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DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

TRIB2 confers resistance to MAPK and mTOR1 inhibitors

Neuton Pedro Gorjão da Silva

Oncobiology Master's Thesis

Supervisors: Dr. Wolfgang Link

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Declaração de autoria do trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Acknowledgements

This last year, at the Cancer Signalling Laboratory, was an amazing experience. It allowed me to grow up as a person and as a researcher, so I would like to express my gratitude to everyone who helped me to accomplish this important step in my life.

My first words go to my Supervisors dr. Wolfgang Link and dr. Richard Hill. Thank you for having accepted me so kindly in his research group and giving me the opportunity to do my master´s thesis in such a remarkable group. Thank you for your support, guidance and help since the first day, and more important, thank you for your friendship.

Thank you to the other Lab members, Inês, Marta, Pedro and Joel for your help, kindness and friendship.

Special thanks to my friend Tânia Saramago that helped me to edit the thesis. Thank you to all my closest friends, Nicole, António Brazio, Andreia Mendes, Mariana, Celso Pinto for your friendship and support.

Thank you to all members of Bragança´s, Patricia Madureira´s and Ana Teresa Maia´s Labs, to Dino, Gonçalo Pinheiro, Daniel, Frederica, Joana Sousa, Om Rathor and Gil Carraco for your advices, support and friendship.

I also would like to express my gratitude to my beloved family. You have been always present even 300 km away.

At least I would like to dedicate this thesis to my girlfriend Cátia Lopes. I cannot imagine finish this journey, without her presence and love.

"It has been a beautiful fight.
Still is."

Charles Bukowski

Resumo

A incidência mundial de cancro é elevadíssima, estima-se que um em cada cinco de nós irá morrer como consequência desta doença. Um estudo feito à escala global demonstra que existem 14.1 milhões de casos por ano, resultando em 8,2 milhões de mortes. Em Portugal, o cancro é a segunda principal causa de morte, a seguir às doenças do aparelho circulatório, em particular o AVC (acidente vascular cerebral). Apesar de se encontrar no grupo restrito de tumores malignos mais facilmente detetáveis, o melanoma em fases mais avançadas da doença, é a forma mais mortal de cancro da pele que conduz a um tempo médio de sobrevivência bastante reduzido.

Do ponto de vista clínico, apesar de existirem pacientes em fases iniciais que poderão ser tratados com sucesso através de cirurgia, em fases mais avançadas a aquisição de resistência à quimioterapia continua a ser um problema bastante comum.
É imperativo encontrar alternativas de tratamento e melhores métodos de diagnóstico para o combate a esta doença.

A medicina personalizada assume-se cada vez mais como uma excelente alternativa aos tratamentos convencionais. Uma vez que o cancro é uma doença bastante heterogénea, a medicina personalizada exige um conhecimento bastante profundo e especifico de cada paciente para que o tratamento possa ser aplicado de uma forma mais individual, ou seja, será necessário ao nível do diagnóstico, uma capacidade de hierarquizar pacientes com base no êxito perante a resposta ao tratamento com um fármaco que seja específico, por oposição às terapias convencionais. Prevê-se então uma mudança de paradigma no tratamento de cancro: dos agentes citotóxicos mais convencionais, até aos agentes mais específicos, concebidos com base nas especificidades de cada tipo de célula cancerígena. Com base neste pressuposto, é necessária a identificação e validação de novos alvos terapêuticos.

O desenvolvimento de novas técnicas de biologia molecular tem alterado a maneira como nós entendemos a origem do cancro. Estudos “large-scale” ou “image-based”, permitem interpretar os diferentes fenótipos resultantes de alterações químicas, genéticas ou epigenéticas pertencentes a cada tipo de tumor. Desta forma é possível

compreender as inúmeras vias moleculares orquestradas por diferentes genes, que dão origem controlam e regulam diferentes tumores.

Numa célula normal, uma via de sinalização é desencadeada quando uma molécula (sinal) se liga a um recetor extracelular. É ativado então uma cascada bioquímica de eventos, evolutivamente conservados. O fim de uma via de sinalização passa pelo núcleo, cujo objetivo fundamental passa pela transcrição de genes que controlam diversos tipos de processos celulares fundamentais. Numa célula tumoral, algumas destas vias moleculares encontram-se alteradas. A partir de screens genéticos, é possível identificar as mutações responsáveis pela desregulação de genes e consequentemente das proteínas constituintes destas vias. As principais vias de sinalização alteradas em melanoma são a PI3K/AKT/m-TOR (principais membros alterados: PTEN (Phosphatase and tensin homolog) - 15–50% deletado, mutado ou silenciado; PI3K (Phosphoinositide 3-kinase) - 6% mutado; recetores membranares - 10%–20% constitutivamente ativados e AKT (Protein kinase B) - 60% amplificado ou ativo), as via MAPK (Mitogen-activated protein kinase), também conhecida como via MEK/ERK (principais membros alterados: RAS (20%) e B-RAF (60%)).

Outro membro com enorme relevo da via PI3K é o gene *tribbles2*. Para além de funcionar como um oncogene, tem sido descrito também como biomarcador para melanoma, característica que permite distinguir e avaliar se um paciente tem ou não desenvolvido este tipo de tumor. Quando expresso, TRIB2 assume também importante destaque na resistência ao tratamento com alguns fármacos e drogas anti tumorais, nomeadamente drogas que atuam ao nível da via PI3K. Tendo em conta todos estes fatores, questionamos se esta proteína também desempenha um papel importante no mecanismo de resistência criado após a administração de agentes inibidores de outro elemento desta via, m-TOR. Outra via de sinalização frequentemente alterada em melanoma é a via MEK/ERK. Para além disso trata-se de uma via alternativa à via PI3K, pelo que testamos a hipótese de que TRIB2 também regula a resistência a inibidores MEK. Examinámos o impacto da sobre expressão de TRIB2 ao nível celular, proteico, RNA e de DNA, na presença de inibidores proteicos MEK ou mTOR.

Tendo como ponto de partida resultados anteriores do nosso laboratório, em que foi possível demonstrar que TRIB2 confere resistência a diferentes tipos de agentes

quimioterapêuticos convencionais, assim como a inibidores de PI3K, começamos por averiguar se TRIB2 também confere resistência em células tratadas com inibidores m-TOR. Foram criadas linhas isogénicas celulares, com o intuito de formar células com diferentes concentrações proteicas: elevados ou baixos níveis de TRIB2.

Após o tratamento com rapamicina, um potente inibidor do complexo mTORC1, verificou-se que apenas as células que contêm elevados níveis de TRIB2, resistem ao tratamento com este tipo de inibição.

De seguida, submetemos de novo linhas isogénicas ao tratamento com um inibidor tanto do complexo mTORC1 como do mTORC2, TORIN1. Os resultados são bastante claros e revelam que TRIB2 não confere qualquer tipo de resistência, quando mTORC2 é inibido.

Quando as células que sobre expressam TRIB2 são tratadas com BAY 766, um poderoso inibidor MEK, são criados mecanismos de evasão e estas células resistem. O mecanismo fundamental pelo qual as células ultrapassam a resistência, é determinada pela interação entre MEK e TRIB2 que formam um complexo proteína- proteína. Estes resultados são enfatizados quando os sinais imunofluorescentes de TRIB2 e MEK de células resistentes se sobrepõem. Observamos também que a ativação da via de sinalização MEK/ERK regulada por TRIB2, reprime a ação de FOXO3a um importante fator de transcrição. Por outro lado, os nossos resultados apontam para que TRIB2 leva a um aumento de afinidade de NF-κB se ligar ao promotor, sugerindo que a sua concentração aumenta no interior do núcleo destas células resistentes a inibidores MEK.

De seguida, testou-se a ativação da via PI3K, verificando se a proteína AKT se encontrava fosforilada. Os resultados são semelhantes para todas as linhas celulares, ocorrendo fosforilação de AKT. Averiguamos de que forma esta proteína seria ativada, e verificou-se que a ativação de AKT ocorre pela fosforilação do resíduo serina na posição 473, sempre que as células sobre expressam TRIB2.

Como é sabido, mTORC2 desempenha um papel importante na ativação de AKT através da fosforilação da serina 473. Um dos elementos fundamentais deste complexo é a proteína RICTOR. Procedemos ao seu knock down, e a resistência criada na presença de diferentes drogas (BEZ235, BAY236 e BAY439), é perdida. O mesmo resultado é obtido quando o Knock down é feito para FOXO3a.

O melanoma, em fases mais evoluídas da doença (estádio IV), tem-se revelado como uma das formas mais mortais de cancro. É imperativo que se consiga encontrar novas formas de combate a este tipo de doença com contornos tão trágicos. A importância biológica deste projeto prende-se exatamente com esta necessidade: tentar perceber qual o papel da proteína TRIB2, do ponto de vista molecular, descrita anteriormente como um biomarcador para melanoma no mecanismo de resistência a múltiplas drogas.

Após o término do projeto, foi possível verificar que TRIB2 confere resistência, em diferentes linhas celulares após a administração de drogas que inibem a estrutura proteica mTOR. TRIB2 funciona como uma molécula ativadora de AKT, uma proteína que desempenha um papel fundamental na via de sinalização PI3K/AKT/mTOR, uma das vias mais alteradas em melanoma. Por sua vez um dos ativadores moleculares de AKT é o complexo mTOR. Verificámos que quando TRIB2 é sobre expresso, a ativação de AKT acontece apenas através do complexo 1 de mTOR e não pelo complexo 2. Indo um pouco mais fundo nesta análise concluímos que AKT é ativado por mTOR1 que tem a capacidade de fosforilar o resíduo serino na posição 473, um dos requisitos para que ocorra a ativação completa de AKT.

Uma outra via de sinalização, que se encontra ativa em melanoma é a via MAPK, mais em concreto a via MEK/ERK. À imagem do que acontece aquando a administração de diferentes inibidores, TRIB2 exerce um papel chave no mecanismo de resistência a inibidores MEK. Em células resistentes, TRIB2 forma um complexo proteína-proteína com MEK, que conduz a ativação de ERK e por consequência de toda a via. Esta ativação, culmina com a presença de vários fatores de transcrição no núcleo, como é exemplo NF-κB, conduzindo à transcrição de diferentes genes fundamentais nos processos de evasão, crescimento celular e proliferação.

Palavras-chave: Melanoma; inibidores MEK; inibidores mTOR; fármaco-resistência; TRIB2

Abstract

Malignant melanoma is the deadliest form of skin cancer that leads to a median survival time of only 6 to 9 months. Besides the patients in early stages that could be successfully treated by surgery alone, actually, there is no efficient diagnostic procedure. It is imperative to find alternatives and better methods of diagnosis for metastatic melanoma. Acquisition of resistance to chemotherapy agents, namely cytotoxic drugs such as dacarbazine, remains a major problem in melanoma therapy.

Since TRIB2 (tribbles2) protein has recently been implicated as a biomarker for melanoma, and as a mediator in the process of resistance to conventional chemotherapeutics or PI3K (Phosphoinositide 3-kinase) inhibitors, we hypothesised that resistance to the inhibition of different components of this and other signalling pathways such as mTOR (mammalian target of rapamycin) and MEK (MAPK/ERK Kinase) is mediated by TRIB2.

In this study, we examined the impact of ectopic expression of TRIB2 in the presence of mTOR or MEK inhibitors.

We demonstrated that in the presence of rapamycin, a potent inhibitor of mTORC1 (mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1), TRIB2 confers resistance to this kind of treatment. However, when we exposed cells to a mTOR2 inhibitor, TORIN1, resistance is not observed. We demonstrated that TRIB2 acts as an adaptor, recruits the mTOR complex 1 that phosphorylates AKT (also known as Protein Kinase B, PKB) on residue Serine 473.

Furthermore, we investigated the effect of TRIB2 expression, when cells where exposed to pharmaceutical inhibition of MEK. We found that TRIB2 expression protects cells against the effect of the MEK inhibitor BAY 766. We have seen that TRIB2 and MEK co-localize, interact and form a protein-protein complex. We also observed that the inhibition of MEK/ERK and PI3K pathways regulated by TRIB2, represses FOXO3 (Forkhead box O3a) a binding to p27 promoter, a well-known and studied transcription factor. On the other hand, high levels of TRIB2 leads to an increase of NF-κB (factor nuclear K B) affinity for promotor. NF-κB enhances the transcription of different genes crucial for melanoma cells proliferation and survival.

This study suggests that TRIB2 is a meaningful component of a drug resistance mechanism in melanoma cells. This molecular process determines and modulates important cell decisions which include cell growth, proliferation and survival.

