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ERK5 is required for melanoma growth and is activated by oncogenic BRAF

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ABSTRACT

ERK5 is required for melanoma growth and is activated by oncogenic BRAF

Malignant melanoma is among the most aggressive cancers and its incidence is increasing worldwide. Although targeted therapies and immunotherapy have improved the survival of patients with metastatic melanoma in the last few years, available treatments are still unsatisfactory showing an urgent need to identify new therapeutic targets. While the role of BRAF-MEK1/2-ERK1/2 pathway in melanoma is well-established, the involvement of the MEK5-ERK5 signaling remains poorly explored. The Hedgehog signaling is an important pathway in melanoma, that has been shown to be required for growth, recurrence and metastasis of melanoma xenografts in mice. Several studies have shown that numerous oncogenic inputs positively modulate the activity of the HH pathway.

In this study, we investigated the function of ERK5 signaling in melanoma, its regulation by oncogenic BRAF and its interplay with the HH pathway. We show that ERK5 is consistently expressed and active in human melanoma cells. Genetic silencing of ERK5 and pharmacological inhibition of the MEK5-ERK5 pathway drastically reduce the growth of melanoma cells harboring wild type (wt) or mutated BRAF (V600E), *in vitro* and *in vivo*.

We also found that oncogenic BRAF positively regulates expression, phosphorylation and nuclear localization of ERK5. Importantly, BRAF enhances ERK5 kinase and transcriptional transactivator activities. Nevertheless, combined pharmacological inhibition of BRAF-V600E and MEK5 is required to decrease nuclear ERK5, that is critical for the regulation of cell proliferation. Accordingly, combination of MEK5 or ERK5 inhibitors with BRAF-V600E inhibitor Vemurafenib is more effective than single treatments in reducing the tumor growth of BRAF-V600E melanoma cells and xenografts.

Moreover, we have also identified the existence of an interplay between the HH pathway and ERK5. By chemical and genetic inhibition of ERK5, we demonstrate that ERK5 positively modulates the HH pathway, increasing transcriptional activity and protein levels of the GLIs transcription factors, the final effectors of HH signaling.

These data support a key role of ERK5 pathway for melanoma growth *in vitro* and *in vivo* and suggest that targeting ERK5, alone or in combination with BRAF-MEK1/2 inhibitors or HH pathway inhibitors, might represent a novel approach for melanoma treatment.

INDEX

ABBREVIATIONS 1

1. INTRODUCTION 6

1.1 Melanoma 6
1.1.1 Melanoma incidence and risk factors 6
1.1.2 Clinical classification and stadiation 7
1.1.3 Signaling pathways altered in melanoma 11
1.1.3.1 The RAS/RAF/MEK/ERK cascade 12
1.1.3.2 Additional pathways deregulated during melanoma progression 14
1.1.4 Targeted therapy in melanoma 16

1.2 The MEK5/ERK5 pathway 20
1.2.1 ERK5 structure and regulation 21
1.2.2 Role of ERK5 in physiological conditions 25
1.2.3 Role of ERK5 in cancer 26
1.2.4 ERK5 inhibitors: clinical relevance 29

1.3 The Hedgehog pathway 34
1.3.1 The GLI transcription factors and their regulation 35
1.3.2 The Hedgehog signaling in cancer 38
1.3.3 Modulation of HH-GLI signaling by oncogenic pathways 40
1.3.4 Crosstalk between HH-GLI and MAPK pathway in melanoma 42
1.3.5 Inhibitors of HH-GLI pathway 44

2. AIM OF THE STUDY 48

3. MATERIALS AND METHODS 49

3.1 Cell culture and melanoma patients samples 49
3.2 Drugs 50
3.3 Plasmids and transfections 50
3.4 Lentiviral vectors, virus production and transductions 51
3.5 Measurement of cell viability and cell cycle phase distribution analysis 52
3.6 Cell lysis, Western blotting and immunoprecipitations 52

3.7 Cell fractionation 55
3.8 ERK5 kinase assay 56
3.9 Luciferase assay 56
3.10 Xenografts 57
3.11 Statistical analysis 58

4. RESULTS 59

4.1 ERK5 is consistently expressed and active in human melanoma 59
4.2 ERK5 is required for melanoma cell proliferation *in vitro* and xenograft growth 62
4.2.1 Genetic inhibition of ERK5 reduces melanoma cells proliferation *in vitro* and *in vivo* 62
4.2.2 Pharmacological inhibition of ERK5 pathway decreases melanoma cell growth 65
4.3 Oncogenic BRAF increases ERK5 activity 70
4.4 The combination of Vemurafenib with ERK5 pathway inhibitors provides enhanced inhibition of melanoma cell growth compared to single treatments 76
4.5 Pharmacological inhibition of ERK5 negatively regulates the transcriptional activity of the Hedgehog-GLI pathway in murine NIH3T3 cells 79
4.6 ERK5 pharmacological inhibition reduces GLI1 levels in murine NIH3T3 cells 80
4.7 Pharmacological and genetic inhibition of ERK5 reduces the transcriptional activity of exogenous GLI1 in melanoma cells 83

5. DISCUSSION 85

6. REFERENCES 91

ABBREVIATIONS

ADBI: assay dilution buffer

AJCC: American Joint Committee on Cancer

AKT: Protein Chinasi B (PKB)

a-MSH: a-Melanocyte Stimulating Hormone

ARF: Alternate Reading Frame

ASIP: Agouti Signaling Protein

ATCC: American Type Culture Collection

BAD: BCL-2 Antagonist of Cell Death

BCC: Basal Cell Carcinoma

BCL-2: B-cell Lymphoma 2

Bmi1: B cell-specific Moloney murine leukemia virus integration site 1

BMK-1: Big Mitogen Activated protein Kinase

BDNF: brain-derived neurotrophic factor

Boc: biregional cell adhesion molecule

BSA: Bovine Serum Albumin

b-TrCP: b-transducin repeat containing protein

JNK1, 2, 3: c-Jun N-terminal kinases 1, 2, 3

CD: Common Docking domain

CCND1: Cyclin D1

CDK: Cyclin-Dependent Kinase

CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A

CDO: Ciliary Dysfunction-Only

CEA: Carcinoembryonic antigen

CEB: Cytoplasmic Extraction Buffer

c-Jun: Jun N-terminal kinase

CIBM: combined inhibition of BRAF and MEK1/2

CK1: Caseine Kinase1

CML: Chronic Myeloid Leukemia

COT: Cancer Osaka Thyroid kinase

CRC: colorectal cancer

CREB: cAMP Response Element-Binding protein

CSC: cancer stem cells

CTCF: corrected total cell fluorescence

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

DFS: Disease Free Survival

DHH: Desert Hedgehog

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

Dyrk1: dual-specificity tyrosine phosphorylation-regulated kinase 1

DUSP: dual-specificity protein phosphatase

E2F: E2 transcription factor

ECM: Extracellular Matrix

EGF: Endothelial Growth Factor

Elk-1: ETS domain-containing protein Elk-1

EMT: Epithelial-Mesenchymal Transition

ERK: Extracellular signal-Regulated Kinase

FAK: Focal Adhesion Kinase

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FGF: Basic Fibroblast Growth Factor

FoxM1: Forkhead box protein M1

GANT: GLI antagonist

Gas1: Growth-arrest-specific 1

GBM: glioblastoma multiforme

GLI- BS: GLI -Binding Site

Gli: glioma-associated oncogene

GLI1deltaN: GLI N-terminal deletion variant

GSK3β: glycogen synthase kinase 3β

HDAC: Histone deacetylase

HEK-293T: Human Embryonic Kidney 293

HES1 TF: hairy and enhancer of split-1 transcription factor

HGF: Hepatocyte Hrowth Factor

HH: Hedgehog

Hhip: HH-interacting protein

HIF 1α: Hypoxia Inducible Factor 1α

HIP: Hedgehog Pathway Inhibitor

HMB-45: Human Melanoma Black 45

IL-6: interleukin 6

IFT: intraflagellar transport

IGF-2: Insulin-like growth factor 2

IHH: Indian Hedgehog

INK4: inhibitor of nuclear kinase 4

KAAD: 3-keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl)

LIF: leukaemia inhibitory factor

MAPK: Mitogen-Activated-Protein-Kinase pathway

MB: medulloblastoma

MBP: Myelin Basic Protein

MC1R: Melanocortin-1 Receptor

MDM2: Murine Double Minute 2

MEB: Membrane Extraction Buffer

MEF: Myocyte Enhancer Factor

MEK: Mitogen-activated protein Kinase

MIM: Missing in Metastasis

MITF: Microphthalmia-associated Transcription Factor

MMPs: metalloproteases

MSCs: Melanoma Stem Cells

MTAP: S-methyl-5'-thioadenosine phosphorylase

mTOR: Mammalian target of rapamycin

NLK: Nemo-like kinase

NEB: Nuclear Extraction Buffer

NES: Nuclear Export Signal or sequence

NF1: neurofibromin 1

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

NGF: Nerve Growth Factor

NHEM: normal human epidermal melanocytes

NLS: Nuclear Localization Signal or sequence

NSCLC: Non-small-cell lung carcinoma

NSE: Neuron Specific Enolase

OPN: osteopontin

OS: Overall Survival

OSCC: Oral Squamous Cell Carcinoma

PDGF: Platelet-Derived Growth Factor

PDGFRα: Alpha-type platelet-derived growth factor receptor

PI3K: Phosphatidylinositol 3-Kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

PR1/PR2: prolin rich domain

PKA: protein kinase A

PML: promyelocytic protein

PTCH: Patched

PTEN: Phosphatase and tensin homolog

Ras: rat sarcoma

RB: Retinoblastoma protein

REN: rennin

ROS: Reactive Oxygen Species

RTK: Receptor Tyrosine Kinase

RSK: ribosomal s6 kinase

S6K1: S6 protein kinase 1

SANT: Smo antagonist

SCF: stem cell factor

SD: Standard deviation

SEM: Standard Error of Mean

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SGK: serum/glucocorticoid-regulated kinase

SH3: SRC Homology 3 Domain

SHH: Sonic Hedgehog

SIP1: Smad interacting protein 1

SMO: Smoothened

STAT: signal transducer and activator of transcription

STK36: Serine/threonine-protein kinase 36

SuFu: Suppressor of Fused

TAD: transcriptional activation domain

TGF-β: Transforming growth factor β

tGLI1: truncated GLI1

TNF-α: Tumor necrosis factor-α

UICC: Union for International Cancer Control

ULK3: unc-51 like kinase 3

VEGF: Vascular Endothelial Growth Factor

ZEB: Zinc finger E-box-Binding homeobox 1

1. INTRODUCTION

1.1 Melanoma

Skin cutaneous melanoma is a tumor that arises from the malignant transformation of melanocytes, the melanin-producing cells present in the deep layer of the epidermis. Melanocytes derive from melanoblasts, which originate from the multipotent cells of the neural crest. During development, these cells migrate to several districts, mainly dermis and hair follicles, where they differentiate into mature melanocytes. In the skin, differentiated melanocytes respond to ultraviolet radiation by synthesizing melanin pigment, then transferred to keratinocytes. A pool of melanocytes with stemness properties (MSCs) remains in the lower permanent portion of skin and the hair follicle, where they ensure a continuous regeneration of mature melanocytes through self-renewal and differentiation (Mort et al., 2015). Maintaining balance between the proliferation of melanocytic stem cells and their differentiation is a very critical process, to which many genetic and environmental factors contribute. Oncogenic events, as the deregulation of genes involved in cell cycle control or in differentiation, might alter this balance providing the basis for melanoma initiation (Regad, 2013). Melanocytes are not confined to the epidermis. Outside the skin, melanocytes are present in considerable number in the uveal tract of the eye and at lower densities in other tissues, such as the meninges, the anogenital tract, basal epidermis layer, mucous membranes and vascular veins. Consequently, melanoma can interest not only the skin, but all body regions where melanocytes are present, giving rise to melanocytic neoplasms different in their clinical, histopathological and genetic characteristics from cutaneous melanomas (Bastian, 2014).

1.1.1 Melanoma incidence and risk factors

Although melanoma constitutes only a small percentage (about 6%) of all skin tumors, it is one of the most aggressive forms of skin cancer due to its propensity to develop metastasis. Indeed, it causes 65% of deaths for skin cancer and represents 5% of all male tumors (5^{th} tumor per relative incidence) and 4% in female sex (7^{th} tumor per relative incidence). It affects mainly subjects between 30 and 60 years of age and rarely interest younger patients. The global incidence of cutaneous melanoma is approximately 200.000 new cases per year, with 5-year survival rates ranging from 15% to 60% in

patients with distant and local metastases, respectively. In recent years, a significant increase in the incidence of the disease has been reported, probably due to the different habits of exposure to UVA and UVB sun exposure, considered one of the major risk factors (Siegel et al., 2017).

As with different types of cancer, both genetic and environmental factors contribute to the development of melanoma. The incidence of melanoma is strongly influenced by UV exposure, pigmentation of the skin (phototype I and II more sensitive to UV demage), geographic parameters (latitude and altitude), presence of congenital or acquired nevi, light immune deficiency and genetic predisposition. Approximately 10% of melanomas occur in a familial context and are characterized by rare deleterious germinal mutations in the cell cycle regulators CDKN2A, cyclin dependent-kinase 4 (CDK4) and in the master gene of melanocyte homeostasis microphthalmia-associated transcription factor (MITF). Additionally, the frequent allelic germinal variants of other genes are associated with low-risk susceptibility (MC1R, ASIP, MTAP and Caspase 8) (Dahl et al., 2007; Chin et al., 2003).

Epidemiologic studies reveal that sun exposure is the major known environmental factor associated with development of melanoma. The high mutation rate in melanoma is largely attributed in particular to mutagenic effects of UVR, with multiple effects in the skin, including genetic changes, formation of reactive oxygen species (ROS), alterations in cutaneous immune function, and production of growth factors (Garibyan et al., 2010). All these events induce accumulation of genetic mutations in melanocyte that activate oncogenes, inactivate tumor suppressor genes and impair DNA repair promoting melanocyte proliferation, blood vessel growth, tumour invasion, evasion of immune response and, lastly, metastasis (De Braud et al., 2003; Thompson et al., 2005).

1.1.2. Clinical classification and stadiation

Melanoma can be classified into 4 different clinical subtypes: superficial spreading melanoma (the most common, represents about 70% of all skin melanomas), lentigo malignant melanoma, acral lentiginous melanoma and nodular melanoma (the most aggressive, represents approximately 10-15% of skin melanomas). Among these subtypes, the first three are characterized by superficial growth and good prognosis. Instead, nodular melanoma is more aggressive and invasive since its early stages. There

are also rare histopathological variants, such as desmoplastic and nevoid melanoma (McCourt et al., 2014).

According to the American Joint Committee on Cancer (AJCC) melanomas are currently classified into four stages, designated from I to IV; whereas stage 0 indicates in situ melanoma, which only affects the top layer of the skin. The melanoma staging recommendations were made on the basis of a multivariate analysis of 30,946 patients with stages I, II, and III melanoma and 7,972 patients with stage IV melanoma. These stages were defined on the basis of the TNM system, which is based on 3 key informations about the tumor: characteristics of the primary tumor (T), involvement of lymph nodes (N) and presence of metastasis (M). The revisions to the AJCC melanoma staging system over time (the 7th edition is the current) reflect progresses in the understanding of the biology of the disease, essential to improve melanoma prognosis of patients (Boland et al., 2016).

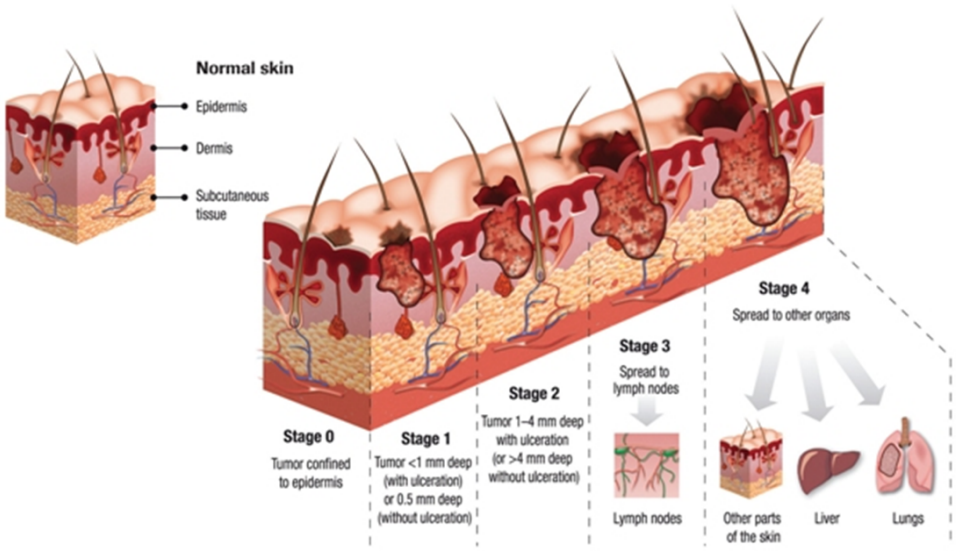


Figure 1.1 Melanoma staging (Image by MedArs.it., 2001).

In the characterization of the primary tumor, the thickness of the lesion is an important parameter to evaluate during the evolution of melanoma, also for the prognosis of the disease. Breslow staging provides a measure of the degree of penetration of melanoma from the superficial granular layer to the deeper and inner level of the skin. The prognosis is good for melanomas less than 1 mm and progressively worsens with increasing thickness.

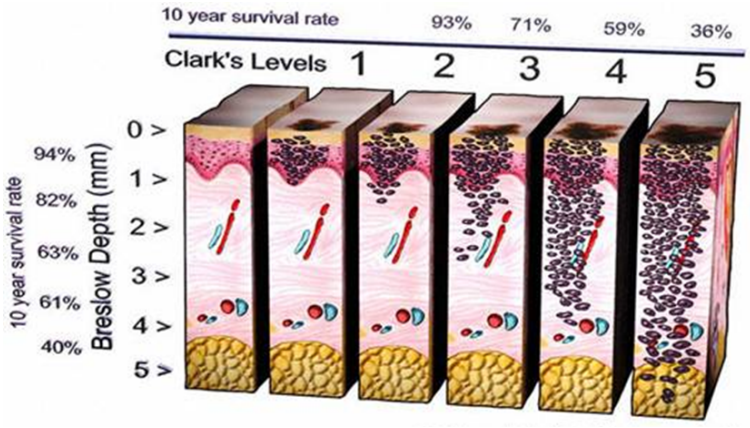


Figure 1.2 Breslow system for tumor thickness evaluation (Image by MedArs.it., 2001).

Other important prognostic indicators are the presence of ulcerations, an unfavorable event that allows to predict a greater chance of metastasis at the visceral and bone. The mitotic index, which indicates proliferative activity, is also associated with unfavorable prognosis. Instead, the degree of lymphocyte infiltration in the tumor is a favorable event but is very low in most patients (Regad, 2013). The prognosis of melanoma is also conditioned by the localization of the tumor mass and by the tumor stage at diagnosis. While early-stage primary melanoma can be successfully treated by surgery, metastatic forms are refractory to all available therapies. To improve the prognosis of the disease, a periodical evaluation of primary lesions is essential to ensure an early diagnosis and an effective therapy. The acronym **ABCDE** summarize some of the features to be evaluated in nevi (McCourt et al., 2014):

A: Asymmetry
B: Border irregularity
C: Colour variation
D: Diameter > 6 mm
E: Evolving

The histologic changes that accompany the progression from normal melanocytes to malignant melanoma are described in the Clark model. The model depicts the stepwise transformation of melanocytes to melanoma as a process of forming nevi and the subsequent development of dysplasia, hyperplasia, invasion and metastasis. The initial phase is characterized by a **radial growth**. The melanocytes can proliferate as

isolated cells or arranged in small nevi, confined at the epidermis or papillary dermis. This phase can last for a few months to years and is characterized by the absence of aggressive behavior and good prognosis. Indeed, melanoma cells at this stage are still dependent on exogenous growth factors due to stimulation by the keratinocytes and are incapable of growing in an anchor-independent manner. The next phase of **vertical growth** represents a more malignant stage of the tumor: the atypical and mitotic tendencies are more apparent and frequent; the neoplasia begins to spread perpendicular to the epidermis, invading the reticular dermis and forming nodules of proliferating malignant cells. At this stage, melanoma cells escape by keratinocytes stimulation and establish close interaction with stromal cells. They acquire autonomous proliferation capacity and cohesive growth, with aggregate or nodule formation extending to the reticular dermis or subcutaneous tissue, acquiring invasive and metastatic properties. The transition from the radial to vertical growth phase constitutes the crucial step in the progression of melanoma, indicative of the acquisition of a metastatic phenotype with worse prognosis (Miller et al., 2006).

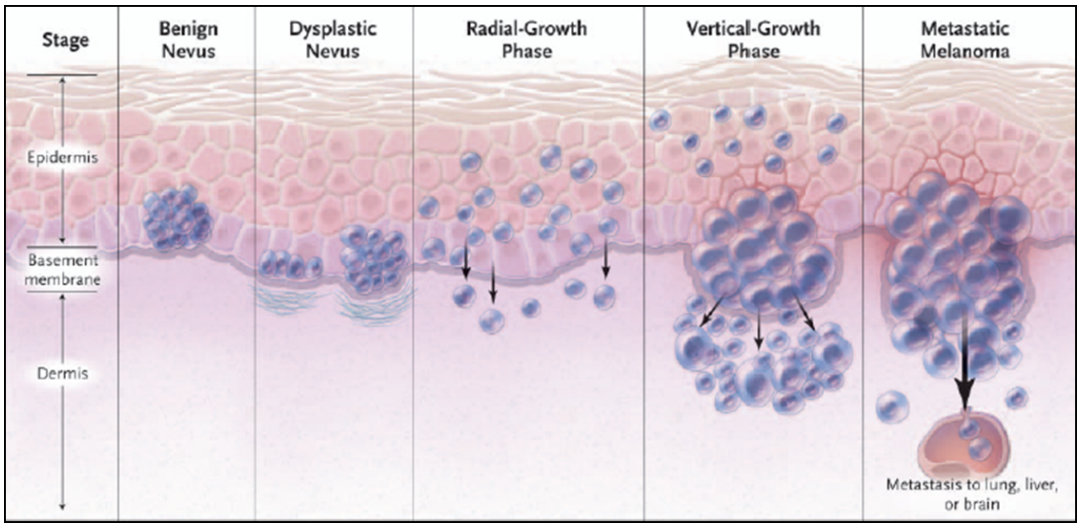


Figure 1.3 Proliferation of melanocytes at different stages of melanoma progression (Modified from Miller et al., 2006).

Several molecular and genetic events have been associated with different stages of melanoma development. The study of the resulting biological events is important to understand the mechanism of progression of this tumor.

1.1.3. Signaling pathways altered in melanoma

Melanoma is a heterogeneous tumor and several molecular events have been identified and associated with its development (Alexandrov et al., 2013). In addition to germline mutations, the main players of melanomagenesis may acquire successive genetic lesions to promote tumors formation. The cascade of genetic events occurring in tumor neoplastic progression can be described following a linear progression pattern, based on the known biological role of these players in melanoma initiation and progression (Bertolotto, 2013).

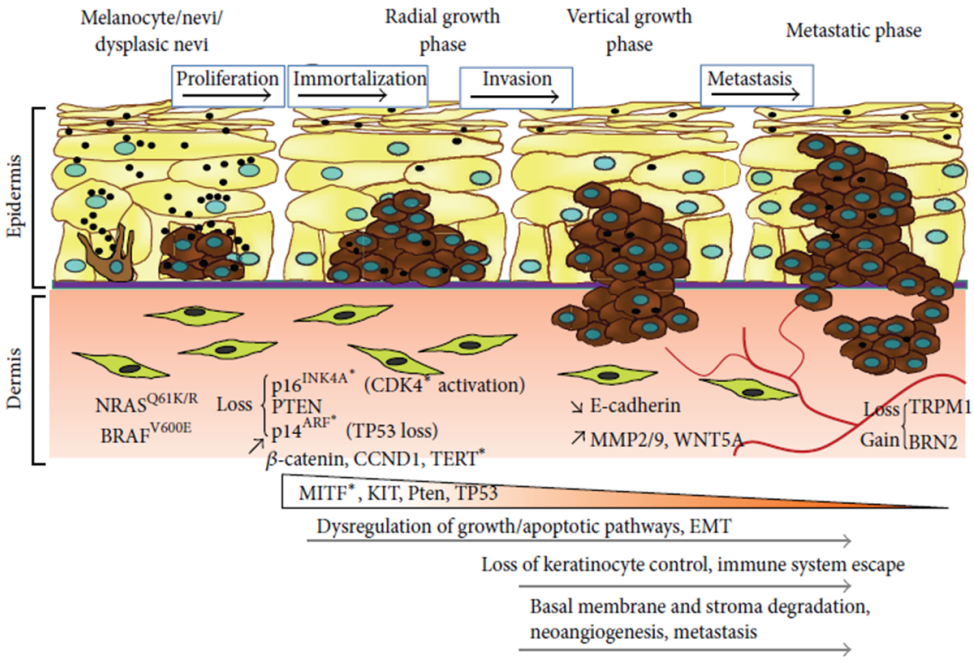


Figure 1.4 Hypothetical model for melanoma progression (Bertolotto, 2013).

The identification of mutations involved in making an individual melanoma cell competent for metastatic spread is crucial to understand melanoma progression. In recent years, genomic sequencing studies of melanoma have uncovered mutations in multiple genes and pathways involved in important cellular processes, such as proliferation, apoptosis, senescence and response to DNA damage (Figure 1.5) (Krauthammer et al., 2015; Hodis et al., 2012; Mar et al., 2013). The study of driver melanoma gene mutations may offer not only the possibility to better understand the molecular basis of melanoma initiation and progression, but importantly it allows to identify new therapeutic approches for melanoma treatment.

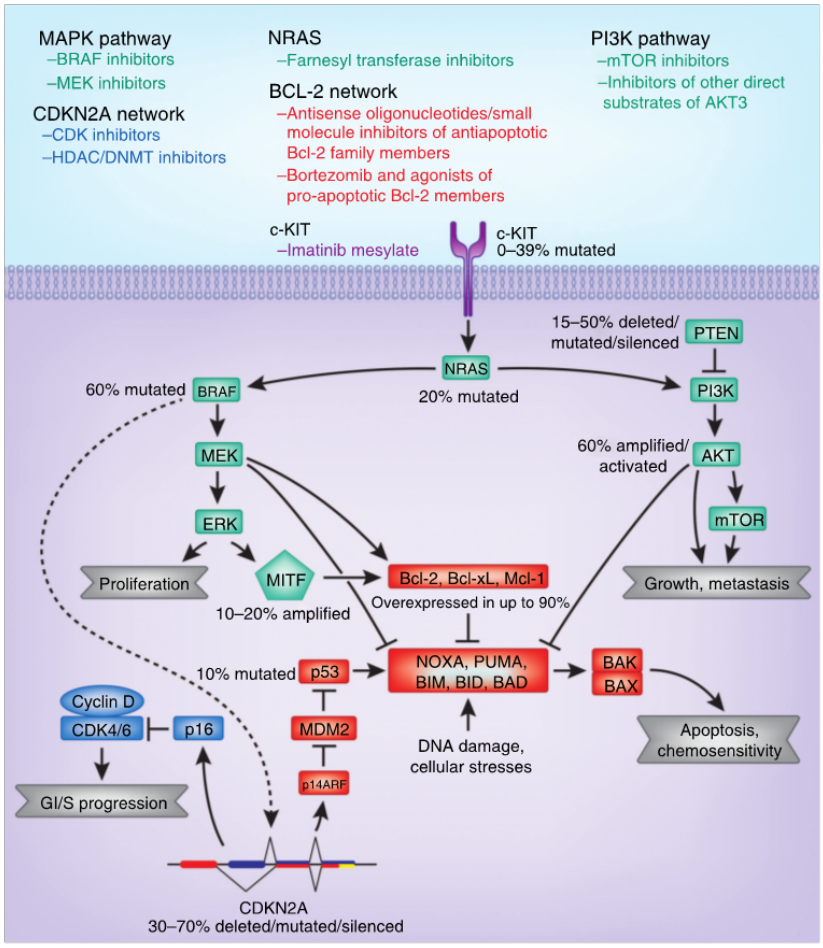


Figure 1.5 Signaling pathways altered in melanoma (Hocker et al., 2008).

1.1.3.1 The RAS/RAF/MEK/ERK cascade

Melanoma can originate from malignant transformation of melanocyte (75% of the cases) or from melanocytic naevi (25% of the cases), that may be congenital or appear throughout the life (acquired). Nevi are benign proliferations of melanocytes, with a very low likelihood of progressing to melanoma (Shain et al., 2016). At a molecular level, the first step in melanomagenesis is the abnormal proliferation of melanocytes sustained by an uncontrolled activation of the mitogen-activated protein kinase (MAPK) signaling pathway. Aberrant activation of this pathway is the result of somatic mutations of N-RAS, associated with about 15% of melanomas, or BRAF, present in about 50% of melanomas (Davies et al., 2002). These mutations, which are mutually exclusive, cause constitutive activation of the serine–threonine kinases in the MEK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) pathway. Both RAS and BRAF are important mediators of the response to extracellular stimuli, including those of the UV rays, and play a central role in regulating cell growth,

survival and proliferation. After UVR exposition, the photoprotection induce not only the skin pigmentation but also the proliferation of melanocytes to increase the melanin production (Mar et al., 2013).

RAS gene family products belong to the small GTPase family, small proteins bound to the cytoplasmic membrane and involved in the intracellular transduction of a plethora of signals. In melanoma, NRAS is the most commonly mutated isoform compared to the other members of the same family (HRAS and KRAS) that are almost never involved. NRAS is mutated in 33% of primary melanomas and 26% in metastatic melanoma. The mutation consists in a substitution of a Glutamine with Arginine, Lysine or Leucine in position 61 (NRASQ61K/R). This mutation impairs the GTP hydrolysis, resulting in a constitutionally activated NRAS. Oncogenic stimulation of NRAS is able to activate two important signaling pathways, thus regulating RAF and PI3K (Phosphatidylinositol 3-Kinase). The RAS-RAF-MEK-ERK pathway is involved in 80-90% of melanomas through mutations on different members (Ghosh et al., 2009).

