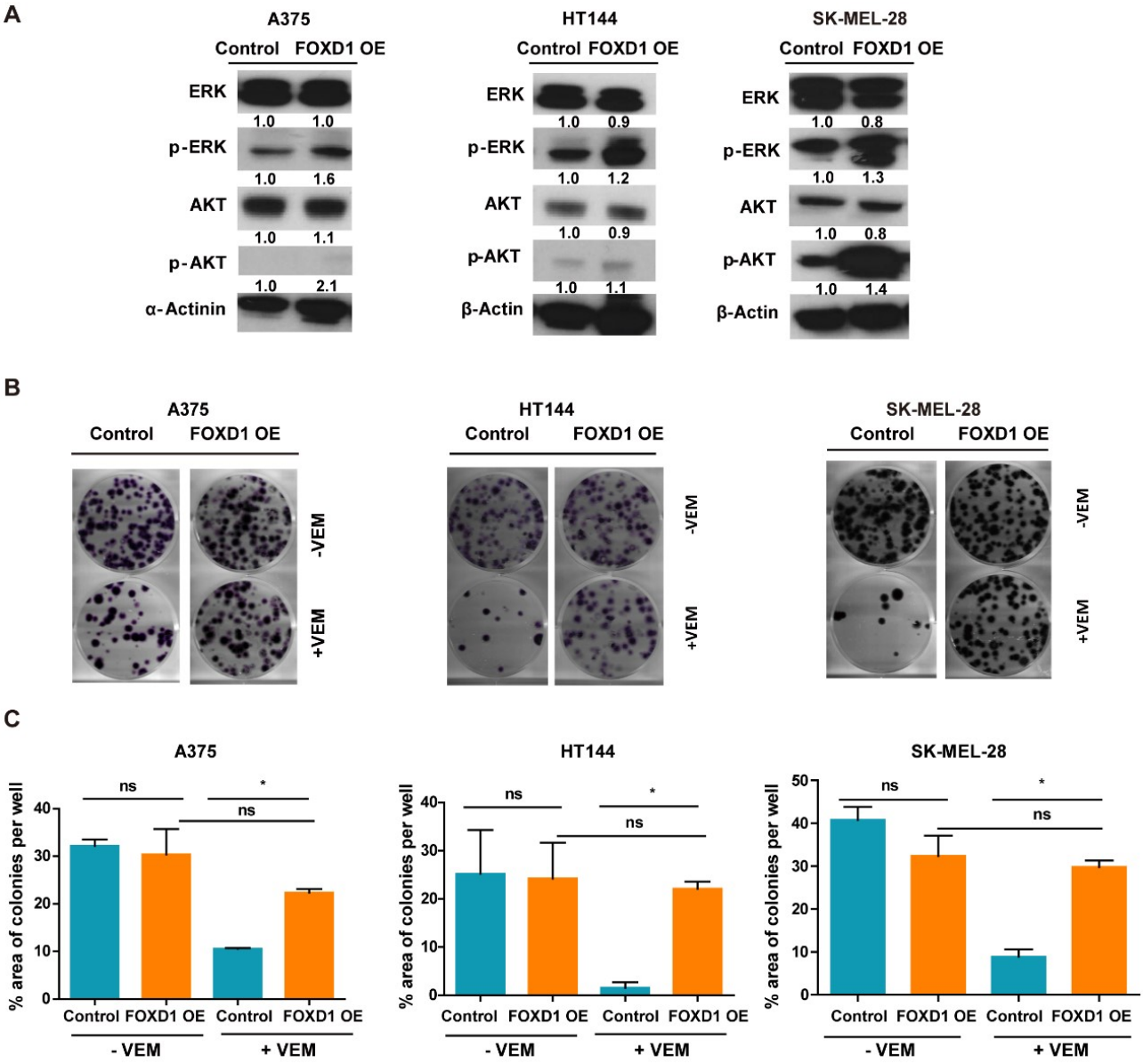


Figure 9. FOXD1 overexpression promotes melanoma cell resistance to BRAFi

A Protein levels of ERK, p-ERK, AKT and p-AKT in A375/HT144/SK-MEL-28 control and FOXD1 OE cells. Densitometric values were normalized to the loading controls β-Actin and α-Actinin. **B** Representative figures of clonogenic assays ± VEM (10 µM) treatment. **C** Clonogenic assay ± VEM (10 µM) treatment.

4.7 FOXD1 OE cells are more resistant towards BRAFi treatment compared to control cells

The colony formation assay shown above indicates that the drug resistance of melanoma cells was increased after overexpression of FOXD1. In order to confirm this result, I performed cell viability experiments using alamar blue. To do this, I seeded the control and FOXD1 OE cells separately and treated them with increasing concentrations of VEM as indicated (0-20 µM). After 72 h, the cells were stained and incubated with alamar blue for 3h and then cell viability was evaluated. As shown in Figure 10, the viability of each cell line was decreased upon VEM treatment. However, HT144 and SK-MEL-28 FOXD1 OE cells showed higher viability than the control groups for each concentration of VEM. A375 FOXD1 OE cells also showed higher viability compared to the control when the VEM concentration was higher than 100 nM (Log [VEM] = 2). Furthermore, the IC50 values also proved that FOXD OE cells were more tolerant to the treatment than control cells. (A375 FOXD OE mean IC50 = 2.39 ± 0.08 µM; HT144 FOXD OE mean IC50 = 2.66 ± 0.08 µM; SK-MEL-28 FOXD OE mean IC50 = 1.54 ± 0.09 µM; A375 control mean IC50 = 3.49 ± 0.06 µM; HT144 control mean IC50 = 15.40 ± 0.10 µM; SK-MEL-28 control mean IC50 = 8.23 ± 0.09 µM). (Supplementary Table S1).

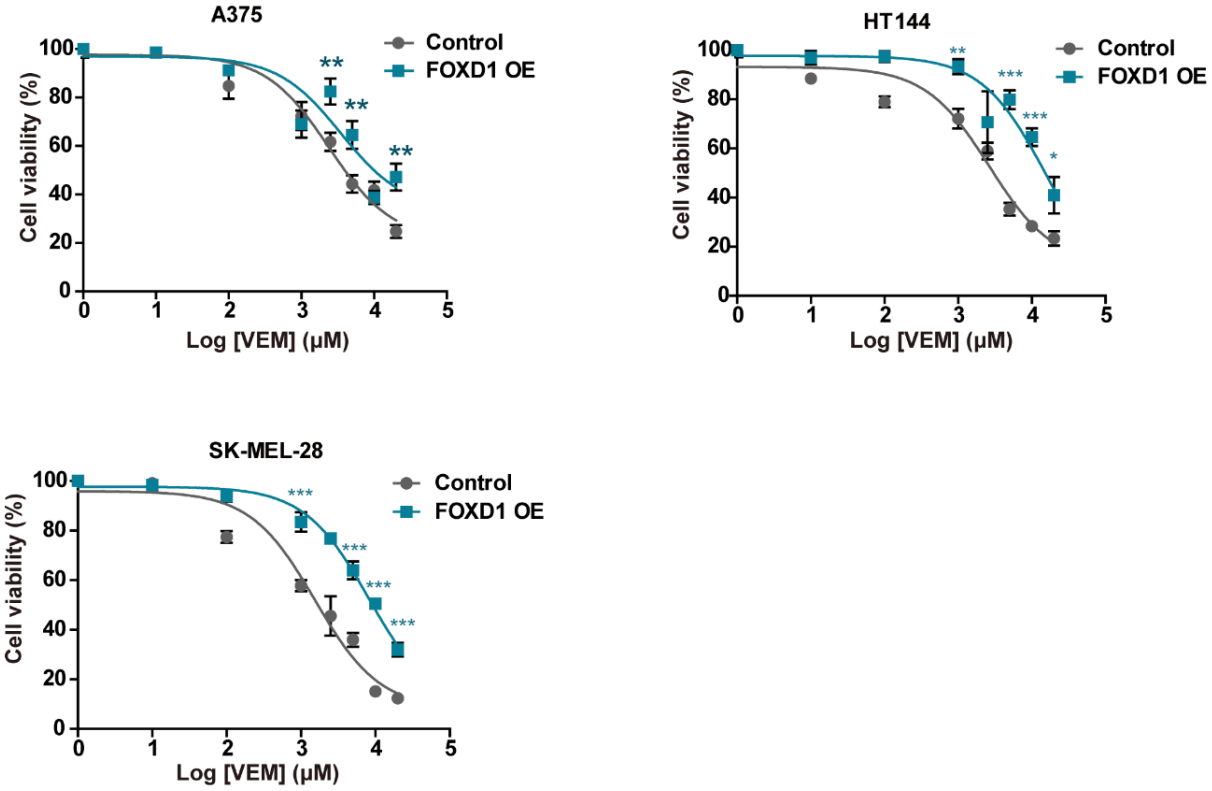
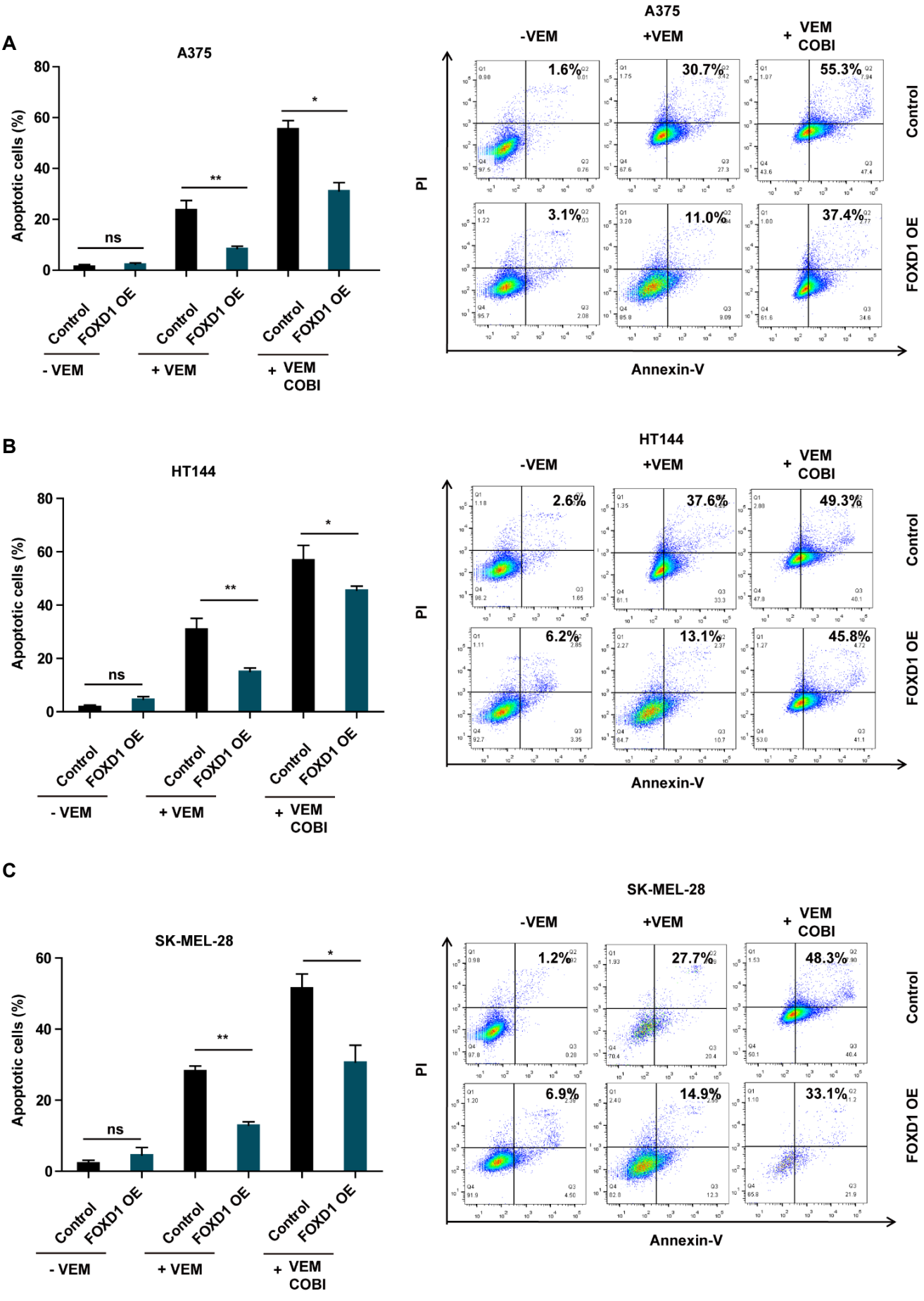


Figure 10. FOXD1 OE cells are more resistant towards BRAFi treatment than control cells
Control and FOXD1 OE cells were treated with VEM (0.0001–25 µM) for 72 h. Cell viability assay was performed using alamar blue.

4.8 FOXD1 overexpression promotes melanoma resistance to BRAFi and MEKi

By comparing the sensitivity to BRAFi treatment between FOXD1 OE and control cells using colony formation assay and cell viability assay, I found that VEM displayed a less inhibitory effect on FOXD1 OE cells. Next, I wanted to investigate if VEM can induce apoptosis in FOXD1 OE and control cells. For this purpose, I stained the OE and control cells with annexin V and PI. Only apoptotic cells will be stained with annexin V and detected by a flow cytometer accordingly. As shown in Figure 11, when these cells were subjected to treatment with 10 μM VEM for 48 h, I detected 30.7%, 37.6%, 27.7% annexin V-positive cells for the A375, HT144, or SK-MEL-28 control group, respectively, whereas the percentage of annexin V-positive cells for the A375, HT144, SK-MEL-28 FOXD1 OE groups were decreased to 11.0%, 13.1%, 14.9%. This clearly suggests that ectopic FOXD1 expression could significantly increase the drug resistance of melanoma cells. Interestingly, the annexin V staining also revealed that ectopic FOXD1 expression markedly decreased the percentage of annexin V-positive cells upon combined administration of the BRAFi and the MEK inhibitor (MEKi), cobimetinib (COBI), compared to the control cells. Furthermore, the combination treatment resulted in more apoptotic melanoma cells compared to VEM monotherapy.


A
A375
Apoptotic cells (%)
80
60
40
20
0
ns
**
*
Control
FOXD1 OE
- VEM
+ VEM
+ VEM COBI
A375
-VEM
+VEM
+ VEM COBI
1.6%
30.7%
55.3%
3.1%
11.0%
37.4%
Control
FOXD1 OE
PI
Annexin-V
B
HT144
Apoptotic cells (%)
80
60
40
20
0
ns
**
*
Control
FOXD1 OE
- VEM
+ VEM
+ VEM COBI
HT144
-VEM
+VEM
+ VEM COBI
2.6%
37.6%
49.3%
6.2%
13.1%
45.8%
Control
FOXD1 OE
PI
Annexin-V
C
SK-MEL-28
Apoptotic cells (%)
60
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ns
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Control
FOXD1 OE
- VEM
+ VEM
+ VEM COBI
SK-MEL-28
-VEM
+VEM
+ VEM COBI
1.2%
27.7%
48.3%
6.9%
14.9%
33.1%
Control
FOXD1 OE
PI
Annexin-V

Figure 11. FOXD1 overexpression promotes melanoma cell resistance to BRAFi and MEKi
A Left: Quantification of apoptosis of A375 cells with Cy5-annexin V/PI staining. A375 control and A375 FOXD1 OE cells were treated with dimethyl sulfoxide (DMSO) or VEM (10 μM) or VEM and the MEKi COBI (10 μM) in combination for 48 h. Right: Representative flow cytometry scatter plots. **B** Left: Quantification of apoptosis of HT144 cells. HT144 control and HT144 FOXD1 OE cells were treated with DMSO or VEM (10 μM) or VEM and COBI (10 μM) in combination for 48 h. Right: Representative flow cytometry scatter plots. **C** Left: Quantification of apoptosis of SK-MEL-28 cells. SK-MEL-28 control and SK-MEL-28 FOXD1 OE cells were treated with DMSO or VEM (10 μM) or VEM and COBI (10 μM) in combination for 48 h. Right: Representative flow cytometry scatter plots.

4.9 FOXD1 promotes melanoma migration and invasion

The migratory and invasive ability are two critical parameters of melanoma cells[142]. To determine if overexpression of FOXD1 could promote melanoma cell migration properties, I performed cell migration assays by using the ibidi culture-inserts. As shown in Figure 12A, compared to control cells, FOXD1 OE cells could reduce the gap within 8 h much more indicating that cells with higher FOXD1 expression level were more migrative. Next, to confirm that the increased expression of FOXD1 could enhance the invasiveness of melanoma cells, I carried out cell invasion assays by using BME cell invasion kit. To do this, I first prepared the cell chamber coated with BME, and then seeded FOXD1 OE or control cells on top of the membrane in FCS-free cell culture medium. At the bottom of the BME chamber, normal cell medium (10% FCS included) was added to attract melanoma cells. After 10 h of incubation, the invasive melanoma cells were able to degrade the BME layer and move down to the bottom of the well. The invasive melanoma cells could be stained and counted. In line with my previous results (Figure 9B), FOXD1 overexpression could drastically increase the invasion abilities of melanoma cells (Figure 12B).