Keywords: Melanoma; MEK inhibitors, drug resistance; mTOR inhibitors; TRIB

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Abbreviations List

AJCC- American Joint Committee on Cancer

AKT- Protein Kinase B

BRAF-B-type Rafkinase

ChIP- Chromatin Imunnoprecipitation

Co-IP Co-Imunnoprecipitation

DNA -Deoxyribonucleic acid

DTIC- Dacarbazine

ERK-Extracellular signal-regulated kinases

FACS -Fluorescent Activated Cell Scanning

FasL- Fas ligand

FDA-Food and Drug Administration

FOXO- Forkhead transcription factor

IGF-1- Insulin-like growth factor 1

IgG- ImmunoglobulinG

Il-2- Interleukin-2

IP- Immunoprecipitation

KD- Kinase Domain

MAPK- Mitogen-activated protein kinases

MAPKK- Mitogen-activated protein kinase kinase

MDM2- Mouse double minute 2 homolog

MEK-MAPK/ERK Kinase

mTOR-Mechanistic target of rapamycin

NF-KB- Nuclear factor KB

PBS-Phosphate buffered saline

PDK1-Pyruvate dehydrogenase lipoamide kinase isozyme 1

PI3K-Phosphatidylinositol 3 kinase

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

pRAS-proline-rich AKT substrate

PTEN-Phosphatase with tensin homology

RICTOR-Rapamycin-Insensitive Companion of mTOR

RTK-Receptor tyrosine kinases

TRIB2- Tribbles pseudokinase 2

USA-United States o fAmerica

CHAPTER 1. *INTRODUCTION*

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1. Cancer

Cancer is a leading cause of death and it is estimated that about one in five of us will die as a consequence of cancer. The worldwide incidence of cancer is extremely high, a 2012 study revealed that there are 14.1 million cases per year, resulting in 8,2 million deaths. It is also estimated that there are 32,6 million people living with cancer (within 5 years of diagnosis). [1]

The evolution of molecular biology techniques has changed the way we see the origin of cancer. Nowadays, the focus of carcinogenesis studies has shifted from the identification of gene mutations to understanding the pathways that these genes control. This paradigm shift has suggested that tumorigenesis, at the molecular level, results after several genetic alterations that have occurred within the cell. It is a multistep process, in which every gene affected encode proteins with different functions.[2,3]

In this way, the most mutations are observed in genes related with cell growth and proliferation (including oncogenes such as RAS, cMYC and SRC), tumour suppressor genes that inhibit cell proliferation (p53, RB and FOXO´s), control of apoptosis (p53, FOXO´s and BCL-2), cell-contact inhibition (E-CADHERIN), promotion of survival (telomerase and PI3K/AKT) and stability genes that are responsible to repair mistakes in genome made during normal DNA replication or induced by exposure to environmental factors (mismatch repair, nucleotide-excision repair and base-excision repair genes).[2]

A cell needs to overcome a number of mechanisms in order to become cancerous. This theory proposes that cells, to achieve transformation, must activate some proliferative signal transduction, evade the growth suppression mechanisms, resistance to apoptosis, enable replicative immortality, induce angiogenesis, activate invasion and promote metastasis.[4,7]

Recently, two other hallmarks were added: reprogramming of energy metabolism and evading immune destruction.[8] Acquisition of drug resistance and avoidance of oncogene (it will be discussed in next sections) and inducing of senescence are also another important features in tumour development.

2. Melanoma

2.1 A clinical perspective

In the last 50 years melanoma has risen faster than any other cancer type and unlike other kind of cancers, melanoma incidence is not strongly dependent on age, and is one of the most common causes of cancer deaths between the ages of 20–35. It remains one of the cancers most resistant to treatment.[9] Melanoma provides one of the best sources to study the relationship between environmental factors and cancer cells. It arises within any anatomic place that contains normal pigment cells (neural crest-derived melanocytes), located on the basement membrane of epithelial surfaces. The main function of melanocytes is the synthesis, storage, and transfer of melanin pigments to surrounding epithelial cells[9].

2.2. Diagnosis

Histological patterns have been well described. Microscopic features that correlate with clinical subgroups (including superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma (it is the most common subtype of melanoma in people with darker skins.), and lentigo malignant melanoma (also known as Hutchinson melanotic freckle., lentigo maligna has a lower rate of transformation to invasive melanoma than the other forms)) have been thoroughly codified. As an example, cutaneous lesions detected as little as 1 mm in Breslow thickness could have microscopic evidence of lymph node metastasis which, when present, confers a significantly increased risk for metastasis.[910]

The traditional Clark model is a multi-step system to predict the progression of melanoma. Several models that try to explain the genetic basis of melanoma development and progression are based on this Clark model. It also predicts that the acquisition of a BRAF mutation can be a founder event in melanocytic neoplasia.

It emphasizes the stepwise transformation of melanocytes to melanoma, from the formation of nevi to the subsequent development of dysplasia, hyperplasia, invasion, and metastasis [9] (Fig.1.1).

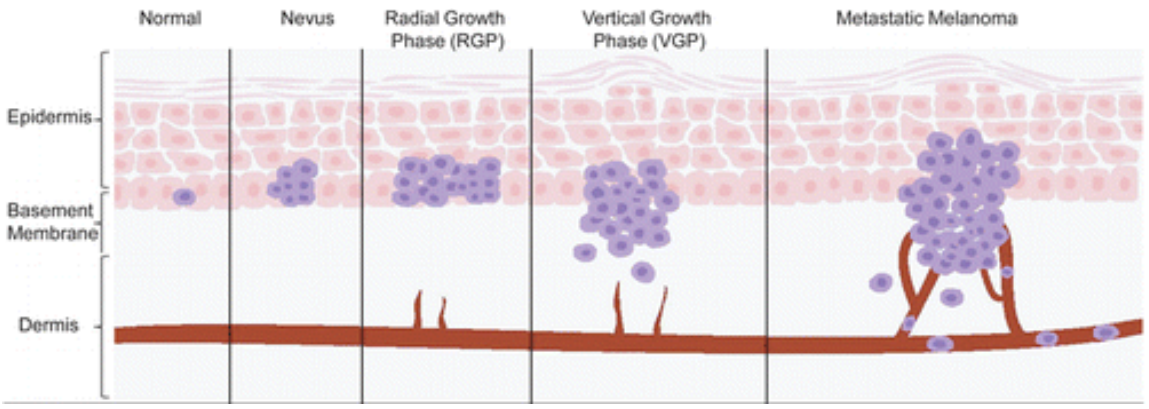

***Figure 1.1-Classification of melanoma development*[55]**
Melanoma can be classified according to the Clark´s model based on the level of invasiveness: I-Epidermis (normal); II to IV- dermis (Nevus, RGP, VGP,); V- subcutaneous tissue (metastatic melanoma). The Breslow approach considers the millimetres of invasion depth, ranging from 0 to 2mm. (representation of a vertical section of skin)

Melanoma progression can also be classified according to different stages of the disease in a clinical grading system (stages 0, I, II, III, and IV).[11]

2.3 Clinical Grading System

Melanoma disease progression is typically evaluated using the American Joint commission on Cancer (AJCC) system (Table 1.1). It is a four stage system that incorporates tumour thickness, presence of ulceration and how widespread the melanoma is in a patient (if it has spread to nearby lymph nodes or any other organs).

The fact is that melanoma diagnosis is very much based on the professional's experience and on complex laboratory tests, being a very subjective factor. Therefore, more accurate ways to diagnose and stage this disease are being searched for.

***Table 1.1-Malignant melanoma staging system approved by AJCC*[11]**

Staging for cutaneous melanoma: a simplified overview	
Stage 0	Melanoma involves the epidermis but has not reached the underlying dermis.
Stages I and II	Melanoma is characterized by tumor thickness and ulceration status. No evidence of regional lymph node or distant metastasis.
Stage III	Melanoma is characterized by lymph node metastasis. No evidence of distant metastasis.
Stage IV	Melanoma is characterized by the location of distant metastases and the level of lactate dehydrogenase.

2.4. Conventional Therapies

The standard therapy options for melanoma are surgery (surgical removal of the tumour and surgical excision margins based on Breslow's tumour thickness) for non-metastatic tumours detected early, surgery, radiation, chemotherapy and supportive care. The median survival time for melanoma patients with stageIV melanoma ranges from 0.7 to 5 months depending on age and performance status (how patients react to treatment).[12]

The chemotherapeutic drugs used in the treatment of several tumours can be classified according to their function: DNA-modifying agents (nitrogen mustard, first used in a patient with lymphoma), anti-metabolites (5-fluorouracil (5-FU)), spindle poisons (paclitaxel), topoisomerase inhibitors (doxorubicin) and cytotoxicantibiotics (bleomycin). Some chemotherapeutic agents have activity in patients with metastatic melanoma, including dacarbazine (DTIC) which is an alkylating agent as well as the nitrosoureas, platinum analogs, vinca alkaloids (vinblastine), and the taxanes (paclitaxel). Numerous trials of single agents or combinations of chemotherapy have been performed, but DTIC remains the standard regimen and the most used chemotherapeutic agent for melanoma. The response rate observed with DTIC chemotherapy alone ranges from 15% to 25%, indicating that improved therapies are desperately required.[12]

Although the clinical criteria for atypical nevi and traditional therapies have been useful in diagnose and improvement of treatment, they are not precise or highly reproducible and contain some pitfalls: for instance, only a small percentage of primary melanomas arise from nevi (10-20%)[9]and chemotherapy for advanced melanoma (stage IV) remains largely palliative with low survival rates after diagnosis.[12]Therefore, new therapy strategies are required.

3. Molecular Pathways in Melanoma

It is crucial to develop new strategies, so over the past 30 years, many groups have helped to decipher the complex genetic networks involved in melanoma proliferation, progression and survival and our understanding has significantly improved. Many of these oncogenic loci and pathways are being well studied and have become crucial targets

for pharmacological drug development. This continuous understanding of the disease can revolutionize the treatment of cancer.[13,14]

In a normal cell, the general signalling can start when a signalling molecule binds to a cell receptor (extracellular signals). The signal molecule can be a growth factor, mitogens, hormones, neurotransmitters or cytokines. It can take place either through direct cell-cell contacts and cell-matrix interactions (integrins and cadherins) or through the action of secreted signalling molecules. Secreted signalling can be classified into endocrine, paracrine and autocrine signalling. In endocrine mechanism, hormones are carried through the circulatory system to act on distant target cells. In paracrine signalling, a molecule released from one cell acts locally to affect nearby target cells and in autocrine signalling, a cell produces a molecule to which it also responds. Then the signal is usually transmitted into a cascade of events evolutionarily conserved, that passes a message into the nucleus. Here, different transcription factors, such as FOXO3a, NF-KB, c-MYC, AP-1, p53 and E2F1 for example, have the capability to enhance transcription. Different genes are transcribed controlling fundamental cellular processes such as proliferation cell growth, apoptosis, cellular energy production, cell transport or homeostasis[10-15]. In tumour cells, these pathways are modified and the scientific community has been performing high throughput genomic screens to identify many of the driver mutations responsible for malignancy. The dysregulation of these genes affecting upstream proteins like cell receptors or downstream proteins plays an important role in the development of melanoma.[10,15]Different studies suggest the involvement of pathways like Notch and WNT/βb-catenin [16,17]. Our attention is directed towards the most mutated molecular signalling pathway implicated in melanoma development. MAPK pathway is the most important pathway in melanoma, and has been reported to be activated in over 80% of all cutaneous melanomas, making it the focus of many scientific studies in the melanoma field. The most common mutations are found in RAS (25%); B-RAF (70%). The second most important signalling axis is PI3K/AKT, with several of its members being mutated or modified are PTEN - 15–50% deleted, mutated or silenced; PI3K – 6% mutated;10%–20% receptor-independent activated and AKT - 60% amplificated or activated.

3.1 RAS/RAF/MAPK molecular pathway

Mitogen-activated protein kinase (MAPK) cascades are key signalling pathways involved in the regulation of cell proliferation, survival and differentiation being activated by a wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases (RTKs), integrins, and ion channels. [20]

The ERK pathway is the best studied of the mammalian MAPK pathways, and is deregulated in approximately, one-third of all human cancers.

The signalling cascade is triggered depending on the molecular stimuli (growth factors and mitogens), the scaffold of the pathway usually includes a set of adaptors (Shc, GRB2, Crk, etc.) linking the receptor to a guanine nucleotide exchange factor (SOS, C3G,

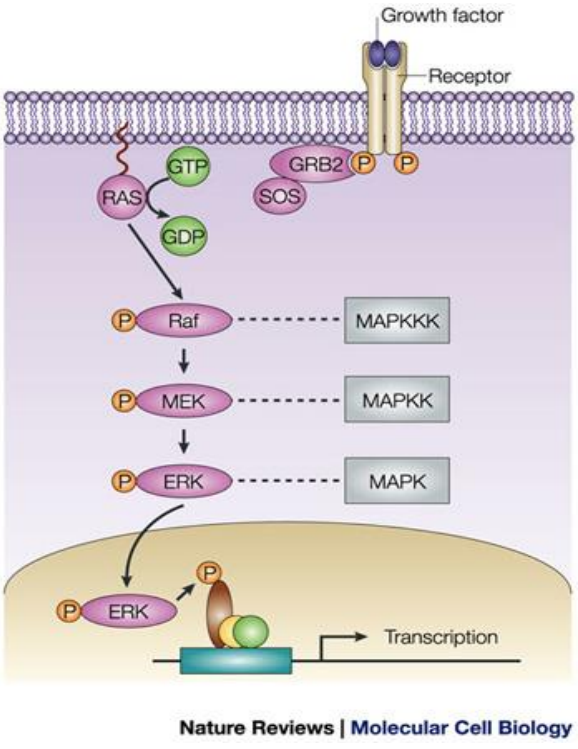

Figure 1.2-MAPK signalling pathway. [54]
Although there are more pathways as shown in this figure, the three most significant arms of the mitogen-activated protein kinase (MAPK) pathway are ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38. They can be activated through different stimulus such as growth factors, integrins and interleukines . The three-tiered kinase dynamic cascade leads to activated MAPKs entering the nucleus to trigger immediate early gene and transcription factor activation for cellular responses such as cytokine production, apoptosis, cell proliferation and migration.

etc.). Then, the signal is transduced to small GTP-binding proteins (RAS). RAS proteins (H-, K- and N-Ras) function as a GDP/ GTP-regulated switch. GDP/GTP cycling is regulated by guanine nucleotide exchange factors (RAS GEFs; e.g., Sos) that promote formation of active RAS-GTP, whereas GTPase-activating proteins (GAPs; e.g., NF1 neurofibromin) stimulate GTP hydrolysis and formation of inactive RAS-GDP. In normal cells, Ras is bound to GDP and inactive. Extracellular stimuli cause transient formation of the active Ras. Activated Ras-GTP binds to downstream effector targets, of which the Raf kinases are the best characterized. The core unit of the cascade of these three kinases: is composed by MAPKKK (Raf-1, B-RAF and A-RAF)) that activates MEK1/2 by phosphorylating serines 218 and 222 in the activation loop, MAPKK (MEK1/2), and MAPK (ERK). An activated ERK dimer can both regulate targets in the cytosol and phosphorylate a variety of transcription factors in the nucleus regulating gene expression. (Fig. 1.2)[20–23]

The second most important molecular network involved in melanomagenesis is the PI3K/AKT/FoxO signalling axis. There is growing evidence that activation of this pathway plays a significant role in melanoma, frequently in the setting of concurrent activation of RAS-RAF-MEK-ERK signalling. This evidence includes the identification of genetic and epigenetic events that activate this pathway in melanoma cell lines.