RAF is a serine threonine kinase which acts as a downstream effector of RAS in the MAPK signaling pathway. The RAF kinase family consists of three proteins (ARAF, BRAF and CRAF), expressed in various human tissues. Several alternative splicing forms are known for BRAF gene, giving rise to at least 10 protein isoforms expressed in a variety of tissues. Forthy-three probable mutations with potential oncogenic activity were described, all located in exons 11 and 15 (Hall et al., 2014). In melanoma, the BRAF gene is mutated in 40-60% of cases (Devies et al., 2002). The prevalent mutation (about 90% of cases) is represented by the replacement of a Valine with Glutamic acid at codon 600 (BRAF-V600E). The BRAF-V600E variant induces a continuous stimulation of cell proliferation and tumor growth through a constitutive ERK activating phosphorylation (Shtivelman et al., 2014). BRAF-V600E is the most common initiating mutation in melanomas. However, in human 81% of melanocytic nevi harbor NRASQ61K/R mutation and 82% of acquired nevi harbor BRAF-V600E mutation. The identification of activating BRAF mutations in benign nevi indicates that its oncogenic activation is a necessary condition in tumor initiation but is not sufficient for melanoma development. In absence of other driver mutations, the mutant BRAF-V600E results in a limited expansion of melanocytes to form a common naevus. These nevi remain as stable lesions that are probably composed of a mixture of permanently arrested (senescent) cells and slowly proliferating cells, evading the immune cell-mediated control. In human melanocytes, mutant BRAF protein induces cell senescence

by increasing the expression of the cell cycle inhibitor of kinase 4A (INK4A) and ARF activity on p53 degradation. This mechanism appears as protective reaction in response to the activation of an uncontrolled mitogenic signal (Pollock et al., 2003). The arrest of the cell cycle caused by INK4A can, however, be overcome by mutations in INK4A itself, as well as other cell cycle factors. Indeed, the expression of INK4 is reduced or absent in about 1/3 of melanomas with BRAF-V600E mutation (Pollock et al., 2003).

1.1.3.2 Additional pathways deregulated during melanoma progression

Benign nevi must acquire additional molecular alterations to progress towards an invasive phenotype. The molecular abnormalities at this stage of progression affect cell growth, DNA repair and the susceptibility to cell death. The PI3K/AKT signaling pathway is involved in cell proliferation and in the apoptosis, through the inhibition of many proapoptotic proteins, such as BAD (antagonist of BCL-2, which hinders the mechanisms of cell death) and the activation of MDM2 (responsible of p53 degradation). The PI3K/AKT signaling pathway is deregulated in advanced melanoma stage. Indeed, PTEN loss is found at high frequency (about 37%) in melanomas, but not in nevi. The levels of other components of PI3K pathway, such as AKT, increase during melanoma development. PTEN loss allows the bypass of senescence induced by p16INK4a loss and triggered activation of the PI3K/AKT proliferative signaling, supporting melanoma progression (Shull et al., 2012). Once malignant neoplastic proliferation has begun, further mutations induce the acquisition of invasion and metastatic capacity. The PI3K/AKT signaling pathway promotes the plasticity of cancer cells by controlling epithelial mesenchymal transition (EMT). This pathway regulates expression and activity of factors involved in cell motility, such as RAC1, and degradation of components of basal laminae, such as the metalloproteases MMP-9, allowing melanoma cells to invade the underlying dermis (Larue et al., 2005).

In some melanomas, MAPK and PI3K pathway dysregulation results from overexpression or hyperactivation of growth factor receptors, such as c-KIT, EGFR or inactivating mutations in neurofibromin 1 (NF1), a negative regulator of Ras (Krauthammer et al., 2015). Activating mutations in GNAQ and GNA11, two G protein α-subunits involved in MAPK signaling, are identified in particular in the uveal melanomas. Other important pathways deregulated in melanoma are the Wnt/β-catenin pathway, which seems to contribute to the inhibition of apoptosis in melanoma progression, and the Hedgehog pathway, which will be further discussed later (Lo et al.,

2014). The frequency of p53 mutations is lowest in melanomas compared to other cancers. Only 1-5% of primary melanoma and 11-25% of metastatic melanoma show mutations or deletions of TP53. Furthermore, it has recently been shown that the tumor microenvironment (altered distribution and chemokine concentration, non-activation of cell-mediated immunity, induction of immunosuppressive mechanisms) and immune system play an important role in the formation and maintenance of melanoma metastases melanoma (Regad, 2013).

This complex mutational landscape shows that melanoma is characterized by different types of gene alterations, flowing into the deregulation of RAS-RAF-MEK-ERK and PI3K-AKT pathways. Interestingly, mutations of NRAS and BRAF or NRAS and PTEN are mutually exclusive. PI3K pathway mutations are present in 9% of NRAS mutant tumors. Co-occurrence of BRAF and PTEN mutations was reported in 17% of melanomas. This is probably because NRAS is able to activate both pathways, while BRAF leads to the activation of the only RAS-RAF-MEK-ERK pathway and confirm the need to other mutation in addition to BRAF for melanoma progression (Miller et al., 2006).

Other genes involved in melanoma, including familial melanoma that represents 8-12% of the total number of cases, are CDKN2A and CDK4.The **CDKN2A** (Cyclin-Dependent Kinase Inhibitor 2A) is mutated in 20-40% of familial melanoma and encodes two tumor suppressor proteins, p16INK4a and p14ARF (Alternative Reading Frame). p16INK4A inhibits Cdk4/6-mediated phosphorylation of the retinoblastoma protein (Rb). In the hypophosphorylated state, Rb binds and represses the E2F transcription factor and prevents G1 to S phase transition in cell cycle. On the other hand, p14ARF directly prevents p53 degradation by the E3 ubiquitin protein ligase MDM2 (Mouse Double Minute 2). In physiological conditions, CDKN2A appears to play a central role in preventing cancer formation by mediating a senescence-like state upon oncogenic stress. In melanoma, the loss of ARF and INK4 activity promotes the proliferation of tumor cells and involves the reduction of p53 protein levels. Consistently, activating mutations of BRAF and loss of functional p16INK4a and p14ARF were detected in the majority of melanomas. Furtheremore, oncogenic mutations in NRAS require concomitant loss of CDKN2A in order to progress melanoma (Nelson et al., 2009).

The gene encoding cyclin dependent kinase 4 (**CDK4A**) is involved in a very small percentage of familial melanoma. The mutation of arginine at position 24 into

cysteine (CDK4R24C) or histidine (CDK4R24C) renders the protein insensitive to regulation by p16INK4a. This results in a constitutive activation of the complex with the Cyclin D and aberrant proliferation, through Rb inactivation and E2F1 activation. CDK4R24C facilitates tumorigenesis of melanocytes transplanted into nude mice and causes escape from cellular senescence (Chin et al., 2003). In addition to these family forms, a number of genes involved in the pigmentation of the skin are related to moderate susceptibility to melanoma development. **MC1R** (Melanocortin-1 Receptor) is a seven-domain trans-membrane domain coupled to protein G. The a-MSH binding promotes the increase in cyclic AMP levels, which promote the activity of MITF, a factor that controls the transcription of genes involved in melanin production. Some MC1R genetic variants are not able to stimulate an appropriate production of melanin, whose job is to defend the skin against the ultraviolet rays damage. The presence of these MC1R variants in combination with intermittent exposure to solar ultraviolet rays is considered to be responsible for BRAF oncogenic activation by increasing intracellular AMP cyclic levels (Tsao et al., 2012). Finally, **MITF** (Microphthalmia-associated Transcription Factor) is amplified in 20% of melanoma patients and represent a negative prognostic factor. MITF controls genes involved in cell cycle regulation, such as CDK2, or antiapoptotic genes belonging to the BCL2 family, which are amplified in 30% of melanomas. HIF1α is also a target of MITF: in the presence of MITF amplification, expression of HIF1α is increased and promotes survival, angiogenesis and metastasis (Dahl et al., 2007).

The recent knowledges and discoveries on the molecular mechanisms involved in melanoma pathogenesis and progression are fundamental to identify markers able to predict both prognosis and therapeutic response to specific treatments in melanoma patients.

1.1.4 Targeted therapy in melanoma

The possibility to efficaciously treat melanoma depends to the tumor stage at diagnosis. Early-stage primary melanoma can be successful treated through surgery, which often eradicates the lesions localized to the skin with a 10-year survival of 95% of patients. The prognosis is poor for patientswith metastatic melanoma, with a median survival of about 10% of patients at 5 years (Ugurel et al., 2017).

The treatment of metastatic melanoma has seen fundamental improvements in recent years. Novel treatment strategies, based on targeted therapy and immunotherapy,

have as main goal the induction of a long-term therapeutic response to significantly improve survival of patients. Before the approval of targeted and immune terapies, conventional chemotherapy was based on the use of alkylating agents such as dacarbazine (Deticene) and IL-2, a cytokine approved by the Food and Drug for melanoma therapy in US. However, only 5% of patients showed an objective response to these chemotherapy drugs with no improvement of overall survival (Tsao et al., 2004).

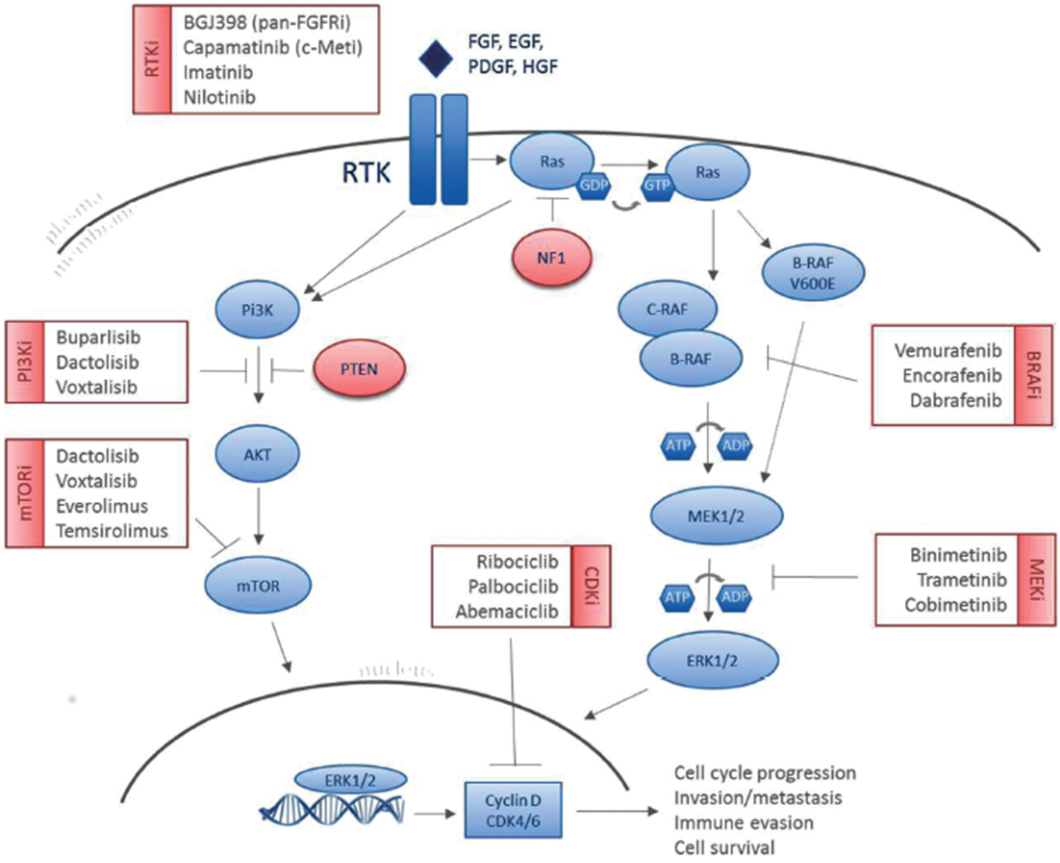


Figure 1.6 Important therapeutic targets in melanoma and the key inhibitors for melanoma therapy (Cosgarea et al., 2017).

The identification of the molecular alterations at the basis of melanoma, particularly in the MAPK signaling pathway, and the possibility to block specific targets with specific inhibitors has significantly broadened the therapeutic horizon and radically improved the therapeutic outcome. In targeted therapy, the characterization of the mutational profile of melanoma patients is crucial to choose the better therapeutic approach (Van Allen et al., 2014). Because of its prevalence in 50% of all melanomas, BRAF-V600E is an oncogenic driver in this tumor and so a key target for melanoma

therapy. The first targeted therapy to demonstrate substantial efficacy against melanoma was Vemurafenib, an ATP-competitive BRAF-V600E inhibitor (Chapman et al., 2011). In recent years, different clinical trials have shown successful inhibition of the MAPK signaling pathway through new highly selective BRAF inhibitors Dabrafenib (GSK2118436) and Encorafenib (Cosgarea et al., 2017).

The treatment of BRAF mutated metastatic melanomas is associated with a rapid therapeutic response in 50–80% of patients as well as a prolonged progression-free survivalof 6–10 months and an overall survival of 16–20 months (Chapman et al., 2011). Based on these successful clinical trials, Vemurafenib and Dabrafenib have been approved in the USA and Europe for the treatment of BRAF-V600 mutated metastatic melanoma. The approval is still pending for Encorafenib. However, BRAF inhibition is not effective in the remaining 50% of BRAF wild-type melanomas, including NRAS mutated tumors (NRASQ61). The treatment of non-mutant BRAF cells with Dabrafenib or Vemurafenib would result in a paradoxical activation of the MAPK pathway, mediated by CRAF (Poulikakos et al., 2010). MEK inhibitor treatment is the only targeted therapy for patients with NRAS-mutated melanoma or wild type BRAF with effective results in clinical trials. Also for MEK inhibitor, resistance has been reported to arise as a result of mutations in the allosteric drug binding pocket or amino-terminal negative inhibitory domain (Das Thakur et al., 2014; Grossman et al., 2001).

BRAF inhibitor monotherapy is not only associated with a rapid therapeutic response but also with the development of resistance within 5–6 months (Wagle et al., 2011). Multiple mechanisms have been reported for BRAF and MEK inhibitor resistance, including upregulation of receptor tyrosine kinase (RTK) signaling, activation of NRAS signaling, amplification of BRAF (Corcoran et al., 2010), alternative splicing of mutant BRAF, emergence of mutations in RAS or MEK concurrently with mutant BRAF (Emery et al., 2009). All the listed resistance mechanisms lead to the reactivation of the MAPK signaling at different levels or to the activation of parallel signaling pathways (Haarberg et al., 2013). To prevent the reactivation of a proliferative signaling, the combination of inhibitors targeting different signaling pathways has become an attractive option, not only to enhance the therapeutic effectiveness but above all to delay the onset of resistance. The combination of BRAF/MEK inhibitors (Dabrafenib and Trametinib) as a strategy to mitigate acquired BRAF inhibitor resistance is clinically effective and was recently approved by the FDA for BRAF-mutant melanomas (Voskoboynik et al., 2014; Volpe et al., 2017). This

therapeutic strategy allows to delay tumor progression, but still does not impair the onset of different mechanism of resistance associated with adirect reactivation of the final kinase of the MAPK pathway, ERK1/2 (Samata et al., 2014). SCH772984 is a potent and selective ERK1/2inhibitor that blocks both ERK kinase activity and its phosphorylation by MEK. SCH772984 shows a strong activity in patients with BRAF, NRAS and KRAS mutant tumors, reducing tumor progression and cell proliferation at nanomolar concentrations also in resistant tumor cells (Morris et al., 2013). However, a long-term exposure of cells to SCH772984 leads to acquired resistance due to a mutation of glycine to asparticacid (G186D) in the Asp-Phe-Gly "DFG"motif of ERK1. The conserved DFG motif is conteined in a pocket adjacent to the ATP binding region and it coordinates the binding of magnesium, which is essential for enzyme catalysis and phosphotransfer (Jha et al., 2016).

In conclusion, although the significant progresses in the melanoma targeted therapy field, a major challenge continues to be forestalling the emergence of resistance. This reveals an urgent need to identify new therapeutic targets to improve the survival of melanoma patients.

1.2 The MEK5/ERK5 pathway

The MAPK (Mitogen Activated Protein Kinase) family is one of the most conserved and expressed extracellular signal transduction pathways in eukaryotes. Different stimuli, including internal metabolic stress, as well as external mitogens, hormones, or neurotransmitters, cell–matrix and cell–cell interactions, lead to the initiation of a phosphorylation cascade culminating in the activation of a final acting MAPK, which transduces the signal into the nucleus. The members of the MAPK family are involved in essential cellular processes such as proliferation, differentiation, migration and apoptosis (Pearson et al., 2001). The importance of the MAPK pathways is highlighted by the observation that their constitutive activation is frequent in multiple human cancers, in particular in melanoma. Three atypical MAPK subfamilies have been identified: ERK3 and ERK4, ERK8 (also known as ERK7) and Nemo-like kinase-NLK. The four conventional MAPK subfamilies include extracellular signal-regulated protein kinases 1/2 (ERK1/2); c-Jun N-terminal kinases 1–3 (JNK1, 2 and 3); p38 MAPKs (α, β, γ and δ p38); and the most recently discovered ERK5 (Cargnello et al., 2011).

The MEK5/ERK5 pathway is the lesser studied among the MAPK pathway members and presents unique structural and functional features distinct from other MAPKs. These peculiar characteristics and the relevance in important cellular functions make this kinase an interesting target for future therapeutics interventions.

The ERK (extracellular-signal-regulated kinase) 5 protein is the effector kinase of a canonical three-tiered MAPK signalling cascade comprising MEKK (MEK kinase) 2/3, MEK (MAPK/ERK kinase) 5 and ERK5 itself. In response to different stimuli, MEKK2/3 binds the N-terminal domain of MEK5 and activates MEK5 by Ser311 and Thr315 phosphorylation. MEK5 protein kinase, encoded by MAP2K5, remains the only known MEK that directly activates ERK5. Alternative splicing results in two isoforms of MEK5 (50 kDa α and 40 kDa β) differing in their relative binding affinities for ERK5. MEK5α is a stronger activator of ERK5 than MEK5β, which lacks the consensus motif crucial to ERK5 binding and complete activation (Hoang et al., 2017). The MEK5 activated form binds to the N-terminal domain of ERK5 and phosphorylates two residues in the TEY sequence, which is similar to the binding sites of ERK1 and ERK2. The dual phosphorylation on Thr218 and Tyr 220 activates ERK5, inducing nuclear translocation (Nithianandarajah-Jones et al., 2012). Known substrates of ERK5 include the transcription factors Sap-1a, c-FOS, c-MYC and MEF2 family members (A,

C and D), as well as kinases, such as ribosomal s6 kinase (RSK) and serum/glucocorticoid-regulated kinase (SGK).

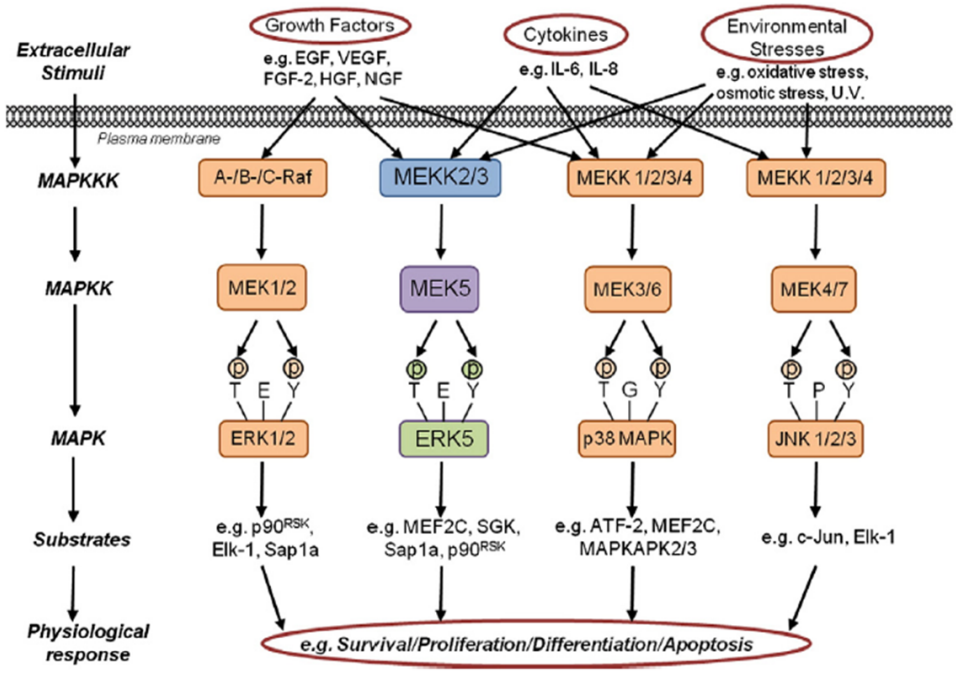


Figure 1.7 MAPK signaling cascade in mammalian cells (Nithianandarajah-Jones et al., 2012).

1.2.1 ERK5 structure and regulation

ERK5 is encoded by the MAPK7 gene, which was first identified and cloned in two independent studies two decades ago (Zhou et al., 1995; Lee et al., 1995). ERK5, also named Big Mitogen Kinase 1 (BMK1), comprises 816 amino acid (aa) residues and is more than twice the molecular weight (110 kDa) compared to the other MAPK family members. Structurally, ERK5 protein contains a N-terminal domain (amino acids 1–406), which is important for the kinase activity, and a large C terminus of 410 amino acids, important for the cellular localization and the transcription regulation.

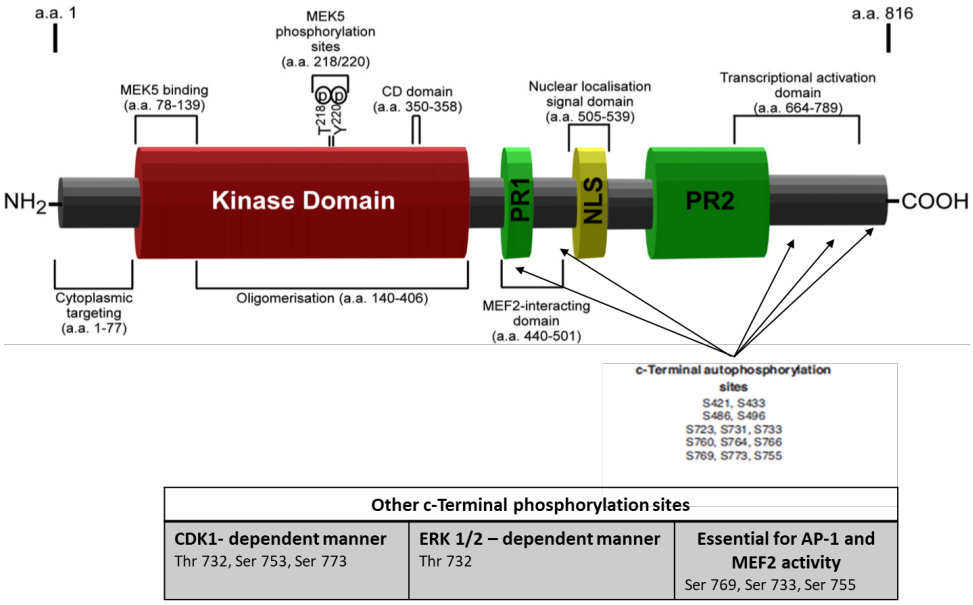


Figure 1.8 ERK5 structure and functions (Modified from Nithianandarajah-Jones et al., 2012).

The N-terminus presents a region required for cytoplasmic targeting (a.a. 1–77), followed by a kinase domain (a.a. 78–406) which shares 66% sequence identity to the kinase domain of ERK2. In the kinase domain there is also a region essential for MEK5 interaction (a.a. 78–139) and for oligomerisation (a.a. 140–406). ERK5 N-terminal region presents a common docking (CD) domain, consisting of a short sequence of negatively-charged amino acid residues (a.a. 350–358) important for the association with substrates containing docking domain. ERK5 differs from other members of the MAPK pathway for the presence of a unique C-terminal domain, which contains a nuclear localization sequence (NLS, a.a. 505–539) important for ERK5 nuclear targeting; two proline-rich domains, PR1 (a.a. 434–465) and PR2 (a.a. 578–701), considered potential binding sites for proteins containing Src-homology 3 (SH3)-domain; and a region for the interaction with the myocyte enhancer factor 2 (MEF2) (a.a. 440–501) (Nithianandarajah-Jones et al., 2012). A potent transcriptional activation domain (TAD) (a.a. 664–789) was identified in the C-terminus of ERK5 (Kasler et al., 2000), through which ERK5 can bind and activate several transcription factors. ERK5 C-terminal domain presents different residues which undergo autophosphorylation by the activated ERK5 itself (Morimoto et al., 2007) or other kinases. The phosphorylation on this region is crucial to regulate ERK5 activity and nuclear localization.

ERK5 is located both in the cytoplasm and in the nucleus. In basal conditions, the inactive form of ERK5 is retained in the cytoplasm, where it is associated with the

co-chaperone Cdc37 and the chaperone Hsp90, which ensures the cytosolic anchorage of the ERK5 protein. The trimeric complex ERK5-Hsp90-Cdc37 stabilizes ERK5 in an inactive conformation that facilitates the MEK5 recognition and activation. In the unphosphorylated inactive form, the N- and C-terminal domains are bound causing a folding structure that hides the nuclear localization sequence (NLS), promoting cytoplasmic retention. In the folded structure, the C-terminal tail masks the CD domain in N-terminus, preventing the interaction of ERK5 with its substrates. The MEK5 phosphorylation on the TEY region initiates the kinase activity of ERK5, that can phosphorylate the C-terminal residues of the protein, promoting the release of Hsp90 from the complex. ERK5 can assume an open conformation, exposing the NES sequence that promotes the nuclear translocation (Gomez et al., 2016).

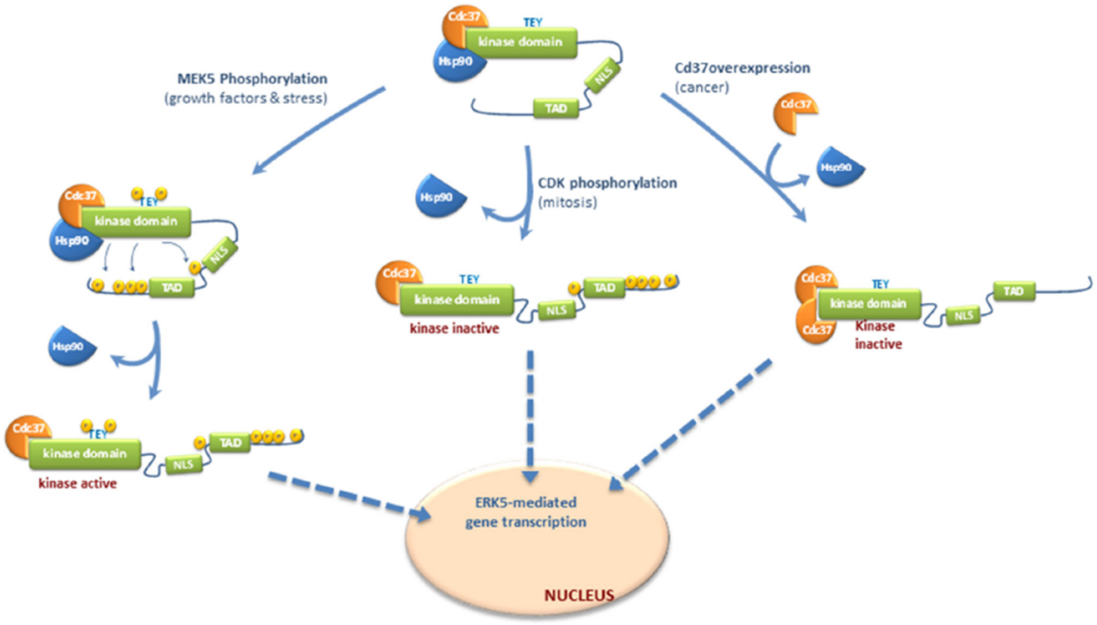


Figure 1.9 Nucleo-cytoplasmic mechanisms of ERK5 translocation (Gomez et al., 2016).

According to this model, the phosphorylation of the C-terminal region is required for the maximal transactivator activity of ERK5, that is exerted through its nuclear localization and the phosphorylation of nuclear targets. It has been shown that gradual truncation of this C-terminal tail gives rise to increased kinase activity of ERK5, suggesting that the tail has an autoinhibitory function. Furthermore, the phosphorylation in this region may influence also the nucleo-cytoplasmic shuttling of the protein. Although full length ERK5 mainly resides in the cytoplasmic compartment and only

partially in the nucleus, the truncation of the last 100 amino acids results in the nuclear accumulation (Buschbeck et al., 2005). Based on these structural features, the kinase activity of ERK5 seems to have two roles in gene expression: the activation of transcription factors by direct phosphorylation and the enhancement of the ERK5 transactivator activity by, presumably, autophosphorylation of its C-terminal-half (Morimoto et al., 2007).

Recently, novel MEK5-independent mechanisms of ERK5 activation and nuclear translocation have been described. It has been shown that nuclear ERK5 devoided of the kinase activity is able to activate transcription (Borges et al., 2007). Once in the nucleus, ERK5 enhances gene transcription by either phosphorylating transcription factors or by interacting with these factors through the transactivation TAD domain located at the C-terminal. Therefore, ERK5 nuclear shuttling requires only C-terminal phosphorylation that may be promoted through the ERK5 autophophorylation or by other kinases. During mitosis, ERK5 phosphorylation is sustained by cyclin-dependent kinase 1 (CDK1), which is important for the G2/M cell cycle transition. Four different residues on C-terminal domain may be phosphorylated during mitosis (Ser753, Thr732, Ser773, Ser706) (Figure 1.8) and these are important for ERK5 nuclear localization of the kinase (Díaz-Rodríguez et al., 2010; Iñesta-Vaquera et al., 2010). Another important residue in C-terminal domain is Thr732, which is phosphorylated by CDK1 and also by ERK1/2. This event induces ERK5 nuclear localization and promotes ERK5-dependent transcription, without affecting the phosphorylation status at TEY or other C-terminal residues. Treatment with a selective MEK1/2 inhibitor (U0126) reduces the Thr732 phosphorylation, suggesting that ERK1/2 can mediate the phosphorylation of ERK5 at Thr732 (Honda et al., 2015).

Beyond the cellular localization and C-terminal phosphorylation, ERK5 activity is regulated by further mechanisms. Three ERK5 splice variants have been identified in mouse (mERK5a, mERK5b and mERK5c): mERK5b and mERK5c, that lack the protein kinase activity, function as dominant negative kinases blocking mERK5a activity and ERK5-mediated MEF2C activation (Hoang et al., 2017; Yan et al., 2001). The duration and the magnitude of MAPK activation are tightly regulated, to assure a physiological and not aberrant signaling. The protein Ser/Thr phosphatases PP1/PP2A (Garcia et al., 2002) and tyrosine-specific phosphatases (PTPs) not only block the ERK5 activation but also effectively impede the translocation of ERK5 to the nucleus

(Buschbeck et al., 2002). Finally, the dual-specificity protein phosphatase DUSP5 and DUSP6 regulate the dephosphorylation of the TEY motif (Arkell et al., 2008).