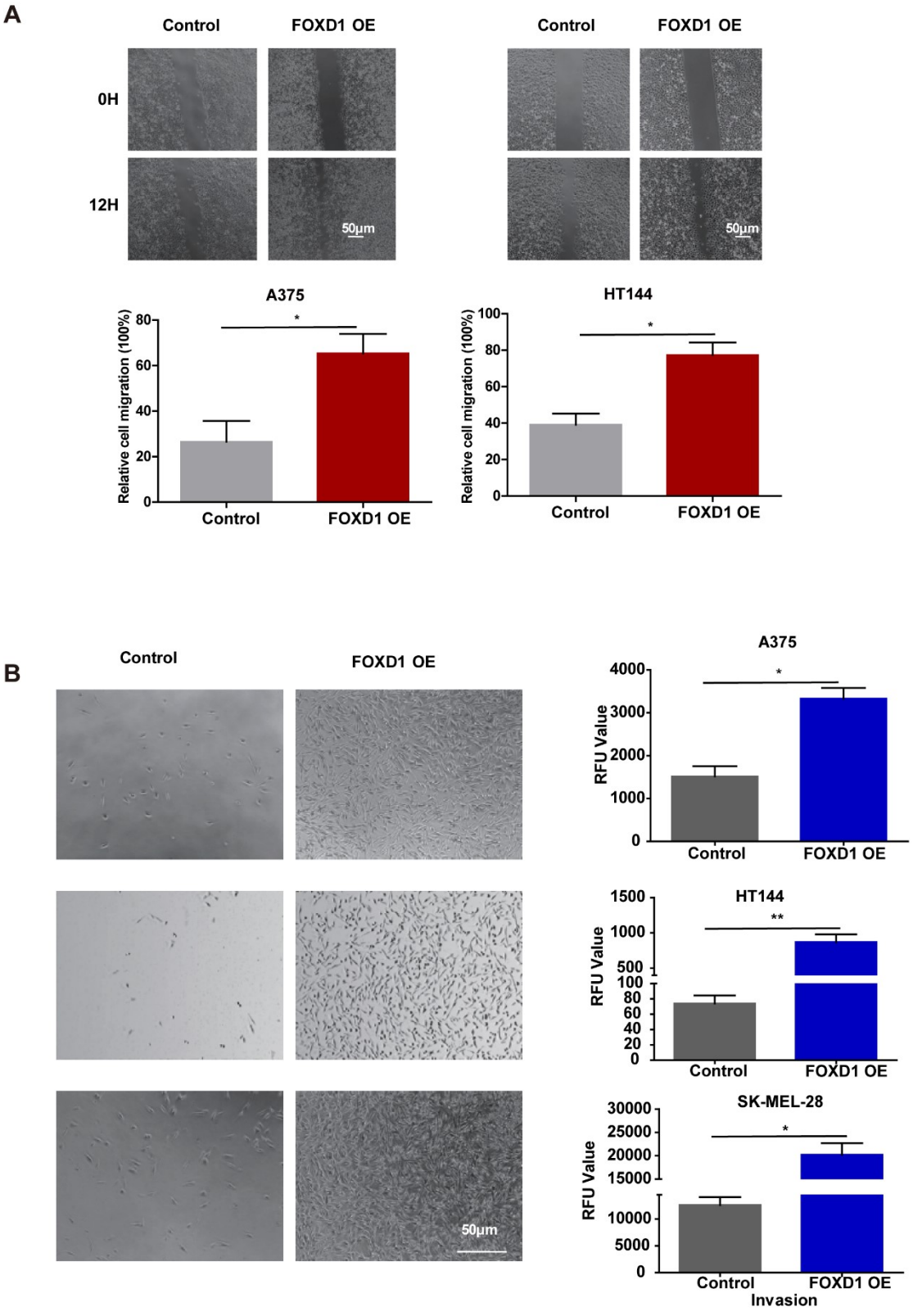


Figure 12. FOXD1 promotes melanoma cell migration and invasion

A Migration assay in all three melanoma cell lines (control and FOXD1 OE groups). **B** Invasion assay in all three melanoma cell lines (control and FOXD1 OE groups).

4.10 FOXD1 expression level is significantly decreased in FOXD1 KD cells

Since FOXD1 overexpression enhanced stemness properties, promoted tumorigenicity and induced the resistance of melanoma cells to BRAFi, it was worthwhile to assess if targeting FOXD1 could be an efficient therapeutic strategy for the treatment of melanoma. For this purpose, all three melanoma cell lines were transfected with two different siRNAs targeting FOXD1. As shown in Figures 13A and 13B, the FOXD1 expression level was significantly decreased upon FOXD1 knockdown compared to the control group.

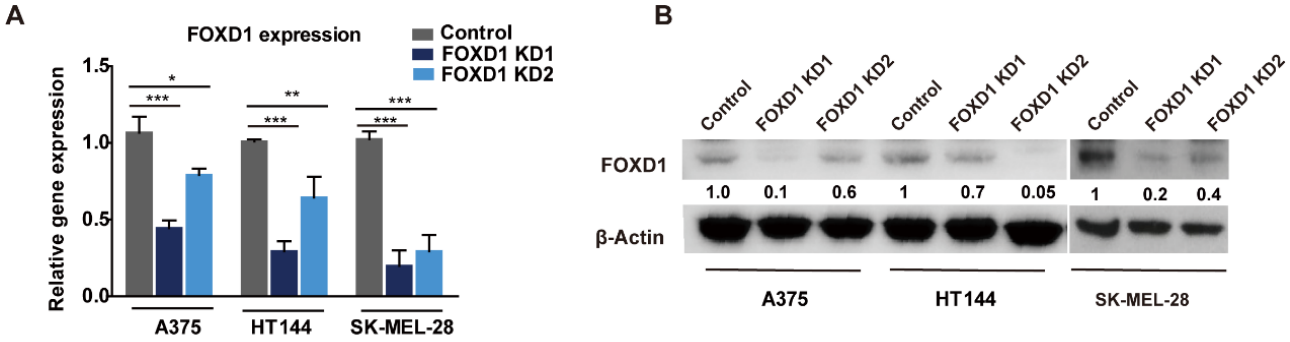


Figure 13. FOXD1 KD cells express significantly less FOXD1 compared to control cells
A Quantification of FOXD1 expression by RT-qPCR in all three different melanoma cells upon FOXD1 knockdown with two siRNAs (FOXD1 KD1, FOXD1 KD2). **B** Protein level of FOXD1 upon transfection of all three melanoma cells with FOXD1-specific siRNAs. Densitometric values were normalized to the loading controls β-Actin.

4.11 FOXD1 KD slightly decreases melanoma cell proliferation.

By comparing the proliferation profiles of FOXD1 OE and control group, I found that FOXD1 exhibited different regulatory effects on different cells: FOXD1 overexpression could only slightly enhance the proliferation rate of A375 and HT144 cells but drastically foster the growth of SK-MEL-28 cells. To further identify the role of FOXD1 in cell proliferation, I conducted an experiment using the Click-iT® EdU kit and evaluated the proliferation rate of different melanoma cell lines after knocking down FOXD1. As shown in Figure 14, FOXD1 knockdown could only marginally reduce the proliferation of all three cell lines.

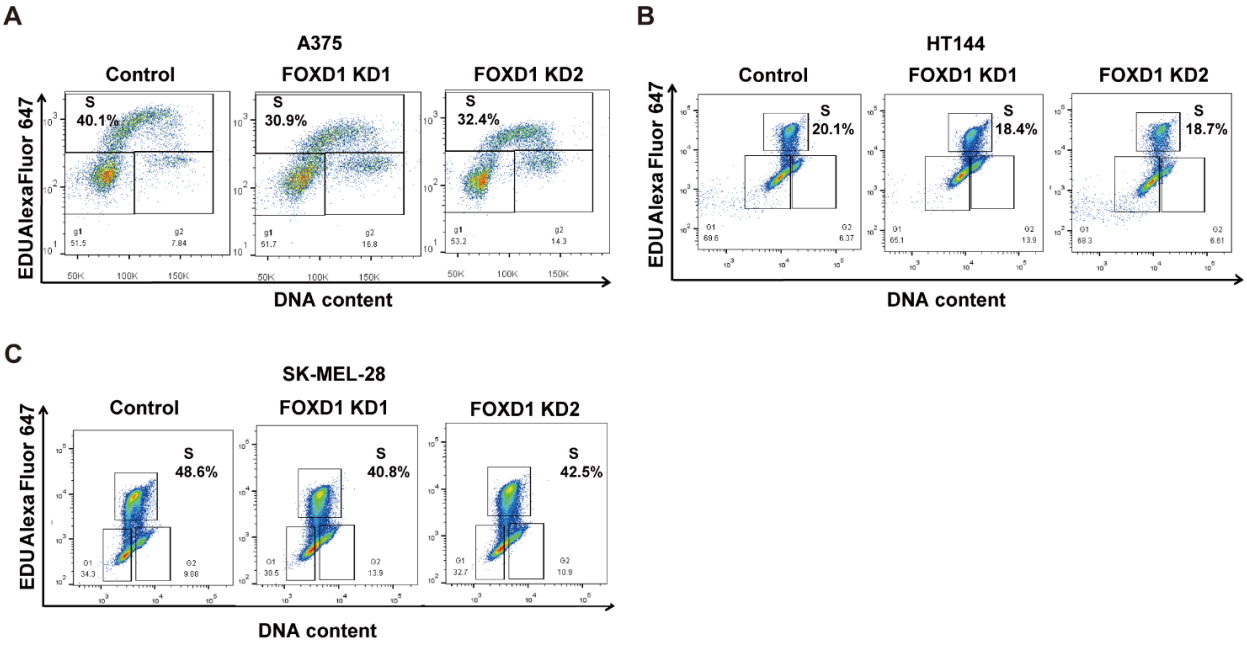


Figure 14. FOXD1 knockdown slightly decreases melanoma cell proliferation

A Proliferation of A375 control and A375 FOXD1 KD cells measured by EdU incorporation and DNA content measurement (PI staining). **B** Proliferation of HT144 control and HT144 FOXD1 KD cells measured by EdU incorporation and DNA content measurement (PI staining). **C** Proliferation of SK-MEL-28 control and FOXD1 KD cells measured by EdU incorporation and DNA content measurement (PI staining).

4.12 Knockdown of FOXD1 decreases the activity of p-ERK, p-AKT and the tolerance towards BRAFi treatment in melanoma cells

Previous data have shown that the MAPK and AKT pathway were reactivated after FOXD1 overexpression. Here, I demonstrated decreased of the p-AKT and p-ERK expression levels after FOXD1 knockdown in comparison to the control group (Figure 15A). To investigate whether knocking down FOXD1 could sensitize melanoma cells to BRAFi, I conducted a colony formation assay. The results showed that downregulating FOXD1 slightly diminished the colony forming capacity of all three cell lines compared to control cells. However, FOXD1 KD cells were more sensitive to vemurafenib as indicated by the fewer number of colonies in comparison to the control cells (Figure 15B).

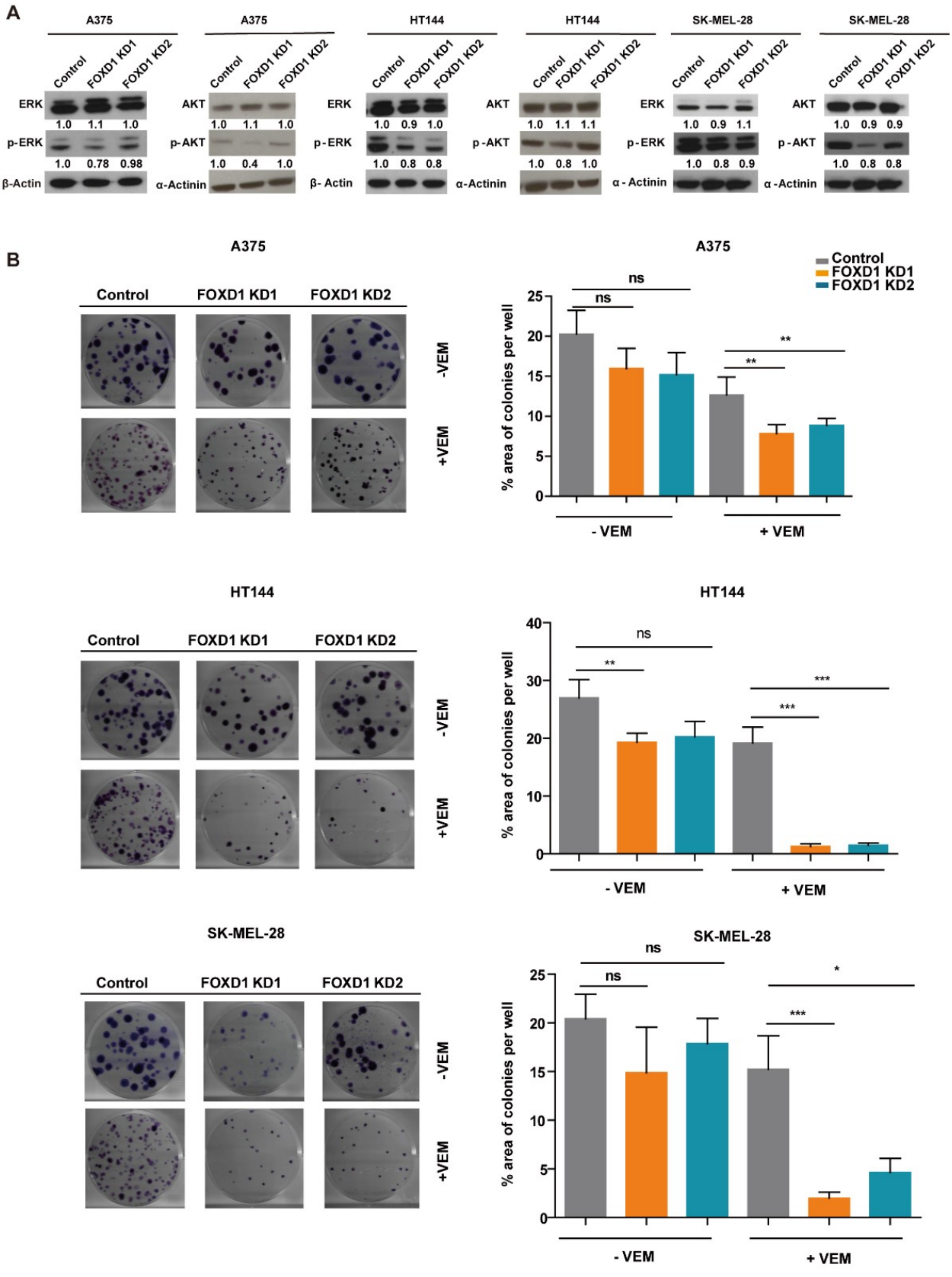

A
A375
A375
HT144
HT144
SK-MEL-28
SK-MEL-28
Control
FOXD1 KD1
FOXD1 KD2
ERK
AKT
p-ERK
p-AKT
β-Actin
α-Actinin
β- Actin
α - Actinin
1.0 1.1 1.0
1.0 1.1 1.0
1.0 0.78 0.98
1.0 0.4 1.0
1.0 0.9 1.0
1.0 0.8 0.8
1.0 1.1 1.1
1.0 0.8 1.0
1.0 0.9 1.1
1.0 0.8 0.9
1.0 0.9 0.9
1.0 0.8 0.8
B
A375
HT144
SK-MEL-28
Control
FOXD1 KD1
FOXD1 KD2
-VEM
+VEM
% area of colonies per well
ns
**
***
*
- VEM
+ VEM

Figure 15. Knockdown of FOXD1 decreases melanoma cell resistance to BRAFi

A ERK, p-ERK, AKT and p-AKT levels in all three cell lines control and FOXD1 KD cells. Densitometric values were normalized to the loading controls β-Actin. The samples are all derived from the same gel/blot and therefore the α-Actinin is the loading control for both panels (shown twice). **B** Left: Representative figures of clonogenic assays ± VEM (10 μM) treatment. Right: Quantification of the clonogenic assays ± VEM (10 μM) treatment.