3.2. PI3K/AKT/FoxO signalling axis

Phosphoinositide 3-kinase (PI3K) is a major component of the signalling pathway and can be activated by receptor tyrosine kinases (RTKs) like HER2 and EGFR or G protein-coupled receptors. The GTPase RAS can also recruit and activate PI3K through direct binding.[18,19]

PI3K is a heterodimer with a catalytic subunit p110 and a regulatory subunit p85 that regulates many normal cellular processes including cell proliferation, survival, growth, and motility. PI3K catalyses the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP2) at the 3′ position on its inositol ring. Pip3 is now able to recruit other downstream molecules such as AKT and PDK1 via their

pleckstrin-homology (PH) domains.18,19 In an opposite way there is a phosphatase PTEN that converts PIP3 back to PIP2 acting as a tumour suppressor (Fig.1.4).

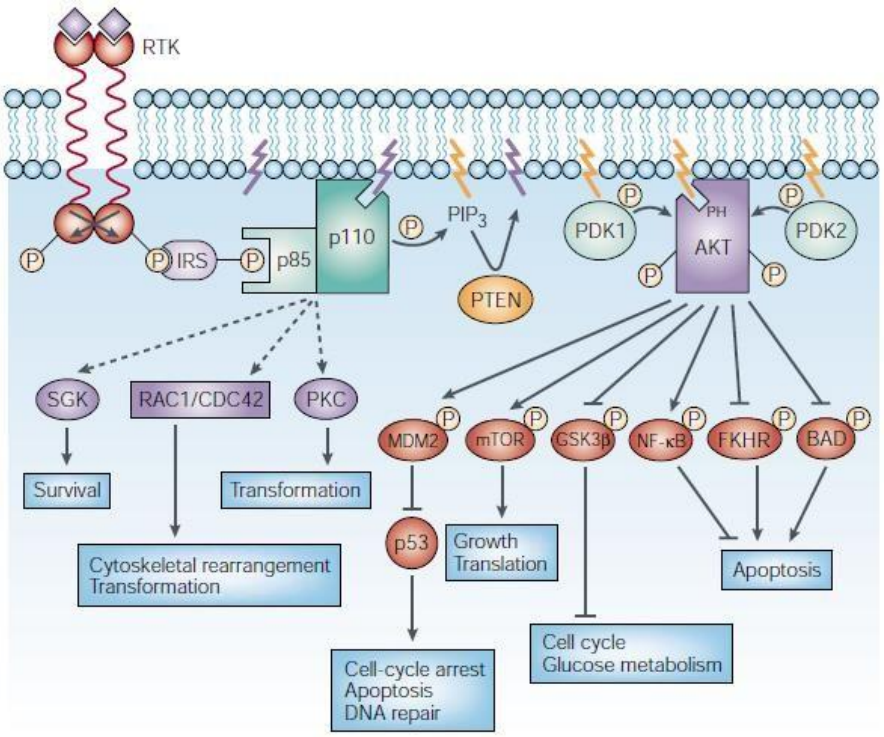

Figure 1.3-The PI3K/Akt/mTOR signaling pathway. [18]

This pathway is up-regulated in melanoma via either direct upstream stimulation (growth factor receptors and their ligands) or indirect activation via cross-talk with RAS. The crucial step in this pathway is the AKT full activation that can regulate members of the apoptotic pathway: inhibit Bad, and FOXO transcription factors and activates NF-kB and CREB that regulate anti-apoptotic genes. It inhibits GSK3β (Glycogen synthase kinase-3) responsible for the degradation of β-catenin resulting in cell cycle progression.

The activation of mTOR leads to regulation of cell growth by controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism.

AKT also targets survival and cell cycle regulation. AKT phosphorylates Mdm2 that controls the levels of p53 in the cell.

AKT can be phosphorylated at two critically conserved residues (threonine 308 and serine 473) and only reaches its full activity if both are phosphorylated. The kinase phosphoinositide- dependent kinase 1 (PDK1) targets the threonine 308 residue whereas the serine 473 residue is phosphorylated by the Mechanistic Target of Rapamycin Complex 2 (mTORC2). When AKT is fully activated, regulates a wide range of different targets with different cell functions (Fig.1.3).

3.3. Mechanistic target of rapamycin (mTOR)

The mechanistic target of rapamycin (mTOR) protein is a 289-kDa serine/threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and is conserved throughout evolution. It has emerged as a critical growth-control node, receiving stimulatory signals from Ras and PI3K downstream from growth factors, as well as nutrient inputs in the form of amino-acid, glucose and oxygen availability Aberrant mTOR signaling is involved in many disease states including cancer, cardiovascular disease, and diabetes. It is present in two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)[67] (figure 1.4)

mTORC1, is composed of regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GbL); proline rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) and is inhibited by rapamycin. After entering the cell, rapamycin binds to FK506-binding protein of 12 kDa (FKBP12) and interacts with the FKBP12- rapamycin binding domain (FRB) of mTOR, thus inhibiting mTORC1 functions. mTORC1 is known for its role in regulating cell growth and proliferation through modulation of protein synthesis. The AKT promotes cell growth (i.e., accumulation of cell mass) predominantly when activation of complex 1 of the mammalian target of rapamycin (mTORC1) occurs Recent research has identified novel mTORC1 cell signalling mechanisms that modulate mitochondrial biogenesis, hypoxia signalling and cell cycle progression.

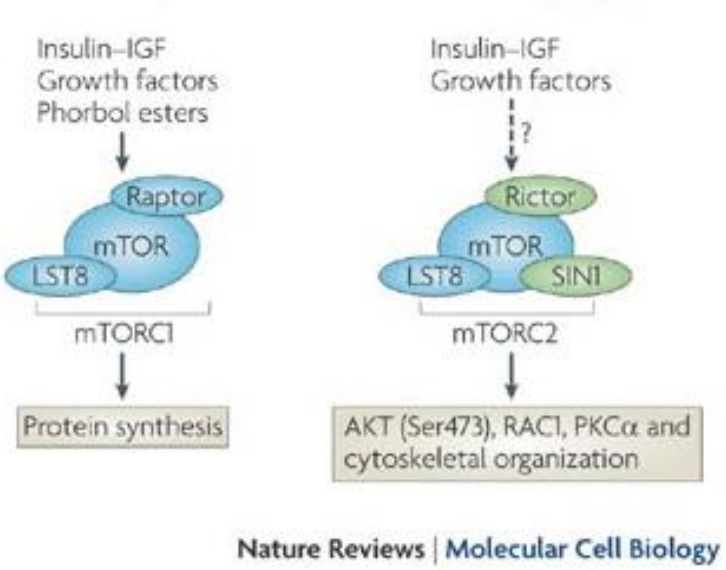

Figure 1.4-mTOR complexes[69].
mTOR nucleates two different complexes: mTOR complex 1 (MTORC1) on the left and mTOR complex 2 (mTORC2) on the right. AKT is activated by mTORC2, through phosphorylation of Ser 473.

The second complex, mTOR complex 2 (mTORC2), is composed of mTOR, RICTOR, GβL, Sin1, PRR5/Protor-1, and DEPTOR. It ca be inhibited with BEZ235 and it is not sensitive to rapamycin. Functions for Mtorc2 is still unclear. However, the first function ascribed to mTORC2, based on the previously known function of TORC2 in yeast, was the regulation of the actin cytoskeleton. Knockdown of mTORC2-specific components in cultured cells results in alteration of the actin cytoskeleton[68]. AKT was identified as a mTORC2 substrate, it was found that phosphorylates Ser473 in the hydrophobic motif of AKT[66]. Although earlier knockdown studies of RICTOR also showed reduced phosphorylation of Thr308 in the activation loop, further studies in knockout mice suggested that phosphorylation of Thr308, by phosphoinositide-dependent kinase 1 (PDK1), does not depend on prior Ser473 phosphorylation[66,67]

3.4. Transcription Factors

3.4.1. Nuclear factor Kappa B (NF-κB)

Increased expression of pro-inflammatory and proangiogenic factors is associated with aggressive tumour growth and decreased survival of patients with cancer. In

particularly, recent genetic and cancer genome studies, support the involvement of nuclear factor Kappa B (NF-κB) transcription factors, and the signalling pathways that control its activity, in human cancer[32,33].

Nuclear factor Kappa B (NF-κB) was first discovered in 1986 as a nuclear factor that binds to the enhancer element of the immunoglobulin kappa light-chain of activated B cells34.This transcription factor has a key role in many physiological processes such as innate and adaptive immune responses, cell proliferation, cell death, and inflammation. It has become clear that aberrant regulation of NF-κB leads to tumour progression, as well as to resistance to chemotherapy and radiotherapy[35].

The genes regulated by NF-κB (NF-κB-dependent transcription) are not only tightly controlled by positive and negative regulatory mechanisms but also closely coordinated with other signalling pathways. The list of NF-κB-dependent target genes can be further extended to regulators of apoptosis (antiapoptotic Bcl family members and inhibitor of apoptosis proteins/IAPs), proliferation (cyclins and growth factors) genes such IκBα, p105, or A20 that generate auto-regulatory feedback loops in the NF-κB response. Other NF-κB target genes are central components of the immune response, e.g., immune receptor subunits or MHC molecules. Inflammatory processes are controlled through NF-κB-dependent transcription of cytokines, chemokines, cell adhesion molecules, factors of the complement cascade, and acute phase proteins This intricate crosstalk is crucial to shaping the diverse biological functions of NF -κB into cell type– and context-specific responses[36].

3.4.2. Forkhead transcription factors of the O class (FOXOs)

One of the most important AKT and ERK targets are the mammalian forkhead transcription factors of the O class (FoxOs) that include FoxO1, FoxO3, FoxO4 and FoxO6. [28–30] FOXO family members have overlapping but different patterns of expression of genes that influence cell proliferation, survival, metabolism and response to stress indicating that they may have redundant as well as distinct functions. These proteins contain multiple levels of regulation including phosphorylation, acetylation/deacetylation, ubiquitination and protein–protein interactions.[28,30] They were first reported in fusion genes in human soft-tissue tumours and leukemias. It was observed that three of the four known FOXO genes were found at chromosomal translocation breakpoints in this type

of tumours. Nowadays it is known that FOXO factors are deregulated in several tumour types including breast cancer, prostate cancer, glioblastoma, rhabdomyosarcoma, melanoma and leukemia. Interestingly, these translocations occur at the identical position, immediately N terminus to the recognition helix (H3) of the FOXO family members and these fusions are no longer controlled by AKT and are constitutively retained in the nucleus.

Another role for FOXO3a inactivation in cellular transformation can also be inferred from the fact that FOXO3a negatively regulates cell survival and cell cycle progression in mammalian cells and that FOXO family members are regulated by the PTEN tumour suppressor. Furthermore, nuclear exclusion of FOXO3a correlates with expression of IKKb or phosphorylated AKT in many primary tumours. It is a good candidate to biomarker, linking poor survival of the patients with breast tumours.

In response to stress stimuli or to nutrient deprivation, FOXO3a has been found to interact with the tumour suppressor p53 *in vitro*. Given that FOXO3a shares similar target genes including *p21, GADD45*, *WIP1* and *PA26* with p53, it suggests that these two proteins may coordinate mechanisms of tumour suppression.[28,30,31] FOXO3a is known as a tumour suppressor and activation of FOXO3a factors by mutation of the phosphorylation sites (thereby restricting the localization of the FOXO proteins to the nucleus) leads to cell-cycle arrest or cell death. Therefore, FOXO3a represent an interesting potential target to develop novel therapeutic approaches for cancer[28]

4. Biomarkers

Biomarkers are tumour related factors that correlate with tumour biological behaviour and patient prognosis. In a very general sense, a biomarker describes any measurable diagnostic indicator that is used to assess the risk or presence of disease. For example, current methods of detection, prognostication, and monitoring of melanoma focus on clinical, morphologic, and histopathologic characteristics of measurable tumour, based on the conventional American Joint Committee on Cancer (AJCC) staging system are Clark and Breslow tumour thickness, presence of ulceration and extent of nodal involvement for primary cutaneous melanoma, and site of metastases for distant metastatic disease.[55] Although this information provides some insight into disease behaviour and outcome, melanoma is still an unpredictable disease. It should be a molecule that can be

measured in blood or other body fluid that are accessible, minimally invasive to the patient and is low cost. Nowadays, the only melanoma biomarker being incorporated in the prognostic classification system established by the AJCC was lactate dehydrogenase (LDH).[56,57]

Given the hypoxic environment of melanoma cells with resultant inability to produce adenosine triphosphate from glucose through oxidative phosphorylation, LDH catalysis the conversion of pyruvate to lactate when oxygen supply is low or absent. It is not a secreted enzyme; thus, an elevated serum level is thought to be secondary to spillage of LDH when melanoma cells outgrow their blood supply. Besides LDH, other circulating tumour markers in melanoma are being tested (melanoma inhibitory activity, lipid bound sialic acid, neuron specific enolase, TA90 immune complex, S-100B protein, 5-S-cysteinyldopa, tyrosinase, cytokines, metalloproteinases).[58]

Modern personalised medicine intends to use individual molecular markers and patterns of markers to subdivide traditional tumour stages into subsets that behave differently from each other, based on current molecular information which indicates that melanoma should be viewed as a heterogeneous group of disorders with molecularly distinct defects in important cellular processes that include cell cycle regulation, cell signalling, cell adhesion, cell differentiation and cell death. Advances in genomics, proteomics, molecular pathology, technologies such as mass spectrometry, protein and DNA arrays, combined with our understanding of the human genome, have generated many candidate biomarkers of potential clinical value. [37]These prognostic and therapeutic markers might help to define molecular targets involved in different pathways, in order to achieve improvements in the clinic.

4.1 TRIB2

TRIB2 is a member of *Tribbles* (Trib) gene Family. The *tribbles* gene was identified first in Drosophila mutational screens for genes that control cell proliferation and migration. The members of this family share a TRIB domain, which is homologous to protein serine-threonine kinases, but lacks the active site lysine lacking a catalytic activity. These proteins function as adaptors in signalling pathways, instead of direct phosphorylation and interact with various transcription factors including ATF4, p65, CtIP.