1.2.2 Role of ERK5 in physiological conditions

In physiological conditions ERK5 is ubiquitously expressed in many tissues, particularly in the brain and the heart. During development, it plays an essential role in the formation of cardiac tissue and blood vessels and is an important mediator of survival signals in nervous system cells. Genetic deletion of ERK5 is embryonic lethal and tissue-restricted deletions have profound effects on erythroid development, cardiac function, and neurogenesis (Hayashi et al., 2004). In adults, it remains active as an important regulator of proliferation and survival, especially in endothelial (Roberts et al., 2009) and immune system cells (Rovida et al., 2008). Initially, ERK5 was identified as a MAPK activated by environmental stresses, such as oxidative and osmotic stress, UV rays, etc. Further studies show that ERK5 is physiologically involved in the response to different stimuli. It is activated by a range of growth factors, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) as well as by cytokines, such as leukaemia inhibitory factor (LIF) and interleukin 6 (IL-6) (Simões et al., 2016). The MEK5/ERK5 signalling pathway has been implicated in the regulation of several cellular processes, including differentiation, proliferation, survival, antiapoptotic signaling and angiogenesis. The identification of downstream effectors of the ERK5 pathway is important to understand its physiological function. The best-characterized ERK5 substrates are the three members of the myocyte enhancer factor (MEF) family of transcription factors. ERK5 plays a crucial role in cell proliferation, inducing the transcription of c-jun through the MEF2C transcriptional activation (Kato et al., 1997) in response to serum or EGF stimulation. Activation of ERK5 pathway induces phosphorylation and stabilization of c-Fos and Fra-1 transcription factors (Lochhead et al., 2012). ERK5 regulates cell cycle progression, in particular the G1/S transition. ERK5 promotes an activating phosphorylation of the serum and glucocorticoid-induced kinase (SGK), which promotes the entry into S phase in response to growth factors. Different studies demonstrate that ERK5 suppresses the expression of the cyclin dependent protein kinase (CDKs) inhibitors p21 and p27 (Perez-Madrigal et al., 2012), promoting cell proliferation. Moreover, ERK5 regulates the expression of the cyclin D1,

involved in a key cell proliferation checkpoint and frequently deregulated in cancer (Wang et al., 2006). ERK5 promotes also the G2/M transition, activating the transcription factor NF-kB, which upregulates genes involved in mitosis such as cyclin B1 and B2 and Cdc25B. During mitosis, ERK5 is involved in cell survival. Indeed, ERK5 prevents caspase activation by binding the pro-apoptotic protein Bim. ERK5 contributes to the survival response in neuronal dorsal root ganglia cells mediating the nerve growth factor (NGR) signaling system. In this pathway, a phosphorylation cascade mediated by ERK5 results in the activation of p90 ribosomal S6 kinase (RSK), which has as ultimately target the Ca2+/cAMP response element binding protein (CREB) (Ranganathan et al., 2006). CREB regulates the transcription of pro-apoptotic and survival genes. Further studies indicate that ERK5 contributes to survival in neurons via activation on transcription factor MEF2, a pro-survival and anti-apoptotic transcription factor (Drew et al., 2012).

1.2.3 Role of ERK5 in cancer

Since its discovery, ERK5 activity and regulation has been studied in cancer because of its implication in essential cellular functions, associated with "the hallmarks of cancer" (Lochhead et al., 2012).

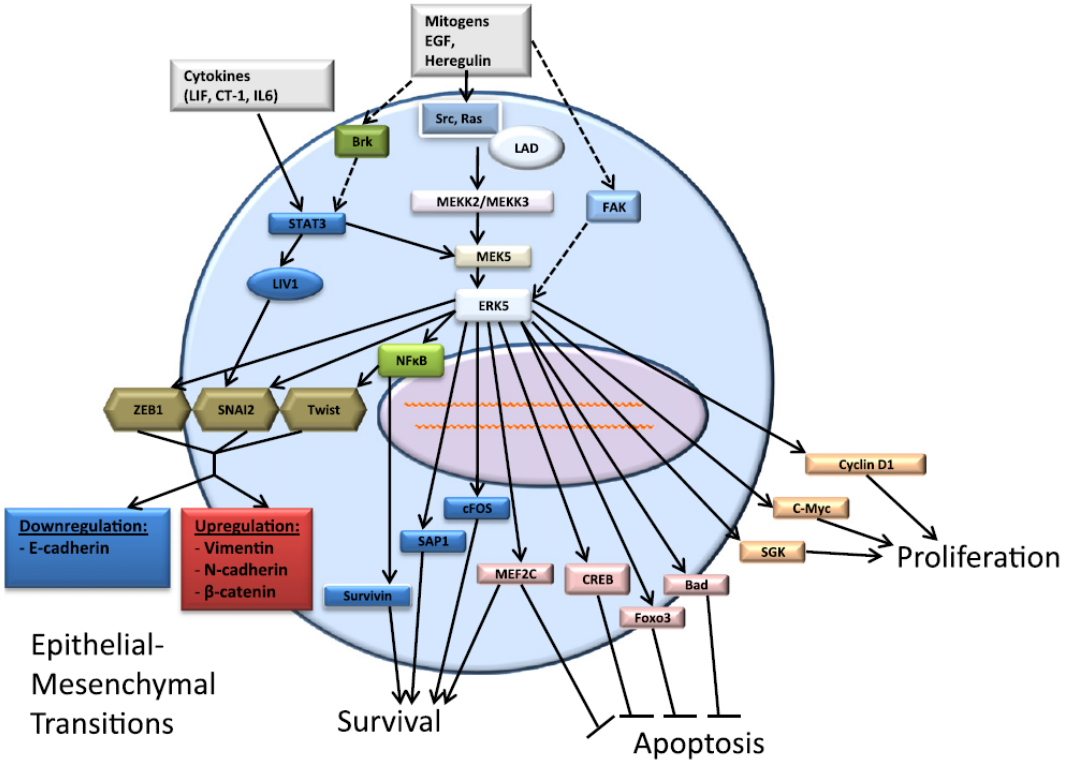


Figure 1.10 The MEK5/ERK5 signaling pathway in tumor cells regulates proliferation, survival, apoptosis and metastatic processes through a complex intracellular signaling system (Drew et al., 2012).

Alterations in the activity and expression of different members of the MERK5/ERK5 pathway seem to be directly involved in various types of cancer. In breast cancer, MEK5 expression is upregulated by constitutive activation of STAT (signal transducer and activator of transcription) 3 that is frequent in patients with advanced breast cancer compared to normal breast epithelial cells (Liu et al., 2017). Furthermore, increased ERK5 protein levels in either TNBC or in HER2+ patients correlate with poorer relapse-free survival (Montero et al., 2009; Ortiz-Ruiz et al., 2014). In prostate and colon cancers, MEK5 and ERK5 are overexpressed and correlate with the presence of bone metastases. The acquisition of a more aggressive and metastatic phenotype correlates with less favorable prognosis (Ramsay et al., 2011; Simões et al., 2016). Furthermore, an ERK5 gene amplification has been identified at 17p11 in approximately 50% of primary hepatocellular carcinomas (HCC). Consistently, in patients with HCC ERK5 is more abundantly expressed in the nucleus compared with normal liver tissue. Genetic or pharmacological inhibition of ERK5 reduces proliferation, migration and invasiveness of HCC cells. Moreover, ERK5 silencing decreases the growth of HCC xenografts (Rovida et al. 2014).

Clinical evidences show that an increase in MEK5/ERK5 signalling may be important for tumor initiation, metastatic progression and drug resistance. ERK5 is responsible for proliferative signaling sustaining the initial stage of cancer through the regulation and induction of cell cycle regulators, including cyclin D1, c-MYC, n-MYC, SGK, RSK2 and NF-kB. Through phosphorylation of MEF2 transcription factors, MEK5 has been shown to regulate the expression of c-JUN, a proto-oncogene essential to cell growth. Moreover, ERK5 can mediate also a survival signaling used by cancer cells to escape apoptosis. In endothelial cells MEK5 activation is responsible for activation of NF-kB and inhibition of caspase 3, resulting in apoptosis inhibition. ERK5 also regulates the activity of transcription factors involved in survival, such as CREB and MEF-2 (Pi et al., 2004). In cancer cells, ERK5 interacts with the promyelocytic protein (PML) and inhibits its tumor suppressor activity. ERK5 mediated-phosphorylation of PML induces the dissociation by MDM2, downregulating the expression of p53 tumor suppression (Yang et al., 2013). ERK5 can sustain cancer cells proliferation not only through the deregulation of its pathway, but also by non-canonical mechanisms. In some cancer types, ERK5 shows a constitutive nuclear localization mediated by the overexpression of Cdc37, which induces the release of the ERK5 cytoplasmic chaperone Hsp90 and the nuclear shuttling of the kinase. Cdc37 acts as an

oncogene, stabilizing other important mediators of cancer proliferation, such as Akt, BRAF and HER-2 (Gomez et al., 2016). Several studies in literature show that silencing or pharmacological inhibition of ERK5 is able to delay cell cycle progression and to reduce proliferation in several types of cancer. However, there are some conflicting reports that challenge the role of ERK5 in cell proliferation (Giurisato et al., 2016; Lin et al., 2016). Among these, a recent study shows that colon cancer cells with KRAS or BRAF mutations do not require ERK5 activity for proliferation at least *in vitro* (Lochhead et al., 2016).

Deregulation of ERK5 pathway is important also in tumor progression and is associated with metastatic risk in prostate, breast, colon, kidney, bone, and oral cancers, with less favorable survival outcome. The MEK5/ERK5 pathway is involved in cellular motility and can therefore play a role in the EMT, one of the key processes in tumor progression towards a metastatic phenotype. It has been shown that transcription of some key genes in EMT (such as Twist, ZEB, Snai2) is under the control of the MEK5/ERK5 pathway (Drew et al., 2011). Moreover, ERK5 is able to form complexes with the αvβ3 integrin and interact with Focal Adhesion Kinase (FAK) to regulate the organization of the cytoskeleton, thus participating in adhesion and motility processes (Sawhney et al., 2009). MEK5 promotes the expression of some extracellular matrix (ECM) metalloproteases, which degrade ECM promoting the migration and metastasis of tumor cells. An experimental system in melanoma A375 cells and prostate cancer demonstrates the ERK5 involvement in the formation of invadopods, cellular cell protrusions that allow migration during metastasis (Ramsay et al., 2011).

Although activating mutations in ERK5 have not been reported so far, the MEK5/ERK5 pathway is altered in several types of cancer. The pathway is under the control of several oncogenes, including RAS, RAF, Src, EGF, VEGF, COT, that promote up-regulation of different downstream MAPKs, including ERK5. COT is a protein kinase involved in the oncogenic transformation of NIH3T3 fibroblasts through a mechanism mediated by a cooperative signal with ERK5 (Chiariello et al., 2000). Oncogenic variants of Src seem to be involved in the activation of ERK5 pathway in fibroblasts, resulting in a transition to an invasive and metastatic phenotype. It would appear that the ERK5 pathway is involved in the transduction of the oncogenic effects of RAS and RAF, which are mutated in most tumors (Nithianandarajah-Jones et al., 2012). Independently of upstream regulators, also other parallel pathways can mediate the deregulation of ERK5 signaling, acting directly on ERK5. The MEK5/ERK5

pathway is one of the alternative pathways reactivated upon chemotherapy, conferring drug resistance to cancer cells. A recent report showed that ERK5 phosphorylation is enhanced in melanoma cells resistant to the combined inhibition of BRAF and MEK1/2 (CIBM), which is the current approach used in therapy. Silencing or pharmacological inhibition of ERK5 impairs the acquisition of resistance to CIBM and sensitizes cancer cell to chemotherapy, restoring the antiproliferative effect of the chemotherapy. The activating phosphorylation of ERK5 in response to CIBM therapy seems to be sustained by a SRC/MEK5 cascade, probably activated upstream by BRAF (Song et al., 2017).

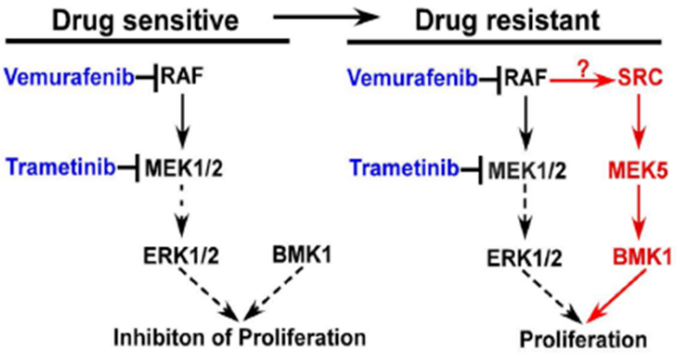


Figure 1.11 Drug resistance to combined inhibition of BRAF and MEK1/2 is mediated by ERK5/BMK1 through SRC/MEK5 cascade (Song et al., 2017).

The emerging key role of the MEK5/ERK5 pathway in several physiological processes and in oncogenesis highlights its potential as target in novel cancer therapeutic strategies.

1.2.4 ERK5 inhibitors: clinical relevance

In recent years interest in developing inhibitory molecules against ERK5 activation and function has emerged, due to the crucial role of ERK5 in tumorigenesis and in drug resistance. The first strategy for ERK5 pathway inhibition is based on the use of microRNAs, particularly miR-143 and miR-145, that physiologically act as tumor suppressors by regulating ERK5 expression levels. In bladder and prostate tumors, the reduced expression of miR-143 seems to be correlated with the high levels of ERK5. The treatment of these tumor cell lines with synthetic analogs of miR-143 and miR-145 shows a reduction in cells proliferation and ERK5 protein levels comparable to the results observed with RNA interference or ERK5 knockdown. However, different studies are still underway to verify that this antitumour activity of these synthetic

miRNAs is due to the selective binding on ERK5, which can also act on different cellular substrates (Clapé et al., 2009¸ Zhou et al., 2017).

Pharmacological molecules actually available for the MEK5/ERK5 signaling inhibition target the central kinases of the pathway (Figure 1.12).

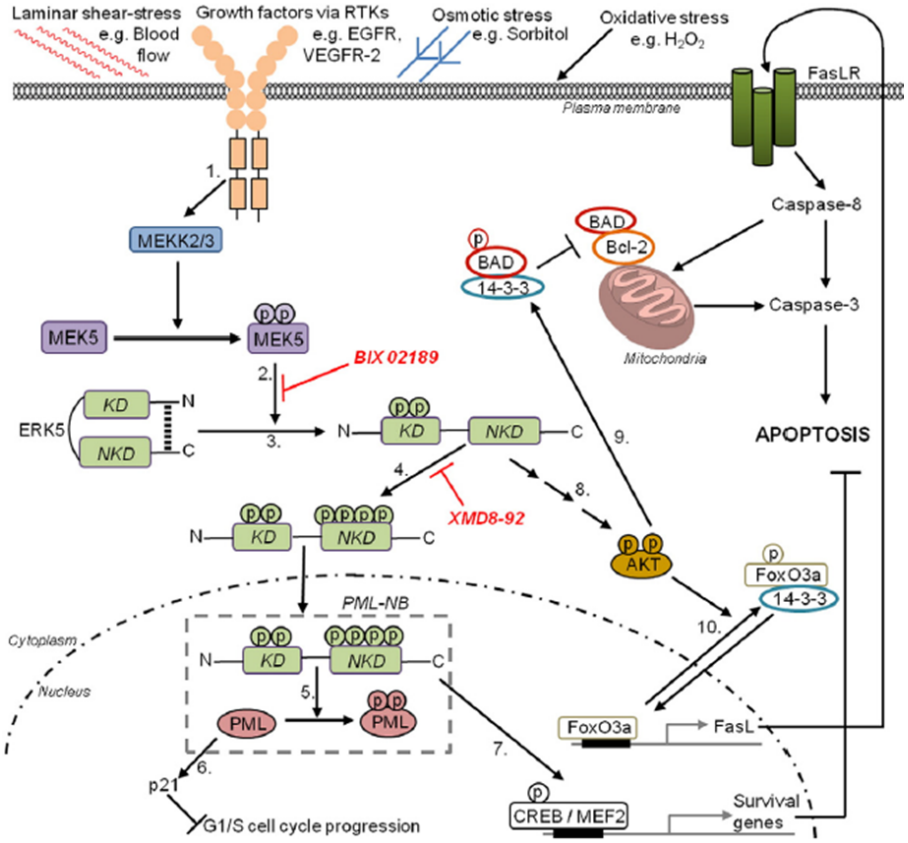


Figure 1.12 Pharmacological inhibition of the MEK5/ERK5 pathway. BIX02189 targets the MEK5, whereas XMD8-92 inhibits the ERK5 kinase activity (Nithianandarajah-Jones et al., 2012).

The first inhibitors described for the ERK5 pathway were the indolinone-6-carboxamides BIX02188 and BIX02189 (Boehringer Ingelheim Pharmaceuticals). These small-molecules compete for the ATP binding site in the MEK5 catalytic domain, blocking its kinase activity with IC50 4.3 and 1.5 nM, respectively. BIX02189 also displayed more potent suppression of ERK5 kinase activity with IC50 59 nM compared to that of BIX02188 (810 nM). Both compounds also inhibited transcriptional activity of MEF2, the downstream substrate of the MEK5/ERK5 signaling cascade, in a dose-dependent manner. These MEK5 inhibitors blocked ERK5 phosphorylation without affecting activation of ERK1/2, p38 MAPK, or JNK (Tatake et al., 2008). According to

a recent study, BIX02189 reduces TGF-β1-induced EMT, cell motility and expression of matrix metalloproteinase-2. BIX02189 strongly blocks the activation of TGF-β1 signaling components independently of MEK5, demonstrating a direct activity on TGF-β1 for BIX02189 (Park et al., 2016).

Furthermore, ERK5 autophosphorylation in its C-terminal domain is fundamental for its activity and nuclear translocation and has been revealed to have a significant influence on the response of cancer cells to chemotherapeutic agents. For these reasons, the inhibition of the final effector of the pathway seems to be a better strategy for clinical application. The benzopyrimido-diazepinone XMD 8-92 is a competitive inhibitor of ATP-site in ERK5 kinase domain, ensuring a total inhibition of ERK5 kinase activity that can no longer phosphorylate either its cellular substrates or itself at the level of the C-terminal domain. To validate the specific activity of XMD8-92 on ERK5, an inhibition profiling of the compound was performed first against a diverse panel of 402 kinases and then against all detectable kinases in HeLa cell line. XMD 8-92 is able to inhibit the ERK5 activation mediated by EGF and significantly reduces ERK5-dependent MEF2C-driven gene expression. No activity against ERK1/2 and MEK5 are observed (Yang et al., 2010). *In vivo*, the IP administration of 50 mg/kg dose of XMD8-92 is efficacy in controlling tumor growth and inflammation with good pharmacokinetics, bioavailability and tolerability (Al-Ejeh et al., 2014). Beyond the promising clinical results obtained with ERK5 chemical inhibition, its clinical application is compromised by the recently discovered role in the direct inhibition of bromodomains (BRDs) (Lin et al., 2016). Although in several studies the results generated through XMD8-92 inhibition were similar to those obtained through specific ERK5-silencing techniques, new chemical inhibitors should be generated to avoid possible unspecific effects related to bromodomain inhibition.

A second generation of ERK5 kinase activity inhibitors was obtained synthetizing derivatives of the benzopyrimidodiazepinone XMD8-92, showing a potent inhibition of ERK5 with IC50 values ranging from 8 to 190 nM. Preliminary data about the application of these new molecules show that the inhibition of ERK5 had no antiproliferative and anti-inflammatory activity (Lin et al., 2016). TG02, an oral pyrimidine-based multi-kinase inhibitor, blocks CDKs 1, 2, 3, 5, and 9 with IC50 values below 10 nM in addition to janus kinase 2 (JAK2), p38d and ERK5 with IC50 values of 19, 56, and 43 nM, respectively. This novel anti-cancer agent inhibited proliferation and survival of multiple myeloma cell linesalso as single agents and has recently completed

phase I of clinical trials for treatment of leukemia and multiple myeloma patients (Álvarez-Fernández et al., 2013). Recently, TG02 has been proposed also in combination with chemotherapy against triple negative breast cancer (Miranda et al., 2015). JWG0-45, another novel ERK5 inhibitor, shows similar biological effects compared to XMD8-92 and much lower affinity toward BRD proteins (William et al., 2016).

Reactivation of ERK5 is involved in drug resistance to cytotoxic agents or target therapy. For this reason the ERK5 inhibitors are proposed in combination therapies. In colorectal cancer the clinical efficacy of the pyrimidine analog 5-fluorouracil (5-FU) is reduced by the development of resistance. Treatment of colon cancer cells HCT116 and SW620 with 5-FU reduced the MEK5 and ERK5 activation. Constitutive activation of MEK5 conferred a survival advantage to HCT116 cells exposed to 5-FU compared to control cells, whereas downregulation of MEK5 signaling with the ERK5 inhibitor XMD8-92, enhanced sensitivity of HCT116 cells to 5-FU-induced cytotoxicity through stimulation of p53-dependent transcriptional activation of p21 and Puma. *In vivo*, combination therapy using 5-FU and XMD8-92 significantly increased apoptosis and reduced tumor burden compared to monotherapy of each compound (Pereira et al., 2016). Consistent with this study, in both HeLa cervical cancer cells and A549 lung cancer cells the combined treatment with XMD8-92 and the chemotherapeutic agent doxorubicin demonstrated a synergistic induction of p53 and significantly promoted tumor regression (Hoang et al., 2017). Recently a compensatory mechanism between the ERK1/2 and ERK5 pathways has been described in colorectal cancer (CRC), with important implication for cancer therapy. According to this model, Ras is constitutively active in CRC and preferentially activates the Raf–MEK1/2–ERK1/2 module. Importantly, ERK1/2 activation also results in the activation of negative feedback mechanisms that suppress its upstream kinases and activate dual specificity phosphatases (DUSPs), which turn off ERK5 kinase activity. Upon MEK1/2 inhibition or genetic knockout of ERK1/2, the lack of negative feedback mechanisms results in upregulation of the Ras–Raf–MEK5–ERK5 module, which maintains cell proliferation under physiological conditions, or supports uncontrolled cell proliferation in colorectal cancer, respectively. Compensatory upregulation of the ERK5 pathway in CRC can be reversed by targeted treatment with its specific inhibitor, XMD8-92, and paves the way for the application of a combined therapy to block ERK5 reactivation (de Jong et al., 2016; see Figure 1.13 for details).

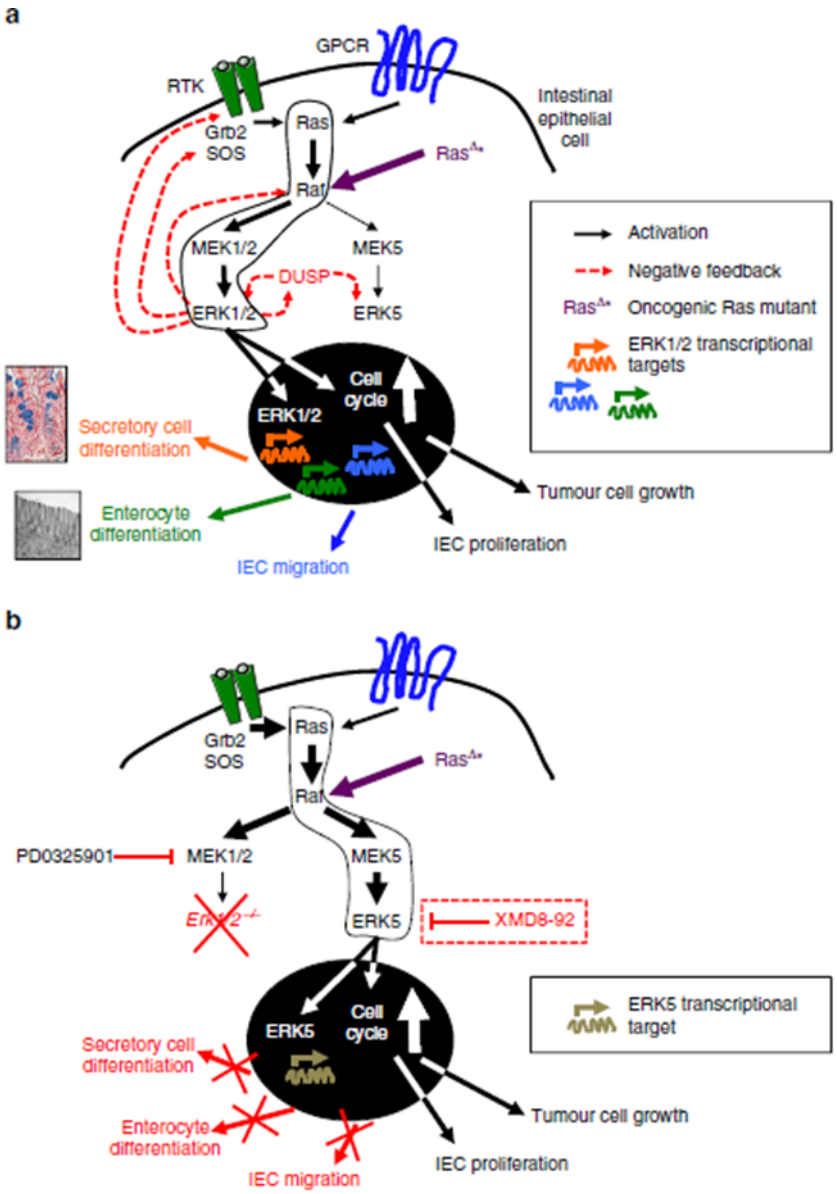


Figure 1.13 Compensatory mechanisms between ERK1/2 and ERK5 pathways in intestinal epithelial cells (a) and colon cancer (b) (de Jong et al., 2016).

Taken together, these results confirm the important role of ERK5 in cancer. The tight correlation between ERK5 and other pathways important in melanoma, such as RAS-RAF-MEK1/2-ERK1/2, prompted us to study ERK5 as novel target in melanoma therapy.

1.3 The Hedgehog pathway

Initially discovered in *Drosophila melanogaster*, the Hedgehog (HH) pathway is a highly conserved signaling in almost all animal kingdoms. It plays a crucial role in embryonic development, particularly during organogenesis in the regulation of cell proliferation, differentiation and tissue patterning. Indeed, HH pathway inactivation causes defects in the development process, while its hyperactivation has been described in various forms of solid and hematologic malignancies. This pathway is also active in the adult, where it is involved in tissues homeostasis, repair and regeneration, and in maintenance of the stem cell pool (Varjosalo et al., 2008).

In mammals, the canonical activation of HH pathway is promoted by three ligands: Sonic hedgehog (SHH), Desert hedgehog (DHH) and Indian hedgehog (IHH). HH ligands are proteins associated with the membrane, synthesized as inactive precursors that meet a number of post-translational modifications before being secreted from the cell in an active form. Despite their high homology, the three HH ligands have different function and localization (Jiang et al., 2008). Sonic HH is the most expressed in mammals and is crucial for the formation of many systems, including limbs, middle brain structures, lumbar spine, thalamus and teeth. It is also involved in the development of many endothelial tissues during organogenesis (Varjosalo et al., 2008). The main actors of the HH pathway are two transmembrane receptors: the 12-pass transmembrane protein receptor Patched (PTCH), which resides at the basis of primary cilium and regulates the activity of Smoothened (SMO), a 7-domain transmembrane receptor coupled to G protein. The mechanism of SMO regulation byPTCH is not yet fully clarified, but presumably involves primary cilia, which play an important role in transmitting this signal. Cilia are projections of the cell membrane present on most vertebrate cells and involved in the detection of chemical and mechanical signals, functioning as a control center for differentiation signals and polarization (Robbins et al., 2012). SMO proteins can exist in three different states: an inactive internalized form (SMOA), which is in balance with an inactive form (SMOB), linked to the cilium. The third activated form (SMOC) is generated from SMOB following phosphorylation in a region rich in arginine; this phosphorylation allows SMO to move into the cilia. In absence of the ligand, PTCH inhibits the conversion of SMO into active form, maintaining the pathway inactive. Binding of HH ligand to PTCH allows to the receptor/ligand complex to be dislocated outside the primary cilium and to be internalized into endosomic vesicles, thus losing its ability to inhibit SMO. SMO is

converted in the active form and can move into the tips of the cilium, activating a cascade of intracellular events. The ligand interaction with PTCH is regulated by several proteins: HH-interacting protein (Hip) competes with PTCH for HH binding and acts as a negative regulator of the pathway. The positive regulators, acting as HH co-receptor, includes Cdo, Boc, Gas1 and Glypican-3 (Heretsch et al., 2010). Following the activation of the HH pathway, SMO promotes the activation of transcription factors GLI, the final effectors of the pathway at the nuclear level.

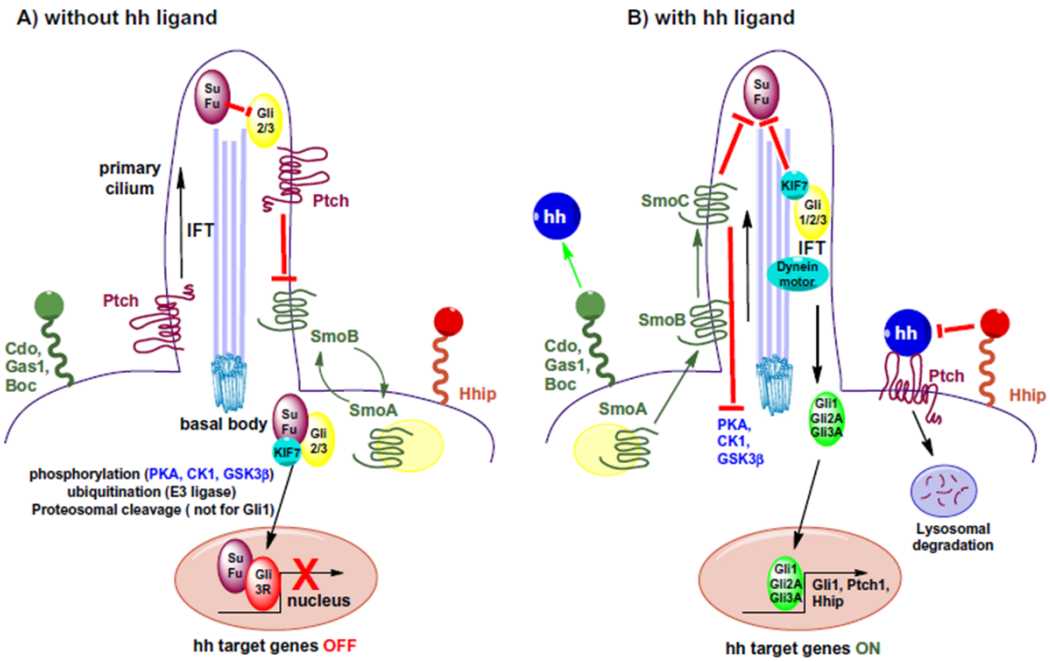


Figure 1.14. The Hedgehog signaling pathway in the primary cilia (Heretsch et al., 2010).