4.13 Knockdown of FOXD1 decreases the tolerance of melanoma cells towards BRAFi treatment

As shown before, cells with increased FOXD1 level displayed more resistance towards vemurafenib treatment. In order to confirm if knockdown of FOXD1 could increase the cell sensitivity to targeted therapy, I performed a cell viability assay using alamar blue. To do this, I treated the melanoma cells with different concentrations (0-20 μM) of vemurafenib for 72 h and stained the cells with alamar blue for 3 h. By analyzing the fluorescence intensity, I found that after receiving the same concentration of vemurafenib, the cell viability of FOXD1 KD groups was significantly lower compared to the control groups, indicating that FOXD1 KD cells are more sensitive to BRAFi (Figure 16). Furthermore, the IC50 values also proved that FOXD KD cells were more sensitive to the treatment compared to control cells: Control cells (A375 mean IC50 = 2.80 ± 0.07 μM; HT144 mean IC50 =1.45 ± 0.06 μM; SK-MEL-28 mean IC50 = 1.41 ± 0.06 μM), FOXD1 KD cells (A375 FOXD1 KD1 mean IC50 = 0.03 ± 0.07 μM; A375 FOXD1 KD2 mean IC50 = 0.07 ± 0.07 μM; HT144 FOXD1 KD1 mean IC50 = 0.08 ± 0.07 μM; HT144 FOXD1 KD2 mean IC50 = 0.04 ± 0.06 μM; SK-MEL-28 FOXD1 KD1 mean IC50 = 0.18 ± 0.08 μM; SK-MEL-28 FOXD1 KD2 mean IC50 = 0.07 ± 0.07 μM) (Supplementary Table S2).

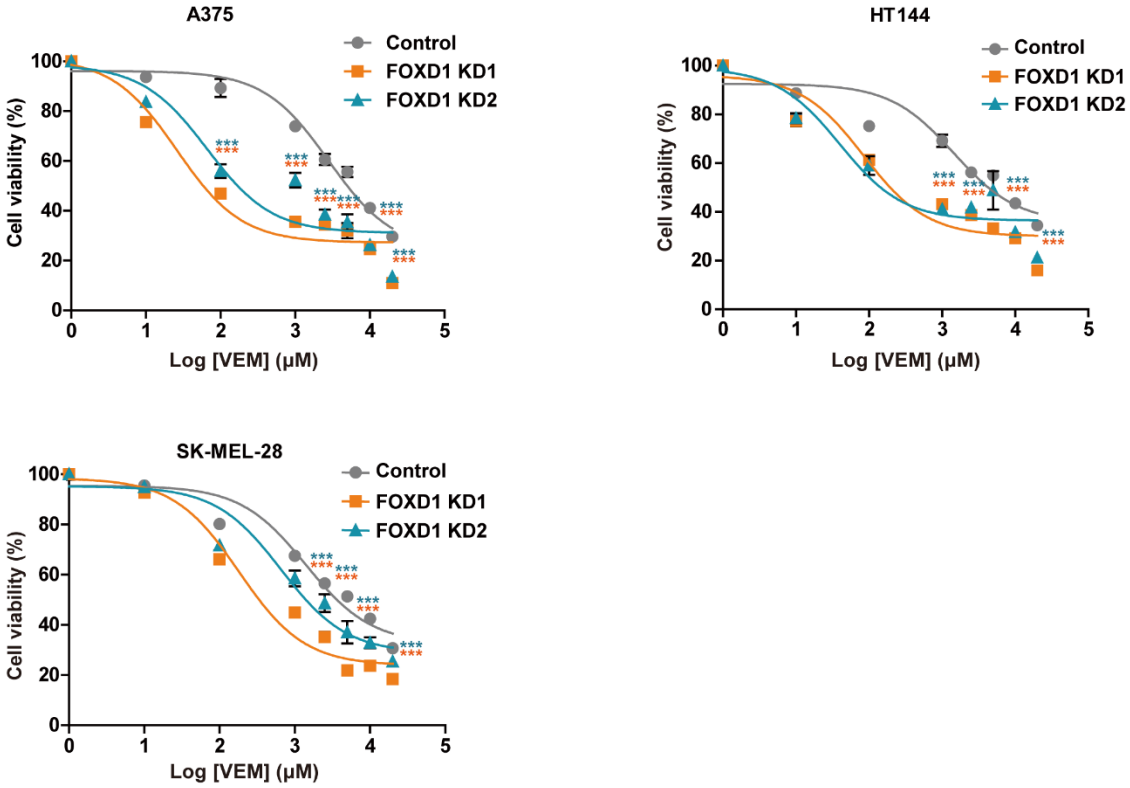


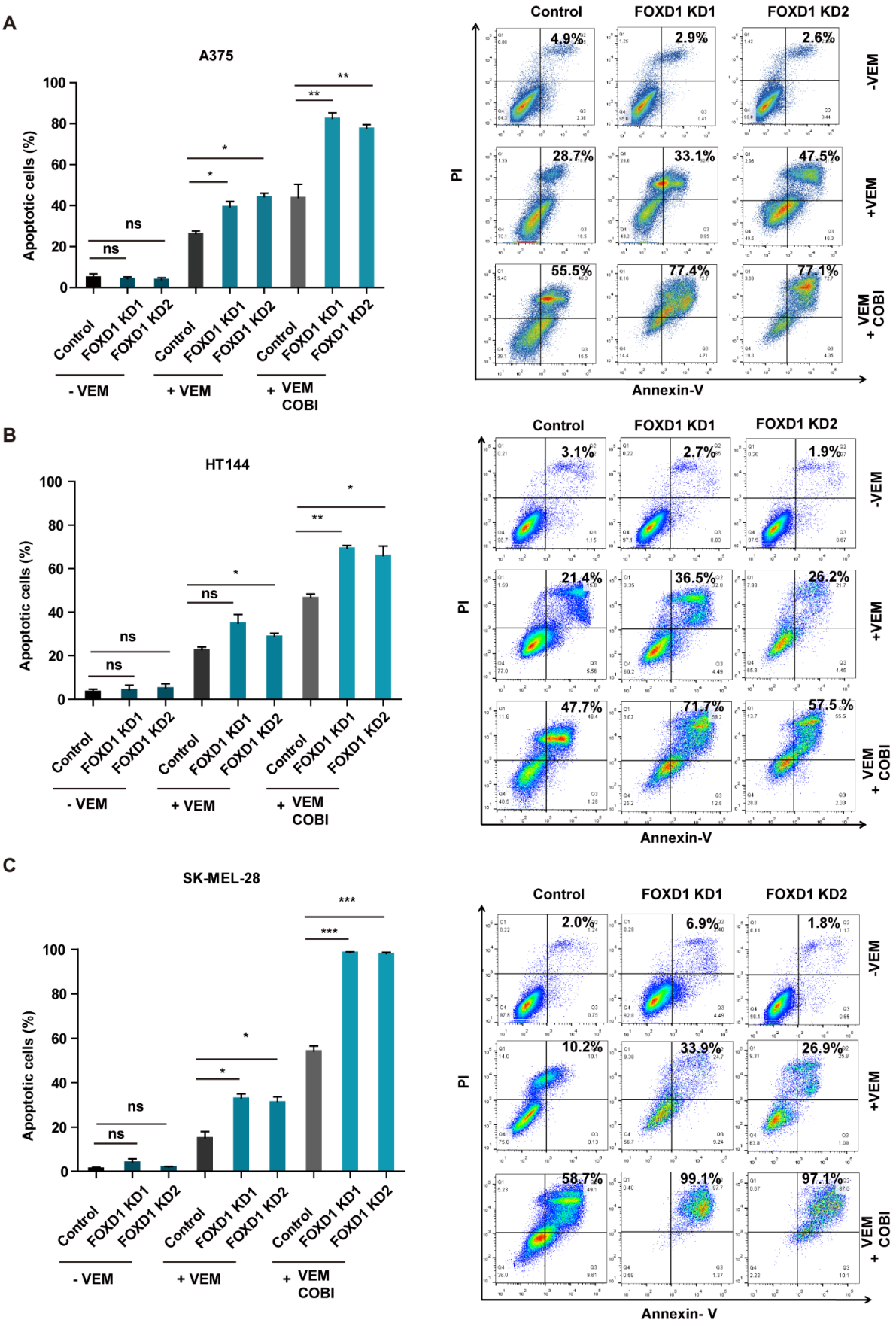
Figure 16. Knockdown of FOXD1 sensitizes melanoma cells to BRAFi treatment

Control and FOXD1 KD cells were treated with VEM (0.0001–25 μM) for 72 h. Cell viability assay was then measured using alamar blue.

4.14 Knockdown of FOXD1 decreases melanoma cell resistance to BRAFi and MEKi

My data above indicate that the FOXD1 KD could resensitize melanoma cells to the inhibitory effects of BRAFi (FOXD1 KD cells have lower IC50). In order to confirm this result, I treated control and FOXD1 KD cells with vemurafenib (monotherapy) or vemurafenib and cobimetinib (combined treatment). After 48 h incubation, I carried out an apoptosis assay through annexin V staining followed by FACS analysis. As shown in Figure 17, I did not observe a conspicuous difference before and after knockdown of FOXD1. However, a drastically elevated proportion of annexin V-positive cells could be detected after FOXD1 KD in combination with BRAFi treatment or a combinatorial treatment with vemurafenib and cobimetinib (Figure 17). These results corroborate that FOXD1 knockdown could sensitize melanoma cells to BRAFi and MEKi treatment *in vitro.* Moreover, caspase 3/7/9 and BAX play vital roles during apoptosis[143]. Here, by using the caspase 3/7 kit in combination with FACS analysis, I found that higher activity of caspase 3/7 in A375 and HT144 FOXD1 KD groups in existence of BRAFi in contrast to the control groups (Figure S1).

Consistently, I also observed a higher protein level of cleaved-caspase 3 and cleaved-caspase 9 as well as BAX in A375 cells after FOXD1 knockdown in combination with BRAFi treatment (Figure S2). These results confirm that silencing FOXD1 could sensitize melanoma cells to targeted therapy.


A
A375
Apoptotic cells (%)
ns
ns
*
*
**
**
Control
FOXD1 KD1
FOXD1 KD2
- VEM
+ VEM
+ VEM COBI
Control
FOXD1 KD1
FOXD2 KD2
4.9%
2.9%
2.6%
28.7%
33.1%
47.5%
55.5%
77.4%
77.1%
-VEM
+VEM
VEM + COBI
PI
Annexin-V
B
HT144
Apoptotic cells (%)
ns
ns
*
ns
*
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*
3.1%
2.7%
1.9%
21.4%
36.5%
26.2%
47.7%
71.7%
57.5 %
Annexin-V
C
SK-MEL-28
Apoptotic cells (%)
***
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ns
ns
2.0%
6.9%
1.8%
10.2%
33.9%
26.9%
58.7%
99.1%
97.1%
Annexin- V

Figure 17. Knockdown of FOXD1 sensitizes melanoma cells to BRAFi and MEKi

A, B, C Left: Apoptosis assay by staining with FITC-annexin V/PI. All three cell lines control and FOXD1 KD cells were treated with DMSO or VEM (10 μM) or VEM and COBI (10 μM) in combination for 48 h. Right: Representative scatter plots of apoptosis assay in all three cell lines.

4.15 FOXD1 expression is upregulated in BRAFi-resistant melanoma cells

According to the data presented so far, FOXD1 seems to be an important factor that may affect the efficacy of targeted therapy. For this reason, I ascertained the FOXD1 expression levels in BRAFi-(vemurafenib) resistant melanoma cells and their parental counterparts using RT-PCR and western blot and found the expression level of FOXD1 is higher in BRAFi-resistant cells in contrast to the non-resistant control groups.

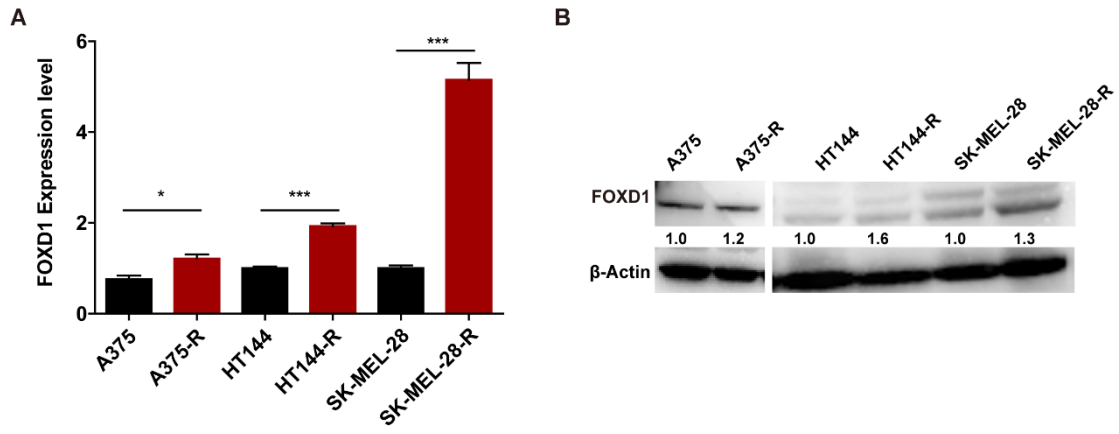


Figure 18. FOXD1 expression is upregulated in BRAFi-resistant melanoma cells

A FOXD1 expression in three BRAFi-resistant cell lines their parental counterparts by RT-qPCR. **B** Western blot analysis of FOXD1 expression in three BRAFi-resistant cell lines and their parental counterparts. Densitometric values were normalized to the loading controls β-Actin.

4.16 FOXD1 knockdown inhibits cell growth of BRAFi-resistant melanoma cells

FOXD1 is a pivotal factor in melanoma stemness as well as sensitivity towards vemurafenib. Since three vemurafenib-resistant cell lines showed higher levels of FOXD1 compared to their non-resistant parental counterparts, I investigated whether FOXD1 expression had an impact on the proliferation of the resistant melanoma cells (Figure 19A, 19B). To do this, I transfected BRAFi-resistant melanoma cells with a FOXD1-specific siRNA (siFOXD1). Using the cell proliferation assay, I could show that the number of S phase cell group was much lower in the resistant cell lines in contrast to their parental counterpart, suggesting that resistant cells were less proliferative. Interestingly, knocking down FOXD1 in BRAFi-resistant cells reduced the percentage of S phase cell populations

even more drastically (Figure 19A). Furthermore, by western blot. I could detect an increased expression of p21, an important marker for cell cycle arrest upon knocking down FOXD1 in all BRAFi-resistant cells (Figure 19B)[144]. The data showed here indicate that FOXD1 is an imperative factor for the growth of drug-resistant melanoma cells.

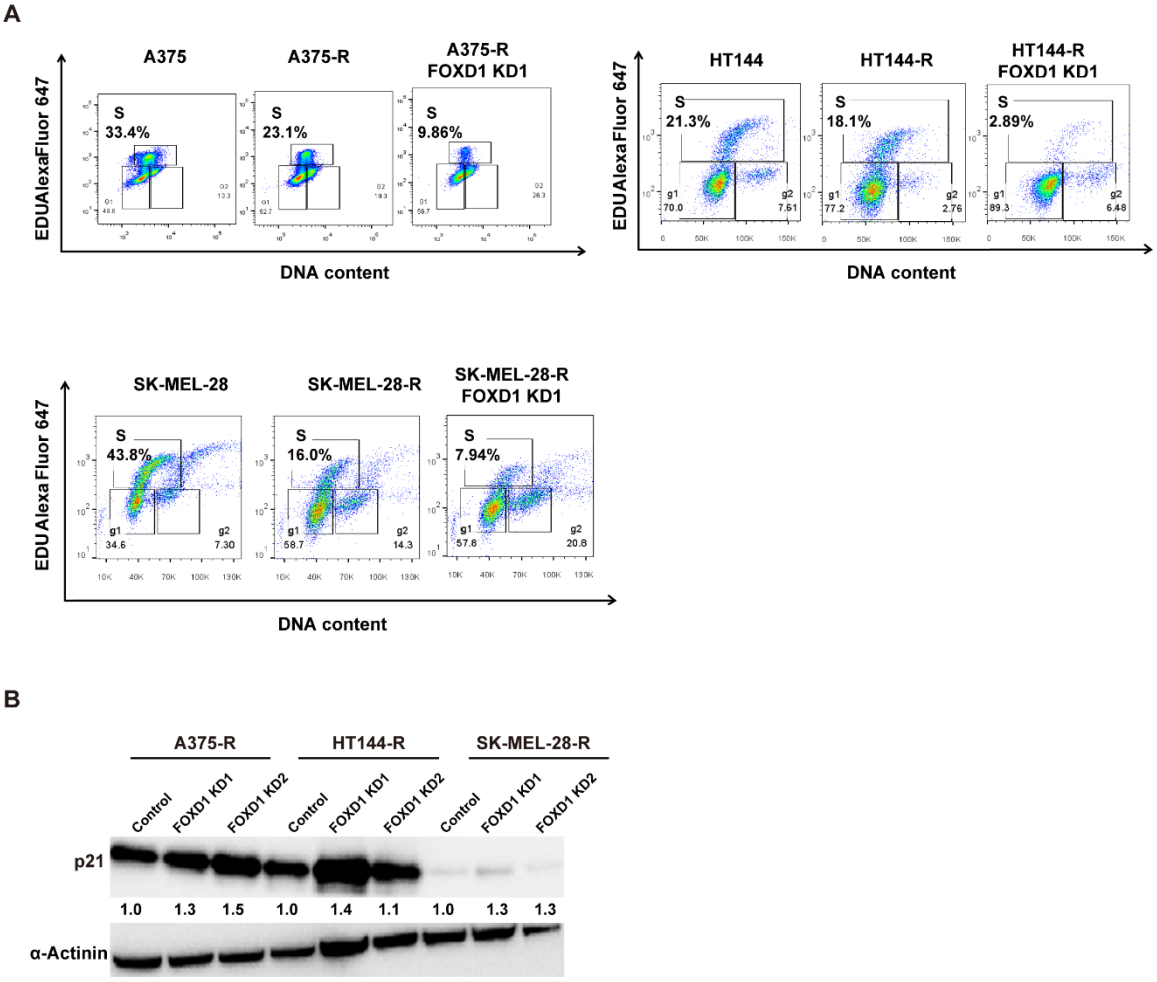


Figure 19. FOXD1 knockdown inhibits cell growth of BRAFi-resistant melanoma cells

A Cell proliferation assay with three parental, BRAFi-resistant and BRAFi-resistant FOXD1 KD cell lines performed by EdU incorporation and DNA content measurement along with FACS analysis. **B** Protein level of p21 in BRAFi-resistant cells upon FOXD1 knockdown. Densitometric values were normalized to the loading controls α-Actinin.