MAPKK and COP1 are also known to interact with TRIB2. They are also involved in a series of non-neoplastic disorders including metabolic, neurological diseases and cancer.

TRIB2 is a FOXO repressor that contributes to the maintenance of oncogenic properties by cells in melanoma such as growth and survival.[38–40] The TRIB2 domain(s) responsible for oncogenic activity are currently unknown but the integrity of the KD is essential for cell proliferation and survival. Mutations in this domain significantly interferes with these activities. [39] TRIB2 is associated with high expression in melanoma and this high expression will induce the repression of FOXO family members, that are known to play a central role in diverse physiological processes including cellular energy storage, growth, and survival and protect the organism from stress and aging[28]. Recently, TRIB2 was found to be a melanoma biomarker that reflects the stage and progression of melanoma and as well the predicting the clinical outcome following patient treatment [52]

TRIB proteins share four motifs: a pseudo kinase domain/TRIB domain (divergent kinase region with undetermined catalytic activity), a catalytic loop, a COP1 site used to direct key target proteins to the proteosome , and a MEK1 site that binds and modulates MAPKK kinase activity [40] (Fig.1.5)

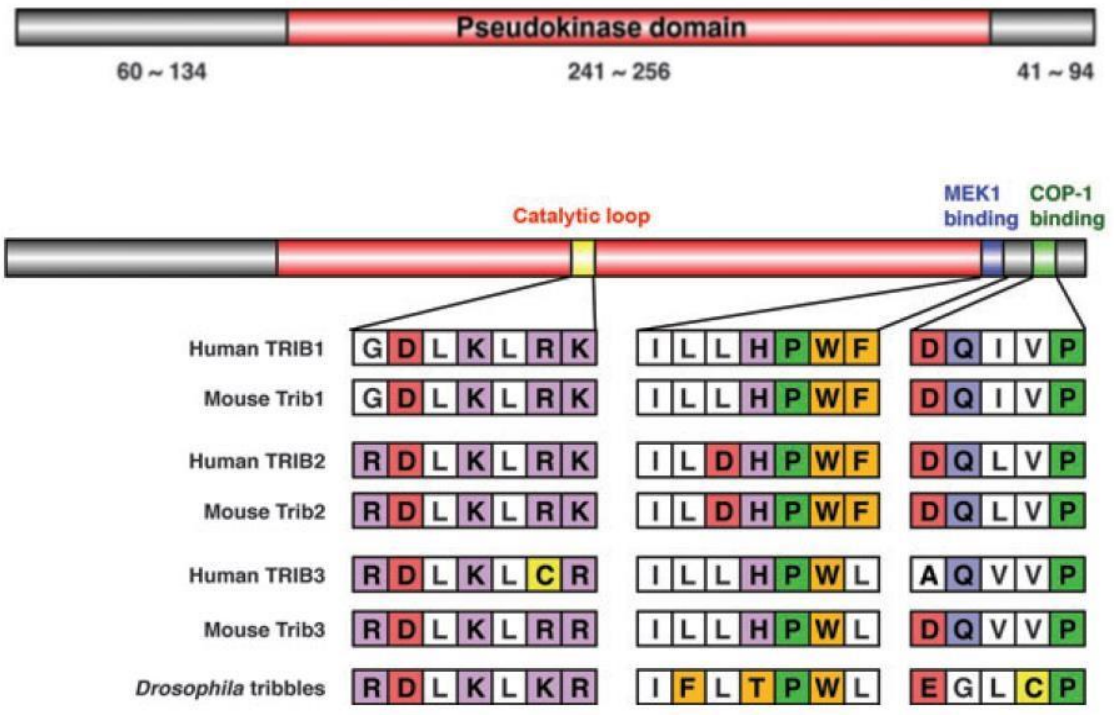

Figure 1.5–Different motifs within the TRIBBLES protein structure.[40]
The tribbles family of genes encodes pseudokinase proteins that share a divergent kinase region (Trib domain), a catalytic loop, a COP1 site used to direct key target proteins to the proteosome for degradation, and (3) a MEK1 site that binds and modulates MAPKK kinase activity. These proteins are highly conserved in evolution as we can see through the similarity between species (Human, Mouse and Drosophila).

The role of TRIB2 in the development of several diseases has been described in the literature: TRIB2 negatively regulates WNT signalling in liver, its over expression induces acute myelogenous leukemia in mice through inactivation of C/EBPα, TRIB2 is over expressed and often amplified in lung cancer and the down regulation of the tumour suppressor gene FOXO3a is mediated by TRIB2 in human melanoma cells[38–40]

5. Melanoma Treatment

Cancer medicine is increasingly moving toward a new era of personalized diagnostics and therapeutics**.** The identification and validation of tumour relevant molecular targets in the different pathways discussed previously, is the first step in the discovery of these therapeutic compounds. Targeted therapeutic agents, includes small molecules or monoclonal antibodies that interact with a molecular target.

From 2011 until now, the U.S. FDA has approved seven novel agents, such as BRAF-inhibitors (vemurafenib 2011, dabrafenib 2013), MEK-inhibitors (trametinib 2013), anti-PD1 antibodies (nivolumab 2014, pembrolizumab 2014), anti-CTLA-4 antibody (ipilimumab 2011), or peginterferon-alfa-2b (2011) In the recent years, the use of traditional chemotherapy for metastatic melanoma has given no evidence for survival benefit. Recent advances have led to the first clinical trials in history where treatment has been associated with a survival benefit[16]. A phase III trial compared vemurafinib, BRAF inhibitor specific to melanomas harbouring the BRAF V600E, to dacarbazine, a traditional chemotherapeutic drug (alkylating agent), in patients with previously untreated melanoma. The vemurafenib arm demonstrated superior overall survival (86% versus 64% at 6 months) and progression-free survival (median 5.3 months versus 1.6 months when compared to dacarbazine alone).[51]

Another two different phase III trials, shows that the median progression-free survival was 5·1 months for dabrafenib and 2·7 months for dacarbazine.[52]

Trametinib (GSK1120212, GlaxoSmithKline Pharmaceuticals) is an orally available, small-molecule, selective inhibitor of MEK1 and MEK2. In 2013, after

treatment cutaneous squamous cell carcinomas were not observed and the median progression-free survival was 5·7 months for these patients.[53]

In table 1.2 is represented the bulk of agents used to target different pathways in melanoma. The majority of these compounds like PI3K/AKT or MEK/RAF inhibitors, are still in experimental development or clinical trials, but they seem to be very promising in melanoma treatment. However, some of this drugs have failed in clinical trials or the tumour has developed mechanisms of resistance by deregulation of other pathways by other oncogenes, suggesting that other points might be more promising to target, and the mechanisms of resistance must be discovered.

5.1 mTOR inhibitors

The mechanistic target of rapamycin (mTOR) is a critical kinase in the regulation of gene translation and has been suggested as a potential target to treat melanoma. So, over the past few years a number of components of the PI3K/mTOR pathway have been the subject of intense drug discovery both in pharmaceutical companies and in academic research. Rapamycin is one of the most efficient inhibitors being tested. It is an allosteric inhibitor of mTOR, and was approved as an immuno-suppressant in 1999. Other drugs such as rapamycin analogues or rapalogs (Temsirolimus- (CCI-779)), competitive inhibitors (PP242) and other mTOR inhibitors like Everolimus (RAD001), are being developed and tested.

Rapamycin and rapalogs have the interesting characteristic that are very specific for mTORC1 complex. Since it is known that PI3K regulates mTOR activity, most of the non-rapalog mTOR inhibitors described to date in the scientific literature were developed to inhibit other enzymes, especially class I PI3Ks. As an example we have BEZ235, used in our experiments and GSK2126458. These two compounds are known to dual inhibit PI3K and mTOR.

A new generation of ATP-competitive inhibitors that directly target the mTOR catalytic site, with potent mTOR inhibition are now being tested in early clinical trials.

5.2 MEK inhibitors

MEK1 and MEK2 are homologous dual specificity kinases that share ERK as their only known catalytic substrate, making MEK an appealing target for cancer drug development. Besides the ATP-binding region that is also present in other proteins, MEK contains a hydrophobic allosteric pocket adjacent to the ATP-binding site that is specific for this kinase allowing the design highly selective inhibitors of MEK. Another important fact is that unaffected PI3K-AKT status may contribute to increased sensitivity to MEK inhibitors in melanomas whose MAPK pathway is activated through oncogenic mutations in BRAF gene.[43] One of the promising MEK inhibitors is AZD6244. *In vitro* studies reported that the majority of cell with mutations in *Raf* gene are sensitive to this drug. In the other way, none of the resistant cell lines possessed a *B-Raf* mutation, suggesting that cell lines containing mutant B-RAF are dependent on MEK activity and therefore sensitive to MEK inhibition. Another study suggests the use of MEK inhibitors in combination with PI3k inhibitors, in the treatment of cancers with coexisting PIK3C and KRAS mutations[44]

Table 1.2- Most common inhibitors being tested (clinical trials) for the treatment of melanoma.

Pathway	Mol. Target	drug	Mode of action	Ref.
PI3K/Akt	c-kit	Imatinib		[39]
PI3K/Akt	PI3K	ZSTK474an	ATP-competitive inhibitor that inhibits all four PI3K isoforms	[40]
PI3K/Akt	PI3K/mToR	BEZ235	Kinase inhibitor(PI3K and mTor)	[40]
PI3K/Akt		GSK2126458	A small-molecule pyridylsulfonamide inhibitor of PI3k	[40]
PI3K/Akt		BYL719	PI3K inhibitor	[40]
PI3K/Akt	PI3K/mToR	CCI-779 (Temsirolimus)	An ester analog of rapamycin. Inhibits mTOR	[41]
PI3K/Akt	PI3K/mToR	RAD001 (Everolimus)	Inhibitior of mTOR	[2]
PI3K/Akt	akt	Isoselenocyanates (ISC-4)	Small molecule that inhibits akt	[42]
MAPK/ERK	Raf-1	Bay439006	Competes with ATP	[43]
MAPK/ERK	^{V600E}b-Raf	Sorafenib (BAY43-9006)	inhibits cell proliferation and vascular development	[44]
MAPK/ERK	^{V600E}b-Raf	PLX4032 (Vemurafenib)	Inhibits directly ^{V600E}b-Raf	[44]
MAPK/ERK		GSK2118436	inhibitor of B-raf	[40]
MAPK/ERK	^{V600E}b-Raf	(dabrafenib)		
MAPK/ERK	Mek	AS703026	small-molecule inhibitor of MEK1 and MEK2	[40]
MAPK/ERK	Mek	AZD6244	ATP-independent inhibitor of MEK1 and MEK2	[40]
MAPK/ERK	Mek	E6201	inhibitor of MEK1 and MEK2	[40]
MAPK/ERK	Mek	GSK1120212	inhibitor of MEK1 and MEK2	[40]
MAPK/ERK	Mek	GDC0973	MEK1 inhibitor	[40]
MAPK/ERK	Mek	MEK162 (binimetinib)	inhibitor of MEK1 and MEK2	[40]
MAPK/ERK	Mek	D0325901	inhibitor of MEK1 and MEK2	[40]

6. Resistance to melanoma treatment

Tumour cells typically adapt to be dependent on their specific driving oncogene and the pathways it regulates; this is termed oncogene/pathway addiction. This process is present in the majority of tumours that develop resistance to inhibitors. As the most mutant melanoma cells are ^{V600E}B-RAF, these tumours are common addicted to ERK1/2 signalling.

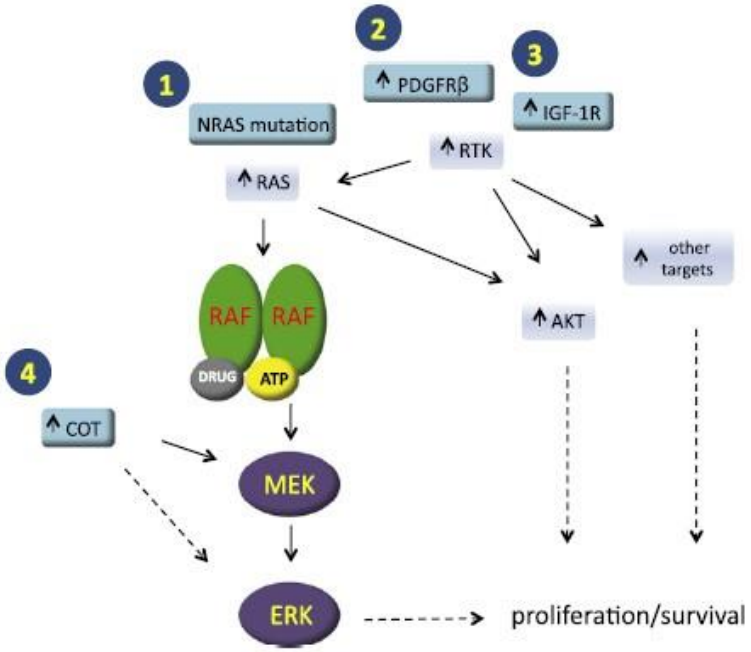

Figure 1.6–Mechanisms of intrinsic resistance, developed by melanomas that carry BRAFV600E mutation due to exposure to B-Raf inhibitors[47]:

1- Activation of molecular pathway by Ras activation or mutation; 2, 3- activation of receptor tyrosine kinases (PDGFRβ and IGF-1R) leads to RAF dimerization and consequent activation of downstream targets. Activation of RTKs also results in activation of other survival pathways, in an MEK-independent way; 4- COT is a kinase that can activate MEK and resist RAF inhibition.

The first approach consisted in creating small molecules to target this pathway (BRAF and MEK inhibitors). Although BRAF and MEK inhibitors have proven clinical benefits in melanoma, most patients develop resistance when submitted to monotherapy. This is an important issue for any targeted therapy and it is crucial to find out the mechanisms of resistance to develop an effective therapeutic strategy for their reversal. In melanoma, response to B.RAF inhibitors are not durable and resistance to

treatment develops in 6-8 months from the initiation of therapy. Resistance is very common, leading to insensitivity of the RAF kinase to the inhibitor.

