Aus der Klinische Kooperationseinheit Dermato-Onkologie des Deutsches Krebsforschungszentrum (DKFZ) an der Klinik für Dermatologie, Venerologie und Allergologie der Medizinischen Fakultät Mannheim
(Direktor: Prof. Dr. med. Jochen Utikal)

The role of ERRFI1 in melanoma progression and resistance towards targeted therapy

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Nina Wang

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Dekan: Prof. Dr. med. Sergij Goerdt
Referent: Prof. Dr. med. Jochen Utikal

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

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ABBREVIATIONS

AKT	v-akt murine thymoma viral oncogene
ATCC	American type culture collection
AXL	AXL receptor tyrosine kinase
BCA	bicinchoninic acid protein assay
BP	biological processes
BRAF	B-RAF proto-oncogene, serine/threonine kinase
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cDNA	complementary DNA
Cobi	cobimetinib
CSD	cumulative solar damage
DKFZ	German Cancer Research Center
DMEM	Dulbecco’s modified eagle’s medium
DMSO	dimethylsulfoxide
ERK	extracellular signal regulated kinase
ERRFI1	ERBB receptor feedback inhibitor 1
etc.	et cetera
FACS	fluorescence activated cell sorting
FC	fold change
FCS	fetal calf serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GO	gene ontology
GRB2	growth factor receptor-bound protein 2
GSEA	gene set enrichment analysis
IHC	immunohistochemistry
KD	knockdown
LC-MS/MS	liquid chromatography tandem mass spectrometry
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MITF	microphthalmia-associated transcription factor
mRNA	messenger ribonucleic acid
NC	neural crest
NCT	National Center for Tumor Diseases, Heidelberg
NEAA	non-essential amino acids
NHM	normal human melanocytes
NRAS	neuroblastoma RAS viral oncogene homolog
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PI	propidum iodide
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PVDF	polyvinylidenfluorid
p53	tumor protein P53
RAS	rat sarcoma
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid

RT	room temperature
RTKs	receptor tyrosine kinases
SD	standard deviation
siRNA	small interfering RNA
SOX10	SRY-box transcription factor 10
TCGA	The Cancer Genome Atlas
TMA	tissue microarray
TRP1	tyrosinase-related protein 1
TYR	tyrosinase
UVR	ultraviolet radiation
V	volt
Vem	vemurafenib
vs.	versus
WB	western blot
WHO	World Health Organization
WT	wild type
18S	18S ribosomal RNA
µ	micro

1 INTRODUCTION

1.1 Malignant melanoma

Melanoma is a type of skin cancer that has a high mortality rate, accounting for more than 50,000 deaths cases worldwide per year[1]. Melanoma arises from the malignant transformation of melanocytes, pigment-producing cells typically found in the skin. Melanoma predominantly occurs in Caucasian populations, with an incidence of about 60 new cases per 100,000 inhabitants in Australia and New Zealand, 30 new cases per 100,000 inhabitants in the USA, 25 new cases per 100,000 inhabitants in Europe[2].

1.1.1 Melanoma subtypes and risk factors

Most melanomas occur on the skin and can be divided into four categories: acral lentiginous, lentigo maligna, nodular, and superficial spreading melanomas[3]. In 2018, the world health organization (WHO) released the 4th edition of their classification of skin tumors, which gives a detailed overview of the molecular characteristics of melanoma and divides melanoma into nine different subtypes[2]. This classification focuses on cumulative solar damage (CSD) and highlights the molecular diversity of melanomas. It presents a more complex system that links sun exposure patterns with specific genetic changes, resulting in different melanoma subtypes. Treatment strategies that target specific mutations and pathways are particularly effective in personalized medicine. The classification divides melanoma into low-CSD melanomas, high-CSD melanomas, and non-CSD-associated[4]. Low-CSD melanomas especially include superficial spreading melanoma, usually in younger individuals. It suffers less damage from sun exposure and is characterized by BRAF mutations. Lentigo maligna melanoma and desmoplastic melanoma belong to high-CSD melanomas and often happen in elderly adults. They receive more ultraviolet radiation damage and are frequently characterized by NF1 or NRAS mutations. Acral melanomas, mucosal melanomas, uveal melanomas, Spitz melanomas, and melanomas arising in congenital or blue naevus are divided into non-CSD melanomas. This type of melanoma usually does not have mutations such as BRAF, NRAS, and NF1, instead, they are characterized by CCND1 gene amplification or KIT mutations[5].

There are numerous risk factors for developing melanoma. The overexposure to ultraviolet radiation (UVR) from natural sunlight and indoor tanning are the most common

risk factors for melanoma[6]. Besides, other risk factors include the high number of naevus, family history of melanoma, non-melanoma skin cancer history, immunosuppression, and xeroderma pigmentosum[2, 7].

1.1.2 Somatic mutations in melanoma

Genetic mutations that modulate cell proliferation, differentiation, and death accumulate, resulting in cutaneous melanoma[8]. The most common genetic mutations in melanoma development affect the genes BRAF, NRAS, NF1, etc.

Specifically, BRAF mutations are detected in about 50% of melanomas and are often linked to UVR exposure[9]. Targeted therapies to inhibit the effects of BRAF V600E mutations have been developed and showed high initial response rates in melanoma patients. In mechanisms, mutations in BRAF can trigger the constitutive activation of the MAPK/ERK signaling pathway, which is vital for cell growth and survival of melanoma cells. These findings demonstrate the importance of BRAF mutations in melanoma pathogenesis.

NRAS mutations, generally exclusive of BRAF mutations, are detected in 15-20% of melanoma cases[5]. This part of melanoma patients tends to be older and suffer chronic sun damage[10]. Additionally, NRAS-mutated melanoma aberrantly activates the MAPK signaling pathway through CRAF[11]. Although it is important in the pathogenesis of melanoma, the current clinical efficacy of targeted therapies against NRAS-mutated melanoma is not ideal. Besides, NRAS-mutated melanoma tends to be highly aggressive, thereby the overall clinical prognosis of NRAS-mutated patients is poor[12].

In addition to the most common mutations in BRAF and NRAS, other gene alterations play a key role in the development of melanoma[13]. Of note, melanoma patients harboring NF1 mutations account for 10-15%[14]. The loss of functional alterations in NF1 causes the activation of the MAPK signaling pathway, contributing to the decreased sensitivity to BRAF inhibitors (BRAFi) in melanomas with BRAF mutations. NF1 mutations are usually detected in elderly melanoma patients and patients who have continued exposure to UVR[15]. In addition, KIT mutations are mainly found in mucosal and acral melanomas and are less common than those mentioned above. Similarly, GNAQ/GNA11 mutations are usually detected in uveal melanomas[16].

1.1.3 Pathways involved in melanoma development

The main signaling pathways dysregulated in melanoma cells include the MAPK/ERK pathway, the PI3K/AKT pathway, and the cell-cycle regulation pathway.

The MAPK/ERK pathway is critical in melanoma development by transmitting signals generated by extracellular stimuli such as growth factors and receptor tyrosine kinases[17]. It is triggered by the binding of extracellular growth factors to receptor tyrosine kinases (RTKs), which initiates a series of intracellular activations of RAS, RAF, MEK, and ERK. The MAPK/ERK pathway is often hyperactivated due to its components' aberrant expression or activation. Although the mutations of NRAS regulate CRAF, they ultimately activate the MAPK signaling pathway, which is similar to BRAF. In addition, NF1 and KIT also contribute to the excessive activation of the MAPK signaling pathway. This pathway is constitutively activated in most cutaneous melanomas and regulates melanoma cell proliferation and survival[18]. Therefore, targeted therapeutic agents against this pathway have begun to be investigated and applied to clinical patients. Among them, BRAFi has a high response rate to initial treatment in melanoma patients. Besides, the combination of BRAFi and MEK inhibitors (MEKi) can reduce cytotoxicity and extend the progression-free survival of patients[19].

The PI3K/AKT pathway affects cell growth, cell survival and metastasis[20]. AKT pathway activation is induced by external growth factors followed by phosphoinositide 3-kinase (PI3K) activation, leading to the increased production of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). This facilitates the transfer of AKT to the plasma membrane, where it undergoes phosphorylation and becomes activated. This pathway is dysregulated in about 70% of melanomas, and its dysregulation is often due to the amplification of AKT3 as well as the loss of PTEN[21, 22]. As the intracellular level of PIP3 is negatively controlled by the phosphatase PTEN, the AKT pathway's dysregulation can also happen due to insufficient PTEN expression or function[20].

Mutations of the genes involved in the cell cycle, such as cyclin-dependent kinase inhibitor 2A (CDKN2A), cyclin D1, and cyclin-dependent kinase 4/6 (CDK4/6), result in uncontrolled cell cycle progression. Specifically, CDKN2A encodes two proteins ($p16^{Ink4a}$ and $p14^{Arf}$). CDK4/6 can mediate retinoblastoma-associated protein (RB) phosphorylation or inactivation, vital for regulating the cell cycle. Based on this, $p16^{Ink4a}$ limits the cell cycle progression by inhibiting CDK4/6 and cyclin D1[23]. Furthermore,

$p14^{Arf}$ regulates the cell cycle by inhibiting the degradation of p53. Collectively, the mutations of CDKN2A contribute to the uncontrolled cell cycle progression[24].

Besides, the pigmentation-related pathway, p53 pathway, Notch and Wnt pathways and so on also play indispensable roles in melanoma carcinogenesis[24].

1.1.4 Phenotype switching and heterogeneity in melanoma progression

Melanoma cells can alter their cellular phenotype, enabling them to adapt to stressful conditions, including therapeutic interventions. This phenotypic plasticity is increasingly recognized and indispensable for melanoma progression and therapy resistance. Phenotypic plasticity significantly contributes to tumor heterogeneity and poses a substantial obstacle to targeted therapies as well as immunotherapies[25, 26]. Termed phenotype switching in melanoma, this phenomenon exhibits dynamic cellular state alterations, including reversible changes in transcription and epigenetic modifications[27, 28].

There are two main phenotypes of melanoma cells characterized by the level of MITF expression. There is the proliferative/differentiated $MITF^{high}$ phenotype (termed the “melanocyte-like” state), and the invasive/undifferentiated $MITF^{low}$ phenotype (known as the “mesenchymal-like” state)[18, 29]. RTKs such as AXL mainly contribute to the shift towards the mesenchymal-like state, with high RTK expression associated with the undifferentiated $MITF^{low}$ phenotype[30, 31].

In addition, the research team led by Graeber performed gene expression profiling of a set of human melanoma cell lines and demonstrated with this analysis that the two main phenotypes described before can be further subdivided into four melanoma subtypes C1-C4[32]. Specifically, C1 refers to the undifferentiated subtype, C2 refers to a neural crest-like subtype, C3 refers to a transitory subtype, and C4 refers to the melanocytic subtype[32].

1.2 Therapeutic options for the treatment of melanoma

Noticeable progress has been made in the treatment of melanoma over the past few decades[33]. The introduction of targeted therapies and immunotherapies has substantially improved the overall survival rate of melanoma patients. Besides, even though new innovative treatment strategies for melanoma, such as microbiome research and neoantigen vaccines, are currently under extensive investigation[5], the established options for melanoma treatment are generally divided into two main categories[34]. On the one hand, there are conventional therapies, which typically involve surgery, systemic

chemotherapy, and radiation therapy. On the other hand, targeted therapies and immunotherapies have contributed to a substantial improvement in melanoma management[35, 36].

1.2.1 Conventional therapies

Surgery is the primary treatment for localized and regional melanoma and usually includes lymph node dissection and biopsy to evaluate if the disease has already spread. Primary melanoma is usually treated with wide excision. The tumor is removed with margins ranging from 0.5 to 2 cm, depending on the depth of invasion[34]. Melanoma patients with sentinel lymph node (SLN) metastasis and clinically detectable locoregional LN metastasis were usually treated with completion lymph node dissection (CLND). However, nowadays, it is only applicable to patients with clinically detectable locoregional LN metastasis due to the limited improvement in survival rate[37]. Patients with thin, non-invasive tumors achieve a high cure rate with surgical treatment. Despite this, surgery is not an option for the treatment of advanced metastatic melanoma.

Systemic chemotherapy for melanoma includes drugs that cause cell death by disrupting the essential cellular structures or processes[38]. It is typically employed in patients with metastatic melanoma that are unable to be cured by surgery. Among these, dacarbazine is the first chemotherapeutic agent approved by the Food and Drug Administration (FDA) for treating melanoma patients. However, the median survival period is 5 to 11 months, and the one-year survival rate is only 27%[39]. Moreover, the associated side effects of standard chemotherapy due to its non-specific effects on healthy cells remain issues. Additionally, the emergence of chemotherapy resistance suggests further research to develop new treatments[40].

Radiation therapy targets melanoma by employing high-energy X-rays or particles. It is commonly used to treat patients with metastatic melanoma. Numerous retrospective research has elucidated that combining radiation therapy and immunotherapy can significantly improve the median overall survival of melanoma patients[41].

1.2.2 Targeted therapy

Melanoma can develop resistance to traditional treatments, resulting in disease recurrence and progression. For this reason, there is a need for novel pharmacological agents that target specific molecular pathways involved in melanoma growth and progression. Targeted therapies aim at obstructing melanoma development with specific

inhibitors that target BRAF, MEK, NRAS, and KIT. This type of therapy provides a personalized approach by inhibiting key molecules[42]. Targeted therapies are commonly used for patients with metastatic melanoma. Among these, BRAF inhibitors (BRAFi) or MEK inhibitors (MEKi) have been shown to improve the clinical outcomes of patients effectively. Specifically, numerous studies have demonstrated that they can prolong the progression-free survival and overall survival of patients with BRAF-mutated melanoma[43, 44]. Advanced melanoma patients with BRAF mutation can be treated with the combination therapy of BRAFi and MEKi. There are three combination therapies of BRAFi and MEKi, such as Vem plus Cobi, dabrafenib plus trametinib, encorafenib plus binimetinib. Patients respond favorably to the initial therapy with these agents and show rapid improvement in clinical symptoms[45-48]. Despite these advances, novel treatments affecting new molecules are still required for patients resistant to current targeted therapies[49, 50].

1.2.3 Immunotherapy

Before the use of immunotherapy, the median survival of advanced melanoma patients was only 6 to 9 months[51]. However, the emergence of immunotherapy has substantially improved the median survival, up to nearly 6 years[52]. Even though it does not have high initial response rates in melanoma patients, it can significantly improve patient outcomes and is suitable for melanoma patients with or without BRAF mutation[53].

Immunotherapy focuses on enhancing the endogenous immune response instead of directly targeting tumor cells. Specifically, the monoclonal antibodies targeting cytotoxic T lymphocyte-associated protein 4 (CTLA-4) prevent the inactivation of T cells, thereby facilitating the identification of tumor cells. Ipilimumab is the first CTLA-4 immune checkpoint inhibitor (ICI) approved to treat advanced melanoma patients. Furthermore, anti-programmed cell death protein 1 (PD-1) monoclonal antibodies such as pembrolizumab and nivolumab mainly focus on preventing the immune evasion of tumor cells and restoring the recognition and destruction of tumor cells. The combination of Ipilimumab and nivolumab is often used to improve the clinical efficacy of advanced melanoma. In addition, other combinations of immune checkpoint inhibitors, such as the lymphocyte-activation gene 3 (LAG-3) inhibitor, have emerged. It focuses on preventing the binding of LAG-3 to MHC class II, thereby inhibiting the immune evasion of tumor cells[54]. Besides, other immunotherapies, including TLR-9 agonists, adoptive cell

therapy, and fecal microbiota transplantation, are still being studied to overcome the resistance to ICI therapies.