4.17 FOXD1 knockdown increases the sensitivity of BRAFi-resistant melanoma cells to BRAFi

FOXD1 knockdown could increase the cell sensitivity to vemurafenib, resulting in an increased apoptotic response upon treatment with this BRAFi. Here, I found a reduced expression level of p-ERK in BRAFi-resistant FOXD1 KD cells compared to BRAFi-resistant cells transfected with a scrambled siRNA control upon exposure to vemurafenib (Figure 20A). Moreover, I conducted colony formation assay and found a reduced clonogenic capacity of BRAFi-resistant FOXD1 KD cells after vemurafenib treatment. Furthermore, FOXD1 KD cells showed more sensitive to the vemurafenib with significantly less colonies in contrast to the control (Figure 20B).

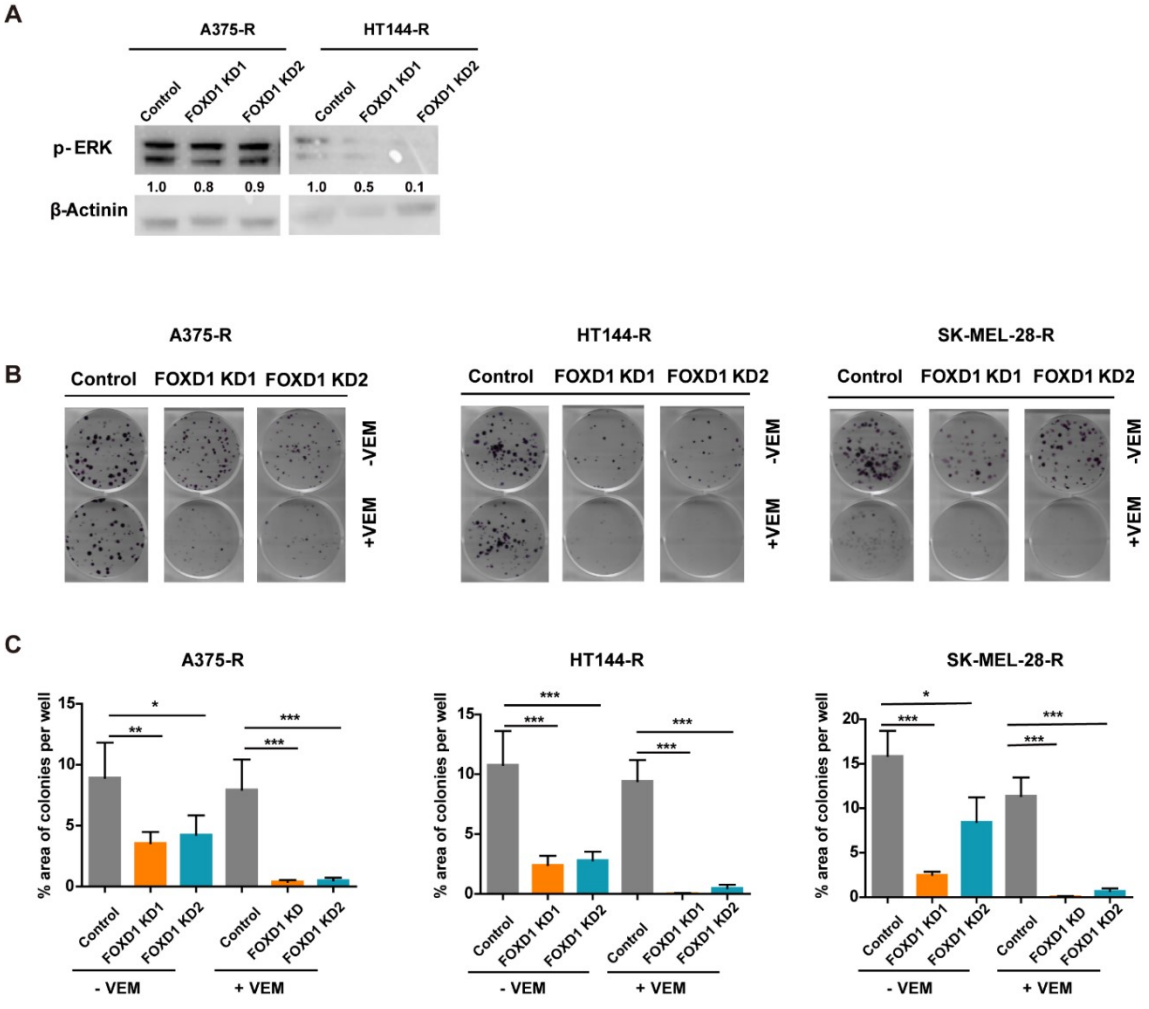


Figure 20. FOXD1 knockdown increases the sensitivity of BRAFi-resistant melanoma cells to BRAFi

A Protein level of p-ERK expression in BRAFi-resistant cells upon FOXD1 knockdown. Densitometric values were normalized to the loading controls α-Actinin. **B**, **C** Clonogenic assay ± VEM (10 μM) treatment.

4.18 FOXD1 knockdown resensitizes BRAFi-resistant melanoma cells to BRAFi treatment (I)

In order to study the impact of FOXD1 knockdown on the susceptibility of BRAFi-resistant melanoma cells to BRAFi treatment, I subjected BRAFi-resistant FOXD1 KD cells to increasing concentrations of vemurafenib. At a concentration of 1 μM or higher I could detect significantly lower viability for the BRAFi-resistant FOXD1 KD cells in comparison to the BRAFi-resistant cells without FOXD1 knockdown. The IC50 values determined for the knockdown and control cell lines confirmed the higher sensitivity of the knockdown cell lines towards vemurafenib (A375 mean IC50 = 17.07 ± 0.11 μM; HT144 mean IC50 = 10.26 ± 0.10 μM; SK-MEL-28 mean IC50 = 7.99 ± 0.08 μM) and BRAFi-resistant FOXD1 KD cells (A375 FOXD1 KD1 mean IC50 = 1.29 ± 0.09 μM; A375 FOXD1 KD2 mean IC50 = 2.59 ± 0.09 μM; HT144 FOXD1 KD1 mean IC50 = 0.42 ± 0.06 μM; HT144 FOXD1 KD2 mean IC50 = 0.07 ± 0.06 μM; SK-MEL-28 FOXD1 KD1 mean IC50 = 0.35 ± 0.06 μM; SK-MEL-28 FOXD1 KD2 mean IC50 = 1.10 ± 0.06 μM) (Supplementary Table S3). (Figure 21).

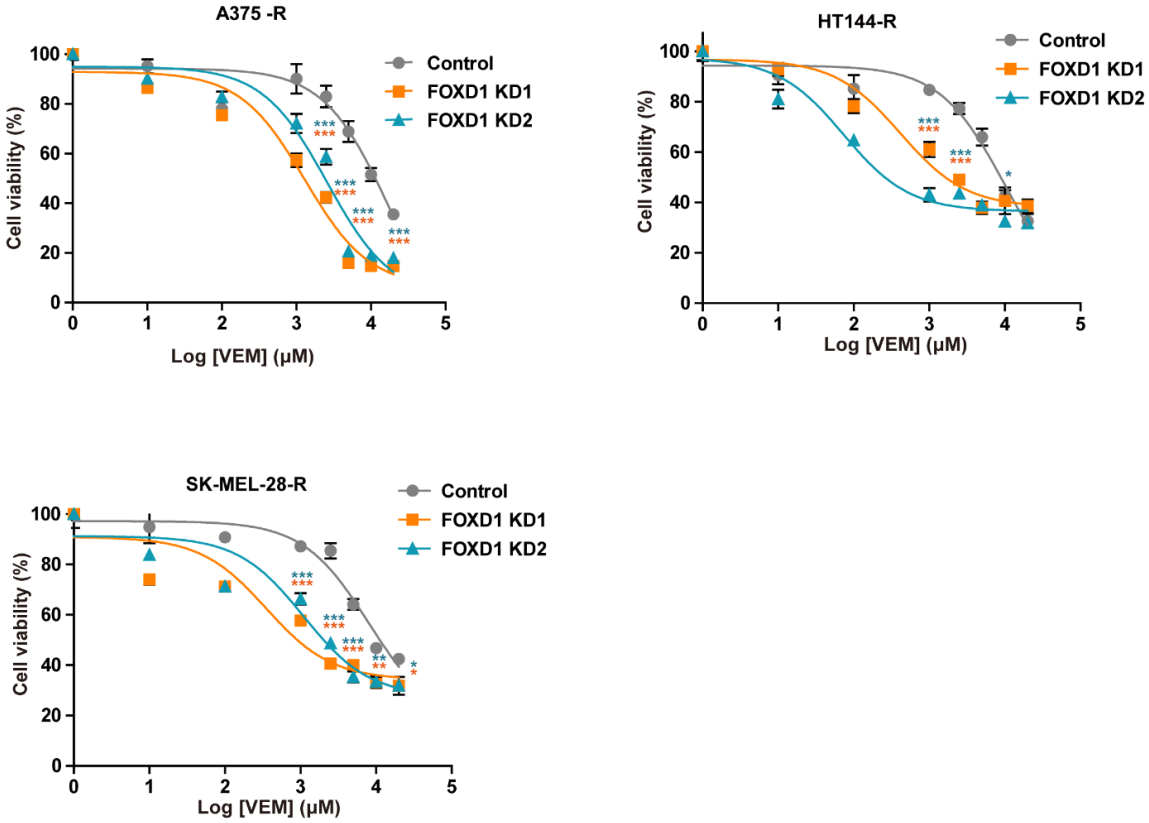
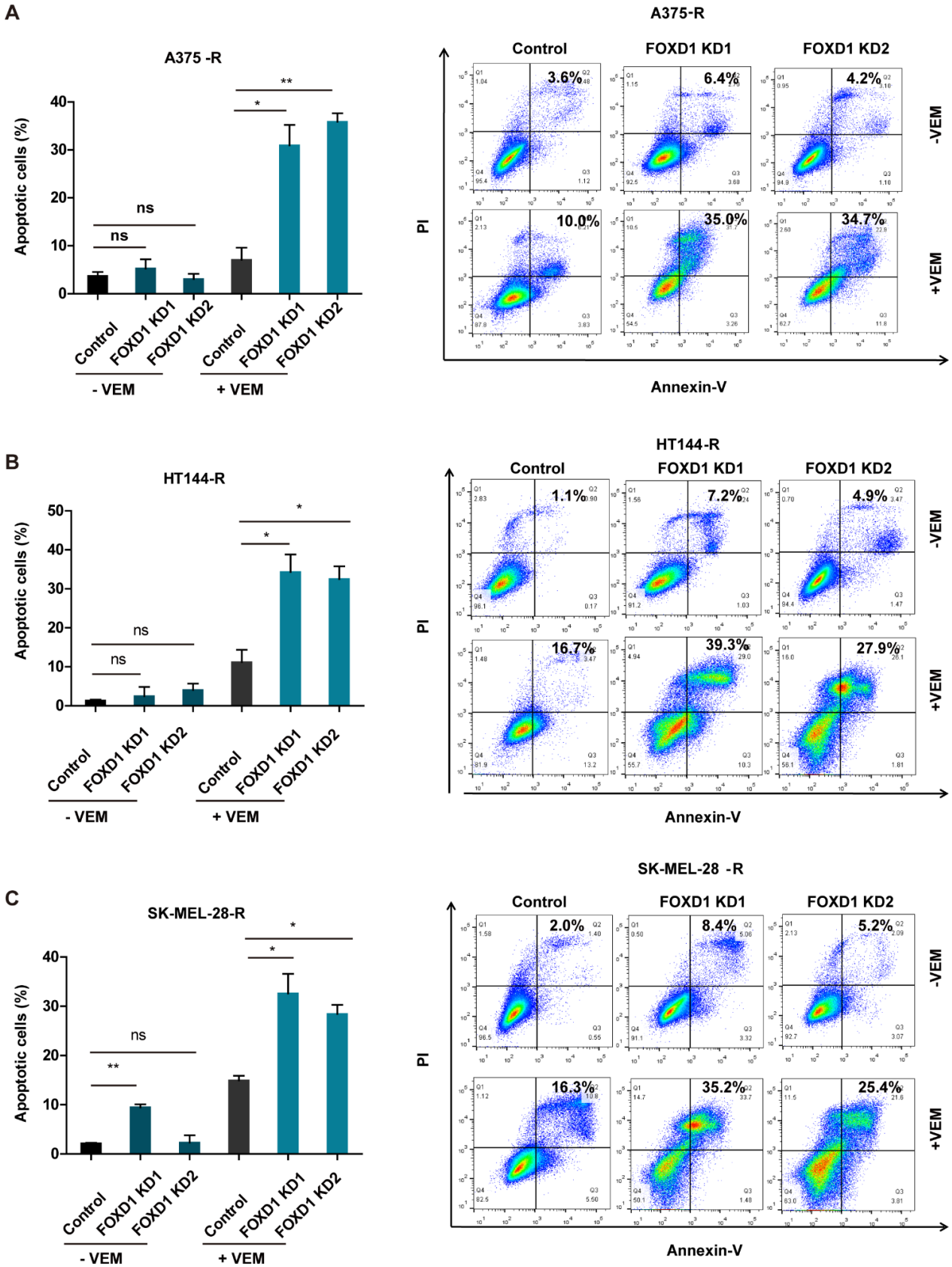


Figure 21. FOXD1 knockdown resensitizes BRAFi-resistant melanoma cells to BRAFi treatment (I) Control and FOXD1 KD cells were treated with VEM (0.0001–25 μM) for 72 h. Cell viability was then measured by using alamar blue.

4.19 FOXD1 knockdown resensitizes BRAFi-resistant melanoma cells to BRAFi treatment (II)

Next, apoptosis assay upon vemurafenib treatment was examined using annexin V kit by FACS. As shown in Figure 22, reducing the expression of FOXD1 in BRAFi-resistant melanoma cells resulted in a slight increase in the number of annexin V-positive cells. Remarkably, combining FOXD1 knockdown with BRAFi treatment drastically increased the percentage of annexin V-positive cells, revealing that FOXD1 expression promoted the resistance of BRAFi-resistant melanoma cells to vemurafenib. Overall, these data demonstrate that cell lines with acquired resistance to BRAFi exhibit a pronounced expression of FOXD1. Knockdown of FOXD1 resensitized BRAFi-resistant melanoma cells to BRAFi treatment.