Two principal mechanisms are suggested: intrinsic and acquired resistance. Some tumours after exposure to AZD6244, show intrinsic resistance resulting in the activation of the PKB pathway. It is achieved through 4 different mechanisms: activation of RAS due to RAS mutations; activation of receptors RTKs (PDGFRβ and IGF-1R) leads to dimerization of RAF compromising the suppression of ERK signalling and promote different/independent survival pathways. As an example, IGF-R Mediates PI3K Signalling in BRAF-Inhibitor Resistant Cells [45]; the last mechanism is the activation of another MEK kinase (COT) (Fig. 1.6). Combination with MEK inhibitors appears to be more effective than single agent treatment.

The acquired resistance is basically composed of two different concepts. The first involves the emergence of mutations in the targeted kinase that abrogate drug binding, such as the "gatekeeper" mutations, it usually makes the kinase constitutive activated. Gatekeeper mutations in the ATP-binding domain often result in increased kinase activity. The second one involves a "kinase switch" in which separate oncoproteins are up-regulated (amplification of a gene, for example) to substitute the driving oncoprotein, re-activating the pathway[43,45]

Although promising findings suggest that simultaneous MEK and IGF-1R/PI3K inhibition leads to cytotoxicity in melanomas resistant to BRAF inhibitors, some tumours create mechanisms of evasion.[22,45–48]This is very surprising, since MEK1/2 mutations are extremely rare in cancer and do not consistently increase MEK1/2 activity. So, a question emerges: if acquired resistance to MEK inhibitors arises, how does it happen?

It is a result of intrinsic and acquired resistance: loss of PTEN tumour suppressor protein, and increased levels of AKT signalling; gene amplification or overexpression of cyclin D1; promoting activation of RAS by silencing NF1; protein kinase D3 is activated leading to PI3K/AKT pathway activation. This way, tumour cell proliferation becomes independent or less dependent on activation of the BRAF-MEK-ERK pathway.[49]
(Fig.1.7)

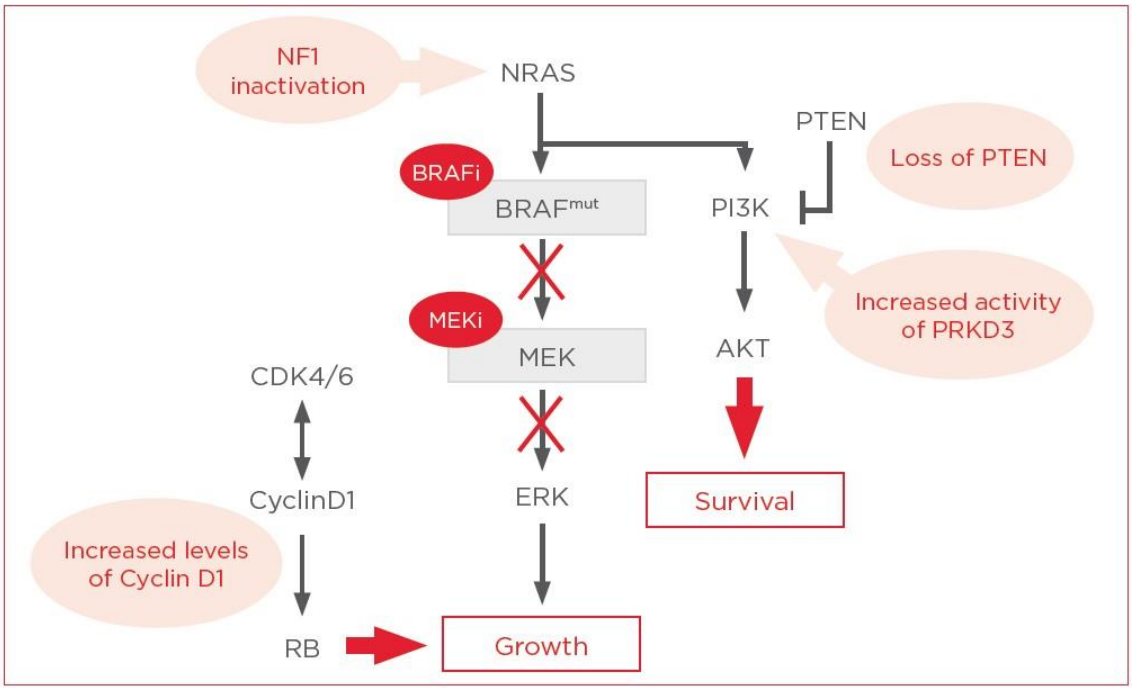

Figure 1.7-Mechanisms of resistance to RAF/MEK inhibitors.[49]
In the presence of BRAF inhibition different pathways are triggered loss of PTEN, a tumour suppressor protein, increases basal levels of AKT that promotes survival. Gene amplification or overexpression of cyclin D1, stimulates the RB pathway. Silencing of the NF1 gene promotes RAS activation and regulates the senescence process and control of cell proliferation. Increased activity of protein kinase D3 (PRKD3) induces activation of the PI3K-AKT signalling;

7. Crosstalking

To restore the intracellular balance, it is considered a more appropriate approach, targeting an entire signalling pathway, as well as the networks in which several proteins from distinct cascades interact which other in a phenomenon called cross talking, rather than a single protein.

In order to understand what mechanisms are behind the resistance in treated tumours and in the parallel activation of different pathways, several studies were performed and reported the existence of cross talking between different proteins, making part of large genetic networks.[11](Fig.1.8)

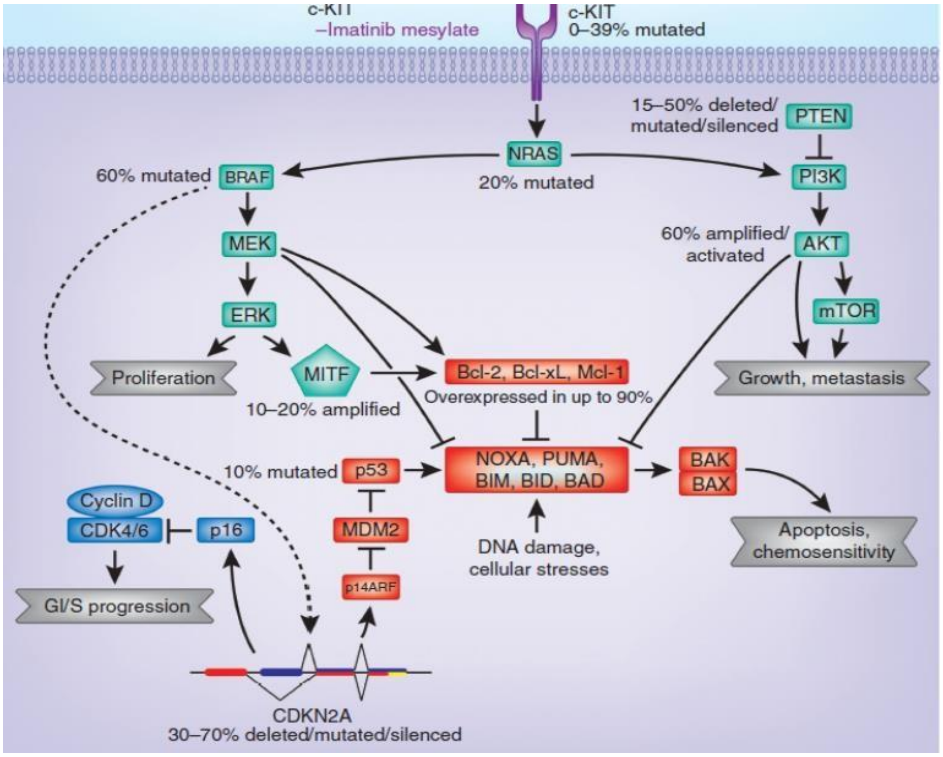

***Figure 1.8-Model that summarizes the different genetic networks that result from the cross talking in melanoma*[13].**
The PI3K/AKT/mTOR and the MAPK pathways are the two most important and mutated genetic networks in melanoma. Cross talking between these two pathways usually occurs.

In most cancers two or more pathways must be activated simultaneously to maintain a positive feedback loop that promotes resistance. For instance, a study revealed that BRAFV600E (V600E mutation results in an amino acid substitution at position 600 in BRAF, from a valine (V) to a glutamic acid (E)), combined with PTEN tumour suppressor gene silencing resulted in development of melanoma with 100% penetrance, with metastases observed in lymph nodes and lungs[41]. Another study reveals that activated ERK increases the level of c-Jun expression by affecting its transcription and stability in human melanoma.[24]. Melanoma cells are also capable of bypassing the negative crosstalk from p38 to ERK that exists in other cancer cells and promote malignancy.[42]

8. Hypothesis

Recent data within our group revealed that TRIB2 is an oncogene and novel biomarker for melanoma. Furthermore, our group has demonstrated that TRIB2 confers resistance to a range of PI3K inhibitors as well as to another anti-cancer therapeutics.

Based on our results regarding TRIB2-resistance to PI3K inhibitors, we hypothesised if TRIB2 conferred resistance to inhibitors that target mTOR, and considering that MEK signals the alternate arm of this network and a putatively identified MEK 1 binding domain within TRIB2 has been identified, we hypothesized, that TRIB2 could confer resistance to MEK inhibitor exposure as well as bind MEK.

Our previous results demonstrate that TRIB2 confers resistance to PI3K, so we also hypothesized if TRIB2 confers resistance to mTOR, another member of PI3K pathway (Fig.1.9).

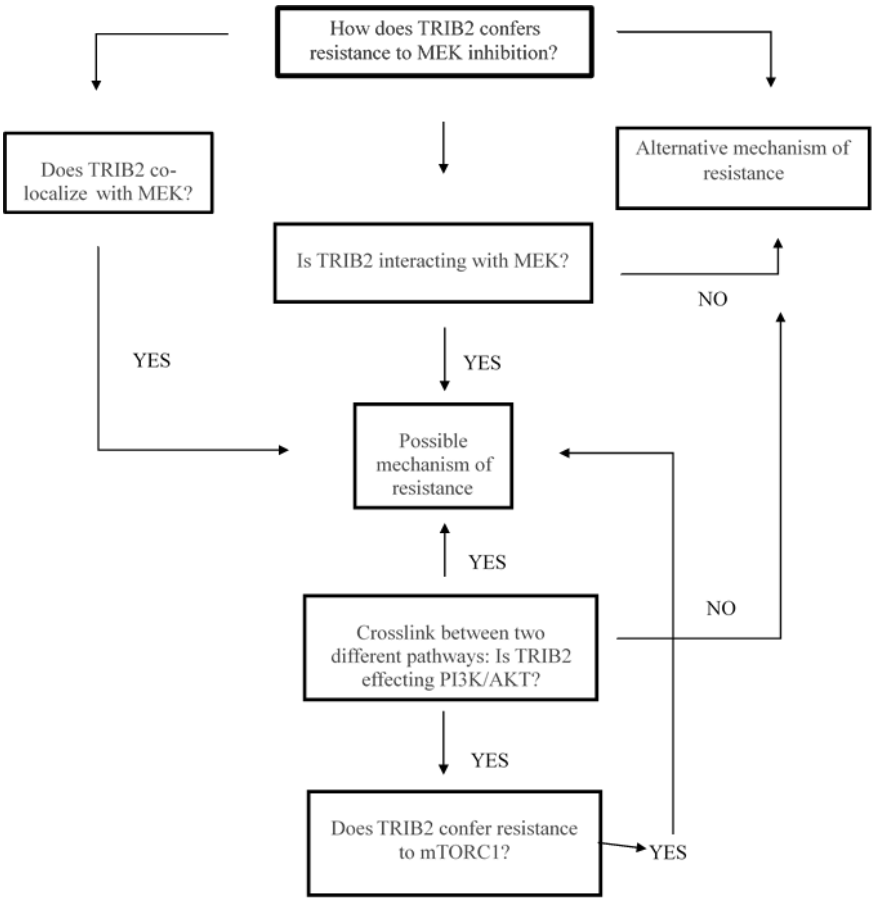

Figure 1.9-Hypothesis model in which TRIB2 confers resistance to mTOR/MEK inhibitors.

CHAPTER 2. *METHODS*

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1. Cell culture and Tissue samples

1.1 Cell Culture

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology, behaviour and biochemistry of cells. In this project we used cell culture to reproduce *in vitro* the effects of several drugs in melanoma cells. The great advantage of this approach is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

The artificial environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O2, CO2), and regulates the physicochemical environment (pH, osmotic pressure, temperature). Most cells are anchorage-dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture)

Isogenic cell lines (Table 2.1) were created in order to express different levels of TRIB2: 293T and U2OS were transfected to overexpress Trib2 because the basal levels of Trib2 in these cells are low. In the other hand, SK-Mel28 and G361 were transfected to knockdown the Trib2 expression (RNAi technique) because these melanoma cell lines have a high expression of TRIB2.

After cell seeding (cell culture hood), the cells grew in DMEM (Sigma) with 10% heat inactivated FCS (Sigma) supplemented with Pen/Strep (Gibco) in T-75 flasks at the humid CO_2 incubator.

Table 2.1-Characteristics of the isogenic cell lines created for experiments.