1.2.4 Emerging therapies

In addition to traditional therapies, targeted therapies, and ICI therapies, some new treatment modalities are under investigation. They have pinpointed potential future therapies, such as oncolytic virus therapy, melanoma vaccines, and photodynamic therapy[55]. However, verifying agent efficacy requires supporting more randomized data in the first, second, and third lines.

1.3 Resistance to targeted therapy in melanoma

Combining BRAFi and MEKi, such as Vem and Cobi, has transformed the treatment landscape for patients with metastatic melanomas harboring the somatic BRAF V600E mutation[56]. Even though they have excellent initial response rates in patients, their usage is still impeded by acquired resistance with clinical relapse[57]. Approximately 50% of patients develop acquired resistance within 1 year and 80% within 5 years[58-60]. Many underlying mechanisms of drug resistance make it difficult to tackle this problem effectively.

Approximately 20% of melanoma patients fail to respond to the initial treatment of BRAFi, resulting from intrinsic resistance[61]. The intrinsic resistance mechanisms usually include the loss of PTEN, loss of NF1, RAC1 mutations, and the amplification of cyclin D, resulting in the activation of substitutive pathways. However, acquired resistance often occurs when the disease progresses after an initial response to targeted therapies. The mechanisms of acquired resistance mainly include the reactivation of the MAPK signaling pathway[62]. MAPK pathway is activated by multiple genetic changes, especially NRAS, BRAF, and MEK1 alterations[63]. BRAF amplification or BRAF splicing can cause BRAF aberrations. Among these, BRAF amplification reactivates ERK in a RAS-dependent manner, further resulting in the reactivation of MAPK signaling pathway. NRAS mutations can ultimately lead to the reactivation of MAPK signaling pathway by activating CRAF during treating melanoma patients with BRAFi. Besides, the resistant mechanisms independent of MAPK modification are also observed, such as activation of alternative RTK-mediated survival pathways[57, 64]. Hyperactivation or overexpression of RTKs not only directly induces RAS to activate the MAPK pathway, but also triggers additional activation of the PI3K/AKT signaling

pathway, which is implicated in resistance towards BRAFi and MEKi. In addition, overexpression of EGFR is detected in melanoma patients resistant to BRAFi or MEKi treatment, suggesting that inhibition of EGFR may restore the sensitivity to targeted therapy[65]. Additionally, the components in the tumor microenvironment, such as the extracellular matrix (ECM), also contribute to the resistance to targeted therapy[3].

1.4 ERRFI1

ERBB receptor feedback inhibitor 1 (ERRFI1) is an adapter/scaffold protein with multiple binding domains that mediate protein-protein interaction. Previous research has demonstrated that ERRFI1 regulates various biological functions and intracellular signaling pathways. As a cytoplasmic protein, multiple studies have shown that ERRFI1 is mainly related to regulating the ERBB receptor signaling pathway[66, 67]. Additionally, the nuclear part of ERRFI1 plays a role in regulating the DNA damage response under genotoxic stresses[68]. These discoveries underscore the expanded functions of this protein[69]. Xu et al. showed that exogenous expression of ERRFI1 inhibits the apoptosis of human breast cancer cells (MCF-7 cells), thereby promoting tumor growth[70], consistent with the conclusions observed by Wendt and colleagues in their study[71]. Park and colleagues demonstrated that after knocking out ERRFI1 through the CRISPR/cas9 method, the proliferation capacity of lung epithelial cells was significantly reduced[72]. In addition, in a study by Kang and others, the upregulation of ERRFI1 in lung cancer cells (PC9 cells) increased the capabilities of cell migration and invasion and promoted epithelial-to-mesenchymal transition[73].

1.4.1 Role of ERRFI1 in cancer

ERRFI1 is differentially expressed in various cancer types[74]. There is controversy about the role of ERRFI1 in cancers, as it has both tumor-promoting and suppressive features[75-77]. However, an expanding collection of research indicates that ERRFI1 is a crucial factor in developing many cancers.

Jäger and colleagues found that the expression of ERRFI1 is significantly upregulated in the tumors of metastatic melanoma patients who died early in contrast to tumors from patients who survived at least 30 months, indicating that high ERRFI1 expression correlates with a poor prognosis[78]. In a study by Kang and others, ERRFI1 acts as an oncoprotein, and the high expression of ERRFI1 is correlated with unfavorable prognosis in lung adenocarcinoma. Overexpression of ERRFI1 promoted cell proliferation

and invasion, as well as EMT[73]. In breast cancer, He and colleagues reported that ERRFI1 is highly expressed and correlates with poor clinical prognosis. In addition, it participated in glycolysis to promote tumor growth in vivo[77]. According to Liu and colleagues in pancreatic cancer, the high levels of ERRFI1 increased the risk of mortality, demonstrating a high degree of predictive value for overall survival[79]. Various studies have confirmed that ERRFI1 expression is increased in different tumors and that its expression correlates with adverse prognosis.

1.4.2 ERRFI1 in therapy resistance

Treatment resistance continues to be a major issue for most of the cancer patients undergoing therapy. Many research efforts have focused on uncovering the mechanisms responsible for drug resistance in cancer. To date, research indicates that ERRFI1 plays a vital role in drug resistance.

Kang and co-workers focused on the role of ERRFI1 in resistance to EGFR-TKI therapies in lung adenocarcinoma. They discovered that the expression levels of ERRFI1 were higher in drug-resistant cell lines, and the depletion of ERRFI1 restored the sensitivity of drug-resistant cell lines to EGFR-TKI treatments[73]. AXL-induced EMT transition is often observed and related to numerous TKI-resistant lung cancers, and the AXL signaling pathway mainly promotes tumor invasion. Conversely, the EGFR signaling pathway is involved in promoting tumor proliferation. A study from Yang and colleagues revealed that ERRFI1 can be positively regulated by AXL, thereby participating in the signal switch of AXL/EGFR[80]. Endo et al. found that high ERRFI1 expression correlates with increased dormancy and decreased treatment efficacy to EGFR-TKI in EGFR-mutated lung cancer cells, as well as impacting patient survival negatively[81]. Furthermore, Izumchenko et al. discussed that during transforming growth factor β (TGF-β)-mediated EMT kinase switching, the expression of microRNAs 200 (miR200) family decreased, and the expression of ERRFI1 upregulated simultaneously. These led to the activation of AKT independent of the state of EGFR, subsequently affecting the drug resistance of tumors to EGFR inhibitors[82]. A study by Cairns and colleagues on lymphoblastoid cell lines noted that the function of ERRFI1 in tumors depended on EGFR expression levels. Specifically, ERRFI1 promoted cell proliferation and chemotherapy resistance by preventing the inactivation of AKT in the situation of EGFR low expression of cells, thereby targeting ERRFI1 could be used as a potential treatment[83]. A case report from Xiao et al. showed that the ovarian cancer female

patient with a detected ERRFI1 mutation received 15 months of progression-free survival (PFS) after being treated with conventional chemotherapy combined with gefitinib, indicating that ERRFI1 can be used as a potential therapeutic target[84]. Additionally, Chang and colleagues found that acquired resistance to erlotinib in many tumor patients was related to the higher ERRFI1/EGFR ratio[85], Zhang et al. also reported that the expression of ERRFI1 was increased in gefitinib-resistant hepatic cancer cells compared to gefitinib-sensitive cells[86]. Furthermore, Kim and colleagues demonstrated ERRFI1 was highly upregulated in radio-resistant rectal cancer cell lines[87]. Together, these findings emphasize the role of ERRFI1 in cell survival and drug resistance in multiple types of cancer.

In summary, the expression of ERRFI1 is elevated in various malignant tumors and correlates with dismal prognosis for clinical patients. These observations indicate that ERRFI1 could be a potential therapeutic target for treating various cancers. To date, there has been limited research about the role of ERRFI1 in conferring resistance to melanoma cells against targeted therapies. Therefore, more studies are required to elucidate the underlying mechanisms.

2 AIM OF THE PROJECT

Melanoma is a malignant tumor that is characterized by a high heterogeneity, which contributes to its resistance to therapies, as previous studies reported. Therefore, it is important to identify novel biomarkers to predict treatment efficacy and patient prognosis.

It has been demonstrated that ERRFI1 is upregulated in many types of cancer and that its expression correlates with prognosis as well as drug resistance. However, there is still little known about the role of ERRFI1 in melanoma progression and resistance.

For this reason, the specific aims of this study are:

1. To investigate the role of ERRFI1 in melanoma progression.

2. To examine the role of ERRFI1 in melanoma resistance to targeted therapy.

3. To explore the underlying mechanisms of ERRFI1-mediated resistance of melanoma cells towards 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
BrdU Cell Proliferation ELISA Kit	Abcam	ab126556
FITC Annexin V Apoptosis Detection Kit I	BD	556547
Immobilion PVDF membrane pore size 0.45 µm	Merck Millipore	IPVH00010
Immobilon Forte Western HRP Substrate	Merck Millipore	WBLUF0100
methanol	Sigma-Aldrich	F4680
miRNeasy Mini Kit (50)	Qiagen	217004
NuPage Gele 4-12% Bis- Tris Protein,1 mm x 10 well	Invitrogen	NP0321BOX
PhosSTOP™ Phosphatase inhibitor Cocktail	Roche diagnostics	04906845001
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
PageRuler Plus Prestained Protein Ladder	Life Technologies	26619
RevertAid First strand cDNA synthesis kit	Thermo Fisher Scientific	K1622
RIPA	Sigma-Aldrich	R0278
RNase-Free Dnase set	Qiagen	79254
RNeasy Plus Mini Kit	Qiagen	74136
Stealth RNAi™ siRNA negative control, high GC	Life Technologies	12935-400
SYBR Green PCR Master mix	Applied Biosystems	4309155
TaqMan™ microRNA Reverse Transcription Kit	Thermo Fisher Scientific	4366596
TaqMan MicroRNA Assays (U6 snRNA)	Thermo Fisher Scientific	4427975

TaqMan MicroRNA Assays (hsa-miR-200c-3p)	Thermo Fisher Scientific	4427975
TaqMan™ Universal Master Mix II, no UNG	Thermo Fisher Scientific	4440043
TritonX-100	Carl Roth	3051.1
Tween® 20	Applichem	A13890500
0,45 µm syringe filters	Carl Roth	P667.1

3.1.2 Cell culture reagents

Product	**Company**	**Catalog#**
DMSO	Carl Roth	A994.2
Dulbeccos's Modified Eagle Medium (DMEM), high glucose	Gibco® Life Technologies	41965-039
fetal bovine serum (FBS)	Biochrom	S0115
non-essential amino acids	Sigma-Aldrich	M7145
Opti-MEM® I reduced serum medium	Gibco®Life Technologies	31985062
PBS	Sigma-Aldrich	D8537
penicillin/streptomycin	Sigma-Aldrich	P4333
RNAiMAX transfection reagent	Life Technologies	13778075
Trypan blue solution	Sigma-Aldrich	93595
trypsin	Sigma-Aldrich	T3924
2-mercaptoethanol	Gibco® Life Technologies	31350010

3.1.3 Antibodies

Product	**Source**	**Company**	**Catalog#**
α-actinin	mouse	Santa Cruz	sc-17829
anti-mouse IgG, HRP-coupled	horse	Cell signaling	7076
anti-rabbit IgG, HRP-coupled	goat	Cell signaling	7074S

AKT	mouse	Cell signaling	2920S
p-AKT	rabbit	Cell signaling	4058
ERK	rabbit	Cell signaling	4695
p-ERK	mouse	Cell signaling	9106S
ERRFI1	rabbit	Atlas antibodies	HPA027206
β-actin	rabbit	Cell signaling	5125

3.1.4 Small molecule inhibitors

Product	**Company**	**Catalog#**
vemurafenib (PLX4032)	Selleckchem	S1267

3.1.5 siRNA

Product	**Company**	**Catalog#**
siERRFI1-1	Thermo Fisher Scientific	1299001
siERRFI1-2	Origene	SR310045

3.1.6 miRNA

Product	**Company**	**Catalog#**
Negative Control 4 miRCURY LNA miRNA Mimic	Qiagen	339173
hsa-miR-200c-3p miRCURY LNA miRNA Mimic	Qiagen	339173

3.1.7 Primers

Target	**Forward sequence**	**Reverse sequence**
AXL	CCGTGGACCTACTCTGGCT	CCTTGGCGTTATGGGCTTC
ERRFI1	GAGCAGTCGCAGTGAGTT	TTGGAAGCATGCCCAAGTG

GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
SOX10	GGCTTTCTGTCTGGCTCACT	TAGAGGGTCATTCCTGGGGG
18S	GAGGATGAGGTGGAACGTGT	TCTTCAGTCGCTCCAGGTCT

3.1.8 Buffers and solutions

Blocking buffer (BSA)	**Cell lysis buffer for protein isolation**
5% BSA	1X PhosphoStop
1X TBST	1X Complete mini protease inhibitor cocktail
	RIPA
Running buffer (pH8.3)	**Transfer buffer (pH8.3)**
25mM glycine	25mM glycine
190mM Tris	190mM Tris
0.1% SDS	20% SDS
dH2O	20% methanol
	dH2O
TBS 10X (pH7.6)	**Washing buffer (1X TBST)**
150mM NaCl	0.02% Tween® 20
50mM Tris	1X TBS
dH2O	

3.1.9 Devices

Device	**Company**
ABI 7500 Real-Time PCR machine	Applied Biosystems
LSR Fortessa HTS	BD Biosciences
NanoDrop ND-1000	Peqlab Biotechnologie

TECAN Infinite M1000 PRO microplate reader	Tecan

3.1.10 Software

Analysis software	**Source**
ABI 7500 Software v2.0.5	Applied Biosystems
Adobe Illustrator 2021	Adobe
FlowJo v10.8.1	FlowJo
GraphPad Prism 9	GraphPad
GSEA	Whitehead Institute Open source
Image Lab Software 6.0.1	BioRad
ImageJ	National Institute of Health

3.1.11 Online database

Database	**Source**
cBioportal	Whitehead Institute Open source
NCBI	National Center for Biotechnology Information
TCGA	National Institute of Health

3.2 Methods

3.2.1 Cell culture and cell lines

Cell line	**Source**	**Cell type**	**Mutation**
HT144	ATCC	melanoma cell line	BRAF V600E
SK-MEL-28	ATCC	melanoma cell line	BRAF V600E
WM9	University of Wroclaw, Poland	melanoma cell line	BRAF V600E

Human melanoma cell lines HT144, SK-MEL-28 and WM9 were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Sigma), 1% non-essential amino acids (NEAA, Sigma), 0.1 mM β-mercaptoethanol (Gibco), and 1% penicillin/streptomycin (Sigma). Cell lines were cultured in a humidified incubator with 5% CO2 at 37℃. Cell medium was replaced every other day, and cells were split every 3-5 days until they reached approximately 80% confluency.