1.3.1 The GLI transcription factors and their regulation

The final mediators of HH pathway belong to the family of Kruppel transcriptional factors. In the eukaryotes, three transcription factors involved in the HH pathway were identified: GLI1, GLI2 and GLI3. GLI proteins are characterized by the presence of five conserved C2-H2 zinc-finger DNA binding domains and a histidine/cysteine linker sequence. The activation of HH signaling promotes the binding to the consensus sequence (5'-TGGGTGGTC-3') on the promoter of target genes, mediating a number of cellular responses (Kinzler et al., 1990).

GLI1 is considered the direct target of HH signaling pathway, while GLI2 and GLI3 act as transcriptional regulators in the late phase. GLI1 acts as a strong transcriptional activator of target genes and is regulated primarily at the transcriptional

level directly by the HH pathway, being a transcriptional target of GLI2 and GLI3 (Kasper et al., 2006). Alternative splicing of the full length (FL) form of GLI1 generates two other possible isoforms: GLI1 deltaN (N-terminal deletion variant) and tGLI1 (truncated GLI1), which differ in function and expression profile. GLI1FL and GLI1 deltaN are expressed both in normal and tumor tissues, whereas tGLI1 is expressed exclusively in tumor cells. tGLI1 is a more powerful transcriptional factor than the GLI1FL form and is highly expressed in various types of cancer, particularly in glioblastoma (GBM), where it is associated with an increase in motility and invasiveness, and in breast cancer (Carpenter et al., 2012). In eukaryotes, the activity of the three transcription factors GLI is context dependent and is differentially regulated by several factors, influencing cellular localization (nuclear or cytoplasmic) and post-translational modifications (phosphorylation, ubiquitination, acetylation and proteolytic degradation) of these proteins. While GLI1 acts exclusively as an activator, GLI2 and GLI3 display both positive and negative transcriptional functions. Modifications occurring at the N-terminal domain of GLI2 and GLI3 are responsible for the function of the two transcription factors. GLI2 has an N-terminal repressive domain and aC-terminal activation domain. It can then act as an activator oras a transcriptional repressor in the C-terminal truncated form (Ruiz i Altaba, 1999). GLI3, on the other hand, can only act as a repressor in its C-terminal truncated form. It has been shown that although GLI1 is predominantly regulated transcriptionally from the HH pathway, binding of GLI2 and GLI3 to the promoter is required for its transcription (Kasper et al., 2006). The subcellular localization of GLI1 is tightly controlled. HH stimulation induces the GLI1 nuclear localization, correlating with high transcriptional activity. In absence of HH pathway activation, GLI1 is retained in the cytoplasm and degraded by the proteasome. SUFU, one of the main negative regulators of HH signalling, interacts with GLI1 both at the N-terminal (amino acids 116–125) and at the C-terminal region and inhibits GLI1 both by retaining it in the cytoplasm and by repressing its transcriptional activity in the nucleus (Merchant et al., 2004). NUMB kinase can also act on GLI1, creating a recognition site for the E3 ubiquitin ligase ITCH, promoting its degradation (Di Marcotullio et al., 2006). In the absence of HH, also GLI2 and GLI3 ligands complex with SUFU protein. They are sequentially phosphorylated by different kinases: PKA (protein kinase A), GSK3β (glycogen synthase kinase 3β) and CK1 (casein kinase1). This modification creates a F-box recognition site for β-TrCP, an E3 ubiquitin ligase that recognizes GLI2/GLI3 and induces a proteasome-dependent

degradation. This proteolysis generates repressive conformations that prevent the expression of target genes. PKA is another important negative regulator of HH pathway: it phosphorylates GLI1 on residue Thr374, hiding the sequence for nuclear localization. PKA also phosphorylates GLI2 and GLI3 in the C-terminal region, thus favoring subsequent phosphorylation by CK1 and GSK3β and the βTrCP dependent protease degradation (Heretsch et al., 2010). The dual specificity Yak-1 related kinases 1 (DYRK1) and 2 (DYRK2) modulate HH pathway in opposite ways. DYRK1 phosphorylates GLI1 in its N- and C-terminal regions, increasing its nuclear retention and transcriptional activity, whereas DYRK2 reduces Gli1 transcriptional activity. Dyrk2 kinase directly phosphorylate GLI2, causing its degradation, and promotes the repressive form of GLI3. The acetylation of GLI1 and GLI2 seems to have an inhibitory effect on their transcriptional activity; this inhibition is removed from the HDAC activity, which promotes the activation of these transcriptional factors and promotes cell proliferation. Among the positive regulators of the HH pathway, the kinase ULK3 phosphorylates the GLI proteins by increasing their activation. STK36 is a Ser/Thr kinase that antagonizes SuFU inhibitory activity, contributing to the activation of the pathway. Missing in Metastasis (MIM) protein is encoded by a HH-responsive gene and is part of the Gli/SuFu complex, which acts by enhancing the transcriptional activity of GLI1 and GLI2 (Choudhry et al., 2014). The activation of the MEK1/ribosomal S6 kinase 2 (RSK2) cascade stabilizes GLI2 protein. RSK2 phosphorylates GSK3β, reducing its activity on GLI2 and its resulting ubiquitination and processing. This supports GLI2 nuclear localization and activation (Liu et al., 2014).

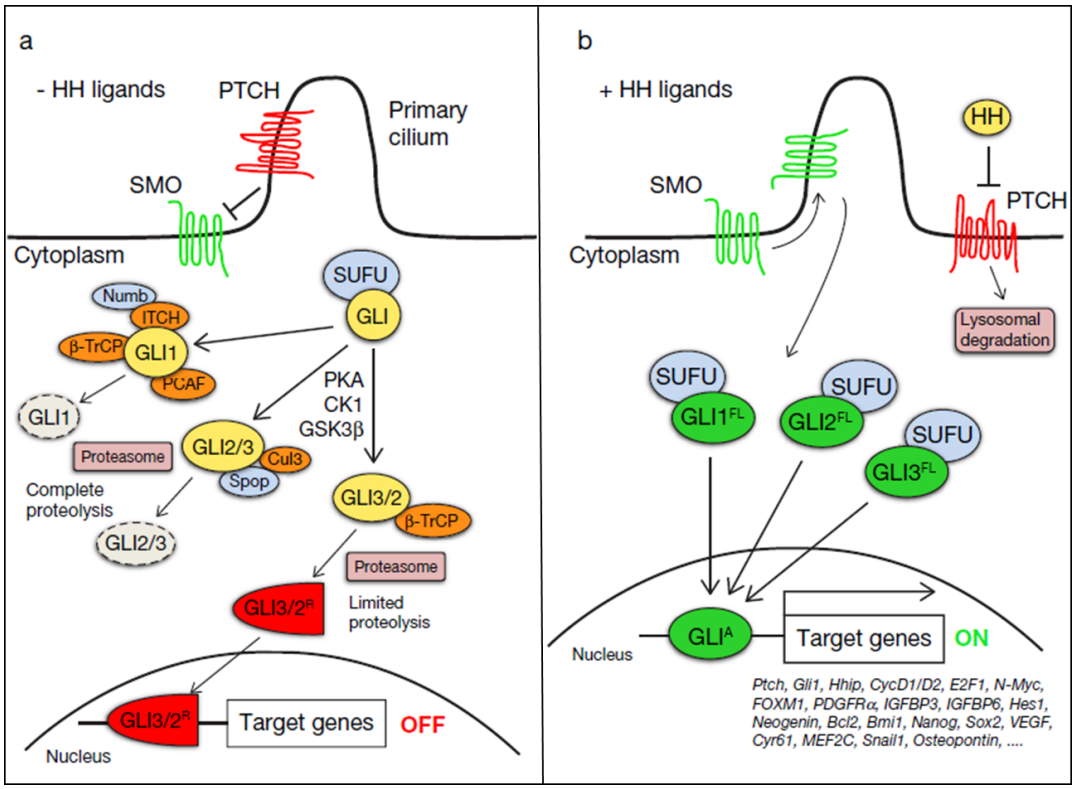


Figure 1.15 Key components of the HH signaling pathway (Pandolfi et al., 2015).

The GLI transcription factors regulate the expression of a number of targets involved in proliferation and differentiation (Cyclin D1 and D2, E2F1, N-Myc, FOXM1, PDGFRα, IGFBP3 and IGFBP6, Hes1, Neogenin), cell survival (BCL-2), self-renewal (Bmi1, Nanog, Sox2), angiogenesis (Vegf, Cyr61), cardiomyogenesis (MEF2C), epithelial–mesenchymal transition (Snail1, Sip1, Elk1 and Msx2) and invasiveness (Osteopontin). Interestingly, GLI transcription factors control the expression of some genes belonging to the HH pathway itself, including PTCH, HIP, Gas1, and GLI1, thus creating a negative feedback control system (through induction of PTCH and HIP) and a positive one (through the activation of GLI1 and Gas1 down-regulation) within the HH signaling pathway itself (Choudhry et al., 2014).

1.3.2 The Hedgehog signaling in cancer

Abnormal activation of the HH-GLI pathway is associated with a variety of tumors, including those of the skin, brain, lungs, prostate, breast, gastrointestinal tract and blood. The constitutive activation of the HH pathway promotes tumorigenesis through various processes. HH pathway promotes tumor cell proliferation and survival, inducing cell cycle regulators such as cyclin D1, cyclin D2, cyclin B1, p21, Bmi1, telomerase

activity and through the regulation of apoptosis genes (Marini et al., 2011). The HH pathway deregulation sustains an aggressive and metastatic tumor phenotype. Indeed, genes involved in EMT, motility and cell adhesion (Snail, E-cadherin, Osteopontin) are under the control of GLI transcription factors. Moreover, this pathway promotes invasiveness through the regulation of extracellular matrix metalloproteases (MMPs). Hedgehog also seems to favor the neo-angiogenesis process by transducing the VEGF signal, which is deregulated in most tumors (Brechbiel et al., 2014).

Multiple mechanisms of HH pathway canonical activation have been described in cancer (Scales et al., 2009). The constitutive HH pathway activation may occur in aligand-independent manner, mediated by loss of function mutations in the negative regulators (PTCH1, SUFU), activating mutations in SMO, or gene amplifications of GLI1and GLI2. This activation mechanism occurs more often in basal cell carcinoma (BCC), medulloblastoma, rhabdomyosarcoma and Gorlin syndrome. In many tumors, the activation of the HH pathway occurs in a ligand dependent manner, through autocrine or paracrine signaling. This mechanism has been identified in several types of cancer, including lung, pancreas, gastrointestinal tract, prostate and colon cancer, glioma and melanoma. Tumor cells are able to secrete and respond to HH ligands and show increased HH ligands expression apparently in absence of genetic alterations of HH pathway components. In the ligand dependent paracrine activation of HH pathway, HH ligands secreted by cancer cells activate HH signaling in the surrounding stroma, as happens physiologically during development. The mechanisms by which the HH signaling and the tumor stroma interact during paracrine signaling are not completely understood. Evidence supporting this mechanism derives from studies in human tumor xenograft models of pancreatic and colorectal cancers. Similarly, in the reverse paracrine HH pathway activation, HH ligands are secreted by the tumor microenvironment and activate the pathway in tumor cells. This mechanism is observed in an experimental model of glioma and in hematological malignancies, such as B-cell lymphoma and mantle cell lymphoma (Scales et al., 2009; Robbins et al., 2012). The HH signaling has also been implicated in the regulation of cancer stem cells (CSC), confirming its critical role in cancer. Activated HH signaling has been identified in CSCs of many solid tumors, such as glioblastoma, breast, colon, pancreatic cancer, melanoma, and hematological malignancies, including CML and multiple myeloma. HH activation increases tumor initiating populations and contribute to self-renewal, growth and tumorigenicity (Takebe et al., 2015).

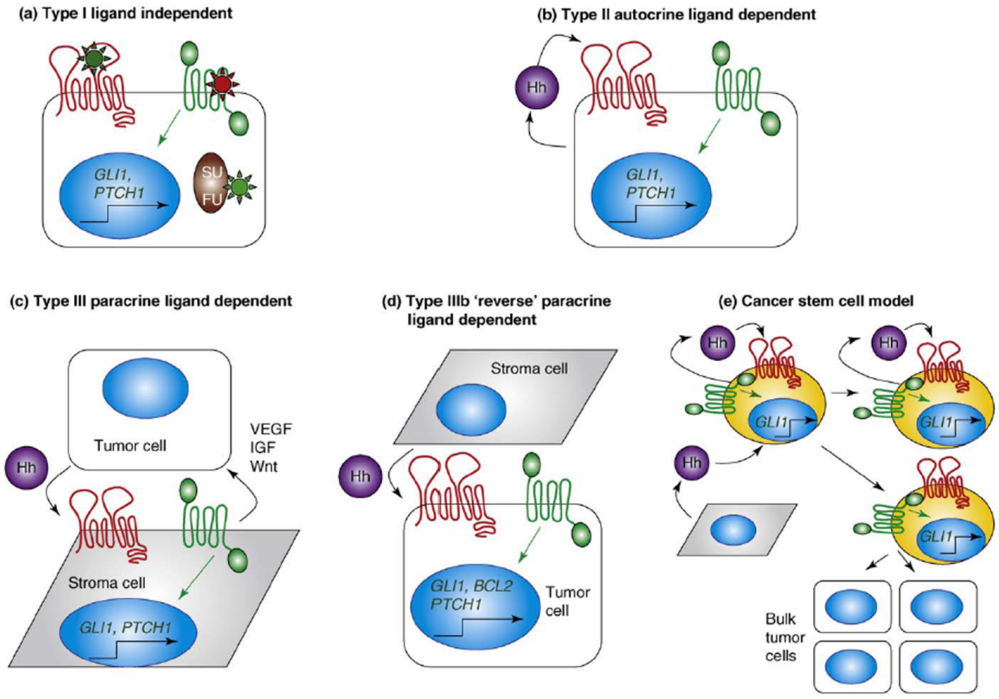


Figure 1.16 Modes of activation of the HH pathway in cancer (Scales et al., 2009).

The activity of HH-GLI pathway in human cancer is the result of its functional cooperation with other signaling pathway and of the direct or indirect regulation on the final effectors of the HH pathway. Several studies suggest that GLI proteins may be regulated by different proliferative and oncogenic inputs, in addition or independently of the canonical HH signaling (Pandolfi et al., 2015). In cancer the acquisition of oncogenic mutations and the inactivation of oncosoppressors alter this balance, leading to a constitutive activation of the pathway (Aberger et al., 2014).

1.3.3 Modulation of HH-GLI signaling by oncogenic pathways

Evidences of a non-canonical activation of HH-GLI signaling by other oncogenic pathways, such as RAS/RAF/MEK, PI3K/AKT/mTOR, Notch, TGFβ, Wnt/βcatenin, have been reported in many types of cancer. AKT signaling pathway promotes nuclear translocation and transcriptional activation of GLI1 in melanoma. The PI3K/AKT pathway inhibits PKA phosphorylation on GLI2, preventing GLI2 degradation and thus increasing GLI2-dependent transcription. PTEN, a negative regulator of PI3K/AKT pathway, is frequently mutated in several tumors and inhibits GLI1 transcriptional activity in glioblastoma. The mTOR/S6K1 pathway, activated by TNF-α, promotes

GLI1 transcriptional activity. S6K1 phosphorylates GLI1 and induce the release by its SUFU inhibitor. TGF-β also promotes the activation of HH pathway by increasing the expression of GLI2 through Smad3 and Smad4, resulting in further increase in GLI1 expression (Brechbiel et al., 2014). It has been identified the existence of a regulatory loop in which p53 and GLI1 negatively control each other. p53 reduces GLI1 activity, nuclear localization and protein levels. The HH pathway inhibits p53 promoting MDM2 activating phosphorylation on Ser166 and Ser186 residues, thus favoring p53 degradation (Stecca et al., 2009). A positive reciprocal loop links NANOG and HH pathway. Notch inhibits the HH signaling pathway through Hes1, a transcription factor that binds GLI1 to the level of the first intron and inhibits its expression. Activation of WNT/β-catenin signaling induces HH-GLI pathway increasing GLI1 expression. In Ewing's sarcoma, the oncogenic activation of GLI1 is directly promoted by the EWS/FLI transcription factor, which results from the translocation between chromosomes 11 and 22 that characterizes this tumor (Pandolfi et al., 2015). Tyrosine kinase receptors of several growth factors, including EGF (epidermal growth factor), PDGF (platelet-derived growth factor), and FGF (fibroblast growth factor) are important GLI positive modulators. It has been shown that HH and these growth factors promote synergistically cell transformation by integrating their signaling at different levels. Furthermore, HH is able to activate the tyrosine kinase receptor, particularly in the case of EGFR. This activation promotes intracellular activation of proliferative pathways of PI3K/AKT and RAF/MEK/MAPK (Aberger et al., 2014). Interplay between HH pathway and RAS/RAF/MEK has also been described in normal cells and tissues and represents one of the most important non-canonical activation ofthe HH signaling.

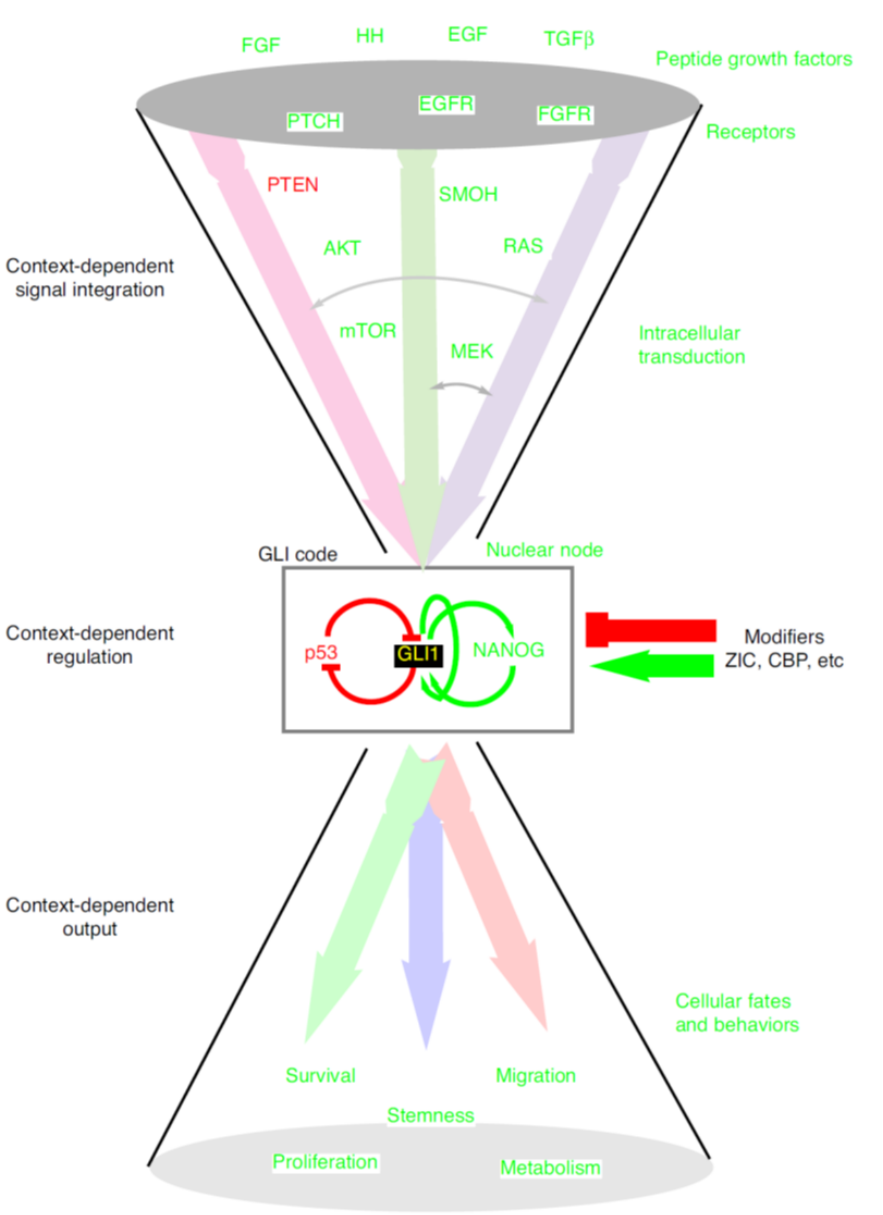


Figure 1.17 Integration of multiple signaling inputs from different pathways converging on HH-GLI pathway (Aberger et al., 2014).

1.3.4 Crosstalk between HH-GLI and MAPK pathway in melanoma

Several studies demonstrate the existence of a crosstalk between HH signaling and MAPK pathway, especially with the MEK1/2-ERK1/2 pathway. Both signaling pathways are crucial in different biological functions and are deregulated in several types of cancers, first of all in melanoma. Constitutively activated ERK1/2 is found in the majority of melanoma, due to the mutually exclusive activating mutations in BRAF (present in 50% of melanomas) and NRAS (found in 15–20% of melanomas). Recent studies indicate that the HH-GLI signaling is active in melanoma and supports growth

and proliferation of human melanoma cells, *in vitro* and *in vivo*. Indeed, SMO antagonists Cyclopamine or Sonidegib treatment reduces proliferation of human melanoma cells and decreases human melanoma xenograft growth in nude mice (Stecca et al., 2007; O'Reilly et al., 2013; Jalili et al., 2013). Interestingly, BRAF mutant cell lines are more sensitive to Sonidegib than BRAF wild type melanoma cells, indicating that the combined inhibition of BRAF (Vemurafenib) and Hedgehog (Sonidegib) can synergistically reduce melanoma cell proliferation (O'Reilly KE et al., 2013). MEK1/2-ERK1/2 signaling acts upstream of HH pathway, regulating the activity of the GLI transcription factors. Oncogenic NRAS (NRASQ61K) and HRAS (HRASV12G) modulate SUFU inhibitory activity and enhance GLI1 function, increasing its transcriptional activity and nuclear localization (Stecca et al., 2007). TGF-β signaling regulates melanoma tumorigenesis and metastasis and is a major inducer of EMT. GLI2 has been identified as a direct transcriptional target of the TGF-β/SMAD pathway in melanoma cells. High GLI2 expression is associated with a more aggressive phenotype, characterized by loss of the cell-cell adhesion molecule E-cadherin, hallmark of cancer progression (Javelaud et al., 2011). Activation of ERK 1/2 promotes cell proliferation and induces expression of SHH, thus activating the HH pathway in a ligand dependent manner. Recent studies have shown that treatment with BRAF inhibitors (BRAFi) induces HH-GLI pathway activation, which is responsible for PDGFR up-regulation following Vemurafenib treatment in human melanoma cells. PDGFR up regulation is one of the resistance mechanisms described in metastatic melanoma following BRAFi treatment (Sabbatino et al., 2014). Therefore, also the HH pathway may activate ERK1/2 by several mechanisms not entirely characterized. The expression of GLI-dependent target genes, such as PDGFR and IRS1, activates downstream ERK1/2 in a GLI-independent manner. Moreover, transactivation of EGFR by SHH has also been described following SMO activation (Pandolfi et al., 2015).

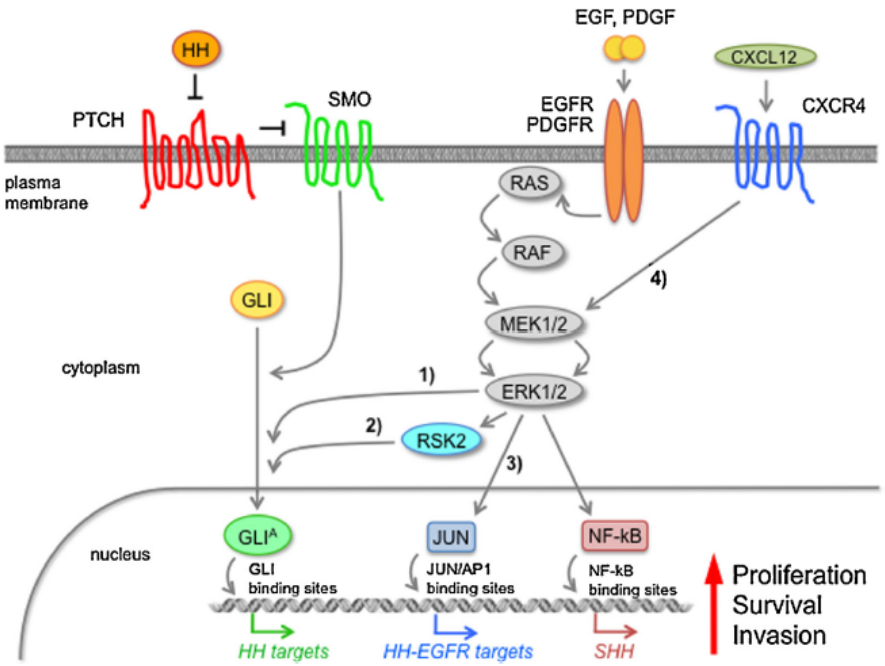


Figure 1.18 Activation of HH signaling by the RAS-RAF-MEK-ERK1/2 pathway (Rovida et al., 2015).

The HH pathway may interact non only with ERK1/2, but also with other final MAPK effectors such as JNK and p38 (Rovida et al., 2015). To date no interplay has been described between ERK5 and HH pathway. However, a report demonstrates that GLI2 and MEF2c, an ERK5 target, activate each other expression. During cardiomyogenesis MEF2c binds to GLI2 promoter, activating its expression (Voronova et al., 2012). The similarity between MEK-ERK1/2 pathway and ERK5 promped us to speculate about an interaction between ERK5 and the HH pathway.

1.3.5 Inhibitors of HH-GLI pathway

Given the important roles played during tumorigenesis, the HH pathway has been a key target for cancer therapy. Many inhibitors currently available can interfere with this pathway by exploiting mainly three strategies:

- inhibition of HH ligand activity;
- inhibition of SMO translocation and activation;
- inhibition of GLI transcription factors.

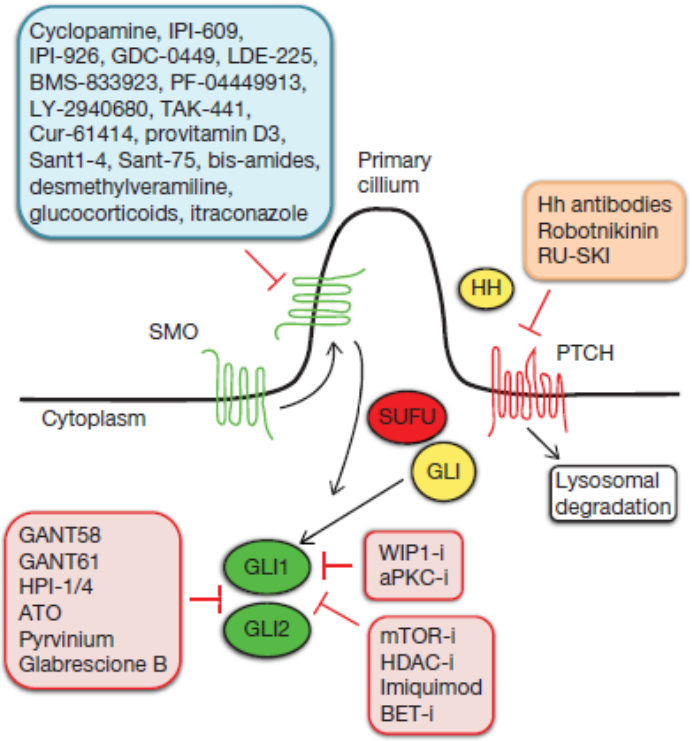


Figure 1.19 HH pathway inhibitors. Inhibitors are classified according to level of the pathway inhibited: SMO translocation and activation (blue), HH/PTCH interaction (orange) and GLI nuclear translocation and transcriptional activity (red) (Pandolfi et al., 2015).

HH ligands inhibition is a poorly used strategy in therapy and is based on the interference between HH ligands and PTCH. Small molecules, such as Robotnikinin, or Sonic HH specific monoclonal antibodies, such as 5E1, attenuate the growth of cancer cells in some tumors, including lung cancer (Stanton et al., 2009). Inhibition of SMO is the most used strategy to target HH pathway. Cyclopamine, an alkaloid extracted from *Veratum Album*, is the natural ligand of SMO extensively used to study HH pathway. Its application as therapeutic agent is hindered by its unfavorable pharmacokinetic properties (poor oral solubility, chemical instability). All SMO inhibitors currently used in clinical trials are semi-synthetic or synthetic cyclopamine derivatives, including the most soluble cyclopamine analogues (IPI-926), KAAD-cyclopamine, agents that inhibit the conversion of SMO into active form (SANT 74-75), agents that inhibit the translocation of the active form of SMO through cilia (SANT 1-4) (Lin et al., 2012). Additional SMO inhibitors with a structure different from Cyclopamine are currently available and many, including Vismodegib (GDC-0449), Sonidegib (LDE-225), BMS-833923, PF-04449913 and LY2940680 are being investigated in clinical trials in a number of advanced cancers. Among these, Vismodegib (GDC-0499,

Genentech/Roche/Curis), is the first Hedgehog signalling antagonist approved by U.S. Food and Drug Administration (FDA) for treatment of advanced or metastatic basal cell carcinoma (BCC). Despite the excellent results reported in clinical trials, in particular for Vismodegib (GDC-0449) and Sonedegib (LDE225), their anticancer activity seems to be limited by the acquisition of resistance due to different mechanisms. The most relevant are the acquisition of mutations in human SMO (D473H); amplification of downstream HH target genes (GLI2 and CyclinD1); increased drug efflux through the adenosine triphosphate (ATP)-binding cassette transporter (ABC); upregulation of other oncogenic signalling, such as PI3K/AKT pathway observed during LDE-225 treatment, leading to a non-canonical activation of the pathway (Pandolfi et al., 2015). Targeting the final effectors of the HH signalling with GLI inhibitors would provide a good approach to block both canonical and non-canonical HH pathway activation and perhaps overcome anti SMO drug resistance. Unfortunately, to date only few molecules acting on GLI proteins have been identified (GANT61 and GANT58, HPI-1, HPI-2) and their use is only limited to preclinical studies. The only GLI inhibitors used in therapy are the arsenic trioxide (ATO), approved by FDA for acute promyelocytic leukaemia, and pyrvinium, an anti-pinworm agent (Onishi et al., 2011). Recently, the identification of the Gli1 zinc fingers involved in DNA binding allowes to synthesize a small molecule (Glabrescione B) that interferes with the interaction of GLI with DNA. Through the strong inhibition of Gli1 activity, Glabrescione B inhibits growth of HH-dependent BCC and MB tumour cells *in vitro* and *in vivo* as well as self-renewal ability and clonogenicity of CSCs (Infante et al., 2015).