Cell line	Amount of protein expressed (TRIB2)
293T Empty(GFP) (Human Renal cancer)	low
293T TRIB2 (Human Renal cancer)	high
U2OS Empty(GFP) (Human Osteosarcoma)	low
U2OS TRIB2 (Human Osteosarcoma)	high
G361 scramble(sc.) sh RNA (Human melanoma)	high
G361 TRIB2 sh RNA (Human melanoma)	low
SK-ML28 scramble(sc.) sh RNA (Human melanoma)	high
SK-ML28 TRIB2 sh RNA (Human melanoma)	low

1.2 Transfection

Transfection is a powerful analytical tool, that introduces foreign nucleic acids (DNA or RNA) into cells to produce genetically modified cells. This technique allows to study the genes and gene products in cells (gene function, regulation and proteins). The transfection

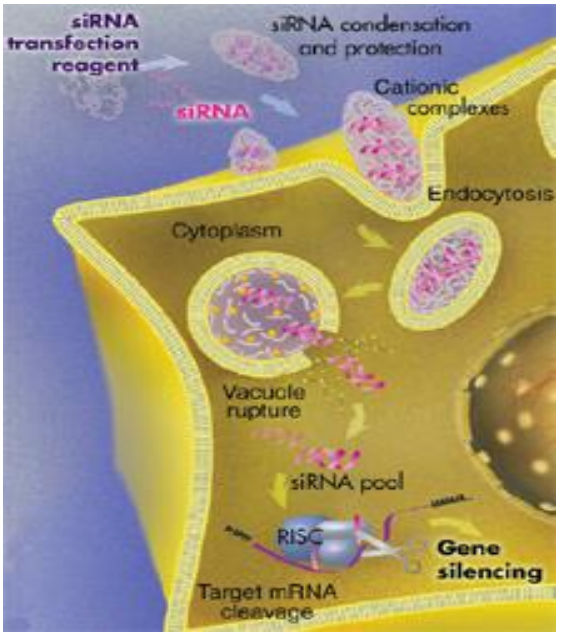

Figure 2.1-Chemical tranfection method.[59]
In this mechanism, DNA/siRNA is wrapped in the transfection reagent and delivered into the cell via endocytosis.

methods are broadly classified into three groups; biological (transduction or virus-mediated), chemical (cationic lipids, cationic polymers, calcium phosphate), and physical (direct injection, electroporation, laser irradiation, magnetic nanoparticles). These methods have advanced to make it possible to deliver nucleic acids to specific subcellular regions of cells by use of a precisely controlled laser-microscope system. However, each method has its own advantages and disadvantages so the optimum method depends on experimental design and objective, and new approaches for transfection methodologies along with improvements to existing protocols have emerged to deliver DNA or RNA into cells, over the last two decades.

One pitfall of numerous cationic lipid carriers that have been developed, is that cationic lipids destabilize the cell membrane, making the process toxic.

In this project we used a new transfection reagent, jetPRIME, that is extremely efficient and contains very low cytotoxicity. It combines the strong interaction with the nucleic acid cargo from lipids, with a natural cell entry pathway (endocytosis) followed by efficient proton sponge-mediated endosome escape (Figure 2.1). As a consequence of tight cargo binding, it can be used equally well for delivery of large plasmids or small siRNA and their mixtures.

The cells were transfected following the JetPrime transfection Protocol (figure 2.2). For optimal DNA transfection conditions, cells were used when 60 to 80% confluent, because few cells grow poorly, they need cell contact. On the other hand, too many cells result in DNA uptake resistance, as well as other essential macromolecules. The number of passages were always low than 20: cells may not respond to the same transfection conditions.

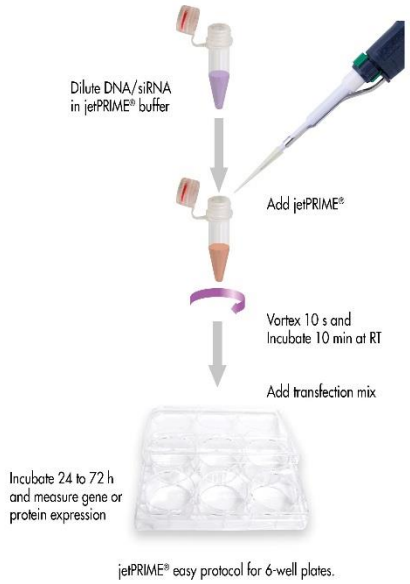

Figure 2.2-DNA transfection protocol.

The first step was to dilute 2 µg of DNA into 200 µl jetPRIME buffer and then vortex. After that, it was added 4 µl of jetPRIME to the mix and it was vortexed for 10s following a short spin. The mix was incubated for 10 minutes at room temperature and then it was added 200 µl of transfection mix (drop wise) per plate of cell culture. Finally, the plates were gently shocked and placed into the incubator for 24 hours.

1.2.1. RNAi technique

RNA interference, most known as RNAi, involving small interfering RNAs (siRNAs) and microRNAs (miRNAs) are noncoding RNAs (20- to 30-nucleotides). Studies started first in plants, flies, worms and RNAi has been discovered as an accidental observation. RNAi pathways confer a qualitative change in the way cellular networks are managed, and their associated proteins control and regulate gene expression in many ways: cell growth, tissue differentiation, heterochromatin formation, and cell proliferation. Another interesting fact, is that miRNAs present in a genome appears to correlate with the complexity of the organism. It is being harnessed by scientists for laboratory research and for the development of new therapies for disease. RNAi dysfunction is linked to cardiovascular disease, neurological disorders, and many types of cancer.[60-62]

In 1998 Andy Fire and Craig Mello investigated the requirements for structure and delivery of the interfering RNA. They showed that injections of double stranded RNA was more effective than single stranded RNA in generating mutant phenotypes. Only a few molecules of double strand RNA are required to affect the cells phenotype.

Apart all implications of these findings in biology and medicine, Andy Fire and Craig Mello discovery leaded to a new paradigm in molecular biology and a technique emerged: RNA interference.[62]

RNA interference (RNAi) has been rapidly adopted as a general method for inhibiting gene expression in most laboratory models and it can be used to induce gene silencing in a diverse range of organisms including fungi, protozoans and metazoan animals.

In figure 2.4, it is possible to see that RNAi does not interact with the DNA of an individual rather it interferes with the translation process of gene expression. The mechanism in which RNA is delivered to the cell is quite complex, dsRNA introduced into a cell is cleaved by the ribonuclease RNase III family member, Dicer, into 21 nucleotide fragments with 5' phosphorylated ends and 2 nucleotides unpaired and unphosphorylated 3' ends. These small interfering RNAs (siRNAs) are subsequently incorporated into a protein.[60]

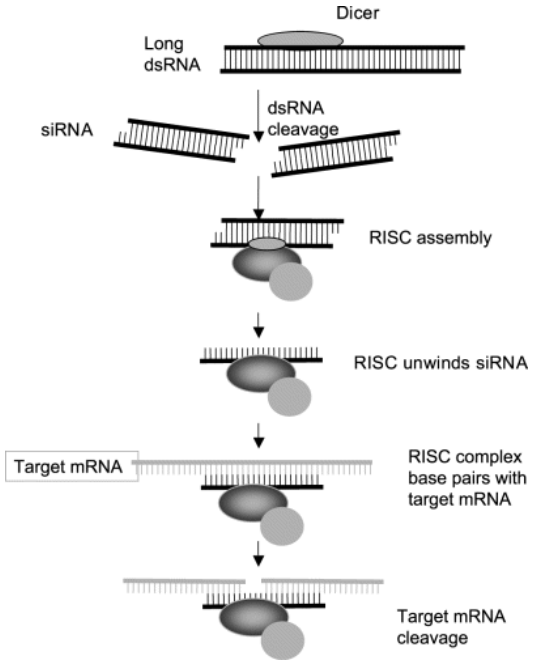

Figure 2.3-RNAi mechanism.[60]
The gene silencing by RNA interference (RNAi) is a post-transcriptional process triggered by the introduction of double-stranded RNA (dsRNA) into the cell.

complex, the RNA-induced silencing complex (RISC), where they guide the cleavage of homologous messenger RNAs (mRNAs), so preventing their expression (figure 2.3).

2. Drug Time Courses

These assays are essential for evaluating compound effects on cells. Traditionally these assays are performed at a specified endpoint after the chosen exposure time. In this study, we monitored compound effects on cell health at various time points during exposure to different drugs (table 2.2) for 12, 24, 48, 72 hours; 1, 6, 12 hours; 48hours or 72 hours depending on the experiment. The cell lines used for drug treatment are represented in table 2.1. After cell culture, cells were observed in the microscope and counted. After the drug treatment, we analysed the protein levels, gene expression or RNA transcripts by some techniques described below

Table 2.2-Chemotherapeutic drugs used in time couses.

Compounds	Target	IC_{50}
TORIN1	mTORC1/2 inhibitor	2 nM/10 nM
BEZ235	dual ATP-competitive PI3K and mTOR inhibitor	4 nM /5 nM /7 nM /75 nM /6 nM
Rapamicyn	mTOR inhibitor	0,1nM
BAY766	inhibitor of MEK 1/2	33nM/762 nM

3. RNA/Protein extraction and FACS (Fluorescence- activated cell sorting) samples

To extract the total protein from each cell line, the cells were trypsinized, resuspended with medium, washed with PBS and collected for falcons. Then, the falcons were spun at 1100 rpm for 5 minutes at 4ºC. After that, we removed supernatant and:

3.1. RNA

We ressuspended with 500 µL of Trizol reagent in eppendorfs and then stored at -80ºC.

3.2. Protein

We ressuspended with 200 µL of lysis buffer (RIPA) in eppendorfs and incubated 30 minutes on ice. After that spun at 1500 rpm for 15 minutes at 5ºC. Then, removed supernadant to new eppendorfs and stored at -80ºC.

To determine the protein concentrations (protein quantification) in each sample we used the Quick Strat™ Bradford Protein Assay (BioRad) and the NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific) following the manufacturers guidelines.

3.2.1. Protein Quantification/Bradford Assay

In our project, some experiments often required a sensitive method for protein quantification. The Bradford Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein: It involves the addition of an acidic dye Coomassie ® Brilliant Blue that binds to the proteins present in the solution. The absorbance ranges from a maximum of 465 nm to 595 nm. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially

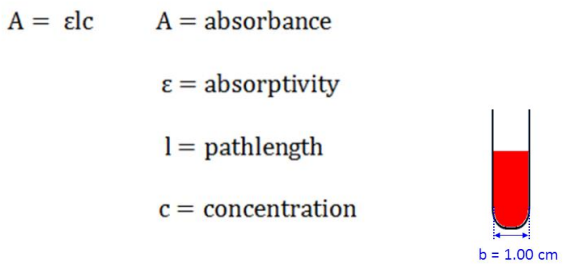

Figure 2.4- Lambert-Beer´s Law. *The Beer´s law is written in the format: y=mx+b, so it is possible to construct a graphic in which concentration is the independent variable and absorvance is the dependent variable***.** The parts of the equation are: A=absorbance, Ɛ=molar absorptivity (constant for a given solute at a given wavelength, C=molar concentration (mol/L))

arginine. Beer's law may be applied for accurate quantitation of protein by selecting an appropriate [63]ratio of dye volume to sample concentration (figure 2.4). Then, it is possible to create a calibration graph, and use it to measure the concentration of an unknown solution (protein concentration).

It was Determined the best fit of the data to a straight line in the form of the equation "y = mx + b" where y = absorbance at 595 nm and x = protein concentration, we used this equation to calculate the concentration of the protein sample based on the measured absorbance. A dilution of the protein sample may be required for the resulting absorbance to fall within the linear range of the assay. Every time the absorbance of the test sample was outside of the absorbance range for the standards, then the assay was repeated with a more appropriate dilution. Samples incubate at room temperature for 10-30 minutes. Then, 99 µl (blank microtube 100 µl) of Bradford reagent was added to each microtube, and let stand 5 minutes at room temperature. We displayed 10 µl of dH2O drops in a parafilm plate and diluted 1 µl of each sample. 1 µl of this dilution was added to the microtubes with Bradford reagent. The last step of this procedure was to measure the absorbance of each sample at 595 nm using a NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific). The data was analysed with Microsoft Office Exccell 2010 ™

3.3. FACS (Fluorescence-activated cell sorting)

FACS is a method used to detect cells that have lost some of their DNA in late stage of apoptosis process following endonucleases activity. Endonucleases degrade DNA in small fragments of about 180 bp, which accumulate in the cell. After ethanol fixation and wash with a phosphatecitrate buffer, these DNA oligimeres leak out of the cell decreasing the DNA content. Using the nucleic acid stain propidium iodide (PI), the number of hypodiploid cells undergoing this process can be counted in subG1 region of PI histogram.

To fix cells, we ressuspended with 500 µL of 70% ethanol in eppendorfs and stored at 4ºC on the fridge at least 24 hours.

Samples were run on FACS after propidium iodide (2.5 mg mL-1) was added to the fixed cells. 10,000 gated, total events were scored per study from triplicate studies. Data was analysed using FACS-express 3 (De Novo software)

4. Western Blotting

Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979. It is now a routine technique for protein analysis. This is an analytical technique used to identify specific proteins using an electrophoresis gel to separate proteins by their density.

The first step in a western blot is to prepare the protein sample by mixing it with a blue solution to stain proteins (loading buffer) that contains a detergent called sodium dodecyl sulfate (SDS), which makes the proteins unfold into linear chains and coats then with a negative charge. Another important component of loading buffer is β-mercaptoethanol that denature proteins. Next, the protein molecules are separated according to their sizes using a method called gel electrophoresis (figure2.5).

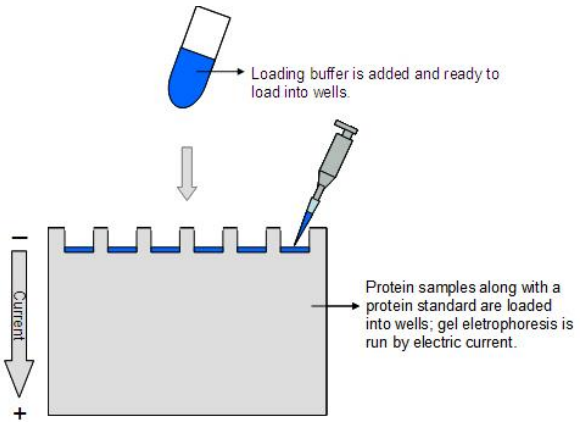

Figure 2.5-Western blot electrophoresis. *After cell lysis, loading buffer is added to supernatant, heated at 95ºC and loaded into the gel wells. Proteins present in solution are separated according to their weight, migrate to the positive pole after running in a two phase agararose gel (stacking gel + resolving gel).*

Following separation, the proteins are transferred from the gel onto a blotting membrane. There are several techniques to transfer the proteins to the membranes (nitrocellulose or PVDF). In this project we used the semi-dry transfer system Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) (figure 2.6).