3.2.2 Vemurafenib-resistant cell lines

To develop vemurafenib-resistant melanoma cell lines, HT144 and SK-MEL-28 cells were exposed to incrementally increasing concentrations of the BRAF V600E inhibitor vemurafenib for six months. Additionally, vemurafenib-sensitive and -resistant WM9 cells were kindly provided by Dr. Ewelina Dratkiewicz from the University of Wroclaw, Poland.

3.2.3 RNA isolation and cDNA synthesis

RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The RNA extraction was performed with DNase digestion on the column. The concentration and quantity of RNA were determined using a NanoDrop ND1000 spectrophotometer. According to the manufacturer's protocol, 500 ng of RNA were used with the RevertAid First Strand cDNA Synthesis Kit for cDNA synthesis.

For the miRNA, total RNA was extracted by using the miRNeasy Mini Kit (QIAGEN) according to the manufacturer´s instructions. cDNA was synthesized with the TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific).

3.2.4 Quantitative real-time polymerase chain reaction

To quantify mRNA expression, qRT-PCR was performed using the ABI® 7500 Real-Time PCR System (Applied Biosystems) and SYBR Green PCR Master Mix (Thermo Fisher Scientific). 18S and GAPDH were used as endogenous controls. The efficiencies of primers used were validated and within the range of 90%-110%. For miRNA, TaqMan Universal Master Mix II (no UNG) and TaqMan MicroRNA assays for miR-200c and control U6 were carried out for each sample. Relative gene expression was calculated from at least three independent experiments using the ΔΔCt method.

3.2.5 Protein extraction

Cells were harvested when they reached confluency and washed with PBS. Proteins were extracted using RIPA buffer supplemented with 1x Complete Mini Protease Inhibitor and 1x PhosStop. Samples were incubated on ice for 30 min, then centrifuged at 14,000 rpm (4℃) for 20 min. Protein quantification was performed with the Pierce BCA protein assay kit (Thermo Fisher Scientific).

3.2.6 Western blot

Between 30-40 µg of protein were loaded per lane on NuPAGE Bis-Tris 4-12% gels. The gel was run at 180 V for 1 h and afterwards the samples were transferred onto a methanol-activated PVDF membrane at 100 V for 1 h. Subsequently, 5% BSA in TBST was used to block the PVDF membrane for 1-2 h at RT. Next, the PVDF membrane was incubated with the primary antibody diluted in blocking buffer overnight at 4℃. The next day, the PVDF membrane was washed three times with 1x TBST for 10 min each, and then incubated with the secondary antibody for 2 h at RT. Protein was detected using the Immobilon Forte Western HRP substrate (Merck) and ChemiDoc Touch Imaging System (BioRAD). Protein quantification was then conducted utilizing Image Lab software.

3.2.7 Mass spectrometry-based proteomics analysis

Melanoma cells (HT144, SK-MEL-28, WM9) transfected with ERRFI1 siRNA for 48 h were used for proteomic analysis. Cells were harvested by scraping and washed with PBS when they reached confluency. Proteins were extracted using RIPA buffer supplemented with 10 mM NaF, 1 mM Na3VO4, 1x Complete EDTA free protease inhibitor, 1x PhosStop, 250 U/ml Benzonase and 10 U/ml DNase. Samples were incubated on ice for 1 h and vortexed every 10-15 min. Next, samples were centrifuged at 20,000 rpm (4℃) for 30 min and afterward the supernatant was transferred to a fresh tube and used for further analyses. Protein quantification was performed with the Pierce BCA protein assay kit (Thermo Fisher Scientific). Peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the DKFZ Genomic and Proteomics Core Facility.

3.2.8 Immunohistochemistry of tissue microarrays (TMA)

Tumor samples from melanoma patients were utilized to prepare TMA slides as reported before[88]. These slides were stained with antibodies against our proteins of

interest and scanned by the NCT Gewebebank facility at the pathology unit of the University of Heidelberg. TMAs were assessed using a scoring system based on the quantity and intensity of the staining as previously described[89]. All analyses were conducted in line with the principles of the Declaration of Helsinki and received approval from the medical Ethics Committee of the Medical Faculty Mannheim, University of Heidelberg.

3.2.9 Transfection of cells

Cells were seeded at approximately 60% confluency in 6-well plates. The next day,

the cells were transfected with two different siRNA targets against ERRFI1 (siERRFI1-1: HSS122815, Thermo Fisher Scientific; siERRFI1-2: SR310045AL, Origene) or a control siRNA (12935400, Thermo Fisher Scientific) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific), following the manufacturer's protocol. 48 h after transfection, RT-qPCR and western blot assays validated the transfection efficiency before proceeding with further experiments.

For miRNA transfection, miR-200c mimics corresponding to the mature microRNA sequence (5´UAAUACUGCCGGGUAAUGAUGGA3´, Qiagen) were transfected using the Lipofectamine RNAiMAX Transfection Reagent and cells were harvested 56 h upon transfection.

3.2.10 BrdU ELISA proliferation assay

Cells (control or knockdown) were seeded at a density of 1-2x10^4 cells per 100 µl of culture medium per well of a 96-well plate. The proliferation assay followed the manufacturer's protocol (Abcam, ab126556). The next day, 20 µl of 1x BrdU were added to each well and incubated for 6-24 h at 37℃. Afterwards, the cell medium was aspirated from each well and replaced with 200 µl fixing solution for 30 min at RT. Next, 100 µl of anti-BrdU monoclonal Detector Antibody were added to each well for 1 h at RT. Then, 100 µl 1x Peroxidase goat anti-mouse IgG conjugate was added to each well for 30 min at RT. Next, 100 µl/well TMB peroxidase substrate were added into the well for 30 min at RT before pipetting 100 µl Stop Solution to each well. Subsequently, absorbance was measured at a dual wavelength of 450/550 nm using a Tecan plate reader.

3.2.11 Cell viability assay

Melanoma cells were seeded at a density of 3,000 to 5,000 cells per well of a 96-well plate and allowed to adhere for 24 h. Cells were then treated with varying

concentrations of BRAFi, ranging from 0.0001 to 25 µM. Cell viability was assessed 48 h later using Alamar Blue. After incubating at 37℃ for 3-4 h, the fluorescence was measured using a Tecan Infinite M1000 PRO at an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

3.2.12 Clonogenic assay

Between 1,000 to 2,000 cells were seeded per well of a 6-well plate. The following day, BRAFi was added to achieve a final concentration of 10 µM. The medium was changed after 24 h. After 10-14 days, cell colonies were stained with 0.5% crystal violet, and the colony count was quantified using ImageJ software.

3.2.13 Cell apoptosis analysis with annexin V and PI staining

Cells were seeded at approximately 60% confluency per well of a 6-well plate for siRNA transfection. After 48 h, BRAFi was added. After 48h of incubation, both adherent and floating cells were harvested and stained with FITC and PI, according to the manufacturer's protocol (BD Biosciences, 556547). Samples were analyzed using LSR Fortessa HTS machine provided by the Flow Cytometry Core Facility at the DKFZ. The data were analyzed with the FlowJo v10.8.1 software.

3.2.14 Dataset analysis

ERRFI1 expression in melanocytes and melanoma cells was analyzed from datasets from the publicly available GSE database (GSE130244, GSE111766), along with the expressions of AXL, SOX10, MITF, TYR, DCT, and MLANA. The expression of ERRFI1 in primary and metastatic melanoma was also assessed using data from The Cancer Genome Atlas (TCGA). Survival analysis was performed using DFCI Science 2015 database (www.cbioportal.org).

3.2.15 miRNA conserved target sites prediction

Putative miRNA target sites in the 3′ UTR of the ERRFI1 gene were identified using miRDB and TargetScan. The analysis focused on the 3′ UTR of the ERRFI1 transcript ENST00000377482.5 to assess potential miRNA binding.

3.2.16 Statistical analysis

Experiments were performed at least three times if not indicated differently. Data were displayed as mean ± SD, and a two-tailed Student´s t-test was used for statistical

analysis. Pearson analysis was employed to define the correlation between two parameters, and the Kaplan-Meier method was applied for survival analysis. Data were processed using Prism 9.0 (GraphPad Software), and statistical significance is indicated by p-values: *$p<0.05$; **$p<0.01$; ***$p<0.001$; “ns” (not significant) $p>0.05$.

4 RESULTS

4.1 ERRFI1 expression positively correlates with AXL expression, but negatively with MITF, SOX10, TYR, DCT, and MLANA expression

One of the challenges of treating metastatic melanoma is the development of resistance to applied therapy. In most cases of acquired resistance, melanoma cells often alter their molecular and cellular phenotypes, known as phenotype switching, adopting a more invasive and aggressive state[90]. Previous research has shown that during acquired resistance to BRAFi, the phenotype switching signature is characterized by the down-regulation of MITF, the master regulator of melanocyte differentiation, and the up-regulation of RTKs such as AXL, which are involved in resistance to therapy[30, 91-93]. Besides, similar to the MITF^{low} phenotype, reduced expression of SOX10 in melanoma has been demonstrated to confer resistance to MAPK pathway inhibition[94, 95]. A previous study from our lab demonstrated that ERRFI1 acts as a NC-related gene, is highly expressed in metastatic melanoma and is associated with a poor prognosis[78]. By examining data from the GEO database (GSE130244, GSE111766) and the cBioportal database (DFCI, Science 2015), I observed that ERRFI1 expression positively correlated with AXL expression but negatively with the expression of SOX10, MITF, as well as melanocytic differentiation markers (TYR, DCT, MLANA) (Figure 1). These findings imply that ERRFI1 may influence the differentiation status of melanoma cells.

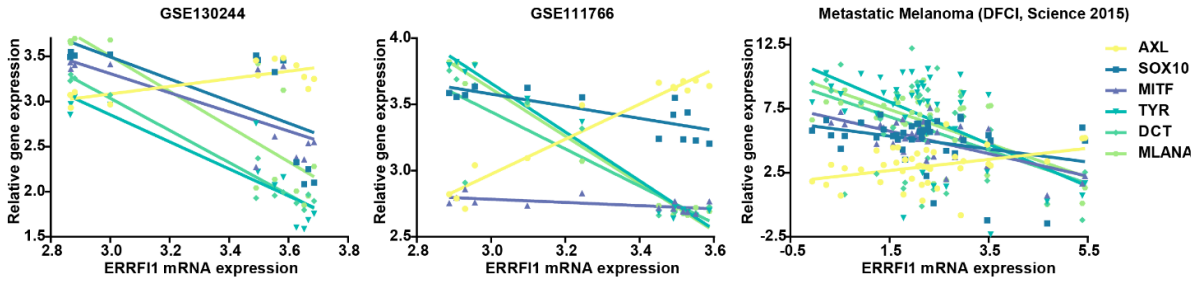

Figure 1. Correlation between ERRFI1 expression and AXL, MITF, SOX10, TYR, DCT and MLANA expression

The scatter plots were generated using the GEO database (GSE130244, GSE111766) and the cBioportal database (DFCI, Science 2015). These data reveal a positive correlation between ERRFI1 expression and AXL as well as a negative correlation between ERRFI1 expression and SOX10/MITF/TYR/DCT/MLANA expression. Detailed information about the Pearson correlation (r) and the corresponding p-value is provided in Supplementary Figure S1.

4.2 ERRFI1 is highly expressed in melanoma and associated with reduced overall survival of melanoma patients

To further investigate the role of ERRFI1 in melanoma, I analyzed clinical samples of melanocytic nevi and primary melanoma. The results indicated a significantly higher expression of ERRFI1 in the melanoma samples (Figure 2A). Additional comparisons were made between melanoma cell lines (HT144, SK-MEL-28, WM9) and normal human melanocytes (NHM). ERRFI1 levels were markedly elevated in melanoma cells compared to NHM (Figure 2B). Data analysis from The Cancer Genome Atlas (TCGA) further revealed that ERRFI1 was upregulated in metastatic melanoma compared to primary melanoma (Figure 2C). The evaluation of data from the cBioportal database on the overall survival of melanoma patients showed that higher intratumoral ERRFI1 levels are associated with decreased overall survival (Figure 2D).

Next, I analyzed differentially expressed genes between melanoma cells and melanocytes using data from two independent melanoma datasets (GSE130244, GSE111766). I identified 5,053 overlapping genes between these two datasets (Figure 2E). Within this subset, 155 genes exhibited consistent up- or down-regulation across both datasets, with an absolute fold change of ≥2 and $p<0.05$. ERRFI1 was found among the top 20 dysregulated genes and to be upregulated in melanoma cells (Figure 2F). Gene Ontology (GO) analysis for biological processes (BP) of these 155 genes revealed that upregulated genes, including ERRFI1, were predominantly associated with processes like apoptosis, cell proliferation, cell differentiation, and NC cell development. Conversely, downregulated genes were linked to melanin biosynthesis and melanocyte differentiation (Figure 2G). The above results suggest that ERRFI1 could be crucial in melanoma progression.

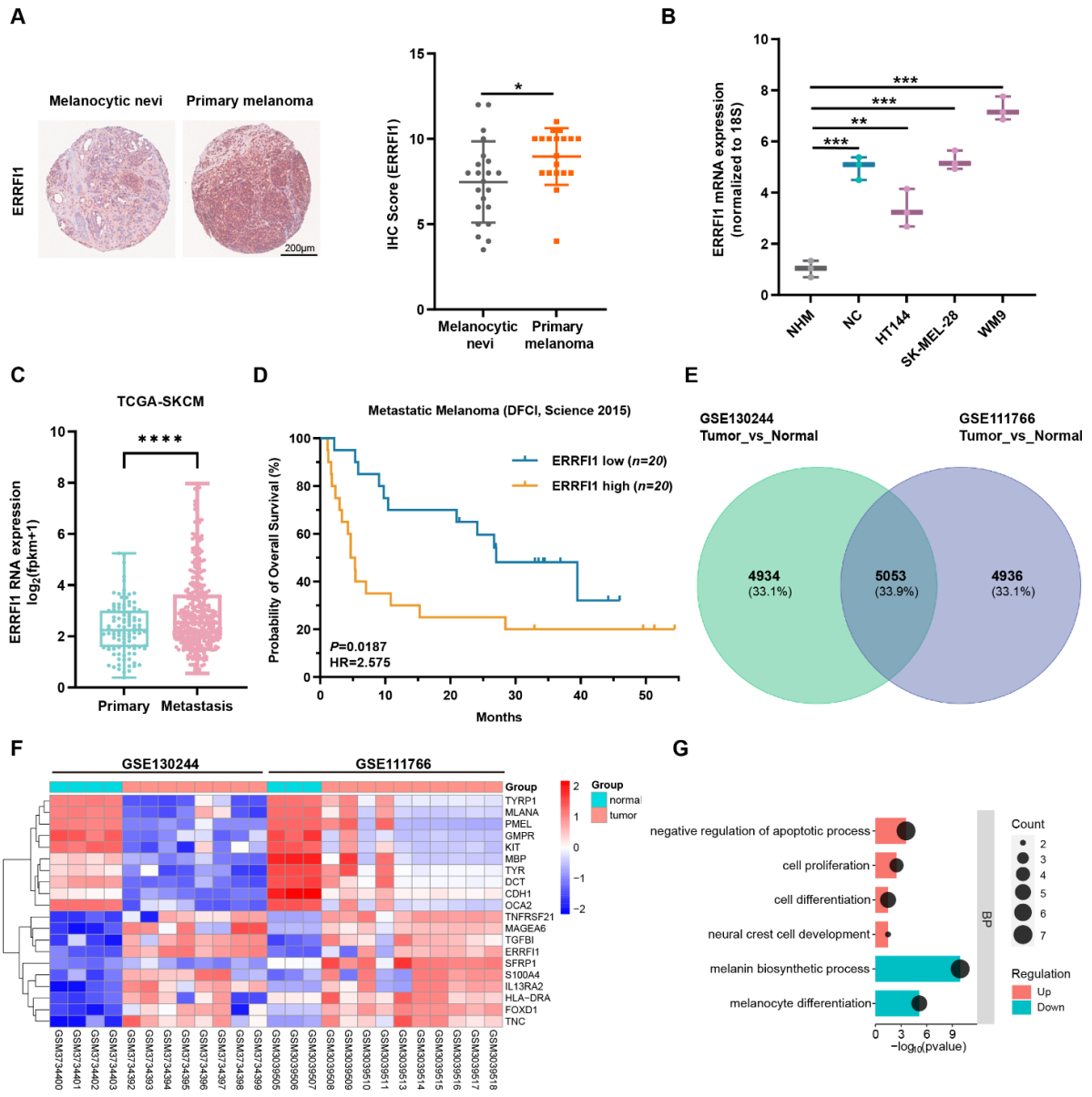

Figure 2. ERRFI1 is overexpressed in melanoma and a high intratumoral expression of ERRFI1 is associated with reduced overall survival of melanoma patients