Based on crosstalk between HH and other pathways, clinical trials and experimental studies have been initiated to evaluate the therapeutic efficacy of the association between SMO or GLI inhibitors and inhibitors of EGFR and MEK, the most interesting regulator of HH pathway. The combination treatment with Cyclopamine and Gefitinib (EGFR inhibitor) has led to a reduction in tumor growth and an increase in apoptosis in pancreatic cancer. Also in the neck head tumors (HNSCC) the association between the SMO inhibitor Saridegib (IPI-926) and Cetuximab has given excellent results, allowing to inhibit tumor cell proliferation and increase the duration of response (Ruch et al., 2013). In glioblastoma, combined therapy between Cyclopamine and Erlotinib (EGFR inhibitor) improved the effectiveness of therapy, reducing the risk of recurrence of the tumor by Cyclopamine administration in a first phase, followed by the association with Erlotinib in a second phase of treatment. This demonstrates that HH

stimulation by EGFR is required for the development of the tumor. In lung cancer (NSCLC) xenograft pretreatment with Vismodegib (SMO inhibitor), Cisplatin and Erlotinib increases the antitumor activity compared with monotherapy. Lastly, in prostate cancer, combined treatment with SMO inhibitors (SANT1) and MEK inhibitors (PD325901 and U0126) shows greater antiproliferative activity than conventional MEK inhibitors (Brechbiel et al., 2014).

The promising results obtained in clinical trials highlight the importance to study the interplay between different pathways, in order to identify new pharmacological targets and develop more effective combined therapies.

2. AIM OF THE STUDY

Malignant melanoma is among the most aggressive cancers and its incidence is increasing worldwide. While early stage melanoma can be cured with surgery, prognosis of metastatic melanoma is still poor. In the last few years targeted therapy and immunotherapy have improved survival of patients with metastatic melanoma, however available treatments are still unsatisfactory, showing an urgent need to identify new therapeutic targets. The BRAF-MEK1/2-ERK1/2 pathway is active in the majority of melanoma and its role has been extensively studied in human as well as in mouse models of melanoma. Another signaling pathway that is required for melanoma cell growth *in vitro* and *in vivo* is the Hedgehog signaling (Stecca et al., 2007; O'Reilly et al., 2013; Jalili et al., 2013). An emerging member of the MAPK family of most recent discovery is the kinase ERK5, which is involved in the regulation of cell survival, anti-apoptotic signaling, angiogenesis, differentiation and proliferation. ERK5 plays a role in the pathogenesis of different types of cancer, including highly aggressive forms of breast and prostate cancers, hepatocellular carcinoma and multiple myeloma (Al-Ejeh et al., 2014; Carvajal-Vergara et al., 2005; Rovida et al., 2015; Yang et al., 2011; Simões et al., 2016). A recent report suggested an association of ERK5 with drug resistance to combined inhibition of BRAF and MEK1/2 in melanoma (Song et al., 2017).

The aim of this study is to explore the role of ERK5 in melanoma and its interplay with oncogenic BRAF and the Hedgehog signaling, with the ultimate objective to identify novel therapeutic options for metastatic melanoma. In particular, we propose the following specific aims:

1. To investigate the role of ERK5 in the proliferation of melanoma cells *in vitro* and *in vivo*, by genetic silencing of ERK5 with specific short hairpin RNAs and by pharmacological inhibition of the MEK5-ERK5 pathway with small molecules.

2. To investigate whether oncogenic BRAF regulates ERK5, since BRAF is mutated in more than half of human melanoma cases. The identification of an interplay between these two kinases may open the possibility to target both pathways as a strategy to inhibit melanoma cell proliferation.

3. To investigate the interplay between ERK5 and the Hedgehog pathway, a signaling that our group first showed critical for growth, recurrence and metastasis of melanoma xenografts in mice. In particular, we will test whether ERK5 affects the activity of the three GLI transcription factors, the last mediator of the Hedgehog signaling.

3. MATERIALS AND METHODS

3.1 Cell culture and melanoma patients samples

A375 (CRL-1619), MeWo (HTB-65), Human Embryonic Kidney 293 (HEK-293T) (CRL-3216) and embryonic fibroblast NIH3T3 (CRL-1658) cell lines were obtained from ATCC (Manassas, VA, www.lgcstandards-atcc.org). SK-Mel-2, SK-Mel-5, SK-Mel-28 and 501-Mel melanoma cell lines were kindly provided by Dr. Laura Poliseno (CRL-ITT, Pisa, Italy). Patient-derived SSM2c and M26c melanoma cells were obtained after protocols approved by the Ethics Committee (Santini et al., 2012; Pandolfi et al., 2013) from patients of the Plastic Surgery Unit of the S.M. Annunziata Hospital (Florence, Italy) and Dermatology Department and Medical-Surgical Critical Area of the University of Florence (Florence, Italy). After mechanical disruption, tumors were incubated for 1 hour at 37°C with 1mg/ml collagenase A and 20μg/ml DNase I (Roche Applied Science, Basel, Switzerland) in DMEM/F12 (Euroclone, Milan, Italy), in order to eliminate residual fat tissue and cellular structures. After dissociation and filtration in 70 μm cell strainers, cells were grown in DMEM/F12 with 10% fetal bovine serum (FBS- Carlo Erba) and epidermal growth factor (EGF) (5ng/ml) (Invitrogen, Carlsbad, CA). SSM2c and M26c cultures were cloned from the original metastases (SSM2 and M26, respectively) by plating one cell per well. Patient-derived melanomas were passaged one to two times prior to RNA extraction and in vitro experiments. The identity of melanoma cells was verified by immunocytochemistry using primary antibodies specific for melanoma: anti-Melan A (A103), anti-S100 (Dako, Glostrup, DK) and anti-Vimentin (V9) (Santa Cruz Biotechnology).

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Euroclone, Milan, Italy, http://www.euroclonegroup.it) supplemented with 10% fetal bovine serum (FBS- Carlo Erba), 1% Penicillin-streptomycin, 1% L-Glutamine (Lonza, Basel, Switzerland). Cell lines were authenticated by cell profiling (Promega PowerPlex Fusion System kit; BMR Genomics s.r.l; Padova, Italy) once a year. Mycoplasma was periodically tested by 4',6-diamidino-2-phenylindole (DAPI) inspection and PCR upon thawing of a new batch of cells, once a month. Cultures are renewed every two months. Genetic alterations of melanoma cell lines used in this study are reported in Table 3.1

Cell line	Mutations
A375	BRAF V600E
SK-Mel-2	NRAS Q61R
SK-Mel-5	BRAF V600E
SK-Mel-28	BRAF V600E
501-Mel	BRAF V600E
MeWo	NF1, p53, CDKN2A trunc/indel
M26c	wt BRAF/ wt NRAS
SSM2c	wt BRAF / wt NRAS

Table 3.1 Genetic alterations of melanoma cell lines used in this work.

3.2 Drugs

Considering that ERK5 was positively regulated by serum and growth factors, before treatment cells were starved for 24 hours and pharmacological treatment was performed in low serum condition. The following drugs were used: ERK5 inhibitors XMD8-92 (Yang et al., 2010) and JWG-045 (Williams et al., 2016) have been developed in Gray's laboratory; MEK5 inhibitor BIX02189 (Tatake et al., 2008), ERK1/2 inhibitor SCH772984 (Morris et al., 2013) and BRAF-V600E inhibitor Vemurafenib (Selleckchem, Italy, www.selleckchem.com) (Sala et al., 2008); CDK1 inhibitor RO-3306 (MedChem Express, www.medchemexpress.com) (Vassilev et al., 2006). For the selection of transduced cells we used puromycin (2µg/ml). Finally, we used SAG (100nM for 48hrs, Enzo Life Sciences, Farmingdale, NY, USA) as agonist of SMO receptor.

3.3 Plasmids and transfections

pcDNA3.1-BRAF-V600E and pcDNA3.1-BRAFwt constructs were a kind gift from Laura Poliseno (CRL-ITT, Pisa, Italy). pcDNA3.1-HA-ERK5wt construct was a kind gift from Atanasio Pandiella (CIC, Salamanca, Spain). The pcMV5-MEK5DD-HA (a constitutively active form of MEK5) was generously provided by Jiing-Dwan Lee (Scripps Institute, La Jolla, CA, USA). HEK-293T or M26c cells were plated in 60 mm diameter dishes ($3x10^5$ cells/dish) and transfected after 24 hours with a total amount of 3µg of plasmid DNA. Transfection was performed in the reduced serum media

OptiMEM (Life Technologies, Carlsbad, CA, USA) using Polyethylenimine (jetPEI reagent, Polypus Transfection, Euroclone, Milan, Italy) or X-tremeGENE (Roche Applied Science) as transfection reagent. Both transfection agents are added to the diluted DNA in a ratio of 3:1. Cells were lysed after 48 hours. When provided, drugs were added 18 hours before lysis.

3.4 Lentiviral vectors, virus production and transductions

Lentiviral vectors for stable knockdown of ERK5 in melanoma cells were TRC1.5-pLKO.1-puro vector containing non targeting sequence shRNA (LV-c), targeting human MAPK7 (NM_139032, NM_139034, NM_002749, NM_139033) clone ID: TRCN0000010275 (LV-shERK5-275) and clone ID:TRCN0000010262(LV-shERK5-262).

HEK293T cells were seeded in 100 mm diameter dishes (2×10^6 cells/dish) in DMEM supplemented with 10% FBS and 2 mM glutamine without antibiotics (complete medium). After 24 hours (40%–70% confluency) medium was replaced with fresh complete medium. The plasmid mixture was prepared as follows: 8 μg of lentiviral vectors encoding for shRNA, 4 μg of pRSV-Rev, 4 μg pMDLg/pRRE and 4 μg pMDG.1-VSV and 150 mM NaCl (Polypus Transfection, Euroclone, Milan, Italy) to a final volume of 250 μL. Transfection was performed using 40 μL of jetPEI reagent (Polypus Transfection, Euroclone, Milan, Italy) following the manufacturer's protocol. Twenty-four hours after transfection, the medium was replaced with fresh complete medium. The following day culture media from HEK293T was collected and fresh complete medium added to the cells. Harvested medium was centrifuged at 1500 rpm for 5 min and filtered through a 0.45 μm filter and either directly added to melanoma cells or stored at −80°C for later use. This procedure was repeated 1 day after. For infection, melanoma cells were seeded in a 60 mm diameter dish (3×10^5 cells/dish). At an optimal confluence of 50%, 2 mL/dish of virus supernatant were added in the presence of 5 μg/mL polybrene. Infected cells were selected with 2 μg/mL puromycin for at least 72 hours.

3.5 Measurement of cell viability and cell cycle phase distribution analysis

The number of viable cells in culture was evaluated by counting trypan blue-negative cells at the indicated time-points with a hemocytometer (Burker counting chamber). For growth curve with MEK5/ERK5 inhibitors we seeded cells in 12-well plates in low serum condition (DMEM 2.5% FBS, 1% Penicillin-streptomycin, 1% L-Glutamine). We seeded 18000 cells/well for M26c and SSM2c cell lines and 12000 cell/well for A375, SK-Mel-5 and 501-Mel cell lines. Viable cells were counted after 72 hours of treatment. IC50 values were calculated using GraphPad Prism software.

For cell cycle phase distribution analysis, we seeded 150000 cells in 60 mm diameter dishes with DMEM 0.5% FBS, 1% Penicillin-streptomycin, 1% L-Glutamine. Cells were harvested after 48 hours and resuspended in 400 µl of an hypotonic solution containing 50 µg/mL propidium iodide, 0.1% w/v trisodium citrate and 0.1% NP40. Cytometric analysis was performed with FACS Canto II (Beckton & Dickinson, San Josè, CA, USA) and analyzed using ModFit LT software (Verity Software House, Topsham, ME).

3.6 Cell lysis, Western blotting and immunoprecipitation

Cells were lysed with different buffers, according to the protein to detect. For GLIs detection, cells were harvested, centrifuged at 1500 rpm and pellet was resuspended in RIPA buffer (1% NP-40, 150mM NaCl, 5mM EDTA, 0.25% NaDOC, 50mM Tris-HCl pH 7.5, 1% SDS) added with 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) and phosphatase inhibitors. After incubation in ice for 20 minutes, lysate was centrifuged for 20 minutes at 14000 rpm. The supernatant containing the whole cell extract (WCE) was recovered and quantified with Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). For Western Blot 80µg of proteins were resolved on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 6% non-fat dry milk (Bio-Rad) diluted in PBS-Tween buffer (PBS and 0.05% Tween 20, PBS-T) for 1h and incubated with the primary antibody of interest overnight at 4°C. After incubation with HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA), bands were visualized by chemiluminescent detection ChemiDoc XRS (Bio-Rad) using

ECL™ Western Blotting Detection Reagents (Sigma Aldrich, GE Healthcare, RPN2209).

For ERK5 detection, cells were washed with 1x PBS and lysed in ice-cold lysis buffer (140 mM NaCl; 10 mM EDTA; 10% glycerol; 1% Nonidet P-40; 20 mM Tris pH 7.0;1% SDS). Immediately before use, the following proteases and phosphatases inhibitors have been added to extraction buffers: 1x Prothease Inhibitor (PI), β-Glycerophosphate 2mM; PMSF 1 mM; sodium phosphate 1 mM and sodium orthovanadate 1 mM. After scraping the cells from the dishes, samples were incubated in ice for 10 minutes and then centrifuged at 14000 rpm at 4 °C for 10 min. Supernatant was transferred into new tubes and quantified with Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). For ERK5 immunoprecipitation, the lysate obtained with the same lysis protocol was incubated with 1µg of ERK5 C7 antibody (sc-398015, Santa Cruz Biotechnology) and Protein A/G PLUS-Agarose (Santa Cruz, sc-2003) beads overnight at 4°C. The immune complexes were recovered by a short centrifugation followed by 3 washes with 1 ml of cold lysis buffer. Samples were then boiled in electrophoresis sample buffer and loaded in SDS-PAGE gels. After transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), these were blocked for 1 h in 5% BSA in PBS-T 0.05% and then incubated overnight with the corresponding antibody. After washing with PBS-T 0.05%, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour, washed and bands visualized by chemiluminescent detection ChemiDoc XRS (Bio-Rad). For the develop, we used Luminata Crescendo Western HRP Substrate (Millipore) a premixed, ready-to-use reagent for chemiluminescent detection in western blotting applications that employ horseradish peroxidase (HRP)- conjugated antibodies. Images were recorded as TIFF files through the Quantity One software (Bio-Rad) and the quantification was performed using ImageJ software. The signal was measured as a corrected total cell fluorescence (CTCF), calculated as product between the Integrated Density, the Area of selected band and the Mean fluorescence of background readings. Each band is normalized on housekeeping and related to control.

Antibodies used are listed above:

Protein	**Source**	**Notes**	**Cat. No.**	**Company**
p21Waf1/Cip1	rabbit monoclonal	12D1	#2947	Cell Signaling Technology, Danvers, MA, USA
BRAF	mouse monoclonal	F-7	sc-5284	Santa Cruz Biotechnology, Santa Cruz, CA, USA

ERK5	rabbit polyclonal		#3372	Cell Signaling Technology, Danvers, MA, USA
ERK5	Mousemonoclonal	C-7	sc-398015	Santa Cruz Biotechnology, Santa Cruz, CA, USA
pERK5-T218/Y220	rabbit polyclonal		#3371	Cell Signaling Technology, Danvers, MA, USA
pERK5-S753	rabbit			Kind gift of Dr. Pandiella
pERK5-T732	rabbit			Kind gift of Dr. Pandiella
pERK1/2-T202/Y204	rabbit polyclonal		#9101	Cell Signaling Technology, Danvers, MA, USA
ERK1/2	rabbit polyclonal	C-16	sc-93	Santa Cruz Biotechnology, Santa Cruz, CA, USA
pMEK1/2-S217/221	rabbit polyclonal		#9121	Cell Signaling Technology, Danvers, MA, USA
pRb-S807/811	rabbit monoclonal	D20B12	#8516	Cell Signaling Technology, Danvers, MA, USA
Actin	mouse monoclonal	AC-15	A1978	Sigma-Aldrich St. Louis, MO, USA
Vinculin	mouse monoclonal		V9131	Sigma-Aldrich St. Louis, MO, USA
HSP90	mouse monoclonal	F-8	sc-13119	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Fibrillarin	goat polyclonal	D-14	sc-11336	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Lamin A	rabbit polyclonal	H-102	sc-20680	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Lamin B1	mouse monoclonal	119D5-F1	sc-56143	Santa Cruz Biotechnology, Santa Cruz, CA, USA
GAPDH	goat polyclonal	V-18	sc-20357	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Cyclin D1	mouse monoclonal	A-12	sc-8396	Santa Cruz Biotechnology, Santa Cruz, CA, USA
CDK1	rabbit polyclonal		sc-954	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Cyclin B	rabbit polyclonal	H-433	sc-752	Santa Cruz Biotechnology, Santa Cruz, CA, USA
pMBP	mouse monoclonal	P12	#05-429	Merck Millipore, Billerica, MA, USA
MBP	mouse monoclonal	F-6	sc-271524	Santa Cruz Biotechnology, Santa Cruz, CA, USA
α Tubulin	mouse monoclonal		sc-32293	Santa Cruz Biotechnology, Santa Cruz, CA, USA

Table 3.2 List of antibodies used in the study.

3.7 Cell fractionation

For cell fractionation, cells were harvested in Trypsin-EDTA (0,05% Trypsin, 0,53 mM EDTA, Invitrogen) to preserve cellular integrity and centrifuged for 5 minutes at 1500 rpm. Pellet was lysed in the buffer A for the cytoplasmic extract (20mM Hepes buffer, 10 mM KCl, 1 mM EDTA, 0.2% NP-40, 10% Glycerol) added with 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) and phosphatase inhibitors. After incubation in ice of 15 minutes, the preparation was centrifuged and the cytoplasmic extract was collected in the resulting supernatant. The pellet (nuclei and membranes) was dissolved in ice-cold lysis buffer (140 mM NaCl; 10 mM EDTA; 10% glycerol; 1% Nonidet P-40; 20 mM Tris pH 7.0;1% SDS, 1x PI,β-Glycerophosphate 2mM; PMSF 1 mM; sodium phosphate 1 mM and sodium orthovanadate 1 mM) and incubated for 10 minutes in ice. The sample was centrifuged for 10 minutes at 14000 rpm and the nuclear protein extract collected from the supernatant. Nucleus-cytoplasm fractions have been quantified with Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and resolved in SDS-PAGE as described above.

In other experiments to obtain also the chromatin bound fraction, the subcellular fractionation was performed using the Subcellular Protein Fractionation Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). The kit includes different buffers that enable stepwise separation and preparation of cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extracts from cultured cells through sequential centrifugations. Immediately before use, protease inhibitors have been added to extraction buffers to maintain extract integrity and function. The adherent cells were harvested with trypsin-EDTA and then centrifuged at 500 g for 5 minutes. The cell pellet was washed with ice-cold PBS 1X and centrifuged at 500 g for 2-3 minutes. The first reagent added to a cell pellet (Cytoplasmic Extraction Buffer- CEB) causes selective permeabilization of cell membrane and the release of soluble cytoplasmic contents. The second reagent (Membrane Extraction Buffer- MEB) dissolves plasma, mitochondria and endoplasmic reticulum/golgi membranes but does not solubilize nuclear membranes. After recovering the intact nuclei by centrifugation, the Nuclear Extraction Buffer (NEB) yields the soluble nuclear extract. Finally, a second nuclear extraction is performed to release the chromatin-bound nuclear proteins by adding 5 µL of 100 mM CaCl2 and 3 µL of Micrococcal Nuclease per 100 µL of room temperature NEB. The recovered insoluble pellet is then extracted with the final reagent to isolate cytoskeletal proteins.

3.8 ERK5 kinase assay

Kinase activity of endogenous ERK5 was measured using a non-radioactive ERK Assay Kit (#17-191, Merck Millipore, Billerica, MA, USA). This *in vitro* kinase assay allows to measure the phosphotransferase activity of an immunoprecipitated MAP Kinase on a specific substrate (myelin basic protein, MBP). The phosphorylated substrate is then analyzed by immunoblot analysis, probing with a monoclonal Phospho-specific MBP antibody; the total MBP antibody was used as control of total MBP levels. ERK5 protein was immunoprecipitated as described above and 500 µg of immunoprecipitated protein was incubated for 30 minutes at 30°C in agitation in presence of Mg2+/ATP cocktail, the assay dilution buffer (ADBI), the Map Kinase substrate cocktail (MBP) and the inhibitor cocktail. The reaction mixture was recovered by a short centrifugation. For SDS-PAGE and immunoblot analysis, we used 5µl of the reaction mixture (approximately 2µg of phosphorylated-MBP), adding 5µl of 1X PBS and 10µl of 2X Laemmli sample buffer. Samples were then boiled and loaded in double in SDS-PAGE gels. After transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), these were blocked with 6% non-fat dry milk in PBS-T 0.05% for 1 hour at room temperature with constant agitation. The nitrocellulose membrane was incubated with 0.5-2µg/ml of anti-phospho-MBP, clone P12, and with the total MBP, clone F-6, overnight with agitation at 4°C. After three washes with PBS-T 0.05%, membranes were incubated with a goat anti-mouse HRP conjugated IgG secondary antibodies for 1 hour and washed. Signal was visualized by chemiluminescent detection by ChemiDoc XRS (Bio-Rad), using Luminata Crescendo Western HRP Substrate (Millipore).

3.9 Luciferase assay

To measure the MEF2 transcriptional activity we used the luciferase reporter 3XMEF2-luc (plasmid# 32967 was a gift from Ron Prywes, Addgene, Teddington, UK) in combination with *Renilla* luciferase pRL-TK reporter vector (Promega, Madison, WI) to control for transfection efficiency and to normalize luciferase activities; pcDNA vector was used to equal DNA amounts. Cells were co-transfected with equimolar amounts of wt ERK5 in combination with the empty vector pCAG, constitutively active MEK5 (MEK5DD) or BRAF-V600E plasmids to evaluate the modulation of the transcriptional activity of MEF2 promoter. To measure GLI transcriptional activity, we used a GLI-responsive luciferase reporter (p8x3GLI-BS, GLI-BS) which contains 8

direct repeats of the GLI consensus sequence GACCACCCA cloned upstream the luciferase gene (kind gift from H. Sasaki) (Sasaki, H. et al., 1997). The activity of the endogenous pathway was evaluated in NIH3T3 cells (seeded 25000 cells/well in 12 well plate in DMEM 2.5% FBS, 1% Penicillin-streptomycin, 1% L-Glutamine). Cells were treated with SAG 100 nM for 48 hours and with increasing doses of XMD8-92 for the last 16 hours. The transcriptional activity of ectopic GLI was evaluated seeding M26c, SSM2c and HEK293T (85000 cells/well in 12 well plate plate in DMEM 2.5% FBS, 1% Penicillin-streptomycin, 1% L-Glutamine) and co-trasfetting cells with the reporter vector p8x3GLI-BS (GLI-BS) (Sasaki, H. et al., 1997) and low amount of GLI1 expression construct. The effect of ERK5 inhibition on transcription was evaluated treating cells with increasing amount of XMD8-92 for 16 hours or cotrasfecting cells with two different shRNA for ERK5 silencing described above (shERK5 262, shERK5 275). After 48 hours from transfection, cells were harvested with Passive Lysis buffer (Promega). Luminescence was measured using the Dual-Glo Luciferase Assay System (Promega) and the GloMax® 20/20 Luminometer (Promega).

3.10 Xenografts

In the first set of experiments, A375 and SSM2c melanoma cells transduced with LV-c or LV-shERK5 were resuspended in Matrigel (Corning, www.corning.com)/DMEM (1/1) and subcutaneously injected (10000 cells/injection) into lateral flanks of adult (8 weeks) female athymic-nude mice (Foxn1 nu/nu) (Harlan Laboratories, Udine, Italy). Number of animals per group was 6 for A375 and SSM2c LV-shERK5 and 7 for SSM2c LV-c. In the second set of experiments, parental A375 cells were subcutaneously injected as above. Once tumors were palpable (4 mm^3), mice were randomized in four groups of 9 mice each and treated intraperitoneally (IP) twice a day for 19 days with vehicle (30% 2-hydroxypropyl-β-cyclodextrin), XMD8-92 (25 mg/Kg), Vemurafenib (20 mg/Kg) or a combination of both drugs. In both experiments subcutaneous tumor size was measured three times a week with a caliper. Tumor volumes were calculated using the formula: $V=W^2 \; x \; L \; x \; 0.5$, where W and L are, respectively, tumor width and length. The experiments were approved by the Italian Ministry of Health (Authorization n. 213/2015-PR) and were in accordance with the Italian guidelines and regulations.

3.11 Statistical analysis

Data represent mean ± SEM or mean ± SD values calculated on at least 3 independent experiments. P-values were calculated using one-way ANOVA when more than two samples were analyzed or Student t-test when two samples were compared. Analysis of *in vivo* combined treatments were performed using the false discovery rate (FDR) adjustment for multiple comparisons. A two-tailed value of $p<0.05$ was considered statistically significant. Number of mice used for each experiment were indicated above.

4. RESULTS

4.1 ERK5 is consistently expressed and active in human melanoma

In the last few years, the involvement of the MEK5/ERK5 pathway has been described in the pathogenesis of different types of cancers (Hoang et al., 2017). However, its role in melanoma is not well established. To assess whether alterations in components of the ERK5 pathway occur in melanoma samples, we have consulted the cBioPortal for Cancer Genomics, an open platform for interactively exploring multidimensional cancer genomics data sets in the context of clinical data and biologic pathways. The cBioPortal provides visualization, analysis and download of large-scale cancer genomics data sets (Cerami et al., 2012; Gao et al., 2013). *In silico* data analysis of primary and metastatic melanomas obtained from the Skin Cutaneous Melanoma data set (TCGA, Provisional) indicated that 47% of human melanomas presented mutations, gene copy number or mRNA alterations in several components of the ERK5 signaling. These alterations involve activators of the pathway, such as MAP3K2, MAP3K3 and MAP2K5 (alternative name for MEK5), MAPK7 itself (the gene encoding for ERK5) and downstream targets of the pathway, including members of the family of MEF2 transcription factors (Kato et al., 1997) (i.e. MEF2A, MEF2B, MEF2C and MEF2D) (Figure 4.1A).

The presence of alterations in the MEK5/ERK5 pathway is relevant for prognosis of melanoma patients, as shown by the Kaplan-Meier curves in Figure 4.1B. Indeed, melanoma patients with MAPK7 alterations (including mRNA upregulation and MAPK7 amplifications, but not deletions) showed reduced disease free survival ($p = 0.042$) and a trend toward shorter overall survival ($p = 4.793e^{-4}$) compared to patients without such alterations (Figure 4.1B).

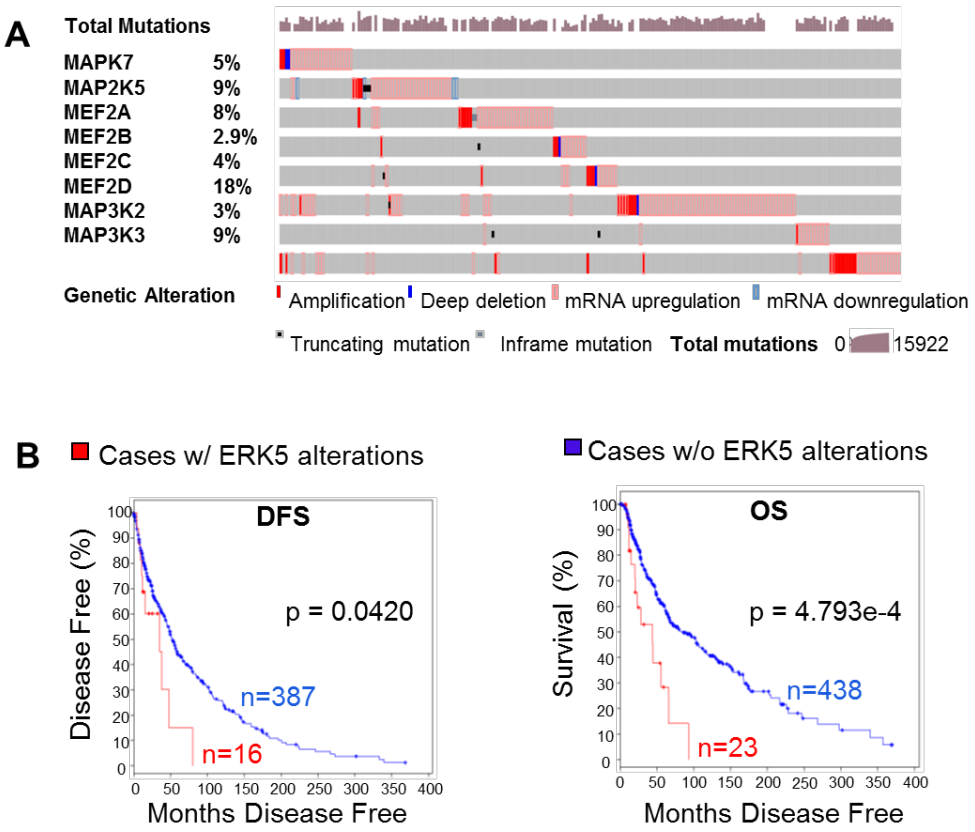


Figure 4.1 Genomic analysis of the ERK5 pathway and survival of melanoma patients with ERK5 alterations. A) Genomic profiles of components of the ERK5 pathway in melanoma patients obtained from the Skin Cutaneous Melanoma data set (TCGA, Provisional) using cBioportal database (http://www.cbioportal.org). Shown are 223 out of 479 (47%) primary and metastatic melanomas with gene copy number and mRNA alterations. Putative passenger mutations are not included. MAPK7 is the gene coding for ERK5/BMK1. MAP2K5 encodes for MEK5. B) Kaplan-Meier Overall Survival (OS) and Disease Free Survival (DFS) in melanoma patients with (red) or without (blue) ERK5 genetic alterations (data set restricted to "AMP EXP >=2", in order to exclude patients harboring MAPK7 deletion). Median months survival: 43.8 vs. 85 ($p = 4.79e^{-4}$, log-rank test); median months disease free: 35 vs. 51.5 ($p = 0.042$, log-rank test). OS and DFS curves were obtained from cBioportal database.

These preliminary data prompted us to investigate the role of ERK5 in melanoma, starting from its expression and activation in this tumor. We analyzed the expression of ERK5 protein level in a panel of 8 melanoma cell lines, including commercial and patient-derived cell lines. Western blot analysis shows that ERK5 is expressed in all melanoma cell lines analyzed and in normal human epidermal melanocytes (NHEM) (Santini et al., 2014) (Figure 4.2). Moreover, it is important to note that the majority of melanoma cell lines tested (SK-Mel-5, SK-Mel-2, MeWo, SSM2c and M26c) presented a slower electrophoretic migration band in ERK5, suggesting that ERK5 is phosphorylated in melanoma cells (Figure 4.2).