A
A375 -R
Apoptotic cells (%)
ns
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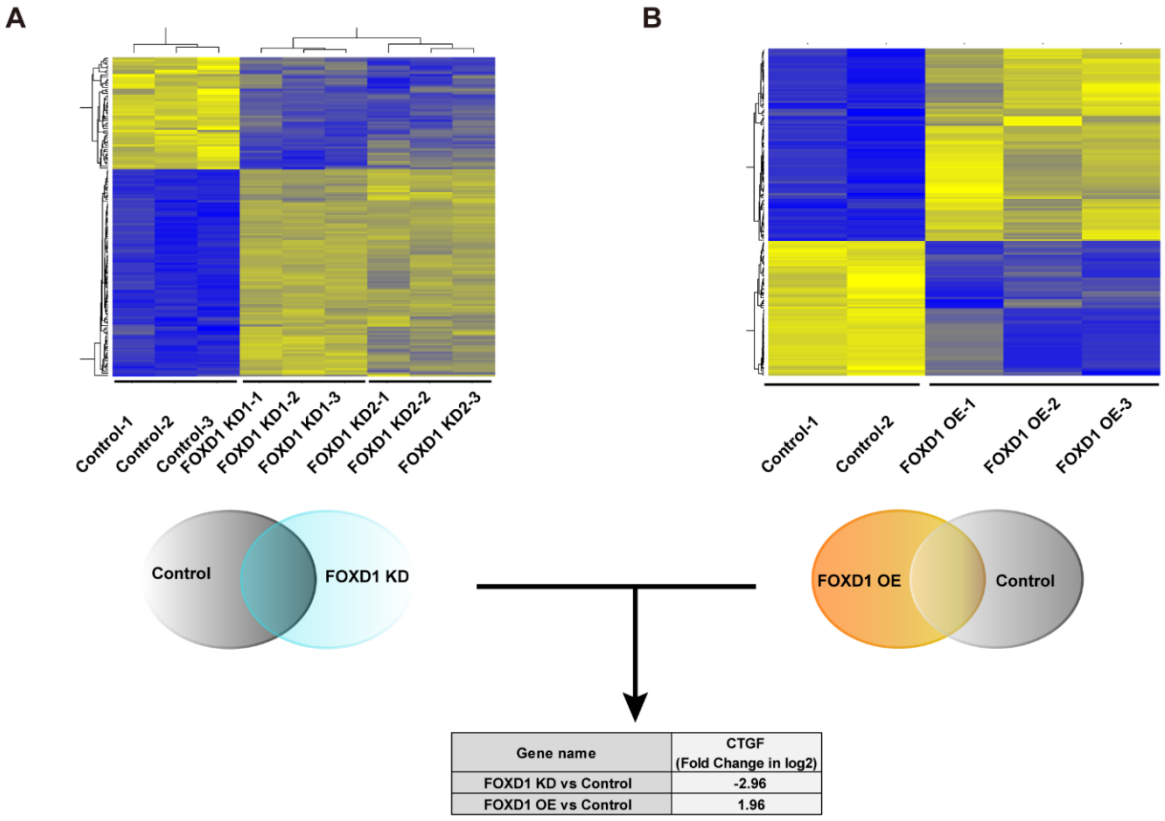
**
Control
FOXD1 KD1
FOXD1 KD2
- VEM
+ VEM
A375-R
Control
FOXD1 KD1
FOXD1 KD2
3.6%
6.4%
4.2%
10.0%
35.0%
34.7%
-VEM
+VEM
PI
Annexin-V
B
HT144-R
HT144-R
1.1%
7.2%
4.9%
16.7%
39.3%
27.9%
C
SK-MEL-28-R
SK-MEL-28 -R
2.0%
8.4%
5.2%
16.3%
35.2%
25.4%

Figure 22. FOXD1 knockdown resensitizes BRAFi-resistant melanoma cells to BRAFi treatment (II) **A**, **B**, **C** Left: apoptosis assay by staining with FITC-annexin V/PI. Three BRAFi-resistant control and FOXD1 KD cells were treated with DMSO or VEM (10 μM) for 48 h. Right: Representative scatter plots of apoptosis assay.

4.20 CTGF is a downstream factor of FOXD1.

To further confirm the molecular mechanisms connecting FOXD1 with cell resistance, I first aimed at identifying potential downstream targets of FOXD1. To this end, microarray was used to investigate the expression of global gene in FOXD1 KD and control cells. In this way, I found that the expression level of the connective tissue growth factor (CTGF/CCN2) differed substantially between these two groups (the log2 fold change of CTGF between FOXD1 KD and control group was -2.27) (Figure 23A). Moreover, comparing the microarray data from FOXD1 OE and control group, CTGF was displayed as one of the top upregulated genes in all FOXD1 OE cell lines (the log2 fold change of CTGF between FOXD1 OE and control group was 1.96) (Figure 23B). CTGF is a secreted and glycosylated protein, which shows different roles in different cancer types. But the function of CTGF in melanoma resistance has not been studied on a large scale yet.



Gene name	CTGF (Fold Change in log2)
FOXD1 KD vs Control	-2.96
FOXD1 OE vs Control	1.96

Figure 23. CTGF is a downstream factor of FOXD1

A, **B** heatmap of microarray assay to compare the global gene expression between FOXD1 KD and control group (left), FOXD1 OE and control group (right). Bottom: fold change of CTGF expression in log 2 between the FOXD1 KD group and the control group from microarray data is -2.96, the fold change in log 2 between the FOXD1 OE group and control group is 1.96.

4.21 CTGF expression positively correlates with FOXD1 expression.

By checking the GEO databases GSE7127 and GSE10196 (Figure 24A, 24B), I found FOXD1 is positively correlated with CTGF in melanoma cells. Moreover, in Cancer Cell Line Encyclopedia (CCLE) database, FOXD1 was also found to positively correlates with CTGF in RNA level in BRAF-mutated melanoma (Figure 24C). Next, I conducted western blot with one melanocyte cell line and eight different melanoma cell lines. I found both FOXD1 and CTGF were higher expressed in melanoma cells than melanocytes. Moreover, cell lines with high FOXD1 expression levels also showed high expression of CTGF (Figure 24D). Additionally, I did TMA staining against CTGF in 85 melanoma specimens and found that FOXD1 and CTGF expression positively correlated (Figure 24E). These results indicate that CTGF might be a downstream factor of FOXD1.

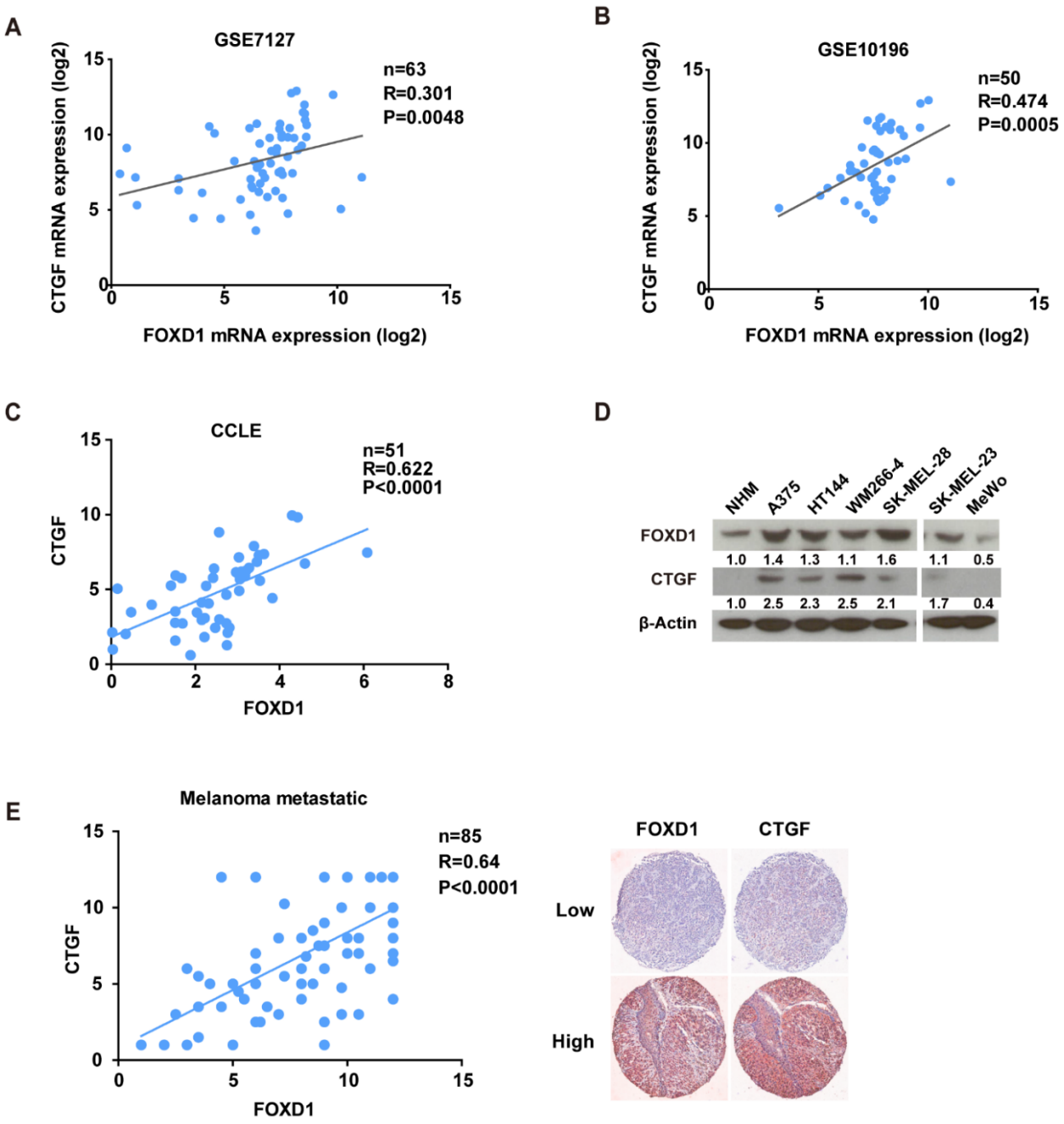


Figure 24. CTGF expression positively correlates with FOXD1 expression

A, **B** FOXD1 is positively correlated with CTGF in two GSE files (GSE7127 and GSE10196). **C** FOXD1 is positively correlated with CTGF in CCLE database. **D** Protein levels of CTGF and FOXD1 in eight melanoma and one melanocyte cell lines. Densitometric values were normalized to the loading control β-Actin. **E** Left: Correlation of the expression of CTGF and FOXD1 in 85 clinical melanoma samples with TMA staining. Right: Representative images of FOXD1 and CTGF TMA staining.

4.22 FOXD1 promotes transcription of the *CTGF* gene

Since CTGF was found as a downstream factor of transcription factor FOXD1. Next, I did chromatin immunoprecipitation (ChIP) assay with A375 cell line. The results showed that

a specific site in the CTGF promoter was bound by FOXD1 protein in A375 cells (Figure 25A). Moreover, to validate if the activity of CTGF promoter can be affected by FOXD1, the dual-luciferase reporter assay was performed. As shown in Figure 25B, the luciferase activity was higher in FOXD1 OE A375 cells in contrast to empty vector control. Furthermore, the CTGF expression was reduced in FOXD1 KD cells and increased in FOXD1 OE cells compared to the control (Figure 25C, 25D). According to these results, I can confirm that FOXD1 could bind to the CTGF promoter and in this way controls the transcription and expression of CTGF.

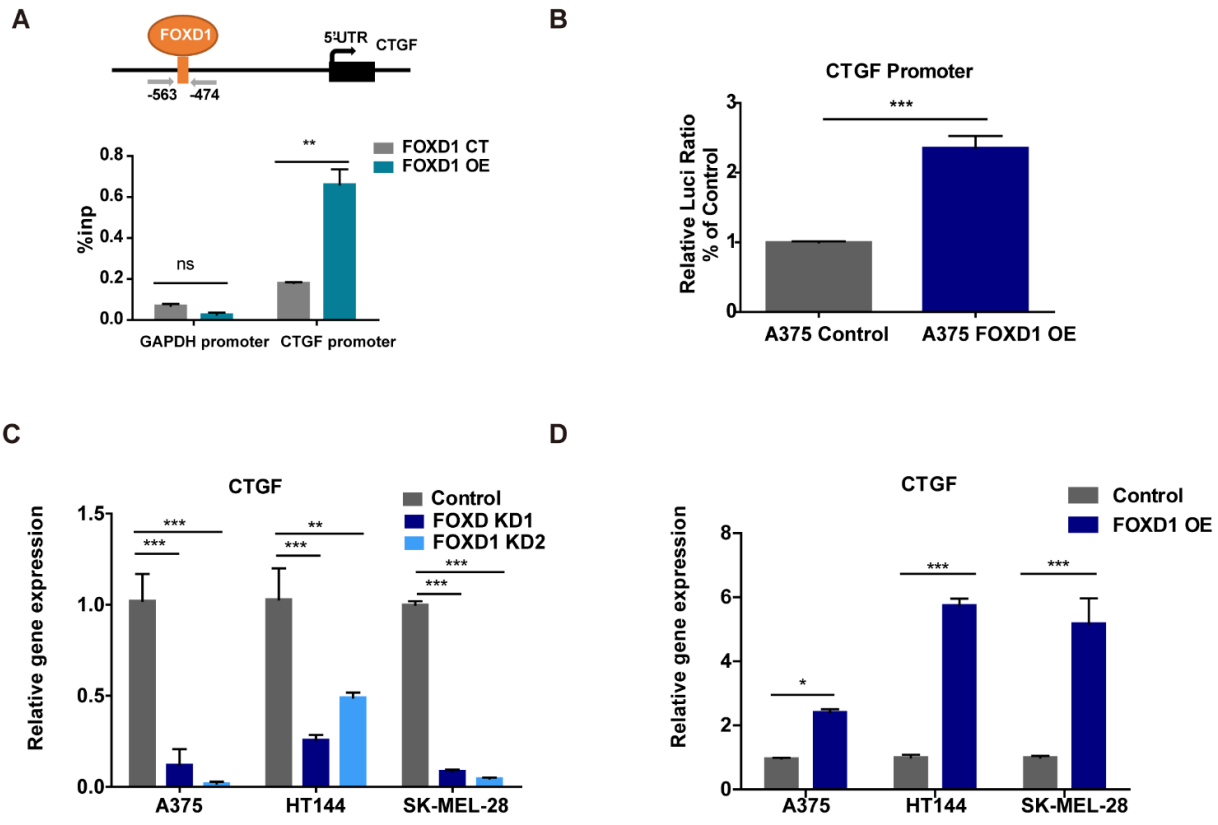


Figure 25. FOXD1 promotes transcription of the *CTGF* gene

A ChIP-qPCR analysis to investigate the recruitment of FOXD1 onto CTGF promoter region. This experiment was conducted in A375 FOXD1 OE cells or A375 control cells and GAPDH promoter was a negative control. **B** A375 cells were cotransfected with a CTGF promoter reporter construct (pcDNA4.10-CTGF) or the empty reporter control (pcDNA4.10) and a FOXD1 OE construct. **C**, **D** CTGF mRNA levels in FOXD1 KD and OE cells.

4.23 CTGF expression is upregulated in BRAFi-resistant melanoma cells

In this study, I could demonstrate that FOXD1 promoted melanoma resistance towards vemurafenib. Moreover, CTGF was identified as a factor whose expression was regulated by FOXD1. A recently published study analyzed the transcriptional profiles of vemurafenib-resistant melanoma cells in contrast to the non-resistant parental cells[132]. Analyzing these published data (GSE68599), I ascertained that FOXD1 and CTGF are among the most significantly upregulated genes in the resistant cells (Figure 26A). In line with this, I also observed higher FOXD1 (Figure 7A) and CTGF protein expression levels in three vemurafenib-resistant cells compared to their non-resistant counterparts (Figure 26B).

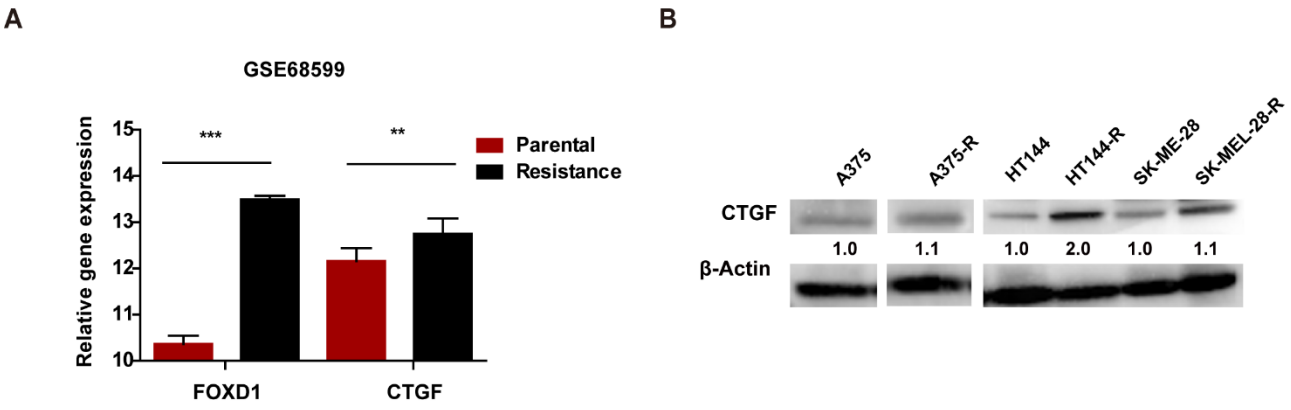


Figure 26. CTGF expression is upregulated in BRAFi resistant melanoma cells

A Expression levels of CTGF and FOXD1 were obtained from the GSE68599 database. **B** Analysis of the CTGF expression by western blot in A375-R, HT144-R, SK-MEL-28-R and their parental counterparts. Densitometric values were normalized to the loading control β-Actin.