(A) Comparison of the expression of ERRFI1 between melanocytic nevi and primary melanoma in clinical patient samples after TMA staining. (B) ERRFI1 mRNA expression levels in NHM, NC, and melanoma cell lines were quantified with RT-PCR. (C) Comparison of ERRFI1 mRNA expression between primary and metastatic melanoma from datasets from the TCGA database. (D) Kaplan-Meier plot comparing overall survival of melanoma patients with high or low intratumoral ERRFI1 expression. (E) Venn diagram depicting the number of overlapping genes differentially expressed between melanoma (Tumor) to melanocytes (Normal) from two GSE datasets. (F) Heatmap generated from two GSE datasets showing the common top 20 dysregulated genes between melanoma cells and melanocytes. (G) GO_BP analysis of 155 genes commonly up- or downregulated (from GSE130244 and GSE111766) with the threshold being set to an absolute fold change of $\geqslant$2 and p<0.05.

4.3 ERRFI1 expression is significantly decreased in ERRFI1 KD cells

To examine the influence of ERRFI1 on melanoma progression and resistance to BRAFi, I employed two different siRNAs to knock down (KD) its expression in melanoma cell lines (HT144, SK-MEL-28, WM9). RT-PCR and western blot analyses 48 h upon transfection confirmed the successful KD of ERRFI1 (Figure 3A, 3B).

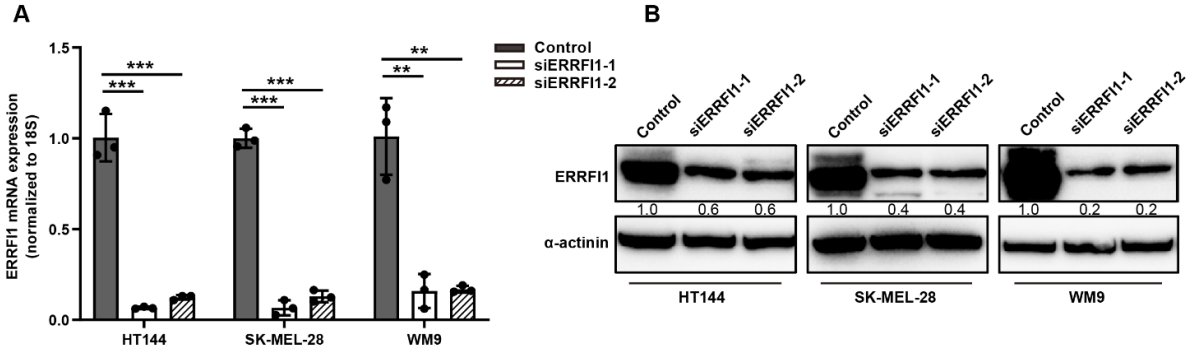

Figure 3. Validation of the ERRFI1 KD upon transfection of melanoma cells with specific siRNA

(A) RT-PCR analysis of ERRFI1 mRNA expression was performed in three melanoma cell lines upon transfection with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). (B) Western blot showing ERRFI1 expression in HT144, SK-MEL-28, and WM9 transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). The experiments were repeated at least three times. Results are shown as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed unpaired Student´s t-test; $^{*}p<0.05$, $^{**}p<0.01$.

4.4 KD of ERRFI1 promotes melanoma cell differentiation

Consistent with our previous findings (Figure 1), ERRFI1 KD increased SOX10 expression and decreased AXL expression across all tested cell lines relative to their control counterparts (Figure 4). As previously noted, SOX10 is closely linked to melanoma cells' differentiation status and drug resistance. Cells exhibiting low ERRFI1 expression show high SOX10 expression, highlighting the critical role of ERRFI1 in driving melanoma cell dedifferentiation and drug resistance. Consistently, decreased AXL expression coupled with elevated SOX10 expression further indicates that ERRFI1 KD cells may show higher sensitivities towards BRAFi than control cells.

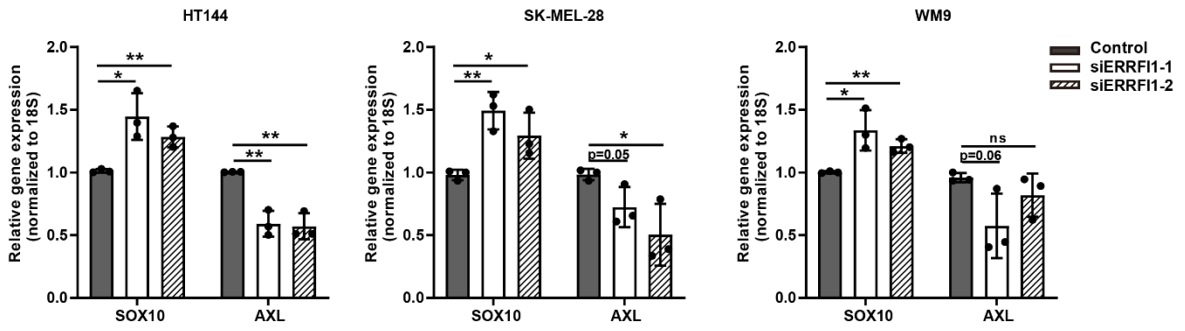

Figure 4. ERRFI1 knockdown triggers the upregulation of SOX10 expression and the downregulation of AXL expression

RT-PCR analysis of SOX10 and AXL mRNA expression was conducted in three melanoma cell lines transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). The experiments were repeated at least three times. Results are shown as mean ± SD from three independent experiments. Statistical significance was performed using a two-tailed unpaired Student´s t-test. Significance is indicated as $^{*}p<0.05$, $^{**}p<0.01$.

4.5 KD of ERRFI1 impairs melanoma cell proliferation

To explore the connection between reduced ERRFI1 expression and melanoma pathogenesis, I explored whether decreased levels of ERRFI1 affect melanoma cell proliferation. To do this, the proliferative capacity of melanoma cells was quantified using the BrdU cell proliferation assay. As shown in Figure 5, ERRFI1 KD melanoma cells exhibited a significantly lower proliferation rate compared to the control.

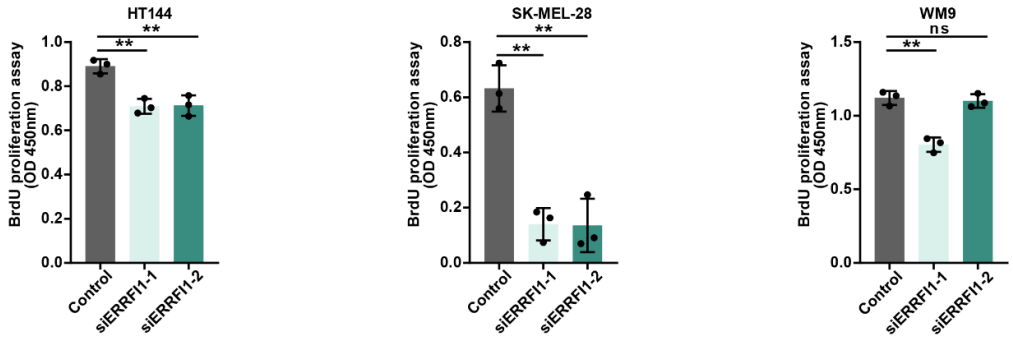

Figure 5. Effect of ERRFI1 on cell proliferation in melanoma cells

BrdU cell proliferation assays were conducted in three melanoma cell lines. Cells were transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control) for 48 h then seeded in a 96-well plate (1-2x10^4 cells/well). BrdU was added for 20 h and then measured using a colorimetric-based ELISA with a TECAN Infinite M1000 PRO microplate reader at a 450/550 nm dual wavelength. The experiments were repeated at least three times. Data are shown as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed unpaired Student´s t-test; $^{*}p<0.05$, $^{**}p<0.01$.

4.6 ERRFI1 KD reduces the colony-forming capacity of melanoma cells and sensitizes them to BRAFi treatment

The correlation between high intratumoral ERRFI1 expression and reduced patient survival prompted me to determine if ERRFI1 KD influences the tumorigenic characteristics of melanoma cells. To pursue this specific objective, a colony formation assay was performed. Melanoma cells were transfected with ERRFI1 siRNA and then treated with either 10 µM DMSO (Control group: -Vem) or 10 µM Vem (+Vem) for 24 h. The ERRFI1 KD group that was not treated with Vem already showed a reduced number of colonies compared to the control group, suggesting that decreased ERRFI1 expression affected the colony-forming capacity of the melanoma cells. Interestingly, ERRFI1 KD in combination with BRAFi treatment reduced the colony-forming capacity even more indicating that ERRFI1 KD sensitized the melanoma cells to the BRAFi (Figure 6A, 6B). Notably, HT144 cells transfected with siERRFI1-1 were unable to form colonies, probably due to the pivotal role of ERRFI1 in cell proliferation and survival.

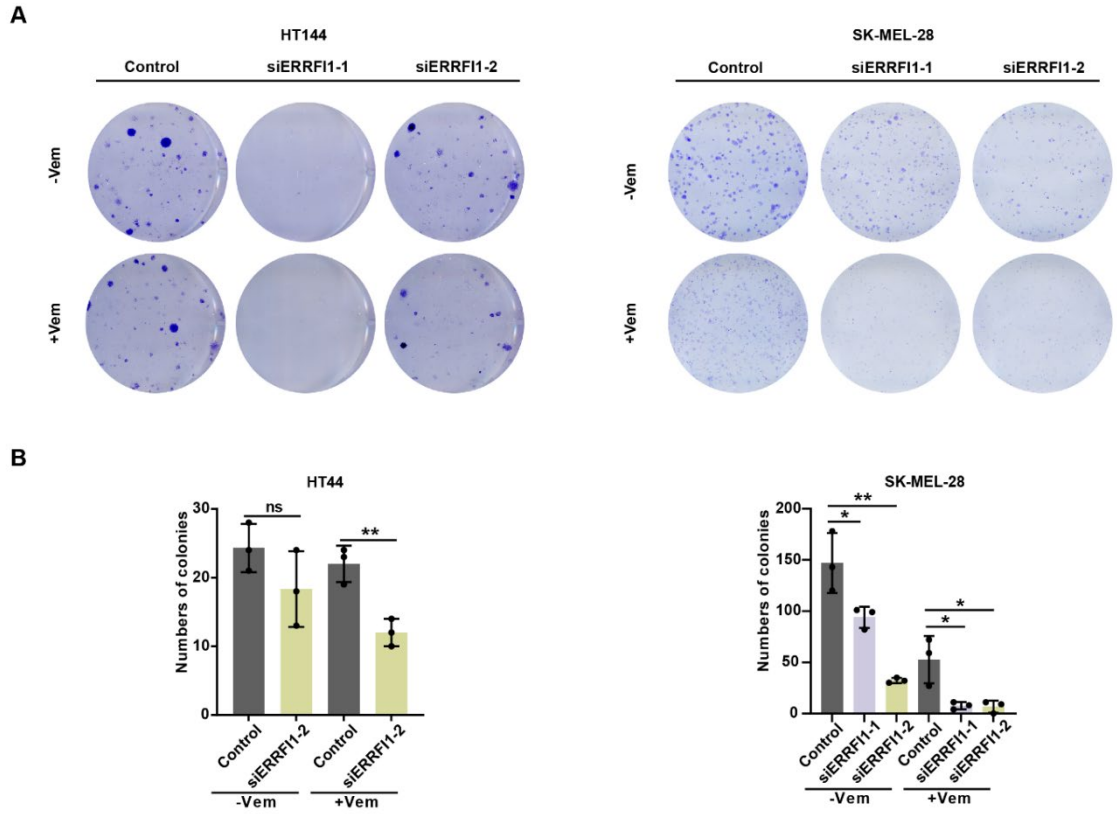

Figure 6. ERRFI1 KD decreases the colony-forming capacity of melanoma cells and sensitizes them toward BRAFi treatment

Colony formation assays were performed with HT144 and SK-MEL-28 cells. Control and ERRFI1 KD melanoma cells were seeded in 6-well plates (0.5-2x10^3 cells/well). Each well was treated with DMSO or Vem (10 µM) for 24 h before changing to fresh medium. Medium was changed twice a week. Surviving cells were cultured for 10-14 days and stained with crystal violet. (A) Representative figures of colony formation assays ± Vem (10 µM) treatment. (B) Quantifying the colony-forming capacity of ERRFI1 KD and control melanoma cells treated with DMSO or Vem (10 µM). The experimental results are presented as the mean ± SD and all experiments were repeated at least three times. Statistical significance was performed using a two-tailed unpaired Student´s t-test. Significance is indicated as $^{*}p<0.05$, $^{**}p<0.01$.

4.7 KD of ERRFI1 shows reduced viabilities towards BRAFi treatment compared to control cells

Next, I compared the cell viability of ERRFI1 KD and control melanoma cells that were additionally treated with gradually increasing concentrations of Vem. As depicted in Figure 7, ERRFI1 KD melanoma cells displayed a much lower cell viability compared with control cells. Moreover, I could demonstrate that ERRFI1 KD significantly lowered the IC50 value of Vem for each cell line tested. These results suggest that ERRFI1 KD sensitized melanoma cells to BRAFi treatment.