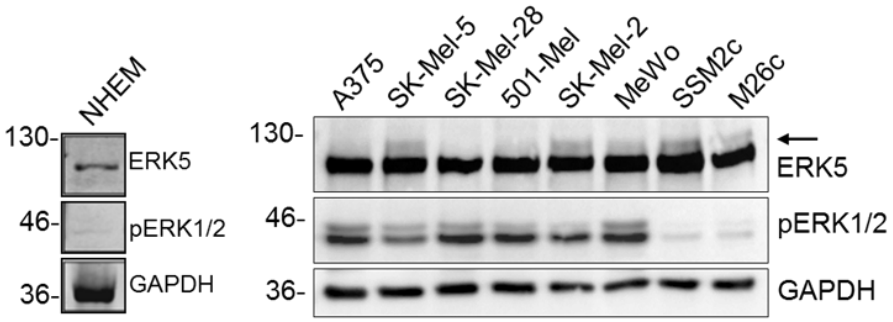


Figure 4.2 Expression of ERK5 in human melanocytes and commercial (lane 1-6) and patient-derived (lanes 7 and 8) melanoma cell lines. Cells were serum starved for 24 hours and total cell lysates were obtained. GAPDH was used as loading control. NHEM: normal human epidermal melanocytes. Arrow indicates phosphorylated forms of ERK5.

The existence of a phosphorylated form of ERK5 indicates that this kinase may be activated in our cells, but it is not enough to prove its activity. To clarify this aspect, we perform an *in vitro* kinase assay on immunoprecipitated ERK5 in four cell lines chosen for subsequent experiments. We used patient-derived SSM2c and M26c melanoma cells, that harbor wt BRAF, and A375 and SK-Mel-5 cell lines, that express a mutated BRAF (V600E). The *in vitro* kinase assay showed that ERK5 is constitutively active in all four cell lines, as evidenced by the phosphorylation of the myelin basic protein (MBP) used as substrate for the assay. The control of the experiment is represented by IgG, which only exhibit the total form of MBP and not the phosphorylated one, to demonstrate that the kinase activity highlighted in the samples is only due to the immunoprecipitated ERK5 (Figure 4.3A).

ERK5 nuclear translocation is another key event for the activity of ERK5, that has to enter in the nucleus in order to phosphorylate target proteins (Gomez et al., 2016). Therefore, we evaluated ERK5 intracellular localization and found that ERK5 was located in both the cytoplasm and the nucleus in our melanoma cell lines (Figure 4.3B).

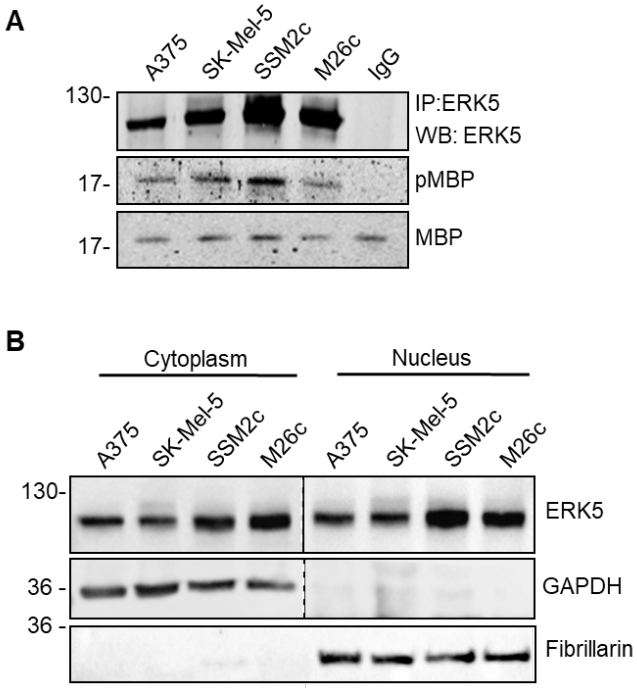


Figure 4.3 ERK5 is active in melanoma cells. A) *In vitro* kinase assay from immunoprecipitated ERK5 in 4 melanoma cell lines. IgG is a control sample without anti-ERK5 antibody. MBP was used as loading control. B) Expression of ERK5 in nuclear and cytoplasmic extracts in 4 melanoma cell lines. GAPDH and Fibrillarin were used as cytoplasmic or nuclear markers, respectively.

All together these data indicate that ERK5 is consistently expressed and active in human melanoma, suggesting that the ERK5 pathway might be important in this tumor.

4.2 ERK5 is required for melanoma cell proliferation *in vitro* and xenograft growth

In order to assess the role of ERK5 in melanoma cell proliferation, we performed genetic silencing of ERK5 using short hairpin RNAs and pharmacological inhibition of ERK5 and MEK5 with specific inhibitors.

4.2.1 Genetic inhibition of ERK5 reduces melanoma cells proliferation *in vitro* and *in vivo*

ERK5 has been reported to play a relevant role in the growth of several types of cancer, including aggressive breast and prostate cancers, hepatocellular carcinoma and multiple myeloma (Al-Ejeh et al., 2014; McCrackenet al., 2008; Carvajal-Vergaraet al., 2005; Rovida et al., 2015; Yang et al., 2011; Simões et al., 2016). To understand the biological role of ERK5 in melanoma, we investigated the effects of ERK5 inhibition in

the proliferation of melanoma cells. A375 and SSM2c cells were transduced with lentiviral vectors expressing shRNA against ERK5 (LV- shERK5) or a non-targeting shRNA (LV-c) (Rovida et al., 2015). Five days post-transduction and 3 days after selection with puromycin, cells were harvested, lysed and analyzed by Western blotting to verify the ERK5 silencing, as showed in Figure 4.4B. ERK5 silencing drastically reduce ERK5 protein levels, without affecting ERK1/2 amount. Using the same lentiviral vectors, we assessed a proliferation assay by viable cell count. ERK5 silencing markedly reduced the growth of melanoma cell lines harboring either wt (SSM2c) or mutated form (V600E) of BRAF (A375), indicating that ERK5 is essential for melanoma cell proliferation *in vitro* (Figure 4.4A).

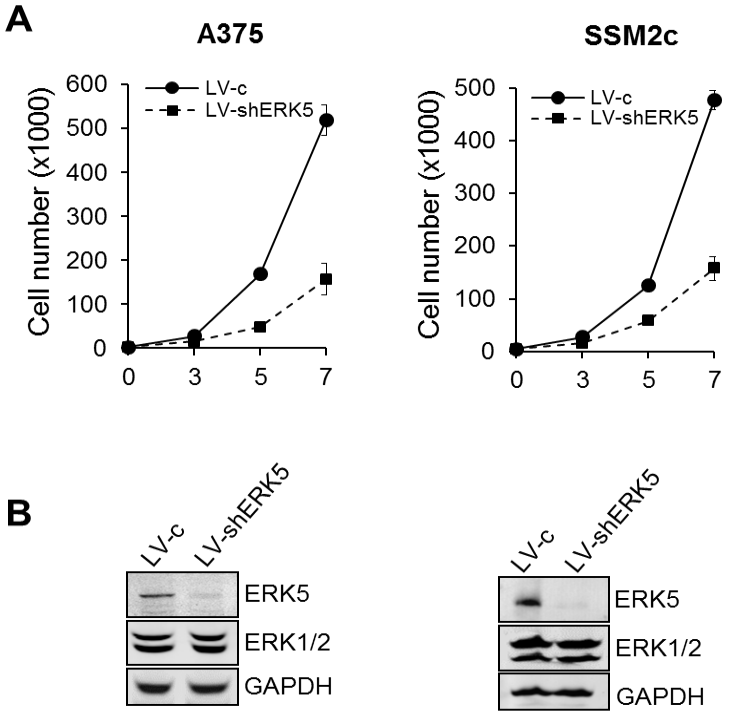
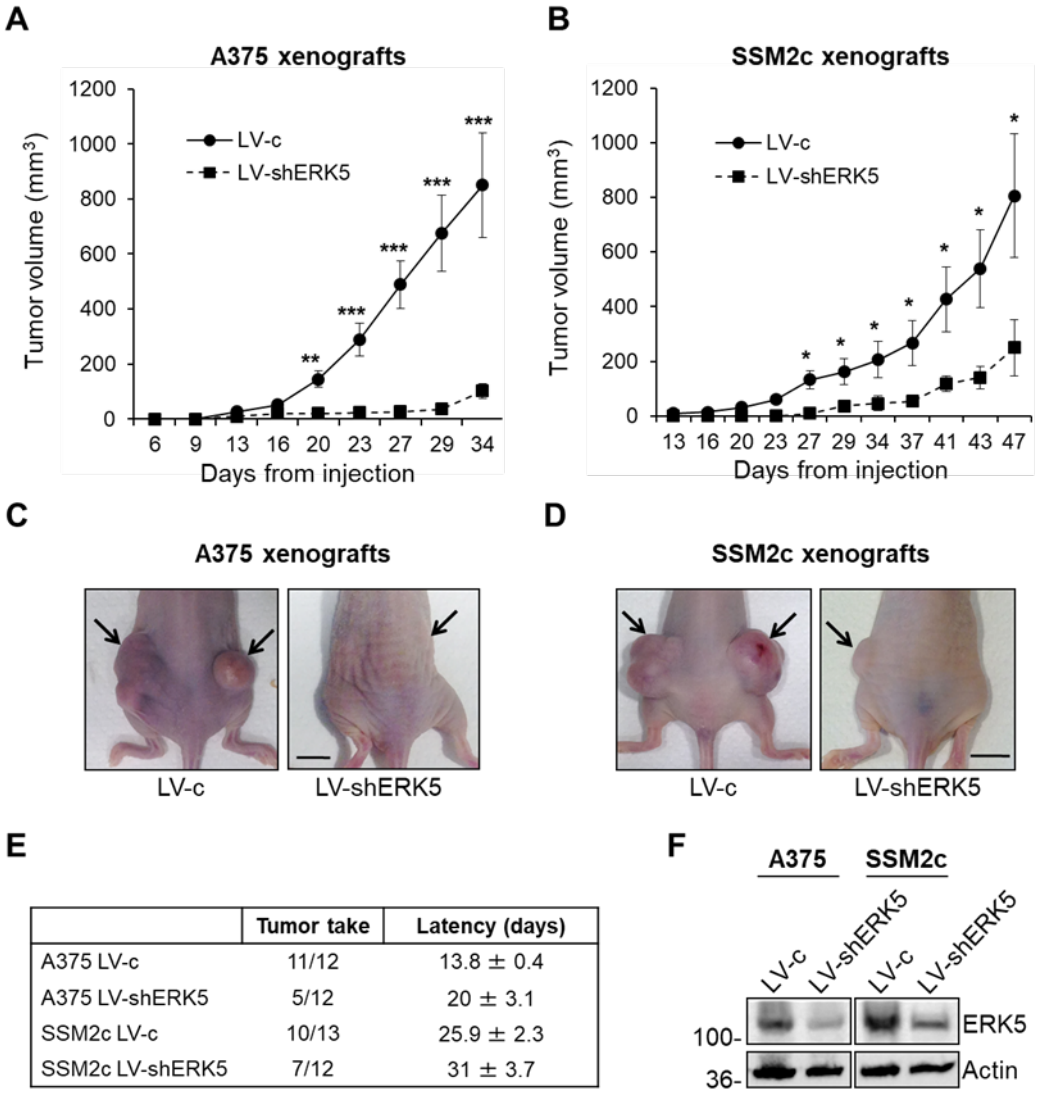


Figure 4.4 Genetic silencing of ERK5 drastically reduces melanoma cell growth *in vitro*. A) Growth curves of A375 and SSM2c cells transduced with LV-shERK5 or LV-c lentiviruses. Data shown are mean ± SD. B) Western blot shows ERK5 silencing efficiency. GAPDH was used as loading control.

To confirm our *in vitro* results, A375 and SSM2c melanoma cells stably transduced with LV-c or LV-shERK5 were subcutaneously injected into athymic nude mice and tumor growth was monitored to investigate whether ERK5 regulates melanoma growth *in vivo*. ERK5 silencing drastically reduced A375 xenograft growth and diminished by 70% SSM2c xenografts compared to LV-c (Figure 4.5A-D). The

table in Figure 4.5 shows that ERK5 silencing reduced also the tumor take, with a delayed tumor appearance in both cell types (Figure 4.5E), supporting the role of ERK5 in melanoma growth. Western blot analysis of tumors dissected at the end of experiments, confirmed the drastic reduction of ERK5 in LV-shERK5 transduced cells (Figure 4.5F).



	Tumor take	Latency (days)
A375 LV-c	11/12	13.8 ± 0.4
A375 LV-shERK5	5/12	20 ± 3.1
SSM2c LV-c	10/13	25.9 ± 2.3
SSM2c LV-shERK5	7/12	31 ± 3.7

Figure 4.5 ERK5 silencing reduces melanoma xenograft growth. A, B) *In vivo* tumor growth after subcutaneous injection of $1x10^4$ A375 or SSM2c melanoma cells transduced with LV-c or LV-shERK5 lentiviruses. Data shown are mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. C, D) Representative images of A375 and SSM2c xenografts in athymic nude mice. Bar = 10 mm. E) Table shows tumor take (number of tumors formed per number of injections) and latency (time from injection to tumor measurability) for each group. Data shown are mean ± SEM. F) Western blot analysis of tumors derived from A375 and SSM2c xenografts. Actin was used as loading control.

Altogether, these results indicate that ERK5 is required for melanoma cell growth *in vitro* and *in vivo*, thus confirming its critical role in melanoma cell proliferation.

4.2.2 Pharmacological inhibition of ERK5 pathway decreases melanoma cell growth

To confirm the promising results obtained with the genetic inhibition of ERK5 and in view of a possible translation to the clinics, we performed pharmacological inhibition of the MEK5/ERK5 pathway using two inhibitors: XMD8-92, that compete for the ATP site of ERK5 reducing its auto-phosphorylation and activation (Yang et al., 2010), and BIX02189, an inhibitor of MEK5, the kinase upstream ERK5 (Tatake at al., 2008). Either drugs, as expected, efficiently reduced ERK5 activation, abolishing ERK5 phosphorylation as shown by disappearance of the slower ERK5 migrating band in the SK-Mel-5 melanoma cell line. Western blot shows that either inhibitors specifically target ERK5, without affect ERK1/2 phosphorylation (Figure 4.6).

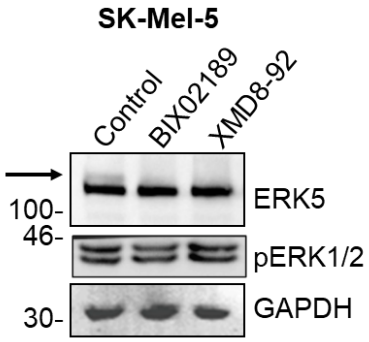
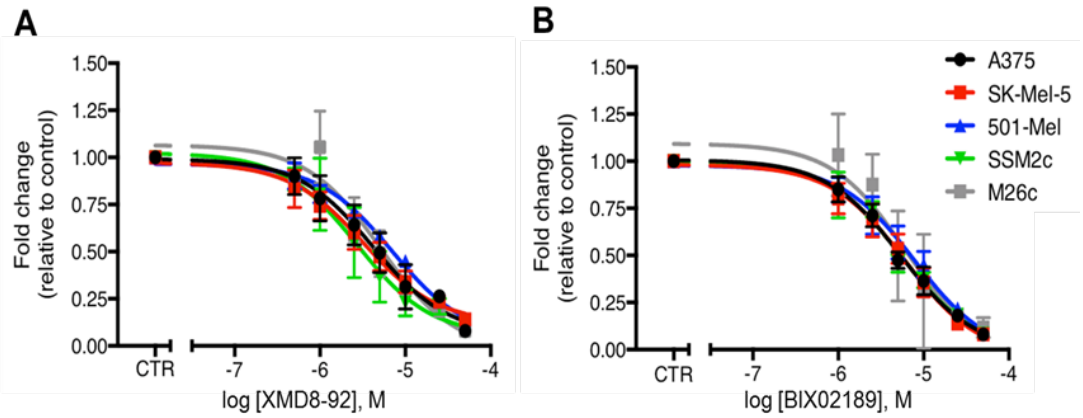


Figure 4.6 Pharmacological inhibition of the MEK5-ERK5 signaling reduces ERK5 phosphorylation. Western blot analysis shows effects of BIX02189 (10 μM) or XMD8-92 (5μM) on ERK5 in SK-Mel-5 melanoma cells. Both treatments abolished the slower migrating form (arrow) of phosphorylated ERK5, compared to vehicle treated cells (DMSO, Control). GAPDH was used as loading control.

Commercial cell lines (A375, SK-Mel-5 and 501-Mel) and patient derived cell lines (SSM2c, M26c) were treated with increasing doses of drugs and viable cells (trypan blue-negative) were counted after 72 hours. Both XMD8-92 and BIX02189 treatments decreased the number of viable cells in a dose dependent manner in several melanoma cell lines expressing either wt (SSM2c, M26c) or BRAF-V600E (A375, SK-Mel-5 and 501-Mel) (Figure 4.7A, B). IC50 values, calculated using GraphPad Prism,

ranged between 2.3 and 3.7 µM for XMD8-92 and from 5.4 to 7.1 µM for BIX02189. The table in Figure 7C shows also the IC50 values for Vemurafenib, the specific inhibitor of BRAF-V600E (Figure 4.7).



C

	XMD8-92 IC50 (µM)	BIX02189 IC50 (µM)	Vem IC50 (nM)
SSM2c	2.3 ± 0.5	5.5 ± 0.7	n.d.
M26c	2.9 ± 0.4	6.3 ± 0.8	n.d.
A375	2.6 ± 0.7	5.4 ± 0.4	58.9 ± 0.7
SK-Mel-5	2.3 ± 0.5	6.1 ± 0.6	65.2 ± 0.5
501-Mel	3.7 ± 0.5	7.1 ± 0.8	57 ± 0.5

Figure 4.7 Effect of pharmacological inhibition of ERK5 pathway in the growth of melanoma cells. A, B) Dose-response curves of XMD8-92 (A) and BIX02189 (B) in a number of melanoma cell lines after 72 hours of treatment; DMSO was used as control (CTR). Data shown are mean ± SD. C) IC50 values for XMD8-92, BIX02189 and Vemurafenib in melanoma cells treated for 72 hours. IC50 values were calculated using GraphPad Prism.

Recent reports suggested the possibility that the efficacy of existing ERK5 or MEK5 inhibitor could be due to secondary effect on BRD protein family (Lin et al., 2016) or on TGFβ (Park et al., 2016). To exclude possible off target effects of XMD8-92 and BIX02189, we used a recently developed and more specific ERK5 inhibitor, JWG-045 (Williams at al., 2016). Compared with the tested inhibitors, we observe an analogous activity of JWG-045 in the reduction of melanoma cells proliferation, with an IC50 value near to the value calculated for BIX02189. In Figure 4.8, we show the treatment of the A375 cell line with JWG-045, but similar results were obtained also for others melanoma cell lines used for proliferation assays.

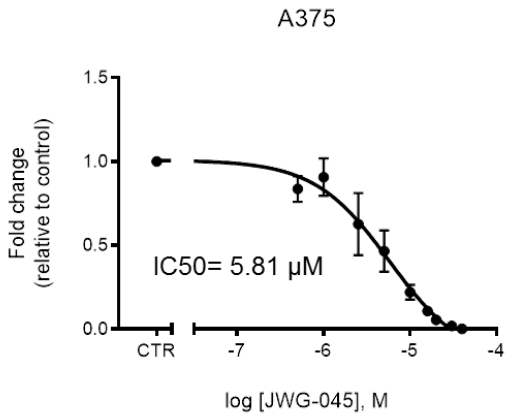
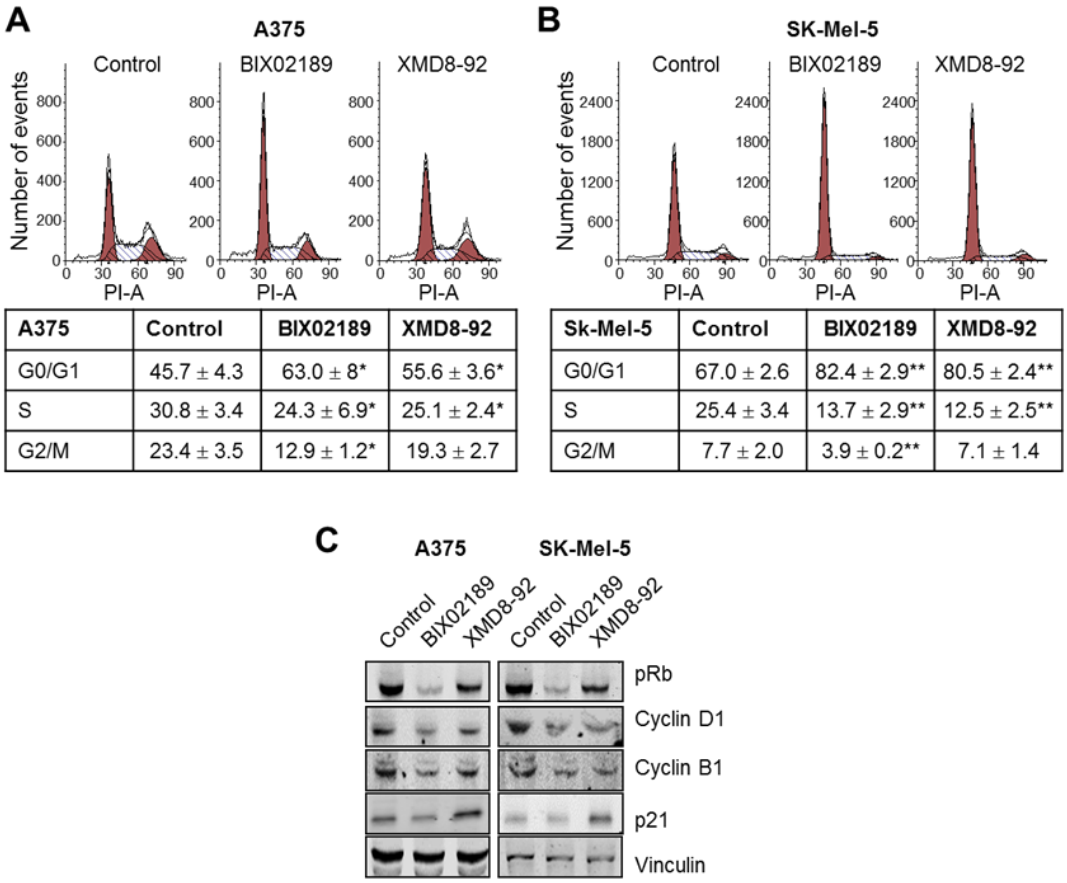


Figure 4.8 Effect of the novel ERK5 inhibitor JWG-045 on the growth of A375 melanoma cells. IC50 of JWG0-45 in A375 cells after 72 hours of treatment using GraphPad Prism software.

Our results indicate that ERK5 pathway inhibition reduces cell growth in melanoma cells. To clarify the mechanism at the basis of this biological effect, we performed cell cycle analysis. In BRAF-V600E-expressing cells (A375 and SK-Mel-5), treatment with either XMD8-92 or BIX02189 markedly reduced the fraction of cells in S phase increasing those in G0/G1 phase. In addition, treatment with BIX02189 significantly reduced the number of cells in G2/M phase (Figure 4.9A, B). Consistently, in BRAF-V600E-expressing cells BIX02189 and, to lesser extent, XMD8-92 reduced the level of pRb (Ser807) and, XMD8-92 in particular, increased the expression of the Cyclin Dependent Kinase inhibitors (CDKi) p21. Finally, the expression of Cyclins D1 and B1 decreased with BIX02189 and, at lower extent, XMD8-92 treatment (Figure 4.9C).

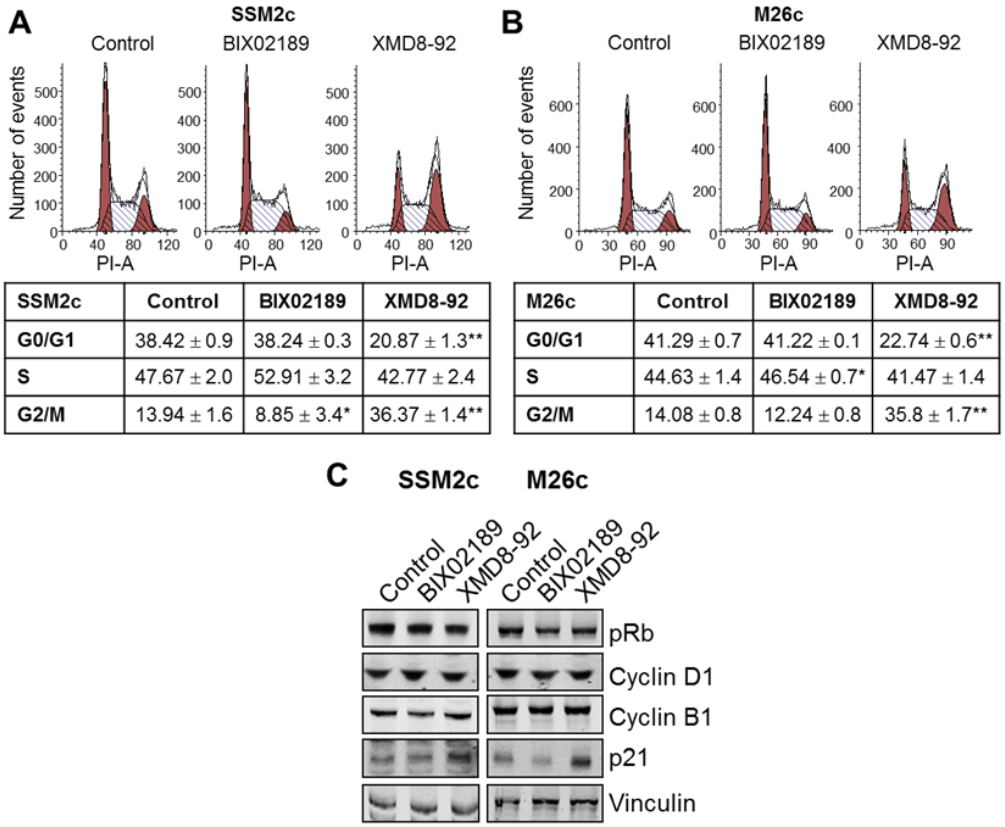


A375	Control	BIX02189	XMD8-92
G0/G1	45.7 ± 4.3	63.0 ± 8*	55.6 ± 3.6*
S	30.8 ± 3.4	24.3 ± 6.9*	25.1 ± 2.4*
G2/M	23.4 ± 3.5	12.9 ± 1.2*	19.3 ± 2.7

Sk-Mel-5	Control	BIX02189	XMD8-92
G0/G1	67.0 ± 2.6	82.4 ± 2.9**	80.5 ± 2.4**
S	25.4 ± 3.4	13.7 ± 2.9**	12.5 ± 2.5**
G2/M	7.7 ± 2.0	3.9 ± 0.2**	7.1 ± 1.4

Figure 4.9 Pharmacological inhibition of MEK5-ERK5 pathway affects cell cycle distribution in melanoma cells harboring BRAF-V600E. A, B) Cell cycle phase distribution plots and values (tables) of BRAF-V600E expressing cells (A375 and SK-Mel-5). Cells were treated for 48 hours with XMD8-92 (5 μM) or BIX02189 (10 μM). Data shown are mean ± SD. *p<0.05, **p<0.01 as determined by Student t-test. C) Expression or phosphorylation status of cell cycle regulators in cells treated for 24 hours with XMD8-92 (5 μM) or BIX02189 (10 μM). Controls were treated with DMSO. Vinculin was used as loading control.

In wt BRAF melanoma cells (SSM2c and M26c), BIX02189 slightly affected cell cycle distribution, with a trend toward an increase of the number of cells in S phase and a reduction of those in G2/M phase. On the other hand, XMD8-92 treatment resulted in a marked accumulation in G2/M phase at the expense of those in G0/G1 and S phases (Figure 4.10 A, B), with an increased expression of p21 in both SSM2c and M26c cells. Accordingly, with XMD8-92 or BIX02189 treatment no differences were found in the phosphorylation of Rb or in the expression of cyclin B1 and D1 (Figure 4.10 C).

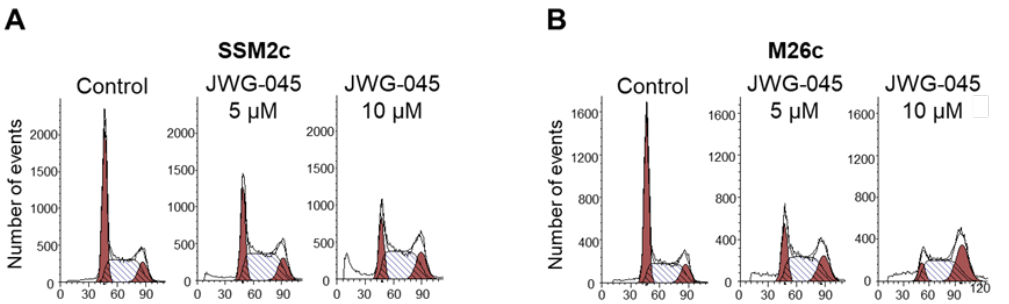


SSM2c	Control	BIX02189	XMD8-92
G0/G1	38.42 ± 0.9	38.24 ± 0.3	20.87 ± 1.3**
S	47.67 ± 2.0	52.91 ± 3.2	42.77 ± 2.4
G2/M	13.94 ± 1.6	8.85 ± 3.4*	36.37 ± 1.4**

M26c	Control	BIX02189	XMD8-92
G0/G1	41.29 ± 0.7	41.22 ± 0.1	22.74 ± 0.6**
S	44.63 ± 1.4	46.54 ± 0.7*	41.47 ± 1.4
G2/M	14.08 ± 0.8	12.24 ± 0.8	35.8 ± 1.7**

Figure 4.10 Pharmacological inhibition of MEK5-ERK5 pathway affects cell cycle distribution in melanoma cells harboring wt BRAF. A, B) Cell cycle phase distribution plots and values (tables) of wt BRAF expressing cells (SSM2c and M26c). Cells were treated for 48 hours with XMD8-92 (5 μM) or BIX02189 (10 μM). Data shown are mean ± SD. *p<0.05, **p<0.01 as determined by Student t-test. C) Expression or phosphorylation status of cell cycle regulators in cells treated for 24 hours with XMD8-92 (5 μM) or BIX02189 (10 μM). Controls were treated with DMSO. Vinculin was used as loading control.