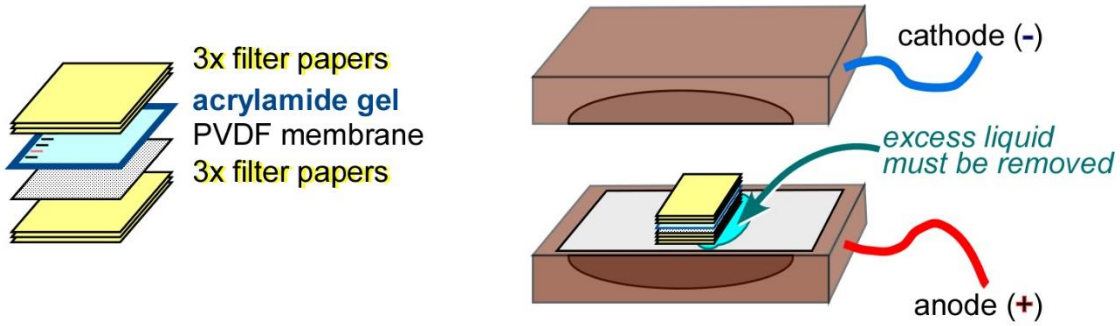

Figure 2.6-Western blot semi-dry transfer apparatus.

Once the transfer is complete, the membrane carries all of the protein bands originally on the gel. Next, the membrane goes through a treatment called blocking, which prevents any nonspecific reactions from occurring. The most common block reagents are BSA and milk that contain caseins (non-immunoresponsive proteins) (figure 2.7).

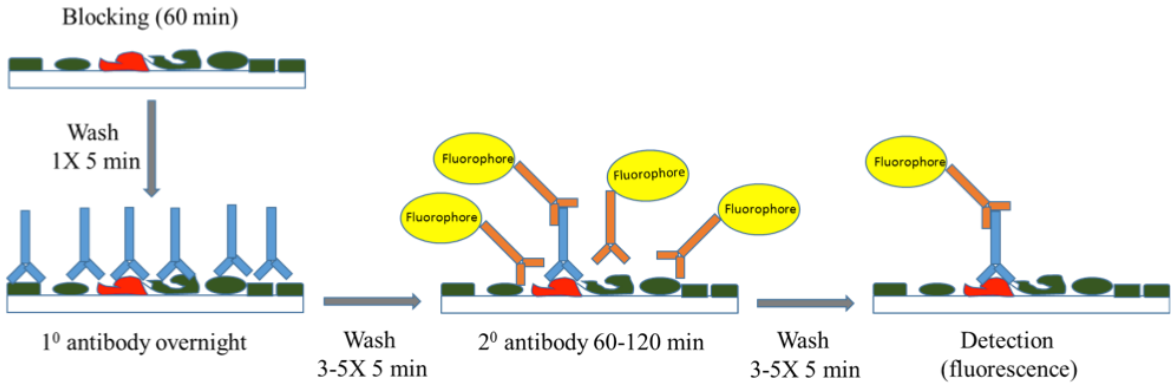

Figure 2.7-Western Blot Blocking and incubation.

After incubation in block solution (milk or BSA), the membrane is incubated with the 1º antibody and 2º antibody, respectively

The membrane is incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. Following incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but this time with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to a reporter enzyme that produces color or light, which

allows it to be easily detected and imaged. For detection we used eenhanced chemiluminescence (ECL), a method which provides highly precise detection of proteins from Western blots. It consists of a chemiluminescence reaction: horseradish peroxidase catalyzes the oxidation of luminol into a reagent which emits light when it decays. Since the oxidation of luminol is catalyzed by horseradish peroxidase (HRP), and it is complexed with the protein of interest on the membrane, the amount and location of light that HRP catalyzes the emission of, is directly correlated with the location and amount of protein on the membrane. These steps permit a specific protein to be detected from among a mixture of proteins.[64]

After protein quantification, our extracted proteins samples were diluted in to 2x lammeli loading buffer and heat at 95ºC for 5 minutes in a thermal block. Then samples were loaded into our 5% SDS-PAGE gels, prepared accordingly with table 2.3. In this gel, proteins are concentrated in gel wells and ready to migrate into the second gel. In second gel, 10% SDS-PAGE (table 2.4) proteins coated with negative charges will migrate to the positive pole After that, separated proteins from the gel were transferred to

Table 2.3-Solutions for Stacking gels for tris-glycimide SDS-polycrimide Gel electrophoresis.

5% gel	**Volume=4ml**
H2O	2,7ml
1,5M Tris (ph=6,8)	0,5ml
10% SDS	0,04ml
10% ammonium sulfate (APS)	0,04ml
30% acrylamide	0,67ml
TEMED	0,004ml

nitrocellulose membranes (Amersham) previously wet with transfer buffer. In this process a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) was used for 60 minutes. Then, the membrane was blocked in 5% milk (in wash solution that contains PBS

Table 2.4-Solutions for resolving gels for Tris-glycimide SDS- polycrimide SDS-polycrimide gel electrophoresis.

10% gel	Volume=10ml
H2O	4,0ml
1,5M Tris (ph=8,8)	2,5ml
10% SDS	0,1ml
10% ammonium sulfate (APS)	0,1ml
30% acrylamide	3,3ml
TEMED	0,004ml

and Tween20) for 1 hour (preventing non-specific antibody binding). After blocking, membranes were immunoblotted with primary antibodies (dilution 1:1000) overnight at 4ºC in a platform shaker. The primary antibodies are specified in Table 2.5.

Table 2.5-Primary antibodies

Primary antibodies	Information
Total MEK	Sc-436; Rabbit; Santa Cruz Biotechnology
Total-ERK	Sc-94; Rabbit; Santa Cruz Biotechnology
P-ERK	Sc-7383; Mouse; Santa Cruz Biotechnology
TRIB2	Sc-292013; Rabbit; Santa Cruz Biotechnology
TRIB2	Homemade
Actin	I-19; sc-1616; Goat; Santa Cruz Biotechnology
Total AKT	C-20; sc-1618; Goat; Santa Cruz Biotechnology
p-AKT	SSer 473; sc-7985; Rabbit; Santa Cruz Biotechnology
Total FOXO (FKHRL1)	N-16; sc-9813; Goat; Santa Cruz Biotechnology
FOXO (p-FKHRL1)	p-Ser253; sc-101683; Rabbit; Santa Cruz Biotechnology
Fas-L	C-178; sc-6237; Rabbit; Santa Cruz Biotechnology
BIM	H-191; sc-11425; Rabbit; Santa Cruz Biotechnology

Table 2.6-Secondary antibodies

Secondary Antibodies	Information
Anti-rabbit	IgG-HRP; sc-2004; Goat; Santa Cruz Biotechnology
Anti-mouse	IgG-HRP; sc-2020; Donkey; Santa Cruz Biotechnology
Anti-goat	IgG-HRP; sc-2314; Donkey; Santa Cruz Biotechnology

After incubation, we washed the membranes 3 times, 10 minutes each one with wash solution (Tris-buffered saline with 0,1% Tween 20 buffer (TBST). Then, we prepared the secondary antibodies (Table 2.6) with block solution (1:1000) and let the membrane incubate 1hour in a platform shaker. We made 3 washes again with wash solution, 10 minutes each one. Finally, we prepared ECL solution (solution I- Luminol Reagent + solution II- Oxidizing Reagent) and we put the membrane in the ECL solution over 5 minutes and watched the images using a Molecular Imager® ChemiDoc™ XRS System (BioRad).

5. Co-Imunnoprecipitation (Co-IP)

Co-Imunnoprecipitation is a technique to identify protein-protein interactions by using protein-specific antibodies to capture proteins that are bound to a specific target protein. The cells were washed with medium and added trypsin to the plates with cultivated cells so the cells remove from the bottom of plates. The solution was collected to new eppendorfs and centrifuged, and the supernatant was collected to new tubes. The protein A/G agarose beads (Sigma) were washed 2 times with PBS and a 50% protein A/G working solution in PBS was made. Each indicated antibody was added to the beads for 1 hour, slightly agitated. After 1 hour the beads were washed (x2) with PBS. 500 µg of total protein lysate was added to each set of beads and incubated overnight at 4ºC. Samples were centrifuged (max speed), the pellet was kept, and washed with pre- chilled PBS (x3). SDS loading buffer was added to beads and the samples heated to

95ºC for 5 minutes. Samples were extracted and run on an appropriate percentage SDS-gel.

6. Chromatin Imunnoprecipitation (ChIP)

Chromatin Immunoprecipitation is a technique used to study the interaction between proteins and DNA in the cell: it is possible to see how transcription factors regulate gene transcription.

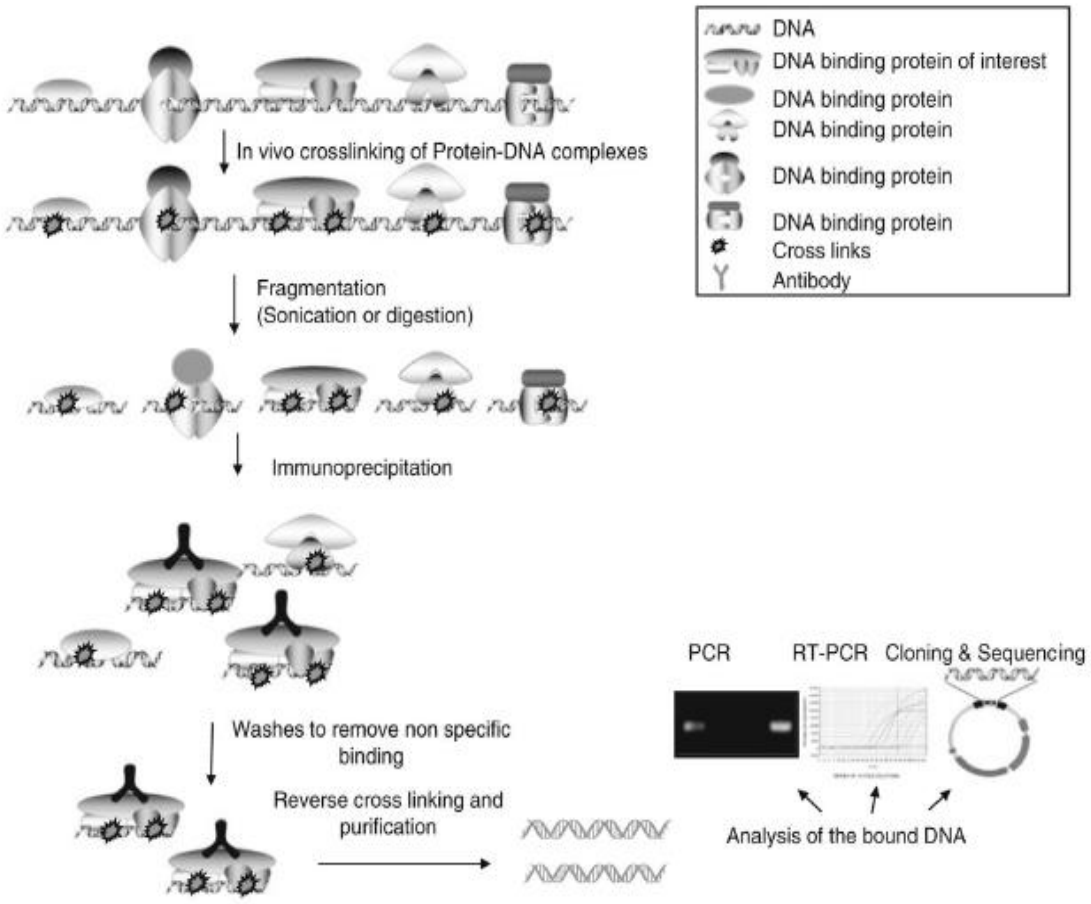

Figure 2.8-Chromatin. Immunoprecipitation Workflow

The plates were washed with medium, and a 1% formaldehyde/PBS solution was added to cross-link proteins to DNA. The solution was removed, and the plates were washed with ice cold PBS (x3). Cells were scraped from the plates with 1M Tris-HCl with 10mM DTT, and transferred to Eppendorf tubes. After centrifugation, the

pellets were washed with Buffer I (10 mM Hepes, pH 6.5; 0.25% Triton X-100; 10 mM EDTA, pH 8.0; 0.5 mM EGTA, pH 8.0) and buffer II (10 mM Hepes, pH 6.5; 200 mM NaCl; 10 mM EDTA, pH 8.0; 0.5 mM EGTA, pH 8.0). After centrifugation, the pellet was resuspended in lysis buffer (made fresh with protease inhibitors (PI) 50 mM Tris, pH 8.1, 1% SDS; 10 mM EDTA, pH 8.0; proteaseinhibitors(1X)). The cells were sonicated (6 times for 10 seconds each sample) on ice, to shear DNA to an average fragment size of 200-500 base pairs. After centrifugation (at max speed for 15 minutes), the supernatant was transferred into a new eppendorf. 300 µl of Buffer D20 mM Tris, pH 8.11%Triton X-100, 2 mMEDTA, pH 8.0, 150 mM NaCl, protease inhibitors (1X). 100 µl input samples were removed at this stage. The input samples were heated overnight at 65°C. To the remaining sample (after the inputs were removed), we added sheared salmon sperm DNA, the antibody of interest and protein G-fast flow beads (Sigma) and Buffer D. After incubation overnight at 4ºC, the beads were pulled down and washed with TSE I (20 mM Tris, pH 8.1, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, pH 8.0, 0.1%SDS), TSE II 20 (mM Tris, pH 8.1 500 mM NaCl 1% Triton X-100 2 mM EDTA, pH 8.0, 0.1%SDS) and TSE III10 mM Tris, pH 8.1, 0.25M LiCl, 1% NP-40, 1% Deoxycholate, 1 mM EDTA, pH 8.0) . Afterwards the beads were washed with ice cold TE. The DNA was extracted with three washes with a solution of NaCHO3 and SDS. Once extracted the samples were transferred to a fresh Eppendorf and heated overnight at 65ºC. Our input and the ChIP samples were loaded into Sigma-PCR clean-up columns and after washing our immunoprecipitated DNA eluted with 30 µl dH2O. Samples were stored at -20ºC.

7. cDNA synthesis

The central dogma of biology states that biological information is transmitted from DNA to RNA to protein. Yet in some situations biological information can be converted from RNA to DNA. Some viruses that have RNA genome are able to convert their RNA genome into DNA thru a polymerase enzyme (reverse transcriptase). This discovery enabled the opportunity of converting mRNA into complementary DNA (or cDNA). Using this enzyme together with a few other components (dNTPs and primers), we are able to reproduce this mechanism in biological research.