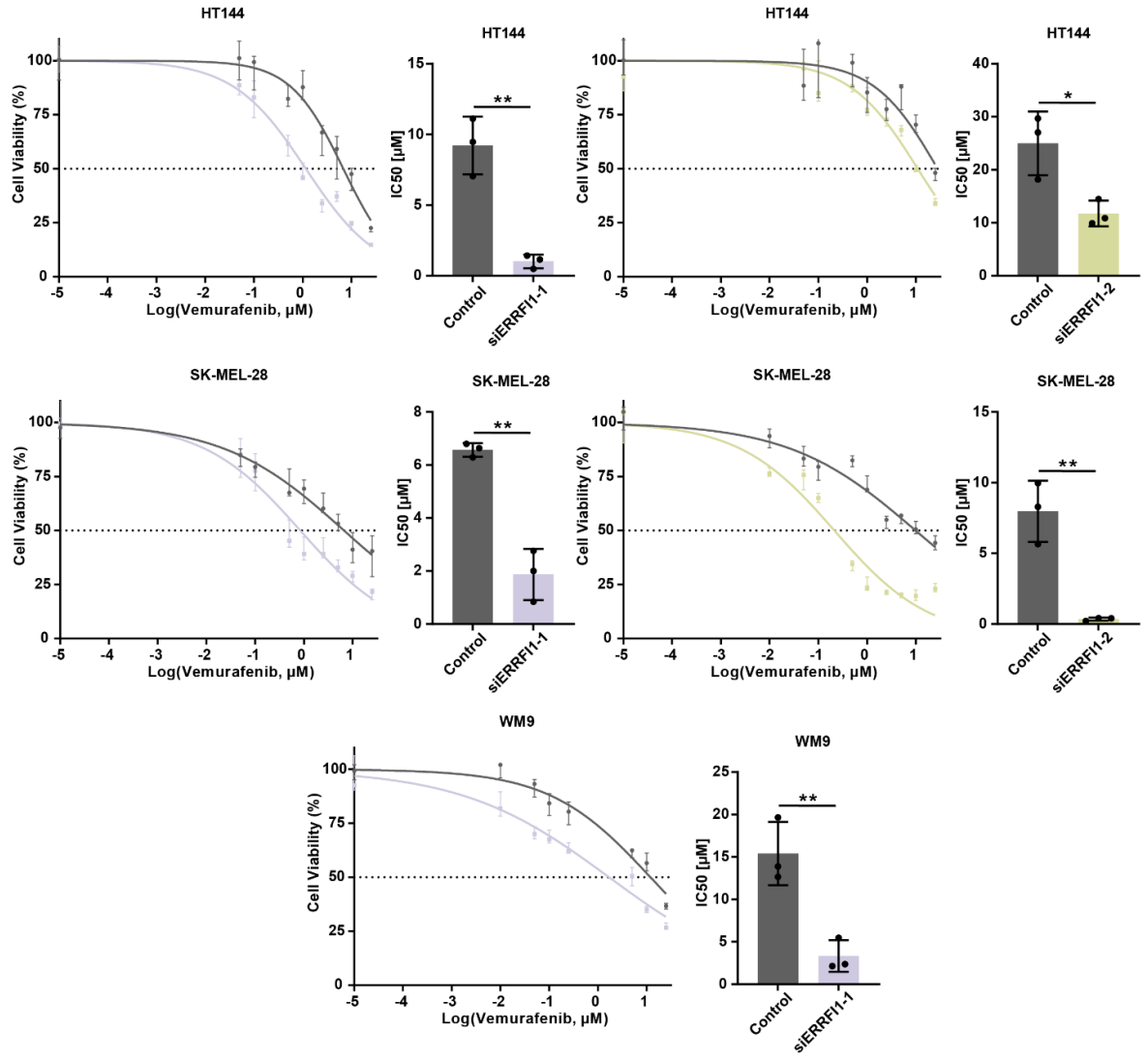

Figure 7. ERRFI1 KD cells are more sensitive toward BRAFi treatment

Cell viability assays were performed with three melanoma cell lines. Cells were transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). 48 h after transfection, 5x10^3 cells were seeded per well of a 96-well plate and treated with Vem concentrations ranging from 0.0001 to 25 µM. Cell viability was measured using the alamar blue assay. Experiments were repeated at least three times independently. Results are displayed as mean ± SD. Statistical significance was determined using a two-tailed unpaired Student´s t-test; $^{*}p<0.05$, $^{**}p<0.01$.

4.8 KD of ERRFI1 increases the apoptosis of melanoma cells to BRAFi

Having shown that ERRFI1 KD sensitized melanoma cells to BRAFi treatment, I wanted to examine if the reduced cell viability of ERRFI1 KD melanoma cells is connected to an increased apoptotic rate of these cells. For this reason, I performed apoptosis assays using ERRFI1 KD melanoma cells that were treated with 10 µM DMSO or 10 µM Vem for 48 h. In this assay, apoptotic cells were specifically labeled with annexin

V-FITC and PI and quantified with a flow cytometer. The combined proportion of early and late apoptotic cells was calculated. As depicted in Figure 8, a significantly higher proportion of apoptotic melanoma cells was found in the ERRFI1 KD group compared with the control group upon treatment with Vem (Figure 8). These results demonstrate that silencing ERRFI1 sensitizes melanoma cells to BRAFi, highlighting its potential therapeutic target for melanoma treatment.

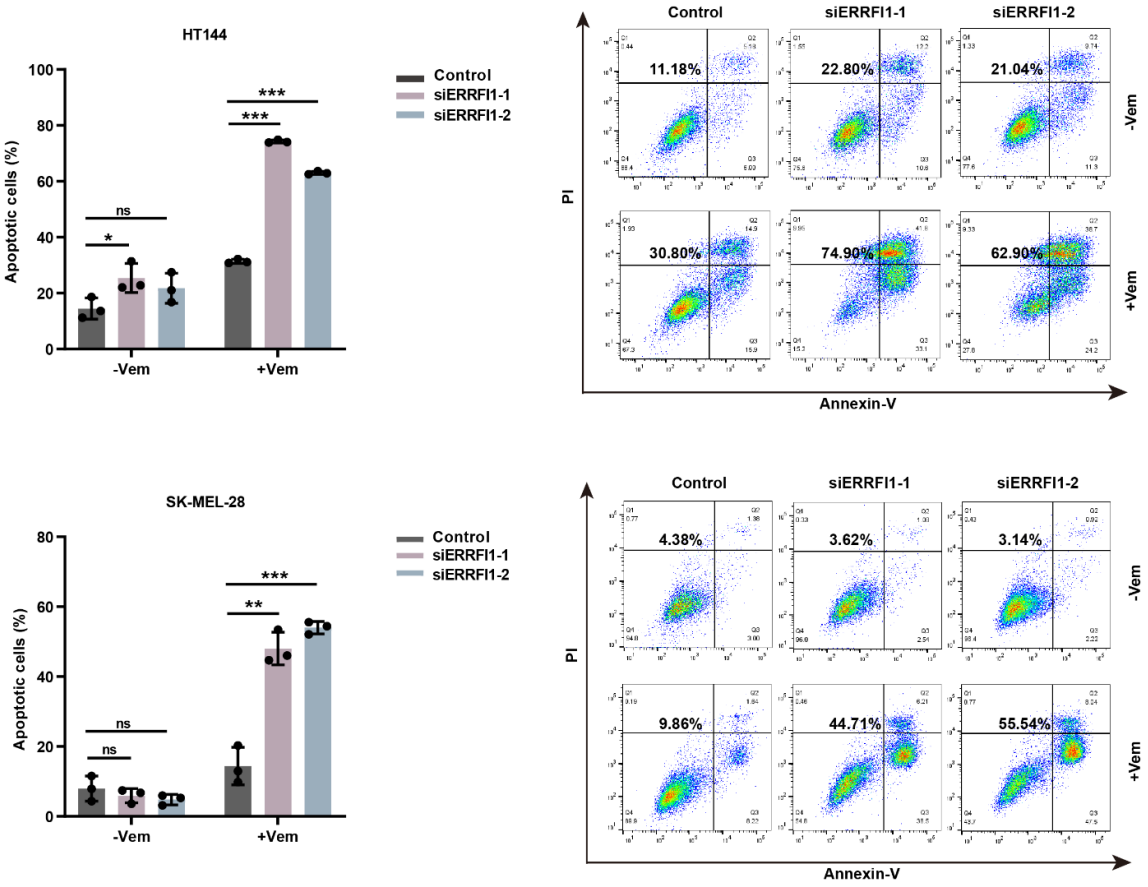

Figure 8. KD of ERRFI1 sensitizes melanoma cells to BRAFi

Apoptosis assays were conducted with HT144 and SK-MEL-28 cells. Cells were transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). 48 h after transfection, the cells were treated with 10 µM DMSO or 10 µM Vem for another 48 h. The proportion of apoptotic cells was measured using an annexin V assay. Left: Quantification of apoptotic cells with annexin V/PI staining. Right: Representative flow cytometry scatter plots. Q1: Necrosis, Q2: Late apoptosis, Q3: Early apoptosis, Q4: Viability. The combined proportion of early and late apoptotic cells was calculated and displayed. The experiments were repeated at least three times. Results are shown as mean ± SD from three independent experiments. Statistical evaluation was performed using a two-tailed unpaired Student´s t-test. Significance is indicated as $^{*}p<0.05$, $^{**}p<0.01$.

4.9 ERRFI1 expression is upregulated in BRAFi-resistant melanoma cells

Based on the results above, ERRFI1 appears to be a significant factor influencing the effectiveness of targeted therapy. To examine the role of ERRFI1 concerning the sensitivity of melanoma cells to BRAFi in more detail, I first compared the expression level of ERRFI1 between BRAFi-sensitive (parental) and BRAFi-resistant melanoma cells. As shown in Figure 9A and 9B, both mRNA and protein levels were elevated in the BRAFi-resistant melanoma cells, further emphasizing the role of ERRFI1 in promoting resistance.

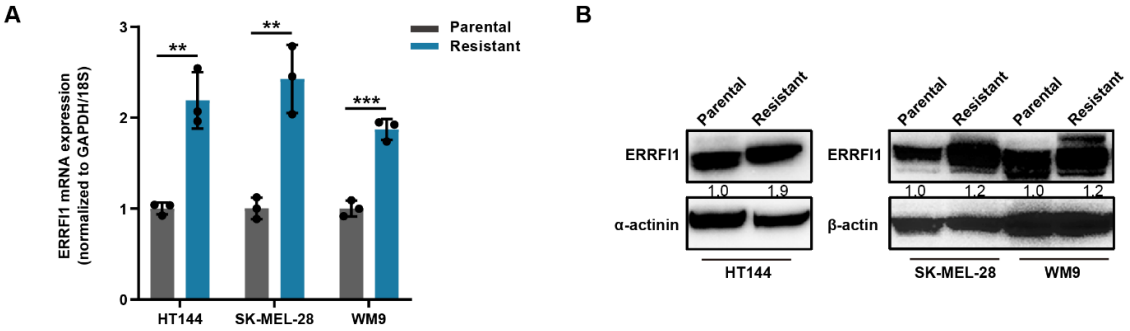

Figure 9. The expression of ERRFI1 is upregulated in BRAFi-resistant melanoma cells

(A) ERRFI1 expression was quantified with RT-qPCR analysis and compared between BRAFi-resistant and -sensitive HT144, SK-MEL-28, and WM9 cells. (B) Western blot analysis of ERRFI1 expression in BRAFi-resistant and -sensitive HT144, SK-MEL-28, and WM9 cells. The experiments were repeated at least three times. Results are shown as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed unpaired Student´s t-test; $^{*}p<0.05$, $^{**}p<0.01$.

4.10 ERRFI1 KD impairs the proliferation of BRAFi-resistant melanoma cells

I could show that ERRFI1 plays a fundamental role in regulating the sensitivity of melanoma cells to BRAFi treatment. In addition, I found that all three Vem-resistant melanoma cell lines utilized in this study exhibited elevated ERRFI1 expression levels compared to their non-resistant parental counterparts. Next, I investigated whether ERRFI1 can influence the proliferation of these BRAFi-resistant melanoma cells. For this, ERRFI1 was knocked down in the BRAFi-resistant cells (HT144-R, SK-MEL-28-R, WM9-R), and the successful KD was confirmed on mRNA and protein level (Figure 10A, 10B). Afterwards, these ERRFI1 KD cells were used for a BrdU proliferation assay. Figure 10C illustrates that a significantly decreased cell proliferation was measured for the ERRFI1 KD groups compared to control groups, suggesting that ERRFI1 is essential for the proliferation of BRAFi-resistant melanoma cells.

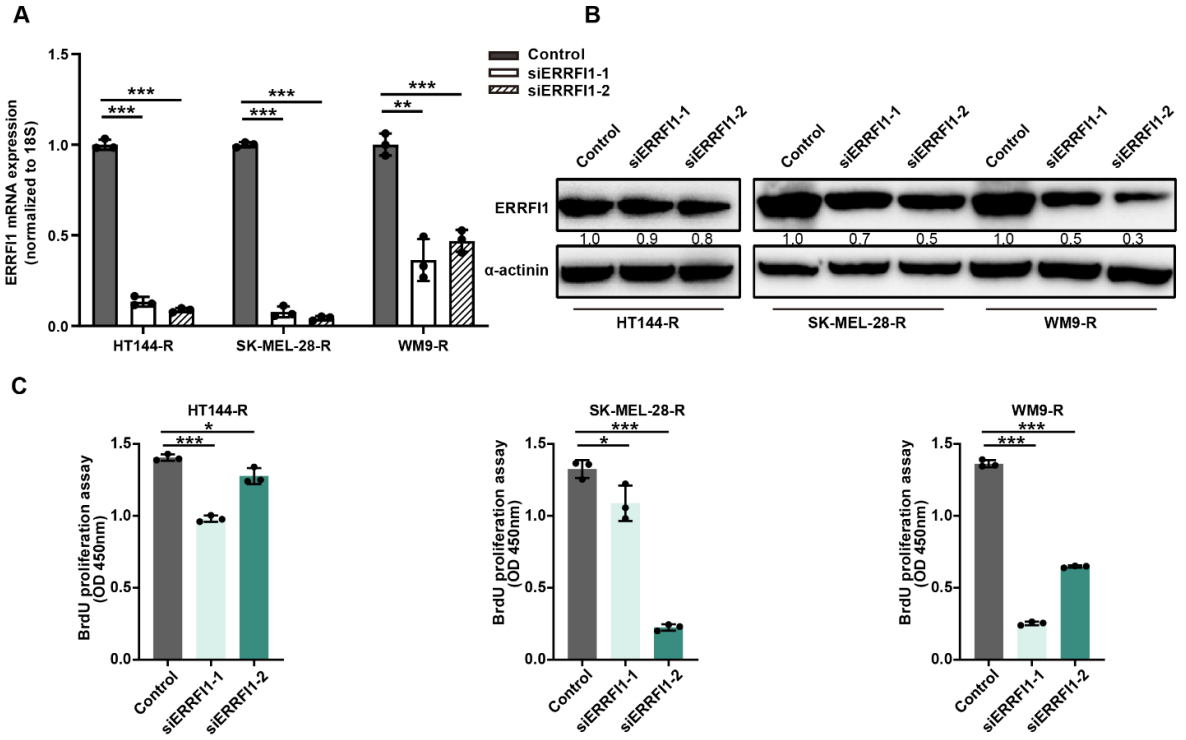

Figure 10. KD of ERRFI1 inhibits the proliferation of BRAFi-resistant melanoma cells

(A) Quantification of ERRFI1 mRNA expression by RT-PCR analysis in three BRAFi-resistant melanoma cell lines upon transfection with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). (B) Western blot analysis of ERRFI1 expression in BRAFi-resistant HT144-R, SK-MEL-28-R, and WM9-R cells upon transfection with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). (C) BrdU assays with BRAFi-resistant melanoma cell lines. Cells were transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). After 48h, 1-2x10^4 cells were seeded per well of a 96-well plate. Cells were incubated with BrdU for 20 h followed by a colorimetric-based ELISA analyzed with a TECAN Infinite M1000 PRO microplate reader at a 450/550 nm dual wavelength. The experiments were repeated at least three times. Data are shown as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed unpaired Student´s t-test; $^{*}p<0.05$, $^{**}p<0.01$.