4.24 CTGF knockdown resensitizes BRAFi-resistant melanoma cells to BRAFi treatment

To investigate the function of the FOXD1-CTGF axis in drug resistance, I first reduced the expression level of CTGF in A375-R and HT144-R cells with esiRNA-CTGF. Then I measured the level of apoptosis upon BRAFi treatment by FACS. To do this, I treated A375-R/HT144-R and A375-R/HT144-R CTGF KD cells with DMSO or 10 µM vemurafenib for 48 h, followed by annexin V and PI staining. As shown in Figures 27A and B, after knocking down CTGF expression in BRAFi-resistant cells, the apoptotic cells were significantly increased suggesting that CTGF serves as an important factor in maintaining the survival of BRAFi-resistant melanoma cells. Additionally, similar to FOXD1 knockdown, the number of apoptotic cells was highly elevated in the A375-R/HT144-R CTGF KD groups after vemurafenib treatment compared to their control counterparts. Overall, these data suggest that the knockdown of CTGF can make BRAFi-resistant melanoma cells become more

sensitive to BRAFi treatment.

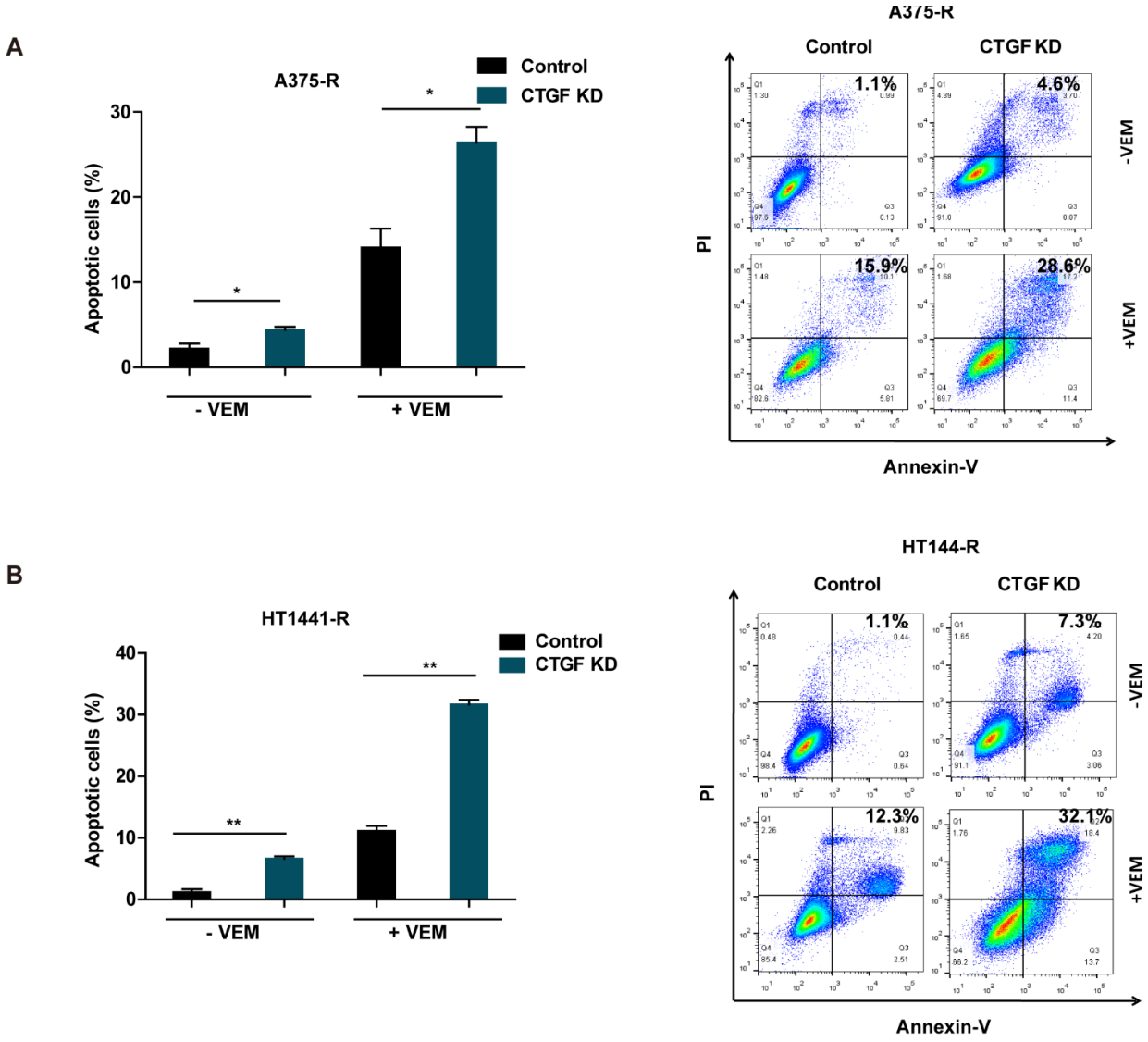


Figure 27. FOXD1 knockdown resensitizes BRAFi-resistant melanoma cells to BRAFi treatment

A, **B** Left: Apoptosis assay by staining with FITC-annexin V/PI. A375-R/HT144-R cells were transfected with the esiRNA targeting CTGF and 48 h later treated with DMSO or VEM (10 μM) for another 48 h. Apoptotic cells were then stained and measured by FACS. Right: Representative scatter plots of apoptosis assay.

4.25 Recombinant CTGF abrogates the effect of FOXD1 knockdown on the sensitivity of BRAFi-resistant melanoma cells to BRAFi treatment

Since CTGF is a downstream factor of FOXD1, a reduction of the level of CTGF could resensitize BRAFi-resistant melanoma cells to vemurafenib. For this reason, I performed a rescue experiment to further verify the importance of the FOXD1-CTGF axis for melanoma resistance. First, I did an apoptosis assay by FACS analysis. Interestingly, as

shown in Figure 28A and B, FOXD1 KD cells treated with 200 ng/ml recombinant CTGF protein were less sensitive to vemurafenib than FOXD1 KD cells that were not treated with CTGF. Second, by using alamar blue assay, I could confirm that recombinant CTGF could reverse the increased sensitivity of melanoma cells induced by FOXD1 knockdown (Figure 28C). These results clearly suggest that FOXD1 promotes melanoma resistance towards vemurafenib by activating the expression of CTGF.

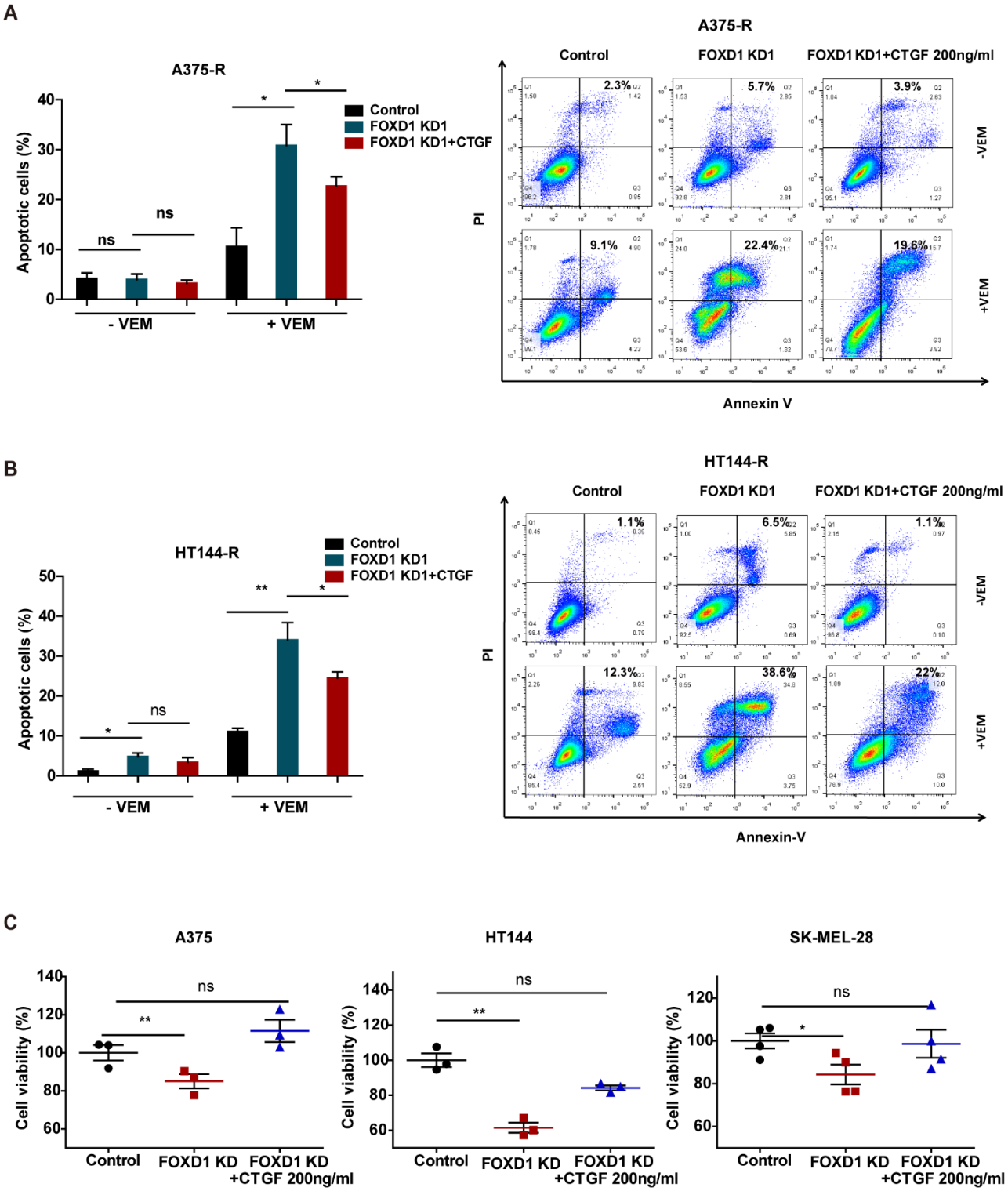


Figure 28. Recombinant CTGF abrogates the effect of FOXD1 knockdown on the sensitivity of BRAFi-resistant melanoma cells to BRAFi treatment

A, **B** Left: Apoptosis assay measured by FACS. Parental A375/HT144, A375-R/HT144-R and A375-R/HT144-R FOXD1 KD cells treated with recombinant CTGF protein (200 ng/ml) were exposed to DMSO or VEM (10 μM) for 48 h. Apoptotic cells were then stained and measured by FACS. Right: Representative scatter plots. **C** A375, HT144, and SK-MEL-28 cells were transfected for 48h with the indicated siRNA targeting FOXD1 and then treated with DMSO or VEM (5 μM), cultured with or without recombinant CTGF (200 ng/ml) for an additional 48 h. Then cell visibilities were measured using alamar blue.

4.26 TGF-β expression correlates with FOXD1 expression in melanoma.

By further analyzing the global gene expression in FOXD1 OE cells and control cells, I found many tumor microenvironment-related factors such as MMP9 or IL6 to be highly upregulated in the FOXD1 OE group (data not shown). Then, I used the IPA (Ingenuity Pathway Analysis) to gain deeper insight into the microarray data. Interestingly, the differentiation marker MITF was found to be inhibited by overexpressing FOXD1 and several pathways such as hypoxic and ILK signaling which are related to the tumor microenvironment were affected by FOXD1 overexpression. Transforming growth factor-β1 (TGF-β1), one of the most important factors in the tumor microenvironment was also shown to be affected by FOXD1 (Figure S3). In order to confirm this result, I first checked the expression data from the GSE7127 and CCLE databases and found a positive correlation between FOXD1 and TGF-β1 (Figure 29A). Next, by using ELISA, I discovered that the secretion of TGF-β1 was highly increased in FOXD1 OE cells (Figure 29B). By using tissue microarrays (TMA) to investigate the expression of TGF- β1 and FOXD1 in melanoma patient samples, I demonstrated that a high level of TGF-β1 went along with a high level of FOXD1. In contrast, patients with tumors with low levels of TGF-β1 also expressed FOXD1 at low levels (Figure 29C). In conclusion, TGF-β1 expression was significantly correlated with FOXD1 expression in melanoma patients' samples.

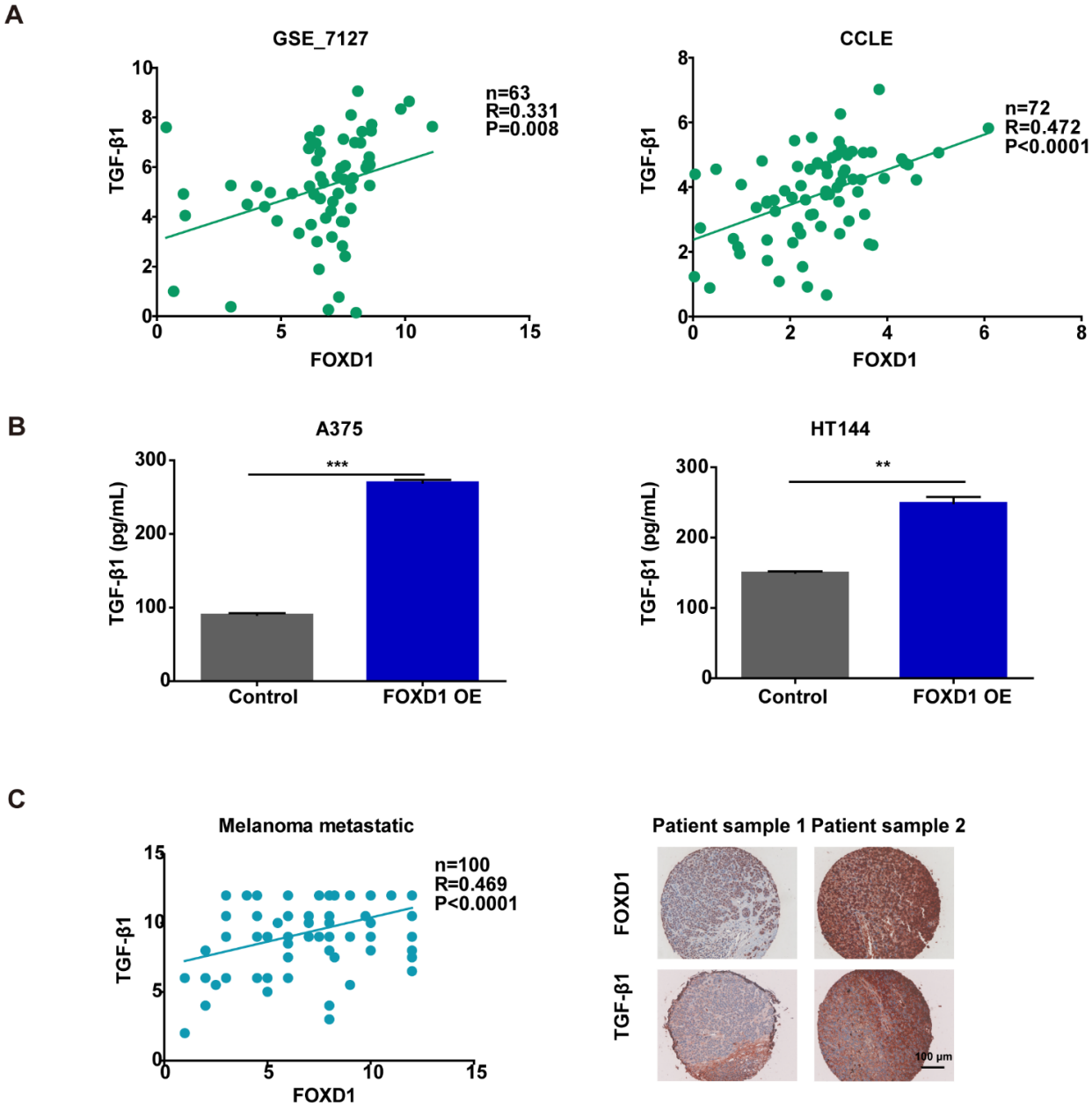


Figure 29. TGF-β expression correlates with FOXD1 expression in melanoma

A Pearson correlation of FOXD1 and TGF-β1 gene expression (data from GSE7127 and CCLE database). **B** TGF-β1 protein expression in A375 and HT144 FOXD1 OE cells measured by ELISA. **C** Left: Pearson correlation of IHC scores for tissue microarrays (TMA) of FOXD1 and TGF-β1, n = 100. Right: IHC staining of samples of melanoma metastases for TGF-β with low FOXD1 score (patient 1) and high FOXD1 score (patient 2).