We confirmed the specific effect of XMD8-92 on ERK5 using the JWG-045, that shows weak binding to BRD4 (Williams et al., 2016). This new inhibitor exhibited an effect similar to that observed for XMD8-92 on cell cycle phase distribution, confirming that the effect of XMD8-92 does not appear to be due to inhibition of BRD proteins (Williams et al., 2016; Lin at al., 2016).



SSM2c	Control	JWG-045 5 µM	JWG-045 10 µM
G0/G1	44.5 ± 1.4	31.33 ± 2.2	22.7 ± 1.0*
S	43.8 ± 1.6	54.6 ± 1.8	59.4 ± 0.7*
G2/M	11.7 ± 0.2	14.1 ± 0.3*	18.0 ± 1.7*

M26c	Control	JWG-045 5 µM	JWG-045 10 µM
G0/G1	47.7 ± 4.0	29.8 ± 8.1	9.5 ± 1.5*
S	40.3 ± 2.6	51.7 ± 3.8	53.7 ± 3.2*
G2/M	12.0 ± 1.4	18.5 ± 4.3*	36.9 ± 1.7*

Figure 4.11 The novel ERK5 inhibitor JWG-045 affects cell cycle distribution. A, B) Cell cycle phase distribution plots and values (tables) of wt BRAF expressing cells (SSM2c and M26c) treated for 48 hours with JWG-045 (5 µM). Data shown are mean ± SD. *p<0.05, **p<0.01 as determined by Student t-test.

All together these results demonstrated that pharmacological inhibition of the MEK5-ERK5 signaling *in vitro* mimics the effects of ERK5 genetic silencing in the reduction of melanoma cell proliferation.

4.3 Oncogenic BRAF increases ERK5 activity

BRAF is mutated in about 50% of melanomas and is one of the most important target for melanoma therapy (Davies et al., 2002). Since our findings indicate that ERK5 is important for melanoma proliferation, we investigated whether BRAF modulates ERK5 activation. To address this point, we first assessed the effect of oncogenic BRAF on ERK5 protein levels by overexpressing BRAF-V600E in HEK-293T and melanoma cells that express wt BRAF. In both cell types, BRAF-V600E significantly increased endogenous ERK5 protein levels (Figure 4.12).

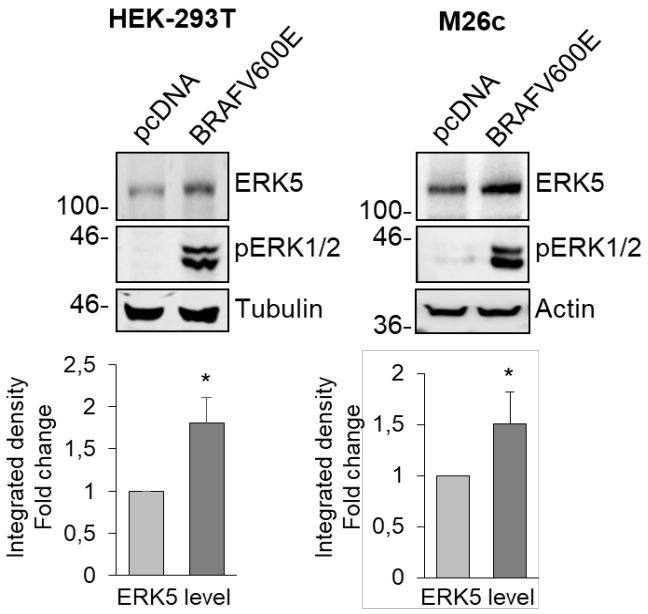


Figure 4.12 Oncogenic BRAF enhances levels of endogenous ERK5 protein in HEK-293T and M26c melanoma cells. Cells were transfected with control empty vector (pcDNA) or BRAF-V600E expressing plasmid and lysed after 24 hours. Western blots were performed with the indicated antibodies. Overexpression of BRAF-V600E is confirmed by marked phosphorylation of ERK1/2. Tubulin or Actin were used as loading control. Quantification of ERK5, normalized for the housekeeping, is reported in the graphs. Histograms represent mean ± SD from three independent experiments. *$p < 0.05$.

To better understand the mechanism by which oncogenic BRAF affects ERK5 protein levels, we studied the effect of its overexpression on ectopic ERK5. Oncogenic BRAF, either overexpressed wt or with V600E mutation, increased both ERK5 protein level and phosphorylation at different residues. Indeed, oncogenic BRAF induced robust phosphorylation of ERK5 at Ser753 and at Thr732 (Figure 4.13), two residues in ERK5 C-terminal region that are putative autophosphorylation sites as well as targets of CDK1 and/or ERK1/2 (Díaz-Rodríguezet al., 2010, Honda et al., 2015). Ectopic expression of constitutively active MEK5 (MEK5DD) induced robust ERK5 phosphorylation at Thr218/Tyr220, as expected, and increased ERK5 phosphorylation at Thr732 (Figure 4.13). Finally, overexpression of oncogenic BRAF induced ERK5 phosphorylation at Thr218/Tyr220, although at a much lower level than that induced by MEK5DD (Figure 4.13). Ectopic expression of wt BRAF elicited similar effects on the expression and phosphorylation of ERK5, although to a lesser extent than BRAF-V600E.

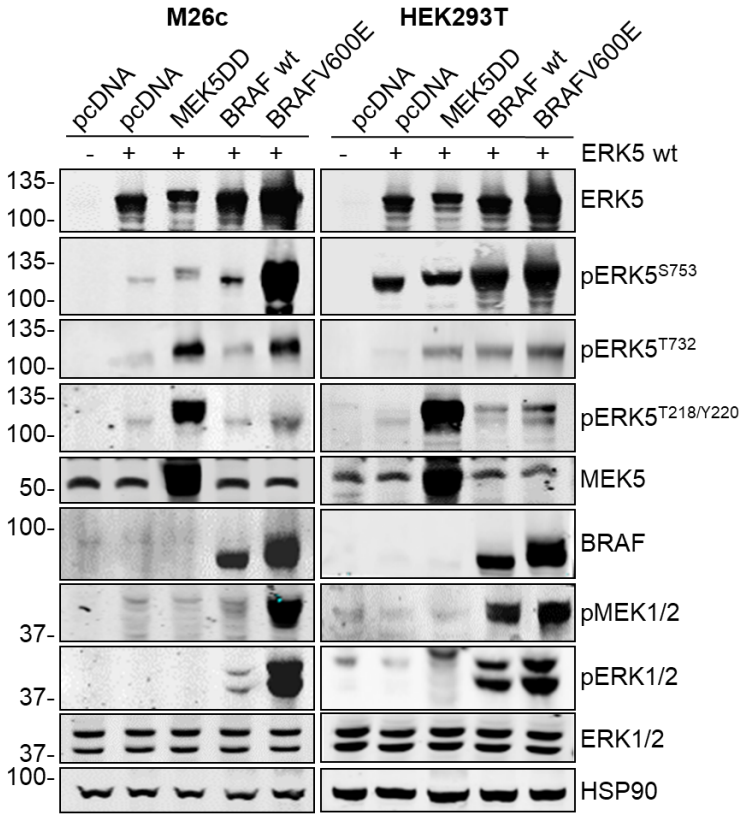


Figure 4.13 Oncogenic BRAF enhances level of expression and phosphorylation of exogenous ERK5. M26c melanoma cells and HEK293T cells were transfected with equimolar amounts of pcDNA (control, -) or wt ERK5 in combination with pcDNA, constitutively active MEK5 (MEK5DD), wt BRAF or BRAF-V600E plasmids. Cells were lysed after 48 hours and Western blot performed with the indicated antibodies. BRAF-V600E markedly increases ERK5 phosphorylation at Thr218/Tyr220, Ser753 and Thr732. HSP90 was used as a loading control.

Because Ser753 can be also phosphorylated by CDK1 (Díaz-Rodríguez et al., 2010), whereas Thr732 is phosphorylated by both CDK1 and ERK1/2 (Honda et al., 2015), we investigated the involvement of these two kinases in BRAF-V600E-dependent regulation of ERK5. Treatment with the ERK1/2 inhibitor SCH772984 or the CDK1 inhibitor RO-3306 reduced ERK5 protein level and phosphorylation at Ser753 and Thr732. Only their combination completely abolishes BRAF-V600E-induced effects (Figure 4.14). Efficacy of SCH772984 on ERK1/2 and of RO-3306 on CDK1 was witnessed, respectively, by decreased phosphorylation of ERK1/2 and increase of Cyclin B, as previously reported (Nithianandarajah-Jones et al., 2012). These data indicate that CDK1 and ERK1/2 are involved in BRAF-dependent regulation of ERK5 phosphorylation and protein levels.

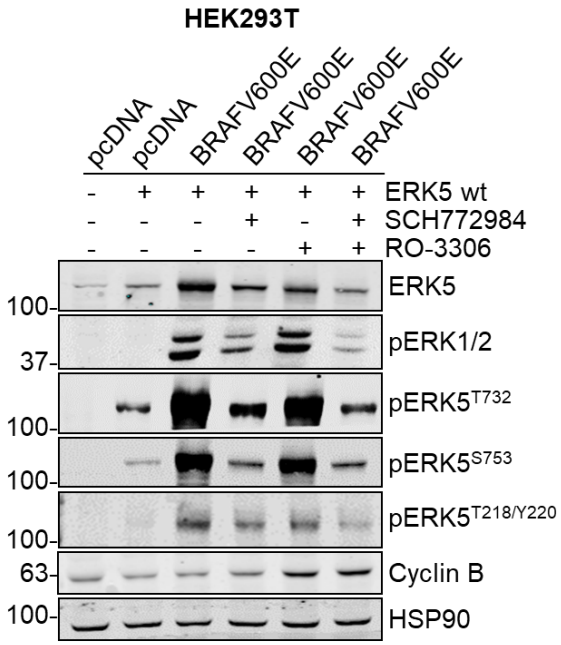


Figure 4.14 Combined inhibition of CDK1 and ERK1/2 decreases ERK5 protein level and phosphorylation. HEK293T cells were transfected with equimolar amounts of pcDNA (control) or wt ERK5 in combination with pcDNA or BRAF-V600E plasmids. Cells were treated with ERK1/2 inhibitor SCH772984 (0.5 μM) and/or CDK1 inhibitor RO-3306 (9 μM) during the last 18 hours of transfection. Cells were lysed after 24 hours and Western blot performed with the indicated antibodies. HSP90 was used as a loading control.

Having established that oncogenic BRAF enhances protein level and phosphorylation of ERK5, we investigated whether also ERK5 functions were affected by this oncogene. First, we performed *in vitro* kinase assay for ERK5 in M26c cells transfected with BRAF-V600E and a constitutively active form of MEK5 (MEK5DD), used as positive control. ERK5 protein was immunoprecipitated and its kinetic activity was measured evaluating the phosphorylation of a specific substrate (myelin basic protein, MBP) by immunoblot analysis of the phosphorylated form of the substrate, probing with a monoclonal phospho-specific MBP antibody. The increase of the pMBP shows that the overexpression of BRAF-V600E enhanced ERK5 kinase activity (Figure 4.15A). Consistently, pharmacological inhibition of BRAF-V600E with Vemurafenib markedly reduced basal ERK5 kinase activity in A375 and SK-Mel-5 cells after 24h of treatment (Figure 4.15B).

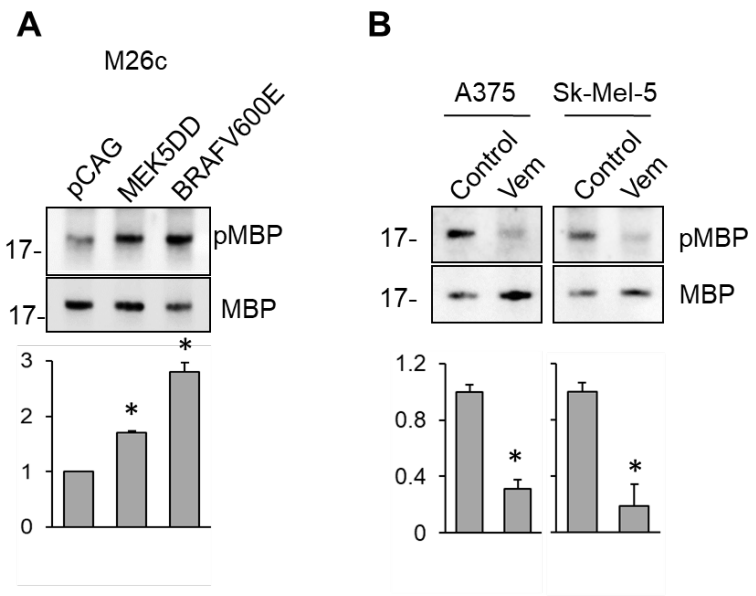


Figure 4.15 Oncogenic BRAF increases ERK5 kinase activity. A) *In vitro* kinase assay for ERK5 immunoprecipitated from M26c cells transfected with equimolar amounts of pCAG, constitutively active MEK5 (MEK5DD) or BRAF-V600E plasmids. MBP was used as a loading control. B) *In vitro* kinase assay for ERK5 immunoprecipitated from A375 or SK-Mel-5 cells treated with 1 μM Vemurafenib (Vem) or DMSO (Control) for 24 h. MBP was used as a loading control. Blots are representative images from at least three independent experiments. Densitometric quantification of blots is reported on the bottom.

ERK5 has to translocate into the nucleus to exert its proliferative activity (Gomez et al., 2016). Therefore, we tested whether BRAF-V600E may affect also ERK5 cellular localization. Overexpression of BRAF-V600E increased the nuclear amount of total and phosphorylated ERK5 at Ser753 and Thr732 (Figure 4.16A). Interestingly, even thug no transcriptional activity has been described for ERK5 so far, oncogenic BRAF increased the amount of ERK5 in the chromatin-bound fraction (Figure 4.16B). To verify whether BRAF-V600E may influence the known transcriptional transactivator activity of ERK5, we performed a luciferase assay using a luciferase reporter for MEF2, a transcription factor regulated by ERK5. In the MEF2-luciferase reporter used in this assay, the firefly luciferase gene is under the control of tandem repeats of the MEF2 transcriptional response element cloned upstream of a c-Fos minimal promoter. The luciferase assay showed that BRAF-V600E enhanced the ability of ERK5 to induce transcription activity of MEF2 (Figure 4.16C), demonstrating that BRAF can also influence this ERK5 function.

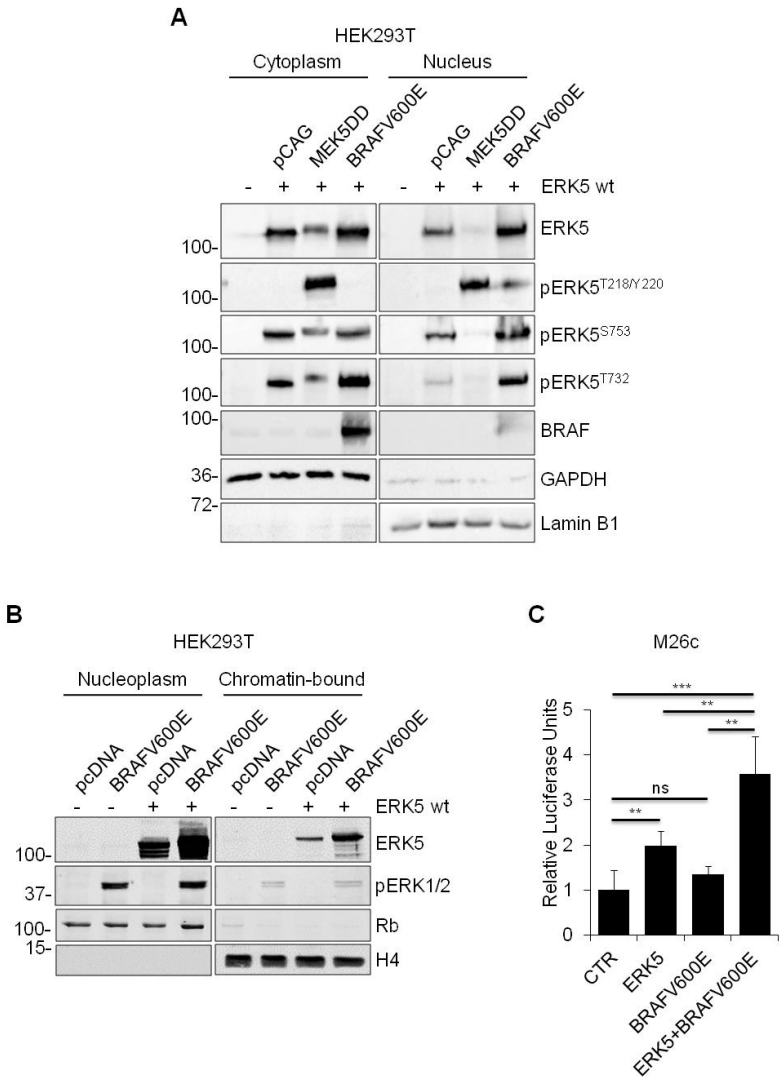


Figure 4.16 Oncogenic BRAF enhances ERK5 nuclear amount and functions. A) Nucleo-cytoplasmic fractionation in HEK293T cells transfected with equimolar amounts of wt ERK5 in combination with the empty vector pCAG, constitutively active MEK5 (MEK5DD) or BRAF-V600E plasmids. BRAF-V600E increases level and phosphorylation of nuclear ERK5. GAPDH and Lamin B1 were used as cytoplasmic or nuclear markers, respectively. B) Nucleoplasm and chromatin-bound fraction from HEK293T cells transfected with equimolar amounts empty vector pcDNA or BRAF-V600E plasmids in presence or not of wt ERK5. Rb and Histone H4 were used as nucleoplasm or chromatin-bound markers, respectively. A and B show representative blots from at least three independent experiments. C) Quantification of dual reporter luciferase assay in M26c melanoma cells showing that BRAF-V600E enhances the transcriptional transactivator activity of ERK5. Relative luciferase activity was firefly/Renilla ratios, with the level induced by control equated to 1. Data represent mean ± SD of three independent experiments. **p<0.01, ***p<0.001 as determined using one-way ANOVA.

Altogether, these data indicate that oncogenic BRAF positively regulates not only the ERK5 protein levels, but also ERK5 activities. Since BRAF influence seems to be crucial for ERK5 in melanoma, the possibility to target either BRAF and ERK5 may be a good strategy to inhibit melanoma cell proliferation.

4.4 The combination of Vemurafenib with ERK5 pathway inhibitors provides enhanced inhibition of melanoma cell growth compared to single treatments

Despite our results showed that BRAF is an upstream activator of ERK5, nuclear ERK5 amount did not decrease after a 24-hour treatment with Vemurafenib (Figure 4.17). This is probably due to a MEK5-dependent phosphorylation of ERK5 upon MEK1/2 inhibition (de Jong et al., 2016), that contributes to preserve the ERK5 protein levels. To assess this hypothesis, we try to inhibit simultaneously MEK5 and MEK1/2 pathway with a combined treatment. Indeed, the ERK5 nuclear amount is reduced in A375 and SK-Mel-5 melanoma cells treated only with a combination of Vemurafenib and BIX02189 (Figure 4.17).

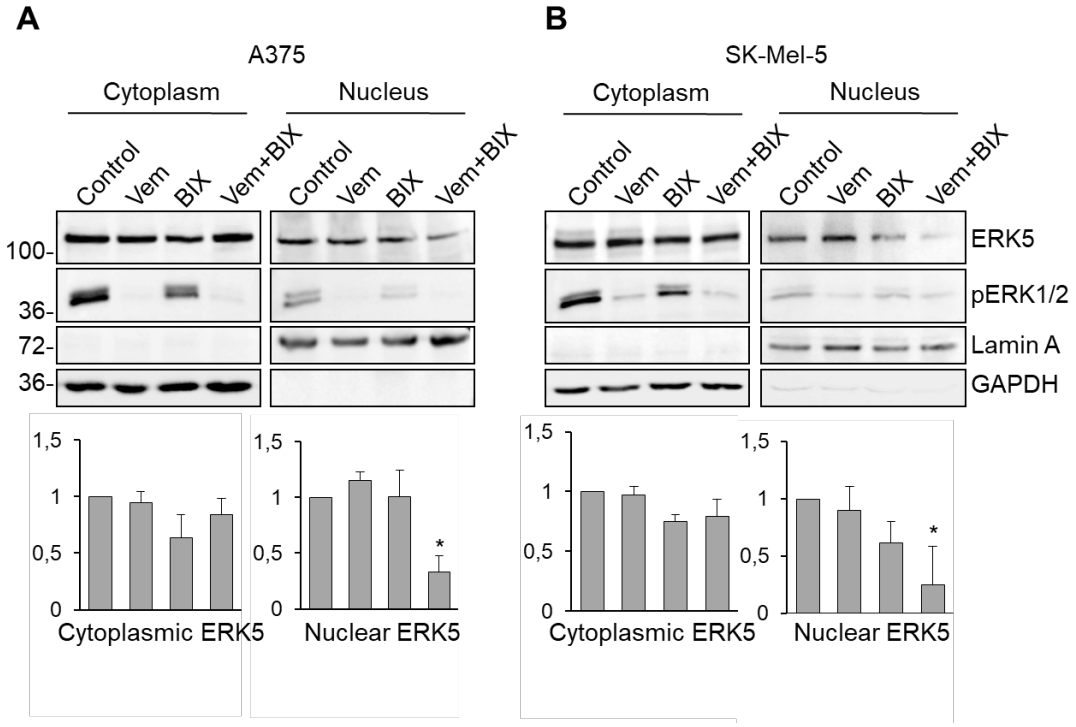


Figure 4.17 Combined inhibition of MEK5 and BRAF-V600E is required to reduce the endogenous level of nuclear ERK5. Cytoplasmic and nuclear extracts of A375 (A) or SK-Mel-5 (B) melanoma cells showing the effect of Vemurafenib (1 μM), BIX02189 (10 μM) or their combination on the expression of endogenous ERK5. GAPDH and Lamin A were used as cytoplasmic or nuclear markers, respectively. Quantification of cytoplasmic and nuclear endogenous ERK5 normalized for loading control from three independent experiments is shown in histograms (mean ± SD). *$p<0.05$ as determined by Student t-test.

To test whether targeting both MEK5-ERK5 pathway and BRAF leads to a better response than single agents also in the reduction of melanoma cell proliferation, we used Vemurafenib in combination with XMD8-92 or BIX02189 at IC50 concentrations. The combination of Vemurafenib with both ERK5 inhibitor displayed additive effects in reducing proliferation of A375 and SK-Mel-5 grown in monolayer (Figure 4.18).

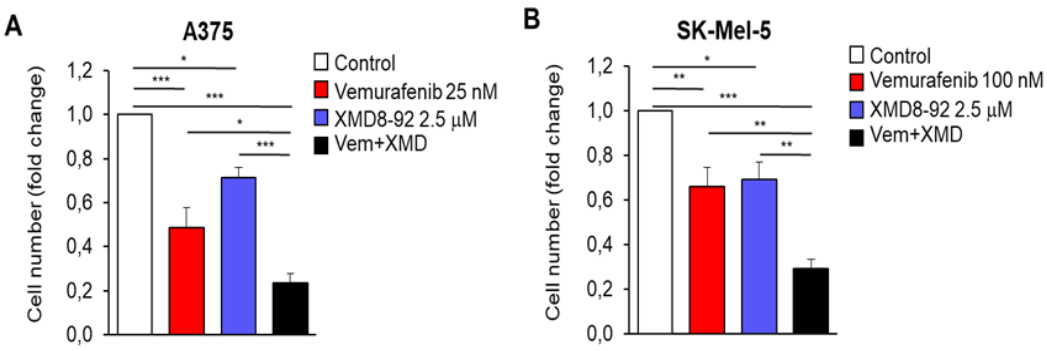


Figure 4.18 Combination of Vemurafenib and XMD8-92 shows additive effects in reducing melanoma cell growth. Number of viable A375 (A) and SK-Mel-5 (B) cells treated for 72 hours with DMSO (Control), Vemurafenib, XMD8-92 or the combination (Vem+XMD) at the indicated concentrations. Histograms represent mean ± SD from one representative experiment out of three performed in triplicate. Bliss Independence indicates additive effects in Vem+XMD vs Vem or XMD. *p<0.05, **p<0.01, ***p<0.001 as determined by one-way ANOVA.

Taken together, these data suggest that combination of Vemurafenib with ERK5 pathway inhibitors provides enhanced effects in reducing melanoma cell growth compared to single treatments.

Finally, we want to confirm the efficacy of Vemurafenib and XMD8-92 combination also in an *in vivo* pre-clinical experiment. BRAF-V600E-expressing melanoma cells A375 were subcutaneously injected into athymic nude mice ($1x10^4$ cells/injection). Treatments started when tumors were palpable and tumor growth was measured every three days and after 19 days of treatment mice were sacrificed. Single treatment with low doses of Vemurafenib (20 mg/Kg) (Yang et al., 2010; Paoluzzi et al., 2016) or XMD8-92 (25 mg/Kg) (Al-Ejeh et al., 2014) twice a day for 19 days produced a 50% tumor growth inhibition compared to the control group. Combination treatment with Vemurafenib (20 mg/Kg) and XMD8-92 (25 mg/Kg) achieved a significantly greater antitumor effect than either agent alone (Combo vs Vem p=0.05;

Combo vs. XMD8-92 p=0.005), in line with *in vitro* assay (Figure 4.19). Treatment with single agents or their combination was generally well tolerated, without significant weight loss or other apparent side effects.

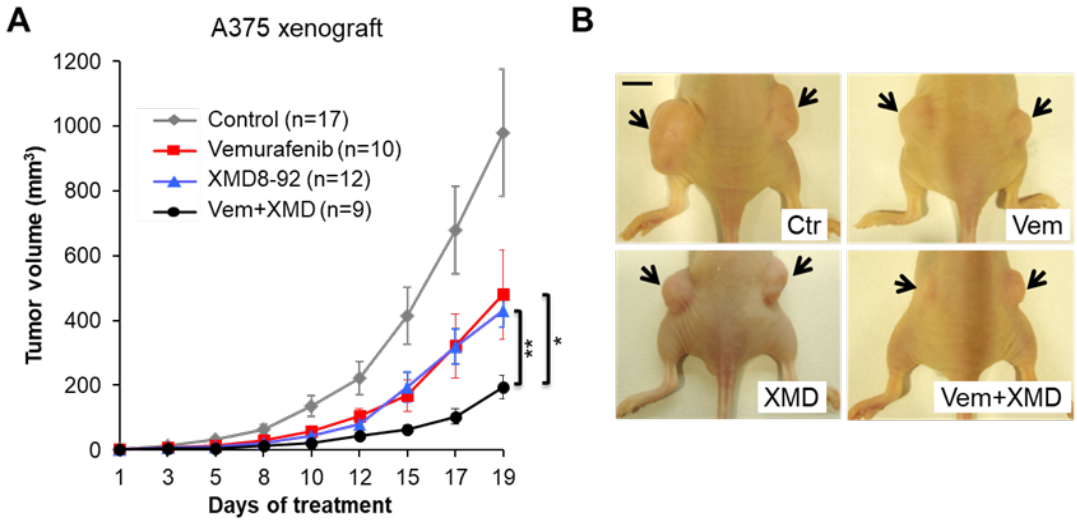


Figure 4.19 Pharmacological inhibition of ERK5 improves the effect of Vemurafenib in reducing melanoma xenograft growth. A) *In vivo* tumor growth of A375 melanoma cells subcutaneously injected ($1x10^4$). Mice were treated at tumor appearance with Vemurafenib (20 mg/Kg), XMD8-92 (25 mg/Kg) or the combination. After 19 days of treatment mice were sacrificed. Data shown are mean±SEM. Combined treatment increased the efficacy of XMD8-92 or Vemurafenib alone. Number of tumors for each group is indicated. *$p \leq 0.05$; **$p < 0.01$ as determined by one-way ANOVA. B) Representative images of A375 xenografts in athymic nude mice. Bar = 10 mm.

Altogether, these results demonstrate that the combination of Vemurafenib and ERK5 pathway inhibitors represents a better therapeutic strategy than single treatments against melanoma growth *in vitro* and *in vivo*.

4.5 Pharmacological inhibition of ERK5 negatively regulates the transcriptional activity of the Hedgehog-GLI pathway in murine NIH3T3 cells

The evidence of a fundamental role of ERK5 in the proliferation of melanoma cells,supported by our results, prompted us to investigate on a possible relationship between ERK5 and HH pathway. The Hedgehog-GLI pathway has been shown to be required for growth, recurrence and metastasis of melanoma xenografts in mice (Stecca et al., 2007; O'Reilly et al., 2013; Jalili et al., 2013). Preliminary data from our laboratory indicated that ERK5 silencing in M26c melanoma cell line reduces the mRNA level of GLI target genes, such as PTCH1, E2F1 and CyD1 and GLI1 itself, suggesting a possible link between the two pathways.

We began to investigate the effect of ERK5 on the HH pathway in physiological conditions, using the HH competent murine NIH3T3 fibroblasts. The treatment of NIH3T3 cells with the synthetic SMO agonist SAG (Chen et al., 2002) allows fully activation of the signaling transduction through the canonical HH pathway, leading to the transactivation of the downstream effectors, the GLI transcription factors. We first tested the effect of ERK5 on the transcriptional activity of the endogenous HH pathway using a luciferase assay. NIH3T3 cells were transfected with the reporter p8x3GLI-BS (GLI-BS) vector (Sasakiet al., 1997), which contains 8 direct repeats of the GLI consensus sequence cloned upstream the luciferase gene. Treatment of NIH3T3 cells with SAG 100nM for 48 hours led to the activation of the transcriptional activity of the endogenous HH pathway, as indicated by the increase of the luciferase activity. The treatment with increasing concentrations of XMD8-92, an ERK5 inhibitor, decreased the transcriptional activity of the endogenous signaling in a dose-dependent manner compared to SAG-treated cells (Figure 4.20).

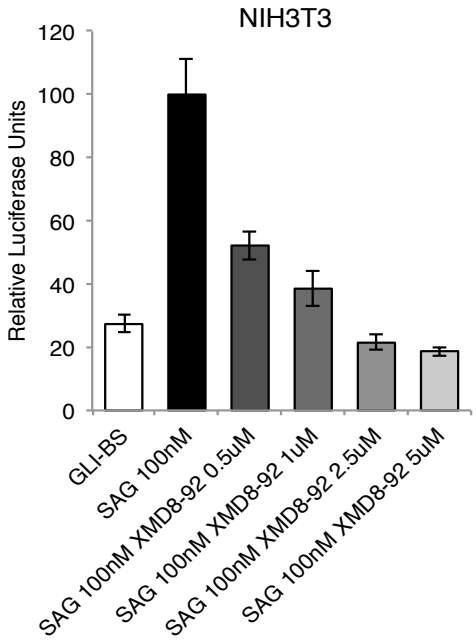


Figure 4.20 Pharmacological inhibition of ERK5 decreases the transcriptional activity of the endogenous HH pathway. Cells were treated with 100 nM of SAG for 48 hours to activate the HH pathway and with increasing doses of XMD 8-92 for 16 hours. Relative Luciferase Units (R.L.U.) is calculated as the ratio between the firefly/renilla signal, considering the activation induced by SAG equated to 100%. Note that ERK5 inhibition reduced the levels of activation of the endogenous HH pathway in NIH3T3 cells in a dose-dependent manner. The data represent mean±SEM of at least 3 independent experiments.