4.11 KD of ERRFI1 resensitizes BRAFi-resistant melanoma cells to BRAFi

I could already demonstrate that the KD of ERRFI1 significantly increased the sensitivity of melanoma cells to the BRAFi Vem. In order to assess the extent of the connection between ERRFI1 expression and sensitivity to BRAFi, I knocked down ERRFI1 in BRAFi-resistant melanoma cells and performed cell viability assays with these cells versus the control with unaltered ERRFI1 expression. After knocking down ERRFI1, the cells were treated with different concentrations of Vem for 48 h. At a Vem concentration of 2.5 µM, a significant decrease in cell viability was observed in the ERRFI1

KD groups of two out of the three BRAFi-resistant melanoma cell lines. Moreover, treatment with increasing concentrations (5 µM, 10 µM) of Vem resulted in a dose-dependent decrease of cell viability of BRAFi-resistant melanoma cells. From this experiment, one can conclude that the KD of ERRFI1 resensitized BRAFi-resistant melanoma cells to the inhibitor.

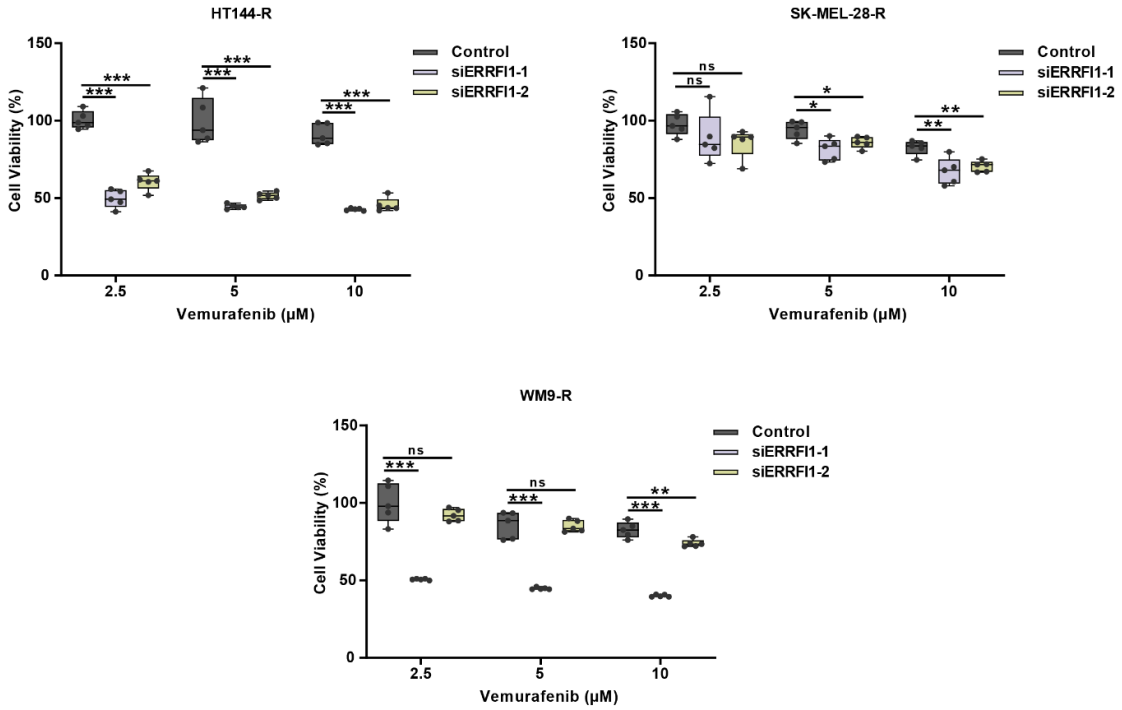

Figure 11. KD of ERRFI1 resensitizes BRAFi-resistant melanoma cells to BRAFi

Cell viability assays of ERRFI1 KD BRAFi-resistant melanoma cells and their respective control groups. Cells were transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). After 48 h, $5x10^3$ cells were seeded per well of a 96-well plate and treated with different concentrations of Vem for 48 h. Cell viability was measured using the alamar blue assay. Experiments were repeated at least three times independently. Statistical analysis was performed using a two-tailed unpaired Student´s t-test. Significance is indicated as $^{*}p<0.05$, $^{**}p<0.01$.

4.12 KD of ERRFI1 in BRAFi-resistant melanoma cells leads to an increased apoptotic rate in response to BRAFi treatment

As shown above, ERRFI1 KD resensitized BRAFi-resistant melanoma cells to BRAFi treatment. To further confirm this observation, I performed apoptosis assays with the BRAFi-resistant melanoma cells upon knocking down ERRFI1. As anticipated, the results from the apoptosis assays confirmed the previously demonstrated resensitization by detecting elevated apoptosis rates for ERRFI1 KD cells treated with 10 µM Vem.

This trend was consistent for all cell lines tested (Figure 12). My findings reveal that BRAFi-resistant melanoma cell lines displayed a significant upregulation of ERRFI1 expression. The KD of ERRFI1 re-sensitized BRAFi-resistant melanoma cells to BRAFi treatment.

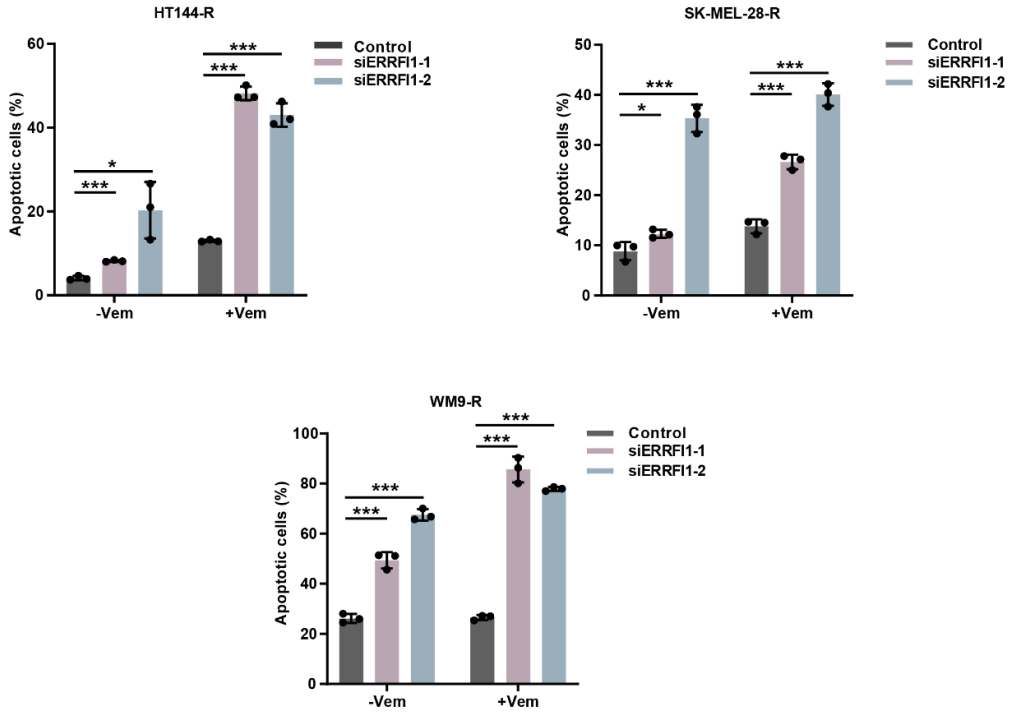

Figure 12. ERRFI1 KD in BRAFi-resistant melanoma cells results in an increased apoptotic rate in response to BRAFi treatment

Apoptosis assays with ERRFI1 KD BRAFi-resistant melanoma cells and their respective control groups. Cells were transfected with two different siRNAs targeting ERRFI1 (ERRFI1 KD) or a non-targeting siRNA (control). 48 h after transfection, the cells were treated with 10 µM DMSO or 10 µM Vem for 48 h. The percentage of apoptotic cells was quantified using an annexin V assay. The combined proportion of early and late apoptotic cells was calculated. The experiments were repeated at least three times. Results are shown as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed unpaired Student´s t-test; $^{*}p<0.05$, $^{**}p<0.01$.

4.13 ERRFI1 KD prevents the reactivation of ERK and AKT signaling pathways in melanoma cells

In the current study, I was able to show that ERRFI1 expression was closely linked to the sensitivity of melanoma cells to BRAFi treatment. This, of course, brought up the question of the molecular mechanisms behind this phenomenon. To address this question, proteomics analysis was employed to uncover differences in global protein expression between ERRFI1 KD and control cells. ERRFI1 KD and control groups from

three melanoma cell lines (HT144, SK-MEL-28, WM9) were analyzed using mass spectrometry. Principal Component Analysis (PCA) demonstrated tightly clustered quadruplicate samples within each group (Figure 13A). A thorough examination utilizing a Venn diagram successfully identified a total of 6,411 proteins that were differentially expressed across all three melanoma cell lines (Figure 13B). Furthermore, a comprehensive circular heatmap highlighted the top 100 dysregulated proteins, clearly visualizing the protein expression alterations. This approach facilitates a deeper understanding of the molecular changes involved (Figure 13C).

In the HT144 cell line, 183 proteins exhibited differential expression, characterized by fold changes greater than 1 or less than -1, coupled with statistically significant values ($p<0.05$). These differentially expressed proteins were prominently enriched in several crucial BP, including cell differentiation, signal transduction, and the regulation of key signaling pathways such as MAPK and PI3K-AKT. The data were effectively illustrated in a detailed heatmap to display these findings visually (Figure 13D). KEGG pathway analysis effectively elucidated significant alterations in several important cellular pathways, including ERBB signaling, p53 signaling, focal adhesion, and PI3K-AKT signaling pathways (Figure 13E). In particular, the analysis detected an upregulation of key regulatory proteins, including CDKN1A and PHLPP1, within the PI3K-AKT pathway (Figure 13F). The elevated expression levels of these regulators indicate an inhibition of the PI3K-AKT signaling pathway, suggesting a potential mechanism that could be targeted for therapeutic purposes. Gene Set Enrichment Analysis (GSEA) further confirmed the inactivation of specific pathways in samples where ERRFI1 was knocked down (Figure 13G). This analysis provided robust support for the observed molecular changes, demonstrating a significant downregulation of the activity of ERK and AKT as a direct consequence of ERRFI1 suppression.

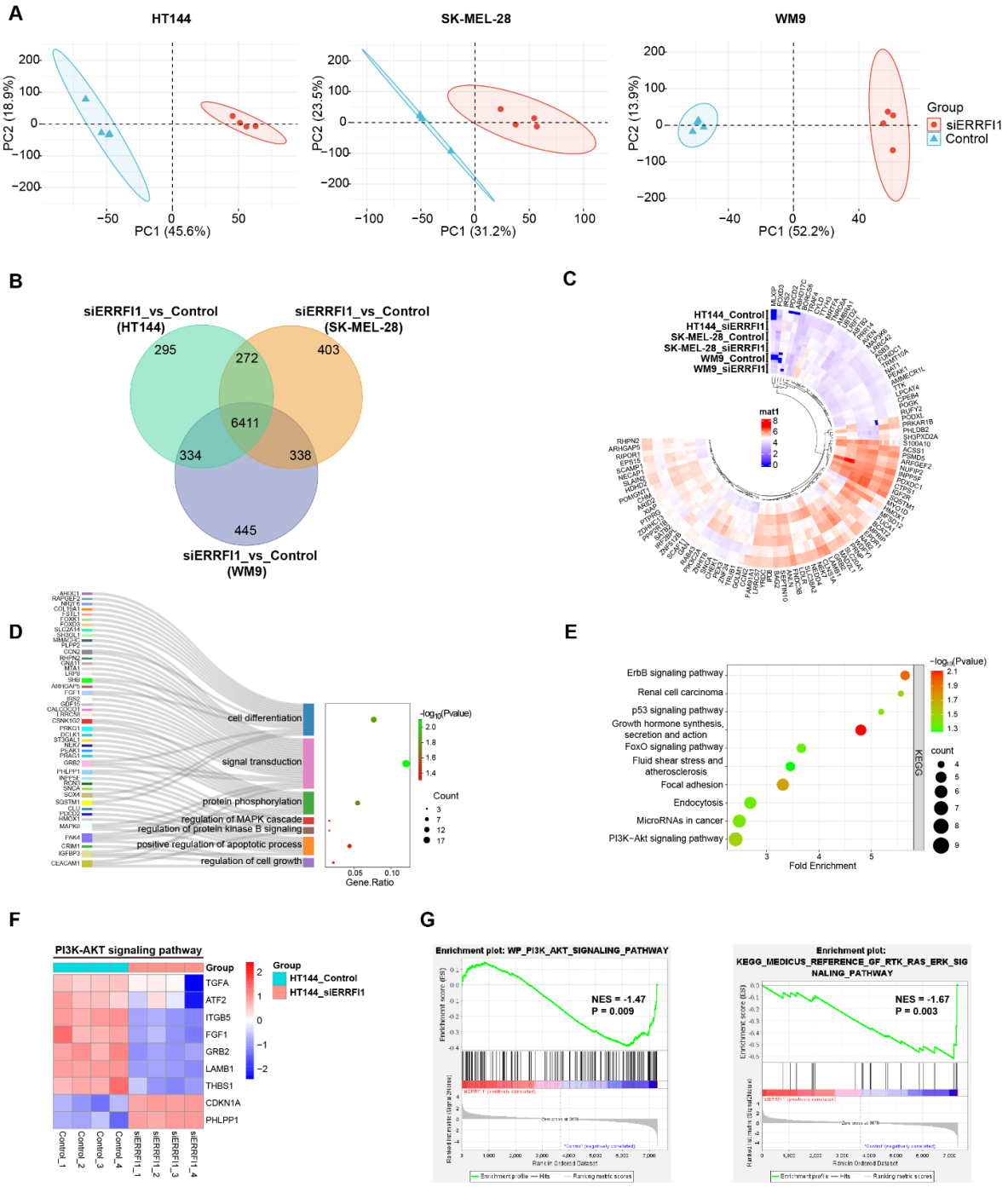
A
HT144
SK-MEL-28
WM9
PC2 (18.9%)
PC1 (45.6%)
PC2 (23.5%)
PC1 (31.2%)
PC2 (13.9%)
PC1 (52.2%)
Group
siERRFI1
Control
B
siERRFI1_vs_Control (HT144)
siERRFI1_vs_Control (SK-MEL-28)
siERRFI1_vs_Control (WM9)
295
272
403
6411
334
338
445
C
HT144_Control
HT144_siERRFI1
SK-MEL-28_Control
SK-MEL-28_siERRFI1
WM9_Control
WM9_siERRFI1
mat1
D
cell differentiation
signal transduction
protein phosphorylation
regulation of MAPK cascade
regulation of protein kinase B signaling
positive regulation of apoptotic process
regulation of cell growth
-log10(Pvalue)
Count
Gene.Ratio
E
ErbB signaling pathway
Renal cell carcinoma
p53 signaling pathway
Growth hormone synthesis, secretion and action
FoxO signaling pathway
Fluid shear stress and atherosclerosis
Focal adhesion
Endocytosis
MicroRNAs in cancer
PI3K−Akt signaling pathway
KEGG
count
Fold Enrichment
F
PI3K-AKT signaling pathway
Group
TGFA
ATF2
ITGB5
FGF1
GRB2
LAMB1
THBS1
CDKN1A
PHLPP1
Control_1
Control_2
Control_3
Control_4
siERRFI1_1
siERRFI1_2
siERRFI1_3
siERRFI1_4
HT144_Control
HT144_siERRFI1
G
Enrichment plot: WP_PI3K_AKT_SIGNALING_PATHWAY
NES = -1.47
P = 0.009
Enrichment score (ES)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
Enrichment plot: KEGG_MEDICUS_REFERENCE_GF_RTK_RAS_ERK_SIGNALING_PATHWAY
NES = -1.67
P = 0.003

Figure 13. ERRFI1 KD prevents the activation of ERK and AKT signaling pathways

(A) PCA plot showing differences between ERRFI1 KD and control samples of HT144, SK-MEL-28, and WM9 cells. (B) Venn diagram depicting the number of overlapping proteins differentially expressed in three melanoma cell lines. (C) Circulation heatmap and hierarchical clustering of differentially expressed proteins in three melanoma cell lines between control and ERRFI1 KD groups (threshold was set with an absolute fold change of >0.5 and p<0.05). (D) GO analysis of 183 differentially expressed proteins (FC>|1|, P<0.05 in HT144 cells) and their involvement in BP function. (E) KEGG analysis shows the top altered pathway after ERRFI1 KD with 183 genes identified in HT144 cells. (F) The DEGs from the KEGG PI3K-AKT signaling pathway were shown. (G) GSEA analysis of mass spectrometry data revealed that MAPK signaling was significantly associated with ERRFI1.