4.27 EGFR and RAS are upregulated in FOXD1 OE cells

Melanoma resistance mechanisms are complex. RTK overexpression and RAS reactivation are two major routes to resistance. EGFR is a key member of the RTKs and I found the protein levels of EGFR (Figure 30A) as well as RAS (Figure 30B) both were

highly increased in FOXD1 OE cells compared to control cells. In addition, by analyzing the gene expression data from GSE7127 and GSE10196, a significant positive correlation between FOXD1 and EGFR expression was found (Figure 30C). Moreover, when comparing the gene expression data from the CCLE database, the strong positive correlation between FOXD1 and EGFR could be confirmed (Figure 30D) (R = 0.666, P < 0.0001). As mentioned before, also a high p-ERK expression was detected in FOXD1 OE cells. These results show that the overexpression of FOXD1 could reactivate the RTK-RAS-MAPK pathway, and hence induce cell resistance.

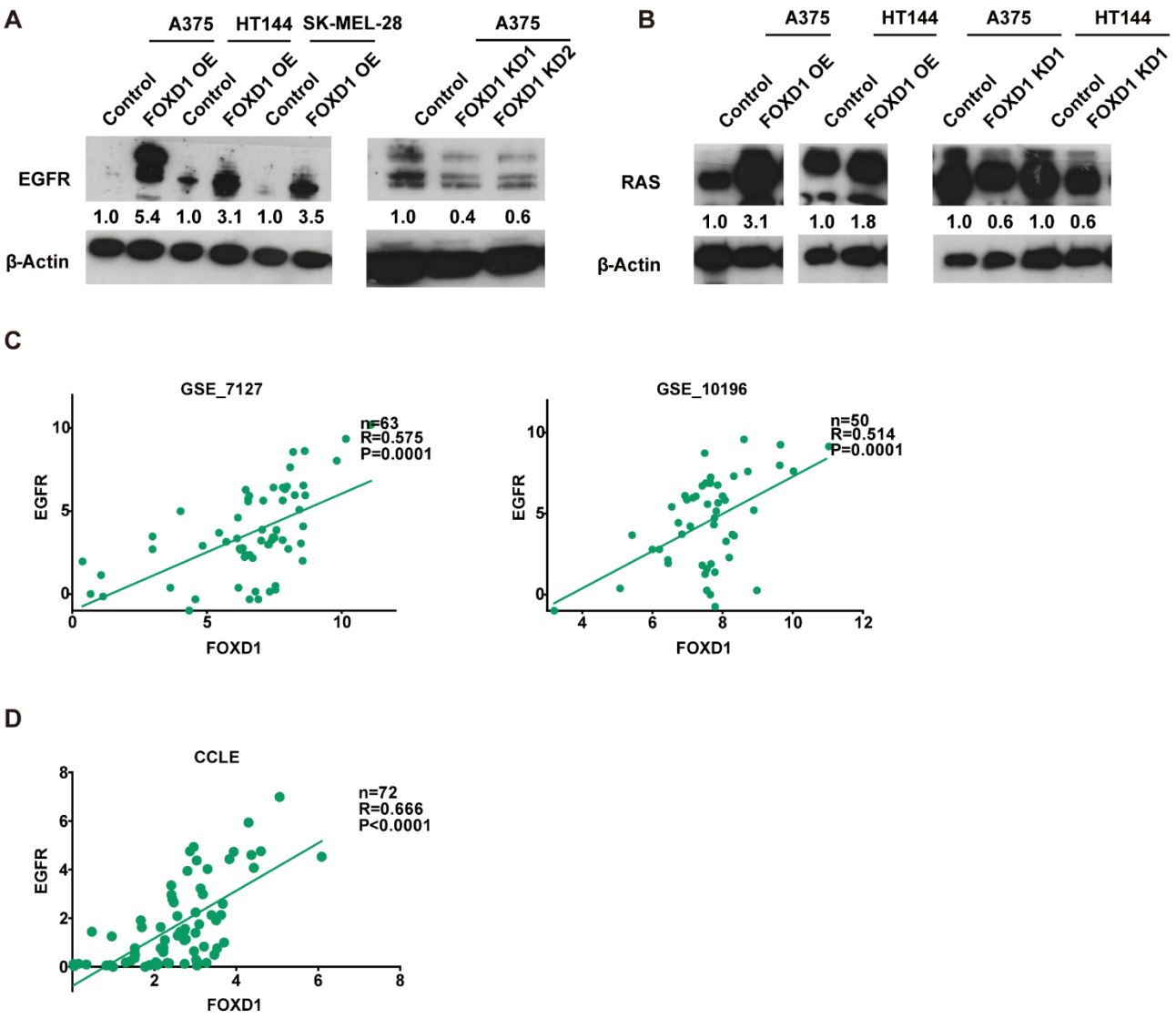


Figure 30. EGFR and RAS are upregulated in FOXD1 OE cells

A Analysis of EGFR expression by western blot in A375, HT144, SK-MEL-28 FOXD1 OE and control cells, A375 FOXD1 KD and control cells. **B** Analysis of RAS expression by western blot in A375, HT144 FOXD1 OE and control cells, A375, HT144 FOXD1 KD and control cells. Densitometric values were normalized to the loading control β-Actin. **C** Pearson correlation of FOXD1 and EGFR gene expression (data from GSE7127 and GSE10196). **D** Pearson correlation of FOXD1 and EGFR gene expression (data from CCLE database).

5. Discussion

5.1 The NC-associated gene FOXD1 plays an important role in melanoma progression

The term "melanocyte" was first introduced by Meyerson in the 19th century. Based on observations and studies over the last two centuries, we have gained extensive and in-depth knowledge of this cell type. It is well established that melanocytes originate from pluripotent neural crest cells and reside, among others, in the epidermis of the skin, the inner ear, the eyes of vertebrate organisms, and the hair follicles. The developmental and differentiation of melanocytes is complex and concludes five stages according to the location of the cells within the body and the expression of key factors. Stage 1: Induction of the neural crest, followed by the emigration of NC cells from the neuroepithelium. Stage 2: Early migration - a subset of neural crest cells is differentiating into melanoblasts and migrates along a dorso–lateral path underneath the ectoderm. Stage 3: Mid migration - the melanoblasts migrate through the dermis and eventually immigrate into the epidermis. Stage 4: Late migration - the melanoblasts expand in the epidermis and some incorporate themselves into developing hair follicles. Stage 5: Upon expansion, the melanoblasts eventually develop into fully differentiated melanocytes and begin to produce pigment.

Melanoma cells and NC cells have many similar characteristic features. These two cell types, for instance, possess certain motility that can detach from an epithelial environment and migrate to distant locations. The microenvironment shows important effects in regulating growth of these two cell types. MITF which is critical for the survival of the cells is also found expressed in melanoma as well as NC cells. Therefore, awareness of melanocyte development possibly provides contributions to understand the formation and progression of melanoma.

Multiple TFs are involved in controlling the development of melanocytes, including SNAIL, ZEB, TWIST, ID3, and others. These NC-associated genes contribute to a gene regulatory network that maintains multipotency and stemness, promotes invasiveness, controls differentiation of NC cells[145–147]. Interestingly, the phenotypic and functional similarities between NC cells and melanoma cells provide convincing evidence that the development of melanoma cells critically depends on genes involved in NC development. In a previous study from our group, for instance, we could show that some NC-associated genes are enriched in melanoma cells and NC cells but not in primary melanocytes. Among these NC-associated genes was ID3 that could mediate melanoma cell migration[148]. Moreover, our previous study also showed that FOXD1 is a NC-related gene whose deletion affected the invasion and migration capacity of melanoma via RAC1B[109].

FOXD1 is an oncogenic TF of the forkhead family. It has a well-defined role in kidney

development[111]. Furthermore, FOXD1 is required for cell reprogramming by mediating self-renewal and differentiation[112]. Many studies have found that FOXD1 acts as an oncogene in multiple cancers. Yael Nemlich noted that FOXD1 could promote melanoma cell proliferation and invasion by inhibiting ADAR1 expression[119]. Chen Li et al. pointed out that FZD3 suppresses the activity of MAPK-mediated signaling pathways by interfering with transcriptional networks controlled by FOXD1, thereby inhibiting the growth and metastasis of melanoma[120]. Another study indicated that FOXD1 is aberrantly expressed in non-small cell lung cancer and correlates with poor survival[122]. Moreover, FOXD1 also promotes chemotherapy resistance of breast cancer[149]. These studies above indicate that the enhanced expression of FOXD1 can affect the behavior of cancer cells such as cell stemness, proliferation, and metastasis as well as drug resistance. Up to now, the role of FOXD1 in melanoma progression remains largely unknown and hence requires further investigation. Here, I found that increased FOXD1 expression correlated with a poor prognosis in melanoma patients **(Figure 4)** and that overexpression of FOXD1 in melanoma cells resulted in increased invasion and migration capacity **(Figure 12)**. These results are in line with our previous study and suggest a key role of FOXD1 in melanoma progression.

5.2 FOXD1 is important for melanoma phenotype switching and resistance.

As noted, the expression level of MITF tightly regulates and controls the biological behavior of melanoma cells[150,151]. Changes in MITF expression levels lead to the transition from a differentiated phenotype (MITF ^{high}) to a stem-like phenotype (MITF ^{low})[152]. Moreover, the progression as well as targeted therapy resistance of melanoma are also deeply affected by the expression level of MITF. Interestingly, previous single-cell profiling revealed positive correlations between MITF and differentiation-related factors. Furthermore, in the same study, MITF was found to negatively correlates with the expression of AXL, which is associated with the invasive, dedifferentiated, drug-resistant phenotype[136]. A previous study of ours demonstrated that FOXD1 was a pro-invasive and pro-migratory NC marker[109]. In this study, I first found that FOXD1 expression negatively correlated with MITF expression but positively correlated with AXL expression **(Figure 5)**. In addition, ectopic expression of FOXD1 in melanoma cells could induce AXL expression but decreased MITF expression levels **(Figure 7)**. SOX10 is one of the important TFs during NC development and its suppression is highly relevant for melanoma resistance to targeted therapies[140]. Moreover, SOX10 is associated with sustaining melanocytic identity through its ability to promote MITF expression[153–155]. Accordingly, the decreased expression of SOX10 and MITF in melanoma cells show a more pronounced "undifferentiated" or stem cell-like phenotype. Interestingly, by analyzing data from GEO databases I could show a negative

correlation between FOXD1 and SOX10 as well as MITF expression **(Figure 5)**. Moreover, melanoma cell insensitivity towards BRAFi was observed when MITF and SOX10 expression were suppressed by ectopically expressing FOXD1 **(Figure 7** and **Figure 9)**. Cell morphology is an important parameter that affects and predicts cell function. In the current study, I observed morphology changes of melanoma cells upon the overexpression of FOXD1: SK-MEL-28 cells overexpressing FOXD1 showed fewer dendritic processes and a less differentiated spindle morphology, which suggests that ectopic FOXD1 could induce the dedifferentiation of melanoma cells. By analyzing the correlation between FOXD1 and melanoma differentiation markers from the online database (e.g. GSE7127, GSE10196 and CCLE), I discovered that cells with high FOXD1 expression showed low expression levels of differentiation markers such as DCT, PMEL, MLANA **(Figure S4)**. These findings together give us compelling evidence that FOXD1 could be essentially involved in melanoma cell dedifferentiation.

Among BRAF-mutated melanomas, consistent activation of MAPK pathway can trigger melanoma progression[156]. The BRAF inhibitor vemurafenib (BRAFi, PLX4032) is considered as the first-line drug for clinical treatment of melanoma carrying BRAF mutation[44]. Despite the high response rate to vemurafenib, patients with melanoma still easily acquire drug resistance[47]. Melanoma cells could establish either intrinsic or acquired drug resistance to a range of anticancer agents. Several potential resistance mechanisms against BRAFi monotherapy and BRAFi/MEKi combination therapy have been proposed including MAPK signaling pathway reactivation, constant activation of RTKs, EGFR overexpression as well as changes in the tumor microenvironment[86–90].

As aforementioned, NC-associated genes are involved in driving melanoma progression. Moreover, increasing evidence indicates that they could also regulate the resistance of melanoma to established therapies. For example, our previous study uncovered that ID3 knockdown could sensitize melanoma cells to vemurafenib treatment[148]. BRN2, a transcription factor from the POU domain family, is a critical NC-associated factor in melanoma invasion and metastasis. Pierce and colleagues found that overexpression of BRN2 can constrain melanoma proliferation and trigger partial resistance of melanoma cells to BRAFi[157]. TWIST1, a basic helix-loop-helix TF, controls melanoma cell invasion, and has recently been found to confer chemo-resistance to various cancer cell types[158–160]. Another NC-associated gene, ZEB1, was identified as a master regulator of stemness as well as resistance in melanoma towards BRAF and MEKi[161]. Another study has shown that the NC-associated gene FOXM1 could confer resistance to human pancreatic cancer cells towards paclitaxel[162]. In addition, FOXD1 is also important to promote drug resistance in breast cancer[127]. Taken together, multiple studies show that NC-associated genes are involved in melanoma progression and resistance and could therefore be utilized as predictive markers. Deciphering the molecular mechanisms controlled by these factors will

advance our understanding of melanoma progression and resistance to targeted therapies. Here, expression data from cell lines and from melanoma patient samples show aberrant expression of FOXD1 in melanoma compared to normal tissues and melanocytes **(Figure 4).** Beyond that, high FOXD1 expression levels went along with a low survival rate, reinforcing the hypothesis that FOXD1 additionally exerts oncogenic functions in melanoma **(Figure 4).** Moreover, increased FOXD1 expression was identified in BRAF inhibitor resistant melanoma cells. **(Figure 18** and **Figure 26A)**. Additionally, further studies noted that ectopic expression of FOXD1 was adequate to promote resistance to BRAFi or BRAFi and MEKi in combination **(Figure 9** – **Figure 11),** whereas FOXD1 knockdown sensitized naive melanoma cells to BRAFi or BRAFi and MEKi in combination **(Figure 15** – **Figure 17)**, reduced the occurrence of resistance upon continuous exposure *in vitro*, and resensitized resistant cells to BRAFi **(Figure 20** - **Figure 22)**.

5.3 FOXD1 promotes melanoma resistance by regulating the expression of the oncogene CTGF.

CTGF is a secreted and glycosylated protein, which contains four common domains shared by the other members of the CCN family. In tumor cells, CTGF expression is associated with cell proliferation, adhesion, migration, invasion, angiogenesis and anoikis [163–168]. In multiple types of cancers, CTGF is considered as an oncogene that could promote drug resistance. For instance, CTGF promotes breast cancer cell resistance to doxorubicin and paclitaxel[169]. Overexpression of CTGF could enhance the resistance of human osteosarcoma cells to cisplatin and paclitaxel[170]. Ectopic expression of CTGF confers resistance to various drugs (such as bortezomib, temozolomide, and others) in U343 glioblastoma multiforme cells[171]. However, the mechanism of how CTGF confers drug resistance to melanoma cells has not been well determined yet.

In this study, I found evidence that FOXD1 promoted resistance by inducing CTGF transcription. I provided several lines of evidence to prove the hypothesis. Firstly, according to the microarray data, I demonstrated that CTGF expression was drastically downregulated upon FOXD1 knockdown and upregulated after FOXD1 overexpression **(Figure 23)**. Secondly, CTGF was found to strongly correlates with FOXD1 expression according to the GEO datasets **(Figure 24A** and **Figure 24B)**. Consistently, the significant positive correlation between the expression of CTGF and FOXD1 was also found from the online database (CCLE) **(Figure 24C)**. Furthermore, on protein level, I confirmed this strong positive correlation **(Figure 24D)**. Interestingly, The TMA assay also revealed a positive interrelation between FOXD1 and CTGF expression **(Figure 24E)**. Most importantly, I verified that FOXD1 is directly bound to a specific region in the CTGF promoter and induced CTGF transcription **(Figure 25A** and **Figure 25B)**. Based on these results, I demonstrated that the overexpression or the knockdown of FOXD1 was sufficient

to increase or decrease CTGF levels in melanoma cells **(Figure 25C** and **Figure 25D)**. Additionally, silencing CTGF expression could reverse the tolerance of melanoma cells to BRAFi induced by FOXD1 overexpression **(Figure 27)**, while treatment with a recombinant CTGF protein could restore resistance to BRAFi even after FOXD1 knockdown **(Figure 28)**. Thus, I conclude that FOXD1 upregulation promoted resistance towards BRAFi via upregulating CTGF.