This result shows that inhibition of ERK5 decreases the transcriptional activity of the HH pathway, suggesting a positive modulation by ERK5.

4.6 ERK5 pharmacological inhibition reduces GLI1 levels in murine NIH3T3 cells

Since Gli1 is the main read-out of an active HH signaling (Lee et al., 1997), we evaluated the effect of ERK5 inhibition on the levels of endogenous Gli1 and Gli2 proteins by Western Blot. NIH3T3 cells were treated for 48h with SAG (100nM), to activate the HH pathway, and with a single dose of XMD8-92 (5μM), which is the highest concentration used to inhibit the pathway, for 24 and 48 hours. Consistent with luciferase assay, SAG induced full activation of the HH pathway, as shown by the strong induction of Gli1 protein. Treatment with XMD8-92 reduced levels of Gli1 protein compared to SAG alone at 24 hours and, even more at 48 hours. The reduction of Gli2 protein level, however, occurred only at 48 hours, probably as a consequence for

the decrease of Gli1 levels. The efficacy of XMD8-92 treatment was demonstrated by the reduction of ERK5 phosphorylation (Figure 4.21).

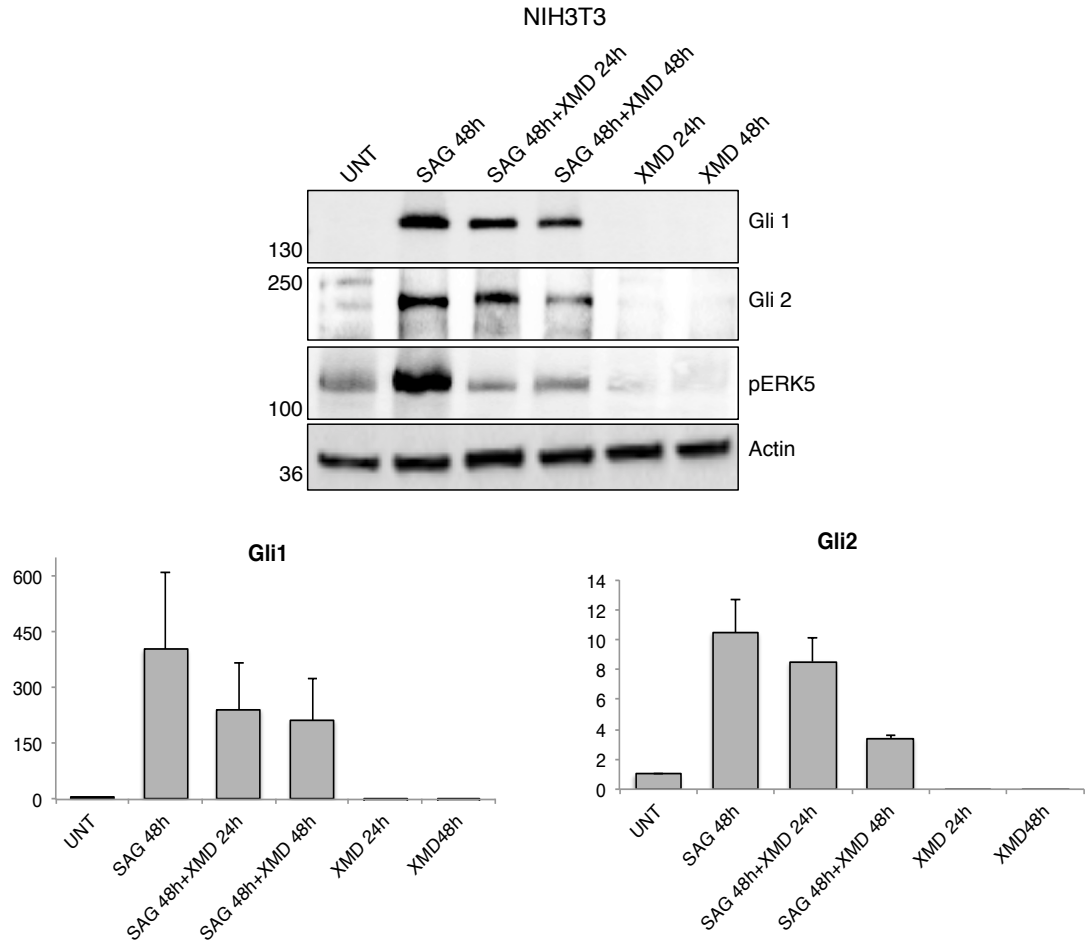


Figure 4.21 Pharmacological inhibition of ERK5 decreases the level of the downstream transcription factors Gli1 and Gli2. Western blot analysis of NIH3T3 cells treated with SAG 100nM for 48 hours and XMD8-92 5μM for 24 and 48 h. Note that ERK5 inhibition progressively reduces Gli1 and Gli2 protein levels. The efficacy of ERK5 inhibition was confirmed by the reduction of ERK5 phosphorylation. Actin was used as a load control. Quantification of Gli1 and Gli2 levels normalized for loading control from three independent experiments is shown in histograms (mean± SD).

To confirm the results obtained with XMD8-92 and to exclude off target effects for this drug, we used the MEK5 inhibitor BIX02189. NIH3T3 cells were treated for 48h with SAG (100nM) and the effect of a single 5μM dose of XMD8-92 for 48 hours was compared with BIX02189, used at 10μM for 24 or 48 hours. The inhibition of MEK5 confirm a robust reduction of Gli1 and Gli2 protein levels at 24 hours and, mostly, at 48 hours. Neither XMD8-92 nor BIX02189 treatment affected the activation

of ERK1/2, confirming the specific involvement of ERK5 in the modulation of Gli levels (Figure 4.22).

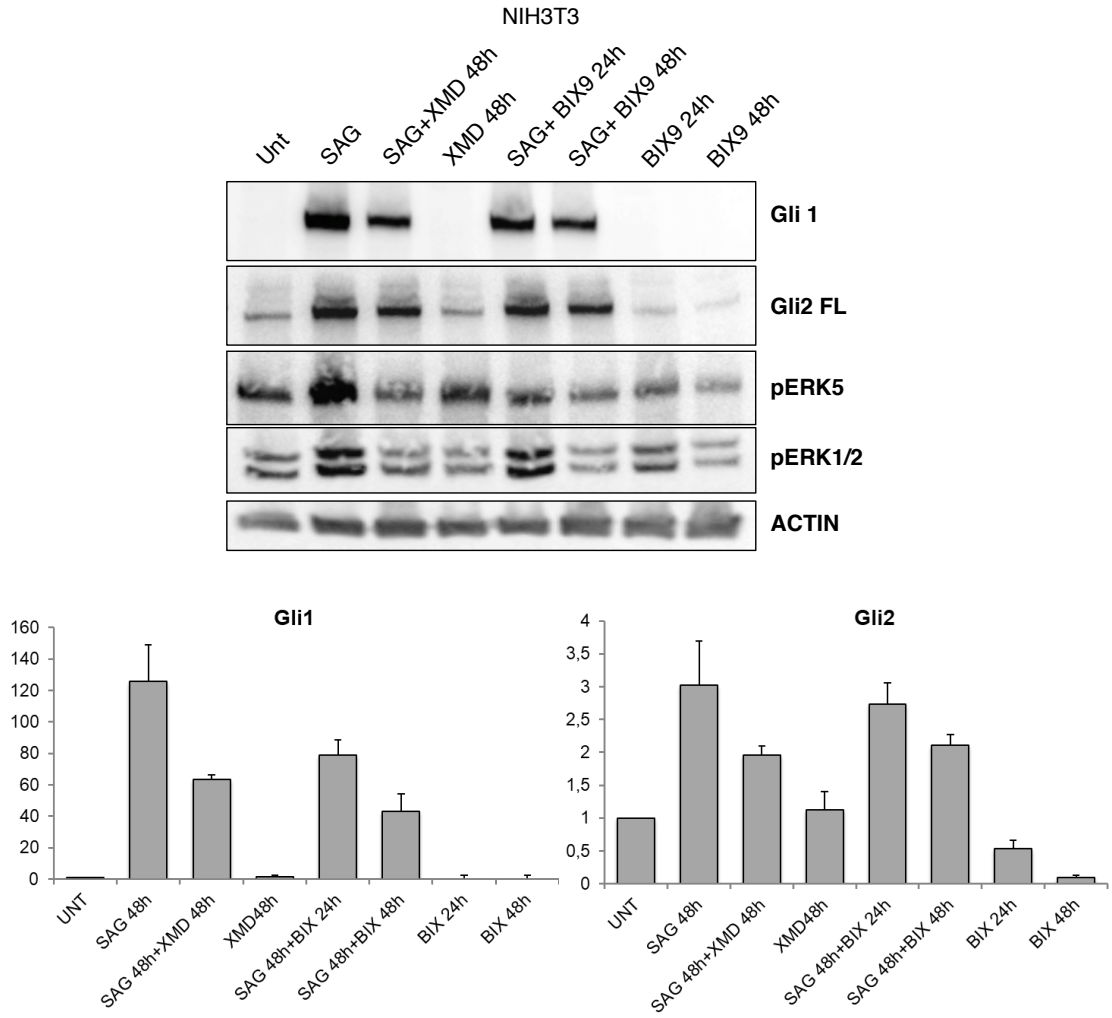


Figure 4.22 Pharmacological inhibition of MEK5 decreases the level of the downstream transcription factors Gli1 and Gli2. Western Blot analysis of NIH3T3 cells treated with SAG 100nM for 48 hours, XMD8-92 5uM for 24 and BIX02189 10uM for 24 and 48 hours. Note that inhibition of MEK5 (BIX 02189) or ERK5 (XMD8-92) reduces Gli1 and Gli2 protein levels. The efficacy of ERK5 inhibition was confirmed by the reduction of ERK5 phosphorylation. Actin was used as a load control. Quantification of Gli1 and Gli2 levels normalized for loading control from three independent experiments is shown in histograms (mean± SD).

All together these results confirm that ERK5 is able to positively modulate the transcriptional activity of HH pathway and the expression of the downstream mediators Gli1 and Gli2. We could hypothesize that the main target of ERK5 regulation is Gli1,

which is directly regulated by the HH pathway and acts as a powerful transcriptional activator of HH target genes.

4.7 Pharmacological and genetic inhibition of ERK5 reduces the transcriptional activity of exogenous GLI1 in melanoma cells

To verify the correlation between ERK5 and GLI1, we monitored the transcriptional activity of ectopic GLI1 following treatment with increasing doses of XMD8-92 in melanoma cells. A luciferase assay was performed in a melanoma BRAF wt cell line (M26c), transfected with the reporter vector p8x3GLI-BS (GLI-BS) (Sasaki et al., 1997). The reporter was activated by co-transfecting GLI-BS with low amount of GLI1 expression construct. Twenty-four hours after transfection, cells were treated with increasing doses of XMD8-92 (0.5, 1, 2.5, 5μM). Pharmacological inhibition of ERK5 reduced GLI1 transcriptional activity in a dose-dependent manner (Figure 4.23).

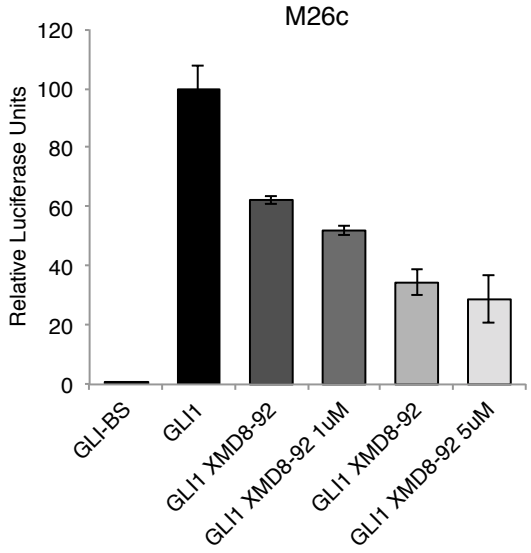


Figure 4.23 Pharmacological inhibition of ERK5 decreases the transcriptional activity of exogenous GLI1. M26 melanoma cells were transfected with a construct for expression of GLI1 to activate the HH pathway and treated with increasing doses of XMD 8-92 for 16 hours. Relative luciferase units (R.L.U.) is calculated as the ratio between the firefly/renilla signal, considering the activation induced by GLI1 equated to 100%. The data represent mean±SEM of at least 3 independent experiments.

These data demonstrate that ERK5 positively modulates the transcriptional activity of GLI1 not only in physiological context of NIH3T3 cells but also in melanoma cells.

After evaluating the effects of the pharmacological inhibition of ERK5 on the transcriptional activity of GLI factors, we verify the results obtained by genetic inhibition of ERK5. To assess GLI1 transcriptional activity, two lentiviral vectors (shERK5 262 and shERK5 275) were used in luciferase assay to silence ERK5. HEK-293T and the melanoma cell lines SSM2c and M26c were co-transfected with p8x3GLI-BS (GLI-BS), GLI1 and with the two lentiviral vectors targeting ERK5 (shERK5 262 or shERK5 275). As shown in Figure 4.24, ERK5 silencing reduces approximately by 50% the GLI1 transcriptional activity in all tested cell lines. Among the two lentiviral vectors, shERK5 275 showed better ability to reduce ERK5 levels (not shown) and GLI1 transcriptional activity compared to shERK5 262.

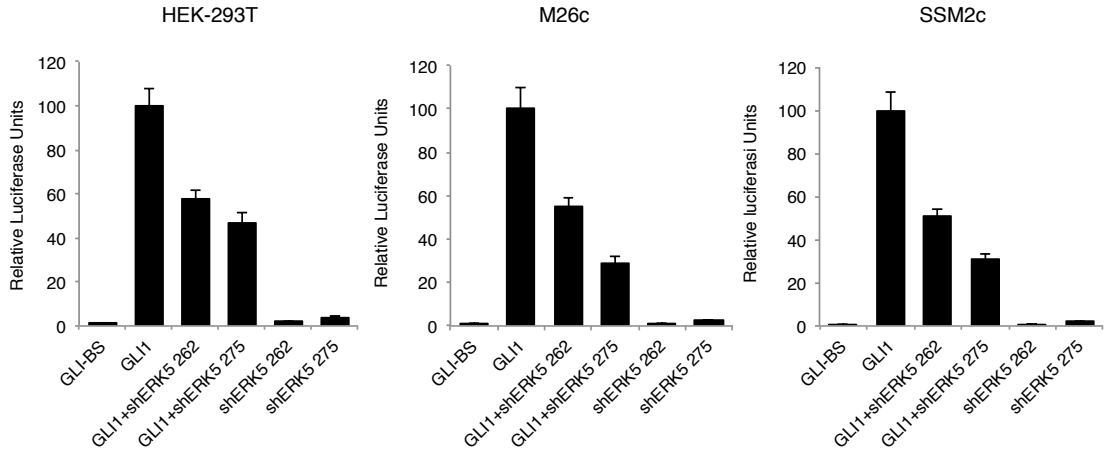


Figure 4.24 Genetic silencing of ERK5 reduces the transcriptional activity of exogenous GLI1 in melanoma cells. M26c and SSM2c melanoma cells and HEK-293T cells were transfected with a construct for expression of GLI1 to activate the HH pathway and with two different shRNA for ERK5 silencing (shERK5 262, shERK5 275). Relative Luceferase Units (R.L.U.) is calculated as the ratio between the firefly/renilla signal, considering the activation induced by GLI1equated to 100%. The data represent mean±SEM of at least 3 independent experiments.

In conclusion, the effect of ERK5 genetic silencing on GLI1 transcriptional activity confirms the results obtained by drug inhibition. However, the experimental data obtained are still preliminary and require further studies to confirm the interplay between the ERK5 and HH pathways.

5. DISCUSSION

Melanoma is the most aggressive form of skin cancer, characterized by high metastatic potential and mortality. The Mitogen-Activated Protein Kinases (MAPK) signaling is the most mutated pathway in melanoma particularly at BRAF level, which presents as prevalent mutation the substitution on V600E residue (Davies et al., 2002; Hodis et al., 2012). Melanoma therapies, based on target therapy and immunotherapy, have improved greatly survival for this disease. However, long-term benefits of targeted therapy are unsatisfactory due to the onset of drug resistance. Moreover, not all patients respond to immunotherapy (Samatar et al., 2014; Flaherty et al., 2012; Teixidó et al., 2015). Therefore, there is an urgent need to identify novel possible targets involved in melanoma growth. ERK5/BMK1 is a member of the MAPK family and regulates cell functions critical for tumor development. Several studies reported a direct involvement of ERK5 in several types of cancer, including aggressive breast and prostate cancers, hepatocellular carcinoma and multiple myeloma (Al-Ejeh at al., 2014; McCracken et al., 2008; Carvajal-Vergara et al., 2005; Rovida et al., 2015; Yang et al., 2011; Simões et al., 2016), but its role in melanoma is less investigated. A study recently demonstrated the involvement of ERK5 in the onset of resistance to combination therapy with BRAF and MEK inhibitors in melanoma cells (Song et al., 2017).

In this study we have investigated the role of ERK5 in melanoma and its interplay with two oncogenic pathways: BRAF-MEK1/2-ERK1/2 and HH signaling. We demonstrated the requirement of the ERK5 pathway for melanoma growth. Indeed, genetic silencing of ERK5 or pharmacological inhibition of ERK5 signaling with two chemically unrelated small molecules dramatically reduce the proliferation of melanoma cells harboring wt or oncogenic BRAF. Importantly, a combination of the BRAF-V600E inhibitor Vemurafenib and ERK5 pathway inhibitors, used at doses able to reduce cell proliferation by half, suppresses melanoma cell proliferation and is more effective than single treatments in reducing growth of human melanoma harboring BRAF-V600E both *in vitro* and *in vivo*. Beyond this biological effects, we showed that oncogenic BRAF positively regulates ERK5 expression, phosphorylation and nuclear localization as well as its kinase and transcriptional transactivator activities. In addition, we demonstrated that ERK5 modulates the activity of GLI1 and GLI2, the last mediators of the Hedgehog signaling.

Our data show that ERK5 is constitutively expressed in several melanoma cell lines and that the presence of ERK5 genetic alterations in melanoma correlates with poor prognosis. Indeed, i*n silico* data analysis indicates that 47% of melanoma patients have alterations in components of the ERK5 pathway, mainly consisting of increased mRNA or gene amplification that are likely responsible for enhanced activation of the pathway. Interestingly, melanoma patients with increased mRNA or amplification of MAPK7, the gene encoding for ERK5, have a shorter disease free survival compared to patients without such alterations. Five out of 479 melanoma patients harbor MAPK7 missense mutations on two potentially phosphorylable sites, including P789S and A424S, which are worth being characterized in future studies to validate their effect on ERK5 activity. Overall, these data identify a wide subgroup of melanoma patients that might benefit from targeting the ERK5 pathway. This prompted us to investigate the role of ERK5 in melanoma cell proliferation.

ERK5 is expressed and active in all melanoma cell lines analyzed, supporting the idea that this kinase is important in melanoma. We show that pharmacological inhibition of the ERK5 pathway with either an ERK5 (XMD8-92) or a MEK5 (BIX02189) inhibitor leads to a strong reduction in melanoma cell growth. In BRAF-V600E-expressing cells, MEK5 or ERK5 inhibitors slow down cell cycle progression with accumulation of cells in G0/G1 phase, likely due to a decreased phosphorylation of Rb, a key regulator of the G1 to S phase transition. Moreover, pharmacological inhibition of MEK5 or ERK5 reduces Cyclin D1 levels and increases p21 expression, as previously reported in other cell types (Perez-Madrigal et al., 2012). In melanoma cells expressing wt BRAF, pharmacological inhibition of ERK5 signaling reduces melanoma cell growth, increasing apoptosis (BIX02189) or blocking cell cycle progression (XMD8-92). Recently, XMD8-92 has been reported to be a dual ERK5/BRD4 inhibitor (Lin et al., 2016). However, our data suggest that the effect of XMD8-92 is mainly on ERK5, since the use of a novel ERK5 inhibitor with much lower affinity toward BRD proteins (JWG0-45, Williams et al., 2016) gave similar results. More importantly, genetic silencing of ERK5 recapitulates, *in vitro* and *in vivo*, the effects of pharmacological inhibition of ERK5 pathway with both XMD8-92 and JWG0-45. All together these data highlight the critical role of ERK5 for growth and proliferation of melanoma cells with either wt or V600E BRAF.

An intriguing point emerging from our data is that the involvement of ERK5 in cancer cell proliferation sustained by oncogenic BRAF or RAS is context-dependent. In

literature, there are conflicting reports that challenge the role of ERK5 in cell proliferation. Indeed, we show here that ERK5 is required for BRAF-V600E-driven proliferation in melanoma *in vitro* and *in vivo*. In addition, it has been reported that hepatocellular carcinoma cells, including HepG2 that harbor mutated NRAS (Q61L), are sensitive to genetic and pharmacological inhibition of ERK5 *in vitro* and *in vivo* (Rovida et al., 2015). In contrast, colon cancer cells with KRAS or BRAF mutations do not appear to be addicted to ERK5 activity for proliferation at least *in vitro* (Lochhead et al., 2016).

Another important finding of this study is the identification of a new mechanism of ERK5 regulation mediated by oncogenic BRAF. Our data indicate that oncogenic BRAF increases ERK5 protein level, phosphorylation at several residues and kinase activity. More importantly, BRAF-V600E enhances ERK5 nuclear localization, including that in the chromatin-bound fraction, and transcriptional transactivator activity. This is at variance with a previous report showing that BRAF-V600E does not enhance ERK5-driven transcriptional activity in presence of overexpressed wt MEK5 in HEK293T cells (Lochhead et al., 2016). As evidenced by the effect on proliferation, even with regard to the transcriptional transactivator activity the effect could be context dependent since our experiments were performed in melanoma cells. Furthermore, the study from Lochhead compares the oncogenic BRAF effect only with MEK5, which itself induces an increase of MEF2D-mediated luciferase activity, probably masking the effect of oncogenic BRAF.

We investigated the mechanism of ERK5 positive modulation by BRAF analyzing the phosphorylation profile of ERK5. According to our model (Figure 5.1), oncogenic BRAF promotes ERK5 phosphorylation at three crucial sites, promoting ERK5 activity and nuclear translocation. First, BRAF increases phosphorylation at Thr732, an event that has been associated with increased ERK5 nuclear localization and ERK5-dependent transcription (Honda et al., 2015). Phosphorylation at this residue is prevented by pharmacological inhibition of ERK1/2 and CDK1, pointing to their possible involvement. Second, BRAF enhances ERK5 phosphorylation at Ser753, an additional CDK1 target residue. Pharmacological inhibition of CDK1 does not completely abolish phosphorylation at this site, leaving open the possibility that Ser753 is an autophosphorylated residue, as is the case for other residues at C-terminus of ERK5 (Morimoto et al., 2007; Buschbeck et al., 2002). The effect of the ERK1/2 inhibitor SCH772984 in abrogating phosphorylation at Ser753 may indicate that

ERK1/2 acts upstream of CDK1, which is consistent with the presence of active CDK1 in oncogenic BRAF overexpressing cells. Finally, overexpression of BRAF induces ERK5 phosphorylation at Thr218/Tyr220, suggesting that MEK5 participates in BRAF-induced ERK5 activation (Figure 5.1). Increased ERK5 phosphorylation at Thr218/Tyr220 by oncogenic BRAF has been showed in a previous report, although the authors reached different conclusions stating that oncogenic BRAF does not stimulate ERK5 phosphorylation (Lochhead et al., 2016). Together, these data indicate that oncogenic BRAF, via CDK1, ERK1/2 and/or MEK5, transduces mitogenic signals to the nucleus through ERK5 (Figure 5.1).

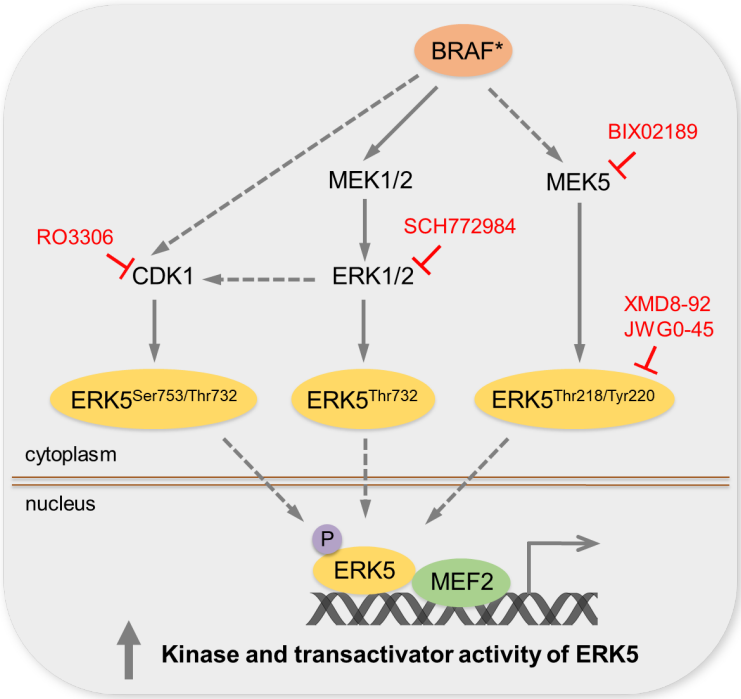


Figure 5.1 Proposed mechanism for oncogenic BRAF-dependent ERK5 activation. Oncogenic BRAF may enhance ERK5 phosphorylation and activity through CDK1 by phosphorylating ERK5 at Ser753 and/or Thr732, through a MEK1/2-ERK1/2-dependent mechanism (Thr732) or through a MEK5-dependent mechanism (Thr218/Tyr220). Solid lines indicate direct established regulatory interactions, whereas broken lines illustrate putative interactions. RO-3306 and SCH772984 are, respectively, CDK1 and ERK1/2 inhibitors, XMD8-92 and JWG-045 are ERK5 inhibitors, BIX02189 is a MEK5 inhibitor.

Our data indicate that Vemurafenib inhibits only ERK5 kinase activity, but is not able to reduce the level of nuclear ERK5. This effect is achieved only with a combined inhibition of BRAF (Vemurafenib) and MEK5 (BIX02189), thus providing the rationale for a combined treatment. Indeed, combination of IC50 doses of XMD8-92 and

Vemurafenib abolished *in vitro* melanoma proliferation and drastically reduced melanoma xenograft growth. The experiments presented here suggest that the combination of ERK5 pathway inhibitors with Vemurafenib could be a good strategy for the treatment of BRAF-mutant melanoma patients. The combination therapy we propose could be also important to prevent the recently reported ERK5-mediated resistance to Vemurafenib-Trametinib treatment in melanoma (Song et al., 2017). Further advocating combination therapy targeting MEK1/2-ERK1/2 and ERK5 pathway, a recent report suggested that pharmacological inhibition of ERK1/2 may induce ERK5 phosphorylation at MEK5 consensus sites in colon cancer cells (de Jong et al., 2016).

Another interesting finding emerging from our study is the positive modulation of the Hedgehog pathway by ERK5. The Hedgehog pathway is required for growth and proliferation of melanoma cells *in vitro* and *in vivo* (Stecca B. et al., 2007; O'Reilly KE et al., 2013; Jalili A et al., 2013). In cancer, constitutive activation of HH signaling may result from canonical activation of the pathway through the binding of Hedgehog ligands to the Smo receptor or from direct activation of the downstream GLI transcription factors by oncogenic inputs (Pandolfi et al., 2015). Several studies have shown that oncogenes (among which RAS/RAF/MEK/ERK pathway and AKT) positively modulate the activity of the HH pathway. Particularly, the MEK1/2-ERK1/2 signaling has been shown to act upstream to the HH pathway, regulating activity and nuclear localization of GLI transcription factors (Rovida et al., 2015). The HH pathway may be also upstream of MAPK, leading to ERK1 and ERK2 activation. Interestingly, HH activation induced by treatment with the BRAF inhibitor Vemurafenib can lead to the induction of PDGFRα sustaining the reactivation of the RAS-ERK1/2 pathway (Sabbatino et al, 2014). This modulatory loop between HH pathway and the final effector of MAPK has important implications for cancer therapy, especially in melanoma where these two signaling play a crucial role.

In this study we identified an interplay between HH signaling and ERK5. In particular, we show that ERK5 is able to positively modulate the activity of Gli1 and Gli2, the final mediators of the Hedgehog pathway. We investigated this regulation both in physiological conditions, using murine fibroblasts which are highly responsive to the canonical activation of the HH pathway, as well as human melanoma cell lines. In both physiological and cancer conditions, pharmacological inhibition of ERK5 reduces the transcriptional activity of Gli1 in a dose dependent manner. These results were also

confirmed with genetic silencing of ERK5 using a lentiviral vectors encoding for a specific ERK5 shRNA. To further clarify this regulation, we investigated whether ERK5 could also affect protein level of GLI1 and GLI2. Consistent with luciferase assay, inhibition of ERK5 with XMD 8-92 or BIX02189 leads a to strong reduction of GLI1 and GLI2 protein levels, suggesting that ERK5 positively regulates transcriptional activity and protein level of GLI1 and GLI2. At the moment we are elucidating whether this regulation occurs at the transcriptional level or through post-translational modifications. In particular, we plan to assess whether ERK5 directly phosphorylates GLI1 or GLI2, or this modulation is mediated by a third factor that is phosphorylated by ERK5. The identification of an interplay between ERK5 and the HH pathway could provide new therapeutic strategies for melanoma patients and open the way for a novel combined therapy for metastatic melanoma.

In conclusion, in this study we have demonstrated the requirement of ERK5 for melanoma cell proliferation, and its modulation by oncogenic BRAF. In addition, preliminary results indicate that ERK5 positively regulates the activity of the GLI1 and GLI2 transcription factors. Our data suggest that targeting ERK5 might be regarded as first-line therapeutic approach for melanoma patients with wt or oncogenic BRAF, or as a potential therapeutic strategy aimed at preventing resistance to BRAF-MEK1/2 inhibitors. The interplay with the HH pathway may be a good target to block reactivation of a proliferative signaling and to obtain a durable response, with an effective improvement of melanoma patient survival.

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