To further confirm the results above, the protein expression levels of key markers of the MAPK and AKT pathway were examined by using western blot. I found that p-ERK and p-AKT expression levels were diminished in the ERRFI1 KD group compared to the control group, indicating that the MAPK and AKT pathways were suppressed upon ERRFI1 silencing (Figure 14).

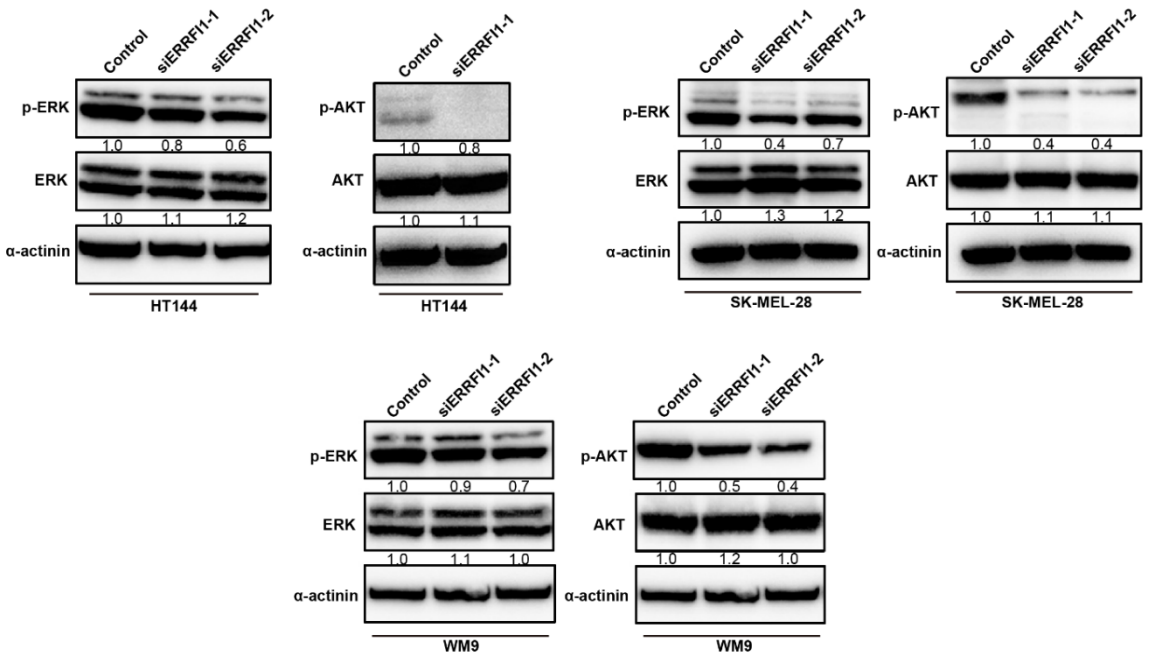

Figure 14. Western blot analysis of ERK and AKT expression after ERRFI1 silencing

Comparison of the protein levels of p-ERK, ERK, p-AKT and AKT in three melanoma cell lines between control and ERRFI1 KD cells. Protein quantification was performed using Image Lab software. Densitometric values were normalized to the loading controls α-actinin. All samples are derived from the same gel/blot and the α-actinin is the loading control for both panels (shown twice).

By performing a protein-protein interaction network analysis, I identified growth factor receptor-bound protein 2 (GRB2) as a hub protein affecting PI3K-AKT signaling (Figure 15). GRB2 is an adaptor protein that plays a crucial role in cell communication, intracellular signal transduction, and cell proliferation, and that contributes to tumor migration, invasion, and metastasis[96-98]. Furthermore, it links various surface receptors to downstream pathways, such as the Ras/MAPK signaling pathway[99-101]. Hao and colleagues demonstrated that GRB2 has a regulatory effect on ERK1/2 pathway, further influencing the proliferation of melanoma cells[102]. Pu et al. elucidated that Hey1 promotes migration and invasion of melanoma cells through the GRB2/PI3K/AKT pathway[103]. ERRFI1 is a typical adapter/scaffold protein, including various protein-protein interaction domains. Previous studies have shown that its SH3 binding domain may regulate the binding of ERRFI1 to some important signaling proteins containing the SH3 domain, of which GRB2 is one[66, 104]. However, the specific interaction between GRB2 and ERRFI1 in regulating melanoma resistance represents a vital area of interest that warrants further investigation to fully elucidate the underlying mechanisms and potential therapeutic implications.

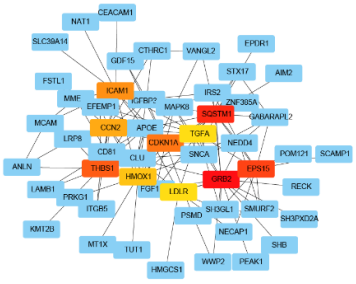

Figure 15. Protein-protein interaction network analysis

By utilizing protein-protein interaction network analysis, GRB2 was identified as a key hub protein among the 183 proteins differentially expressed between ERRFI1 KD and control melanoma cells.

4.14 miR-200c inhibits the expression of ERRFI1 and increases the sensitivity of BRAFi-resistant melanoma cells to BRAFi

MicroRNAs (miRNAs) are small noncoding nucleotide sequences that regulate gene expression by inhibiting protein translation and promoting the degradation of messenger RNA (mRNA). Increasing evidence suggests that altered expression levels of miRNAs trigger drug-resistance in tumor cells. Conversely, normalizing dysregulated miRNAs could reinstate drug-sensitivity in tumor cells. The bioinformatic tools TargetScan

and miRDB were used to conduct predictive analyses, identifying miR-200c as a tumor-suppressive miRNA targeting the ERRFI1 gene (Figure 16A).

Notably, miR-200c is downregulated in melanoma relative to nevi and has been associated with decreased drug resistance. Following transfection with miR-200c mimics, the expression level of miR-200c was substantially increased (Figure 16B). Consequently, the mRNA level of ERRFI1 was reduced in HT144-R and SK-MEL-28-R cells, and the protein level of ERRFI1 was decreased in HT144-R (Figure 16C). Next, I performed cell viability assays with BRAFi-resistant cells treated with Vem upon transfection with miR-200c. These cells showed an increased sensitivity to BRAFi (Figure 16D), indicating that the miR-200c-ERRFI1 axis could enhance the effectiveness of targeted therapy in melanoma.

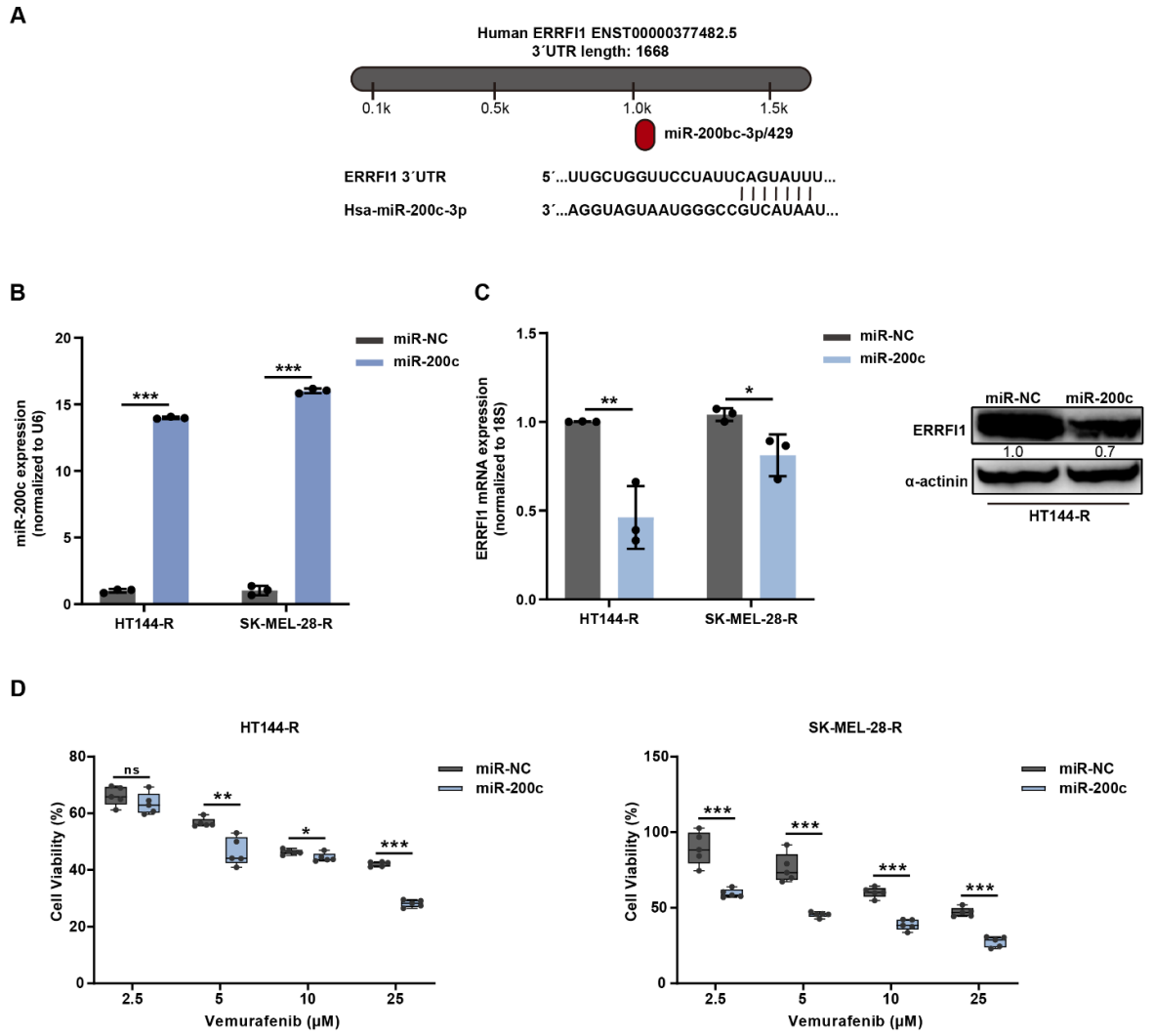

Figure 16. miR-200c inhibits the expression of ERRFI1 and increases the sensitivity of BRAFi-resistant melanoma cells to BRAFi

(A) ERRFI1 was predicted as a target of miR-200c using TargetScan and microRNA database. (B) RT-qPCR analysis of the expression of miR-200c was performed in HT144-R and SK-MEL-28-R cells 56h upon transfection with a miRNA mimic targeting miR-200c or a non-targeting miRNA mimic. (C) Analysis of mRNA and protein expression levels of ERRFI1 in BRAFi-resistant melanoma cells 56h upon transfection with a miRNA mimic targeting miR-200c or a non-targeting miRNA mimic. (D) Cell viability assays with BRAFi-resistant melanoma cells and their respective control groups. HT144-R and SK-MEL-28-R cells were transfected with miRNA mimic targeting miR-200c or a non-targeting miRNA mimic. 56 h later, the cells were seeded in a 96-well plate, then treated with different concentrations of Vem for 72 h. Cell viability was measured using the alamar blue assay. The experiments were repeated at least three times. Results are shown as mean ± SD from three independent experiments. Statistical analysis was performed using a two-tailed unpaired Student´s t-test. Significance is indicated as $^{*}p<0.05$, $^{**}p<0.01$.

5 DISCUSSION

5.1 The NC-associated gene ERRFI1 is crucial for melanoma progression

Cutaneous melanoma is a malignant neoplasm that originates from melanocytes. During embryonic development, melanocytic precursors arise from the NC[105]. In the embryo, NC cells derive from the ectoderm and migrate along defined paths toward their final location, where they differentiate into several cell types. During this process, the NC cells switch from an epithelial to a more migratory, mesenchymal phenotype[105-107]. The melanocytic precursor cells, also known as melanoblasts, proliferate and cross the basement membrane during their migration through the dermis, eventually reaching the epidermis's basal layer. Here, they differentiate into melanocytes and revert to an epithelial, non-migratory phenotype[108, 109].

A transcription factor network, including PAX3, MSX1, SOX10, and MITF, regulates NC formation, NC cell migration and melanocytic maturation. The NC-specific factor MSX1 is pivotal for NC induction as it induces the expression of early NC markers SLUG, SNAIL, PAX3, and FOXD3[110, 111]. Previous studies showed that MSX1 mediates dedifferentiation, promotes melanoma progression and induces phenotype switching[112, 113]. Specifically, Heppt et al. found that ectopic MSX1 expression can switch melanoma cells towards an invasive and metastasis-enhancing phenotype, and patients with high MSX1 expression show unsatisfied overall survival[112]. MITF is a well-researched master regulator of melanogenesis and is pivotal in transitioning from pluripotent NC cells to differentiated melanocytes. Carreira et al. demonstrated that high MITF expression is associated with a differentiated, proliferative phenotype, while low MITF expression can be connected to a stem cell-like, invasive phenotype[114]. SOX10 initiates before the migration period of NC cells and plays a fundamental role in the survival of migrating NC cells and their differentiation into melanocytes[115]. Furthermore, SOX10 is integral in melanoma development and phenotype switching, and its loss can enhance the stemness and invasiveness of melanoma cells[94, 95].