5.4 FOXD1 increases the expression and secretion of TGF-β

TGF-β is an important microenvironment cytokine and highly expressed in advanced stages of melanoma[172]. It serves as an autocrine stimulator that promotes melanoma progression. Chon Sun indicated that the TGF-β signaling pathway is activated after SOX10 suppression[93]. Moreover, melanoma cells treated with recombinant TGF-β are more resistant to vemurafenib than the control group, suggesting an important role of TGF-β in vemurafenib resistance. In this study, I analyzed microarray data (FOXD1 OE vs Control) using IPA **(Figure S3)** and found that TGF-β might be a downstream factor of FOXD1. Besides, I detected a positive correlation between FOXD1 and TGF-β expression by evaluating data from the online databases CCLE and GSE10196 **(Figure 29A)**. Moreover, I observed an increased secretion of TGF-β in FOXD1 OE cells compared to control cells using ELISA **(Figure 29B)**. Consistently, TMA stainings also confirmed a positive correlation between FOXD1 and TGF-β expression in patients with metastatic melanoma. Taking these data together, I conclude that TGF-β might be an effector of FOXD1. However, further studies are still needed to confirm if FOXD1 can regulate melanoma resistance via TGF-β.

5.5 The EGFR-RAS-MAPK pathway is regulated by FOXD1

A study from Real et al. revealed that high expression levels of EGFR are found in less differentiated melanomas[173]. In BRAF-mutated colorectal cancer cell lines, the overexpression of EGFR could trigger melanoma cells to develop resistance to vemurafenib[173]. Also, EGFR was found to regulate melanoma resistance to BRAFi through PI3K/AKT pathway[174]. Interestingly, Ji and colleagues discovered a high expression of EGFR and low levels of MITF in samples from patients with recurrent melanoma[175]. RAS is a downstream factor of EGFR, and the reactivation of RAS is another mechanism of melanoma cells to develop resistance to BRAFi. Indeed, multiple mechanisms (such as NRAS, MEK1 and MEK2 gene mutations, overexpression of PDGFRs and EGFR and other tyrosine kinase receptors, loss of PTEN, etc.) have been identified as key mechanisms that leading to drug resistance in melanoma. Here, I ascertained increased expression of EGFR and RAS in FOXD1 OE cells compared to control cells by using western blot **(Figure**

30A and **Figure 30B)**. Furthermore, MAPK and AKT pathways were both reactivated upon ectopically expressing FOXD1 **(Figure 9A)**. In contrast, these two pathways were both inactivated when FOXD1 was silenced **(Figure 15A)**. Furthermore, by evaluating the gene expression data from online databases, a positive correlation between FOXD1 and EGFR was also detected **(Figure 30C** and **Figure 30D)**. These results together emphasize that FOXD1 could be a potent driver of “phenotypic” resistance of melanoma.

6. Conclusions

In conclusion, my research work uncovered that FOXD1 regulated the reversible transition from a drug-sensitive, differentiated state to a drug-resistant, dedifferentiated phenotype, associated with downregulation of MITF and SOX10 (Figure 31). I demonstrated that FOXD1 promoted the resistance of human melanoma cells to targeted therapy via upregulating CTGF. Silencing either of the molecules rendered cells sensitive *in vitro*. Hence, I conclude that the FOXD1-CTGF axis might be a potential therapeutic target for the treatment of melanoma. In addition, FOXD1 also showed important effects on the EGFR-RAS-MAPK pathway and TGF-β expression. These results indicate that FOXD1 is an important NC-related TF regulating melanoma resistance towards targeted therapy.

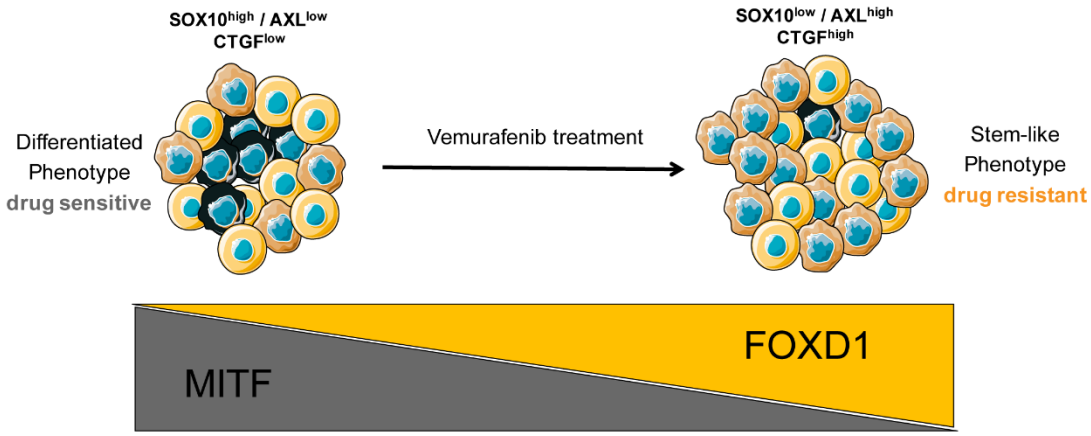


Figure 31. Schematic depiction of the effect of FOXD1 on the phenotype of melanoma cells
Melanoma cells with low FOXD1 expression levels exhibit a drug-sensitive, differentiated phenotype. These cells express high amounts of MITF and SOX10 and only a little CTGF and AXL. When melanoma cells are exposed to BRAFi long term, they will switch to the drug-resistant, dedifferentiated phenotype. These cells show increased expression of FOXD1, CTGF and AXL and low expression of MITF and SOX10.

7. References

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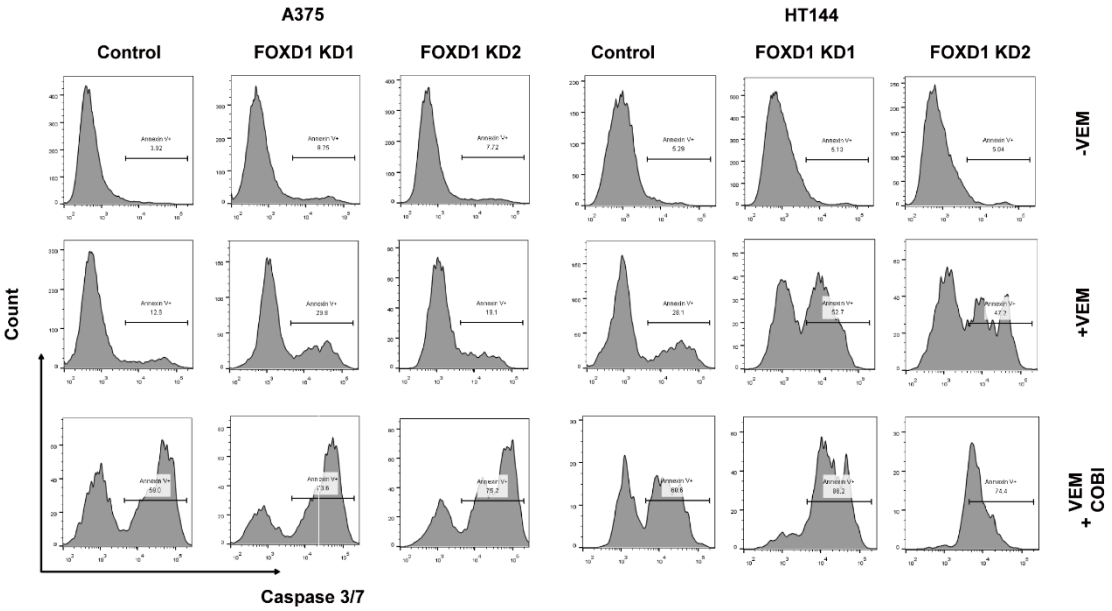
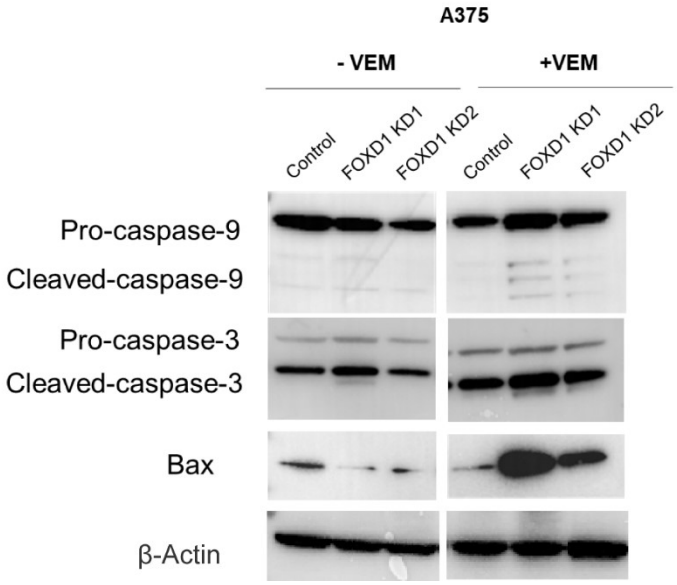
8. Supplemental material

8.1 Supplementary figures



Supplementary Figure S1. FOXD1 knockdown increases caspase 3/7 activity after treatment with BRAFi or BRAFi and MEKi in combination

A375/HT144 control and A375/HT144 FOXD1 KD cells were treated with DMSO or VEM (10 µM) or VEM and COBI (10 µM) for 48 h. After treatment, cells were collected and stained with caspase 3/7 antibodies and measured by FACS.



Supplementary Figure S2. FOXD1 knockdown increases the protein levels of factors related to apoptosis upon BRAFi treatment

Protein levels of cleaved-caspase-3/9 and BAX in A375 cells upon FOXD1 knockdown and BRAFi treatment. β-Actin was used as a loading control.

Top Canonical Pathways

Name	p-value	Overlap
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.15E-04	3.9 % 7/181
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	4.28E-03	6.1 % 3/49
Paxillin Signaling	6.34E-03	3.6 % 4/110
HIF1 Signaling	7.63E-03	3.4 % 4/116
ILK Signaling	9.38E-03	2.6 % 5/192

Top Upstream Regulators

Upstream Regulators

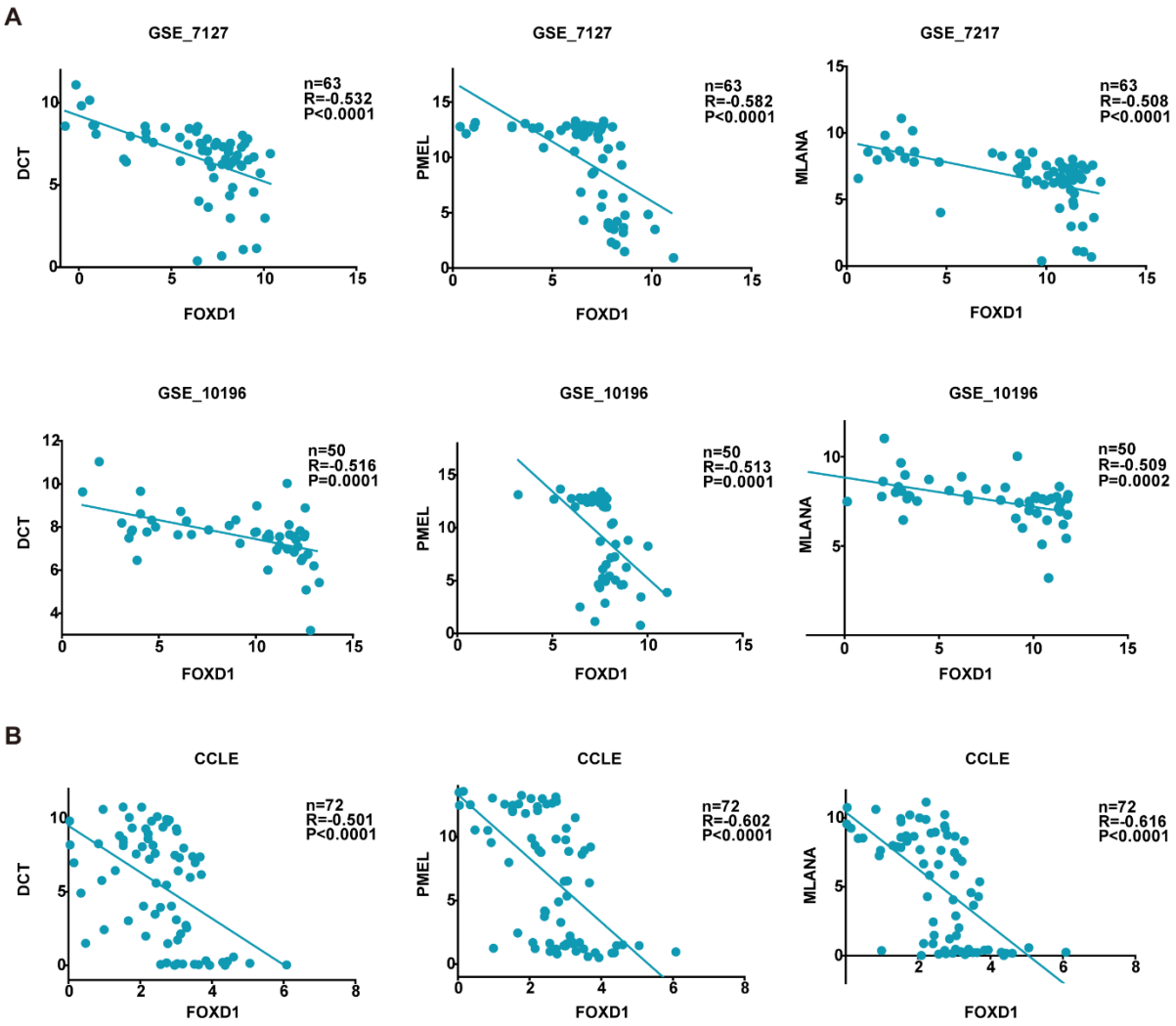
Upstream Regulator	p-value of overlap	Predicted Activation
KIAA1524	2.36E-06	
TP53	2.97E-06	Activated
HIF1A	4.28E-06	
TGFB1	8.26E-06	
MITF	2.59E-05	Inhibited

Causal Networks

Name	p-value of overlap	Predicted Activation
PKM	6.01E-10	Inhibited
TGFB1	1.84E-09	
PRL	2.94E-09	
Mek	4.15E-09	
Gsk3	1.39E-08	

Supplementary Figure S3. Important factors and pathways regulated in A375 FOXD1 OE cells compared with control cells

Microenvironment-related pathways such as HIF1 and ILK signaling are top canonical pathways affected by FOXD1 overexpression. MITF is predicted to be inhibited in FOXD1 OE cells. TGF-β is predicted to be activated in FOXD1 OE cells. The analysis was conducted using IPA.



Supplementary Figure S4. Correlation between the expressions of FOXD1 and DCT, PMEL and MLANA

A FOXD1 expression negatively correlated with the expression of DCT, PMEL and MLANA in the two GSE files (GSE7127 and GSE 10196). **B** FOXD1 expression negatively correlated with the expression of DCT, PMEL and MLANA in CCLE database.

8.2 Supplementary tables

Table S1. IC50 values of vemurafenib of control and FOXD1 OE in three melanoma cells lines

Cells / IC50	IC50 values (vemurafenib μM)	
	Control	FOXD1 OE
A375	2.39 ± 0.08	3.49 ± 0.06
HT144	2.66 ± 0.08	15.40 ± 0.10
SK-MEL-28	1.54 ± 0.09	8.23 ± 0.09

Table S2. IC50 values of vemurafenib of control and FOXD1 KD in three melanoma cells lines

Cells / IC50	IC50 values (vemurafenib μM)		
	Control	FOXD1 KD1	FOXD1 KD2
A375	2.80 ± 0.07	0.03 ± 0.07	0.07 ± 0.07
HT144	1.45 ± 0.06	0.08 ± 0.07	0.04 ± 0.06
SK-MEL-28	1.41 ± 0.06	0.18 ± 0.08	0.07 ± 0.06

Table S3. IC50 values of vemurafenib for A375-R, HT144-R and SK-MEL-28-R (control) and BRAFi-resistant FOXD1 KD cells

Cells / IC50	IC50 values (vemurafenib μM)		
	Control	FOXD1 KD1	FOXD1 KD2
A375-R	17.07 ± 0.11	1.29 ± 0.09	2.59 ± 0.09
HT144-R	10.26 ± 0.10	0.42 ± 0.06	0.07 ± 0.06
SK-MEL-28-R	7.99 ± 0.08	0.35 ± 0.06	1.10 ± 0.06

9. Acknowledgement

Doing PhD research is something that requires long-term persistence, the courage to face all kinds of difficulties, and learn to face setbacks independently. The four years of doctoral studies have been a very important time in my life. Thanks to the help of my family, supervisor, colleagues and friends, I was able to get to complete this task step by step. In this last part of my thesis, I want to send my most heartfelt thanks and blessings to those who encouraged and helped me.

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