Melanoma cells and NC cells share many characteristic features. Melanoma is a highly lethal skin cancer characterized by high intratumoral heterogeneity due to a variety of tumor cell subpopulations expressing various gene signatures with different phenotypes, allowing it to adapt much faster to their environment. This adaptability is one of the reasons why melanomas often develop drug resistances[116, 117]. Heterogeneity is a

dynamic process that allows cells to switch back and forth between different phenotypes. The aggressiveness and resistance of melanoma is partly due to their origin and development during embryogenesis[118]. Numerous studies have demonstrated that the transcription factors involved in NC cells development and melanocyte formation are also expressed in melanoma and contribute to the plasticity of melanoma cells, further resulting in drug resistance[119]. In a prior study conducted by our group, we demonstrated that some NC-related genes are upregulated in melanoma cells but not in melanocytes. For instance, FOXD1 is an NC-associated gene, and the modulation of its expression affected melanoma invasion, migration, and resistance to targeted therapy[120, 121]. Moreover, a study from Sachindra and colleagues reported that the NC-associated gene ID3 regulates melanoma cell migration and promotes drug resistance[122].

ERRFI1 is a protein that is mainly located in the cytoplasm. Many studies have demonstrated that high expression of ERRFI1 affects certain characteristics of tumor cells, such as metastatic capacity and drug resistance. Our lab previously revealed that ERRFI1 is a NC-related gene upregulated in melanoma cells but not in melanocytes and correlates with poor prognosis[78]. The role of ERRFI1 in melanoma progression remains largely unclear, necessitating further investigation. In this study, I could show that ERRFI1 was highly expressed in melanoma cells, especially in BRAFi-resistant melanoma cells (Figure 2, Figure 9). Additionally, increased ERRFI1 expression correlated with a poor prognosis of melanoma patients, which is also in line with the previous study. Consequently, the results above suggest a pivotal role of ERRFI1 in melanoma progression.

5.2 ERRFI1 plays a pivotal role in melanoma phenotype switching and therapy resistance

Heterogeneity was observed in both primary melanoma and cutaneous metastases. MITF plays a key role in mediating melanoma intratumoral heterogeneity, plasticity, as well as phenotype switching[123]. Previous studies have confirmed the existence of $MITF^{high}$ and $MITF^{low}$ subpopulations within melanoma samples[123, 124]. In addition, it has been demonstrated that the cell state characterized by $MITF^{low}$ AXL^{high} shows an invasive phenotype and is associated with resistance to BRAFi and MEKi[18, 30, 125]. Besides, SOX10 acts as a direct upstream regulator of MITF and is also associated with the proliferative $MITF^{high}$ phenotype[124]. In my work, by analyzing datasets from the GEO and cBioportal database, I found that ERRFI1 expression positively correlated

with AXL expression but negatively with MITF and SXO10 expression (Figure 1). Besides, melanoma cell susceptibility towards BRAFi was detected when the expression of AXL was decreased, and the expression of SOX10 was increased (Figure 4, Figure 7). Additionally, by examining datasets from different databases (GSE130244, GSE111766, DFCI Science 2015), I found that cells with high ERRFI1 expression exhibited low expression of melanocytic differentiation markers such as TYR, DCT, MLANA (Figure 1). These findings provide evidence that ERRFI1 might promote the dedifferentiation of melanoma cells.

Numerous studies have shown that ERRFI1 acts as an oncoprotein and is highly expressed in many cancers. Moreover, the high expression of ERRFI1 correlates with poor clinical prognosis[73, 77, 79]. In this study, ERRFI1 was overexpressed in melanoma cell lines and clinical samples from melanoma patients compared to normal tissues and melanocytes (Figure 2). Our hypothesis that ERRFI1 has oncogenic functions in melanoma was further supported by the negative correlation between the expression level of ERRFI1 and the overall survival rates (Figure 1). Additionally, studies to date have demonstrated that ERRFI1 also plays an important role in the drug resistance of tumor cells. The expression of ERRFI1 was reported to be increased in gefitinib-resistant hepatic cancer cells compared to gefitinib-sensitive cells[86]. It was also observed to be highly upregulated in radio-resistant rectal cancer cells[87]. Besides, Kang et al. found that the downregulation of ERRFI1 resensitized drug-resistant lung cancer cells to EGFR-TKI treatments[73]. In my work, elevated ERRFI1 expression was detected in BRAFi-resistant melanoma cells (Figure 9). Furthermore, additional experiments indicated that downregulation of ERRFI1 enhanced the responsiveness of melanoma cells to BRAFi (Figure 6, Figure 7, Figure 8). Additionally, ERRFI1 silencing restored the sensitivity of BRAFi-resistant melanoma cells to BRAFi (Figure 11, Figure 12).

5.3 ERRFI1 enhances melanoma sensitivity by inhibiting ERK and AKT signaling pathways

The resistance mechanisms of melanoma to targeted therapy are complex and diverse. Multiple studies have indicated that the reactivation of p-ERK and p-AKT serve as the primary mechanisms for acquired resistance to targeted therapy in melanoma[126, 127]. In BRAF-mutated melanoma, the continuous activation of the MAPK pathway may initiate the progression of melanoma[128]. Vemurafenib belongs to the BRAFi and is recognized as the first-line small-molecule inhibitor for treating melanoma patients with BRAF mutation. However, melanoma patients quickly develop drug

resistance even though they have excellent responses to vemurafenib[61, 129]. Numerous potential resistance mechanisms to BRAFi alone or the combination therapy with MEKi have been suggested, such as reactivation of MAPK pathway, reactivation of PI3K/AKT pathway, persistent activation of RTKs, EGFR overexpression, and alterations in the tumor microenvironment[129-131].

In this study, I demonstrated that ERRFI1 was connected to BRAFi resistance, and I investigated the mechanism behind this resistance. I used mass spectrometry-based proteomic analysis to identify the commonly differentially expressed proteins between ERRFI1 KD and control cells for three BRAF-mutated melanoma cell lines (HT144, SK-MEL-28, WM9) (Figure 14). Next, the proteins selected by thresholding FC and p-value were further proceeded with GO-BP analysis. The results revealed that processes such as cell differentiation, signal transduction, and regulation of the MAPK and PI3K-AKT pathways were enriched after ERRFI1 KD (Figure 15). KEGG pathway analysis further highlighted alterations in the PI3K-AKT signaling pathway, with key regulators such as CDKN1A and PHLPP1 upregulated (Figure 15). Furthermore, I performed GSEA analysis and confirmed that ERK and AKT signaling were suppressed after ERRFI1 KD (Figure 15). Additionally, I examined the decreased expression level of p-ERK and p-AKT by western blot (Figure 15). Based on the above results, I conclude that ERRFI1 silencing promoted susceptibility towards BRAFi by diminishing the activation of ERK and AKT signaling.

5.4 miR-200c restores melanoma cell sensitivity to BRAFi by reducing the expression level of ERRFI1

Targeted and immune therapies have prolonged the overall and progression-free survival of melanoma patients significantly. However, these treatments face limitations due to the heterogeneity and phenotypic plasticity of melanoma cells, which lead to acquired resistance to BRAF and MEK inhibitors in melanoma patients[132]. MicroRNAs (miRNAs) are short noncoding nucleotide sequences that control gene expression by blocking protein translation and promoting messenger RNA (mRNA) degradation[133]. miRNAs have emerged as molecular regulators in melanoma development, and altered expression of miRNAs has been detected in various stages of melanoma progression[134-136]. Increasing evidence suggests that altered expression levels of miRNAs trigger drug resistance in tumor cells, and thus, normalizing dysregulated miRNAs could reinstate drug sensitivity in tumor cells[135]. miR-200c belongs to miR-200 family,

whose members can modulate EMT and resistance to chemotherapy[137-139]. Previous studies demonstrated that miR-200c expression is associated with cell proliferation, migration, self-renewal, cell survival, metastasis, as well as drug resistance[140-142]. Additionally, miR-200c was reported to be downregulated in melanoma, and reduced expression of miR-200c is usually associated with a poorer clinical outcome for patients with melanoma[143-145]. However, it is still not known how miR-200c mediates the drug sensitivity of melanoma cells.

In this study, I discovered that miR-200c regulated the expression of ERRFI1, thereby modulating melanoma cell sensitivity to BRAFi. Since many miRNAs play a critical role in cancer progression and drug resistance, I performed a target prediction using TargetScan and miRDB. I identified miR-200c as a tumor-suppressive miRNA targeting ERRFI1 (Figure 18). Notably, previous research has indicated that miR-200c is downregulated in melanoma relative to nevi and has been associated with decreased drug resistance[137, 140, 146]. To investigate this, I transfected BRAFi-resistant melanoma cells with miR-200c mimics, leading to a substantial overexpression of miR-200c (Figure 19A). Importantly, both the mRNA and protein levels of ERRFI1 were reduced after miR-200c overexpression, confirming the regulatory effect of miR-200c on ERRFI1 (Figure 19B). Additionally, miR-200c transfection of BRAFi-resistant melanoma cells also restored sensitivity to BRAFi (Figure 20). Considering these results, I conclude that the miR-200c-ERRFI1 axis represents a potential target for therapeutic intervention against melanoma.

In conclusion, my study reveals that ERRFI1 was upregulated in melanoma cells and contributed to melanoma progression. By using the KD approach, I demonstrated that ERRFI1 silencing modulated the reversible phenotype transition from a drug-sensitive, differentiated state to a drug-resistant, dedifferentiated phenotype, characterized by a downregulation of AXL as well as an upregulation of SOX10. Moreover, I showed that ERRFI1 KD increased the sensitivity of human melanoma cells towards targeted therapy by diminishing the activity of ERK and AKT signaling. Furthermore, I demonstrated that the miR-200c targeted the 3´UTR of ERRFI1 and reduced its expression, ultimately resulting in the resensitization of BRAFi-resistant melanoma cells to BRAFi. Consequently, I conclude that the miR-200c-ERRFI1 axis might be a potential target for treating melanoma as it regulates the susceptibility of melanoma cells toward targeted therapy.

6 SUMMARY

Melanoma is the most lethal type of skin cancer that originates from melanocytes. Targeted therapy, as one of the main therapeutic methods for melanoma, achieves great clinical efficiency at the beginning of treatment. However, drug resistance inevitably arises due to mechanisms such as the reactivation of the MAPK pathway. Our lab previously demonstrated that ERBB receptor feedback inhibitor 1 (ERRFI1), a neural crest-associated gene, is highly expressed in metastatic melanoma and correlates with poor prognosis. In this study, I validated that ERRFI1 expression was upregulated in melanoma and demonstrated that it positively correlated with AXL expression, but negatively correlated with SOX10 and MITF expression, as well as with melanocytic differentiation markers, including TYR, DCT, and MLANA. Downregulation of ERRFI1 increased the sensitivity of melanoma cells to vemurafenib (BRAF inhibitor). Furthermore, high ERRFI1 expression levels were found in BRAF inhibitor (BRAFi)-resistant cells. Loss of ERRFI1 resensitized BRAFi-resistant melanoma cells to vemurafenib. Mass spectrometry-based proteomic analysis between ERRFI1 knockdown (KD) and control samples revealed that silencing ERRFI1 inhibited the reactivation of ERK and AKT signaling pathways, which usually contribute to promoting drug resistance. Furthermore, miR-200c was identified as a tumor-suppressive microRNA that targeted the 3' UTR of ERRFI1, resulting in its downregulation. This also resensitized BRAFi-resistant melanoma cells to vemurafenib. This study highlights the critical role of ERRFI1 in melanoma progression. These findings suggest that ERRFI1 is a promising therapeutic target for treating melanoma and offers potential strategies for overcoming drug resistance.

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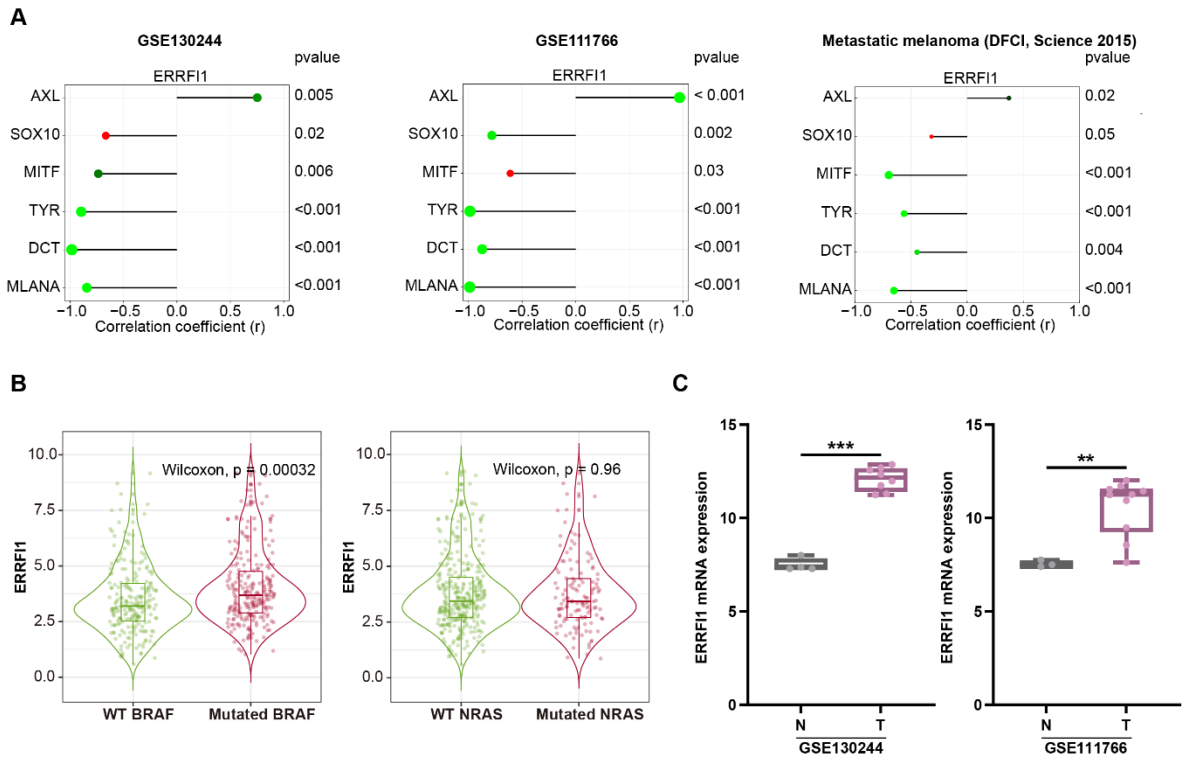
8 SUPPLEMENTARY MATERIALS

8.1 Supplementary figures



Supplementary Figure S1. ERRFI1 is upregulated in melanoma with BRAF mutation

(A) Detailed information about the Pearson correlation (r) and the corresponding p-value is shown. (B) Comparison of the expression level of ERRFI1 in BRAF- and NRAS-mutated melanomas compared to wild type (WT). (C) Comparison of the ERRFI1 mRNA expression between melanocytes (Normal) and melanoma (Tumor) from two independent datasets (GSE130244, GSE111766).

9 CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Nina Wang
Date of birth:	03.12.1992
Place of birth:	Zhejiang, China

SCHOOL CAREER

2007 – 2010	Zhejiang Xianju High School

UNIVERSITY CAREER

2010 – 2015	Kangda College of Nanjing Medical University Bachelor of Medicine
2015 – 2018	Shanghai Jiao Tong University Master of Clinical Medicine
02.12.2016	Physician license (China)
15.11.2018	Certificate of Resident Standardization Training (Pediatrics) (China)
Since SS2021	Doctoral Student, University of Heidelberg, Germany

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