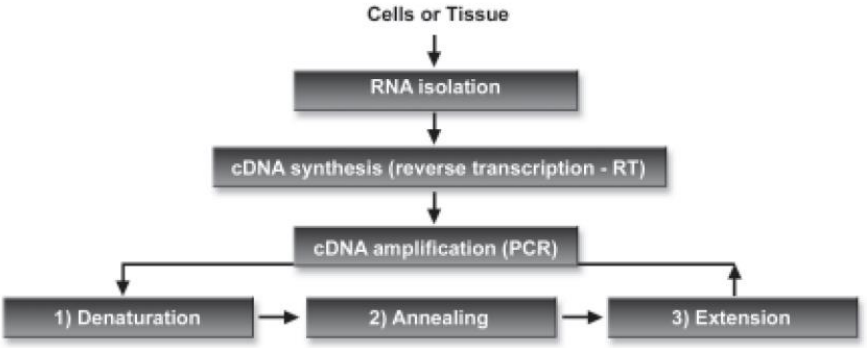

Figure 2.9- cDNA synthesis Workflow.[65]

For the cDNA synthesis, NZY First-Strand cDNA Synthesis Kit (nzytech) was used. Each sample was prepared in a microcentrifuge tube with the amounts of reaction components present in Table 2.7

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Table 2.7–Reagents necessary to cDNA synthesis.

NZY Master Mix preparation	
NZYRT 2x Master Mix	10µl
NZYRT Enzyme Mix	2µl
RNA samples	5µl
DEPC-treated H_2O	3µl

The solutions were then gently mixed and inserted in C1000 Thermal Cycler (BioRad) with the following steps: incubation at 25ºC for 10mins; Incubation at 50ºC for 30mins; Reaction inactivation by 85ºC heating for 5mins. The samples were then put on ice and 1µl NZY RNAse H (E.coli) was added. A last incubation at 37ºC was done for 20mins. The cDNA product was afterwards used in PCR and further stored at -20ºC until required.

8. Real Time Polymerase Chain Reaction (qRT-PCR)

Polymerase Chain Reaction (PCR) is a DNA amplification technique and considerate to be a cornerstone in modern molecular biology. Since DNA is commonly difficult to analyse in cellular concentrations, this technique allows us to acquire DNA amounts high enough to be detectable in signal quantification. The technique is centred in a specific DNA reproduction thru a polymerase enzyme that is controlled by temperature changes: the method begins with the temperature being raised to 95ºC so the double stranded DNA detaches into single strands (denaturation); the temperature is then lowered to 60ºC, allowing primers to bind to the selective DNA sites (annealing); since the polymerase enzyme has now sites to bind to, temperature is raised to the polymerases optimal working temperature (around 72ºC) and replication takes place (extension). These steps are repeated about 40 times (cycles) and in each cycle the amount of DNA is duplicated.

RT-PCR is founded on the same principle, yet instead of analysing the DNA amplification in an agarose gel, the process is monitored by fluorescence the growing in a real-time PCR machine.

Table 2.8-RT-PCR reagents

Reagent	Volume (µl)
2xluminolCt SYBR Green qPCR Ready Mix	10
25µM Forward Primer	1
25µM Reverse Primer	1
DNA	2
1x internal reference dye	1
dH2O	5
Final Volume	20

The procedure was based on RT-PCR protocol (sigma): after the reagents and samples were added to a plate well according to Table 2.8, they were put in the RT-PCR machine (CFX96TM Real Time System – BioRad).

8.1 DNA electrophoresis

DNA gel electrophoresis has the same background as the Westernblot gel electrophoresis, yet for DNA instead of proteins: In this method, negatively charged molecules (as DNA and proteins) are able to migrate thru a pored gel from the negative pole (cathode) to the positive pole (anode) of the electrophorator. Since the small weight molecules migrate faster than the larger ones there will be a sized separation of the all fragments. The results can then be visualized using UV light that illuminates a previously added fluorescent dye (for instance SYBR Green or ethidium bromide).

In this procedure a 1% agarose gel with two comb lines was used. After assembling the support, the gel was prepared: 2gr of agarose were added to 200ml 1x TAE and mixed. The mixture was melted in the microwave during 30 seconds pulses on maximum power and further mixed until all agarose was dissolved. 1µl of Green safe (nzyTech) was added to the agarose buffer before shedding it into the support. Afterwards, the combs were placed in their rightful position. After the gels polymerization, it was placed in the gel box and filled with 1x TAE. For each sample, 1 µl DNA loading buffer was placed on *Parafilm* for further dilution with 10µl of the sample solution. 10 µl of this dilution were then placed in the wells, together with one well of loading marker. The gel was run at 100v during 30mins and the results were observed using Molecular Imager ChemiDoc^{TM} XRS System (BioRad)

9. Dual staining Microscopy

Cell staining is a technique that can be used to better visualize cells and cell components under a microscope. By using different stains, one can preferentially stain certain cell components, such as a nucleus or a cell wall, or the entire cell. Most stains can be used on fixed, or non-living cells, while only some can be used on living cells; some stains can be used on either living or non-living cells.

Cell staining techniques and preparation depend on the type of stain and analysis used. One or more of the following procedures may be required to prepare a sample: Permeabilization- treatment of cells, generally with a mild surfactant, which dissolves cell membranes in order to allow larger dye molecules to enter inside the cell. Fixation - serves to "fix" or preserve cell or tissue morphology through the preparation process.

This process may involve several steps, but most fixation procedures involve adding a chemical fixative that creates chemical bonds between proteins to increase their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid. Mounting - involves attaching samples to a glass microscope slide for observation and analysis. Cells may either be grown directly to the slide or loose cells can be applied to a slide using a sterile technique. Thin sections (slices) of material such as tissue may also be applied to a microscope slide for observation. Staining application of stain to a sample to color cells, tissues, components, or metabolic processes. This process may involve immersing the sample (before or after fixation or mounting) in a dye solution and then rinsing and observing the sample under a microscope. Some dyes require the use of a mordant, which is a chemical compound that reacts with the stain to form an insoluble, colored precipitate. The stainning will remain on/in the sample when excess dye solution is washed away.

Slides containing each sample, disposed face up, were observed in the Microscope to check cell confluence. If they are all ok, we can now proceed with the dual staining protocol. Each sample was put in a 6 well-plate (35mm), one lamella for each well with PBS solution. They were washed with 1x PBS and block solution was prepared (1:30). Drop equal amounts (+/- 200ul) of blocking solution in the glass with the parafilm paper for each lamella. Slides were incubated 37ºC, 30mins. Lamellas were removed from the Humified Chamber and turned face up in the six plate wells and washed with PBS (1x) three times. The remaining block solution were removed from the glass with parafilm. Primary antibody was added in a 1:500 concentration (in PBS) and left in a humidified chamber for 1h at 37ºC. The samples were washed three times with PBS and the secondary antibody was added for 30mins (also at 37ºC in a humidified chamber) and with a 1:1000 concentration. Again the samples were washed three times with PBS. The slides were prepared according to Invitrogen Clean Mount™ Mounting solution protocol. To avoid bubbles, bubbles were removed from the tip of pipette, the surface of the non-sample side of the coverslip was cleaned prior to imaging, in order to remove fingerprints, residual salts, and other contaminants that can diminish image quality. A small amount of mounting medium was applied to the surface of the slide. The coverslip containing the sample was removed from the buffer, blotted the excess of buffer and slowly we tip the coverslip onto the mounting medium. The slides were then incubated at 60ºC for 30 minutes.

CHAPTER 3. *RESULTS*

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1. TRIB2 confers resistance to mTOR1 complex inhibition

Previous data from our Laboratory shows that TRIB2 confers resistance to BEZ235, a potent PI3K/mTOR inhibitor. Based on this data, we wanted to address if TRIB2 could confer resistance to specific targeted therapeutics within this large signalling cascade. We hypothesised that TRIB2 is also conferring resistance to inhibition of the mTORC complex, another component of the PI3K/AKT pathway.

To investigate how TRIB2 expression confers resistance to mTOR1 inhibition, we used isogenic cell lines with matched TRIB2 protein expression (high/low levels). Isogenic lines were treated with rapamycin a potent inhibitor of mTORC1 in a drug time course.

We noticed that 72 hours post treatment, some significant changes occur, based on the concentration of TRIB2 within the cells. In order to be seen in the SubG1 area, in FACS output, a cell must have lost enough DNA to appear there. This means that, if cells enter apoptosis from the S or G2/M phase of the cell cycle or if there is an

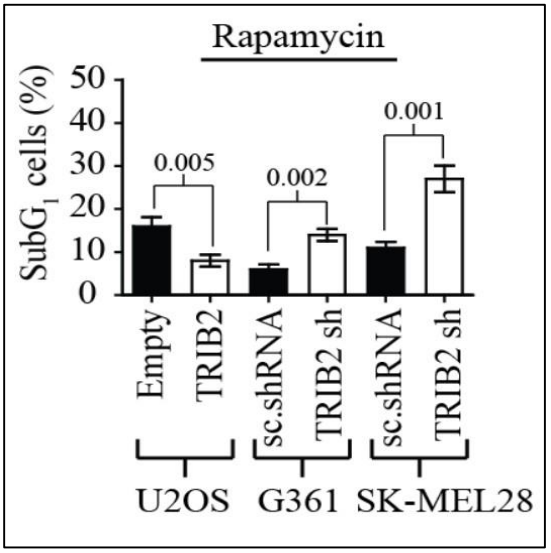

Figure 3.1- TRIB2 status confers resistance to rapamycin.
Different isogenic cell lines U2OS, G361 and SK-MeL28 were treated with 1 nM rapamycin and 72 hours post exposure. SubG1 population was determined by FACS analysis (PI staining). N=6, 50,000 total events counted per samples. Stats are two-tailed ANOVA and P values are shown above each analysis bracket. U2OS-Empty (black bar), G361-TRIB2shRNA and SK-MEL28-TRIB2shRNA (white bars) represent the cells that contain low levels of TRIB2. U2OS-TRIB2 (white bar), G361-sc.shRNA and SK-MEL28-sc.shRNA (black bars) represent the cells that contain high levels of TRIB2.

aneuploid population undergoing apoptosis, they may not appear in the SubG1 peak. The remaining cells that have lost DNA for any other reason, will appear in the SubG1 region. It is possible to see that the U2OS-TRIB2 (high expression of TRIB2) cell line has a significantly lower percentage of Sub-G1 cells compared with U2OS-Empty cells (low expression of TRIB2) (Fig. 3.2). G361-*TRIB2*shRNA and SK-MEL28-*TRIB2*shRNA (that have low levels of TRIB2) show the opposite pattern. This data suggests that the presence of TRIB2 negatively correlates with the number of SubG1 cells (dead cells), and subsequently the cells resist treatment. This means that, when TRIB2 is present, we have more viable cells, while rapamycin treatment is performed.

2. TRIB2 does not confer resistance to mTORC2 complex inhibition.

To test the hypothesis that TRIB2 is conferring resistance to mTORC2 inhibition, we submitted isogenic cell lines to a time course up to 72 hours with TORIN1 and performed FACS analysis as described in methods section. Surprisingly, we noted that the treatment profiles were completely different compared to rapamycin treatment (Fig. 3.2). We expected that TRIB2 could confer resistance to mTORC2 inhibition, but this is not the case: the over expression of TRIB2 produces no effect on cells when exposed to TORIN1. The percentage of Sub-G_1 cells for cell lines that overexpress TRIB2 (U2OS TRIB2; 293T TRIB2 and SK-MeL28 scramble) is higher when compared with cells with endogenous levels or shRNA knocked down TRIB2 , (U2OS Empty, 293T Empty and SK-MeL28 shRNA). This data suggests that TRIB2 does not confer resistance to mTORC2 complex inhibition.

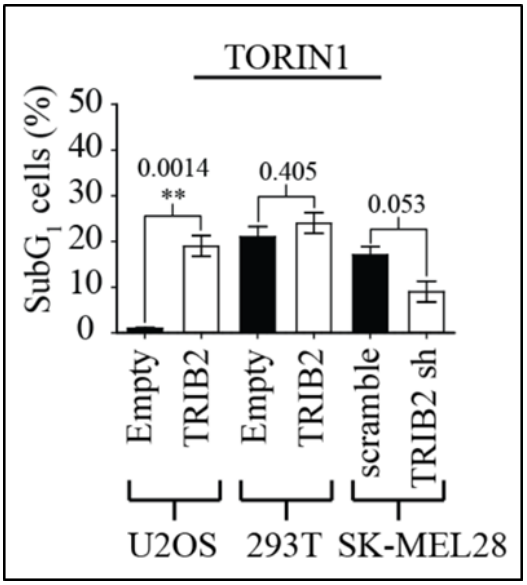

Figure 3.2- TRIB2 do not promote resistance when cells are treated with mTORC2 inhibitors.
Isogenic cell lines treated with TORIN1 (100 nM) at 72h post treatment, the percentage of SubG1 cells were analysed by FACS (PI staining). 50,000 total events counted per samples. Stats are two -tailed ANOVA and P values are shown above each analysis bracket. U2OS-Empty (black bar), G361-TRIB2shRNA and SK-MEL28-TRIB2shRNA (white bars) represent the cells that contain low levels of TRIB2. U2OS-TRIB2 (white bars), G361-sc shRNA and SK-MEL28-sc.shRNA (black bars) represent the cells that contain high levels of TRIB2

To validate these results, the following step was to evaluate if the inhibitors that we use were functional, as well as if the cell lines with TRIB2 shRNA knock down was effective (and that TRIB2 was indeed knocked down). We performed the protein expression analysis using SDS-PAGE immunoblotting, according with the protocol described in methods section. It is possible to see that TRIB2 is only expressed in the scramble sequence cell lines (Fig. 3.3). In contrast, the two different TRIB2 shRNA sequences were highly effective at knocking down TRIB2 (in two cell lines with high endogenous TRIB2 protein expression). We can conclude that the transfection and the respective Knock down of TRIB2 were effective.

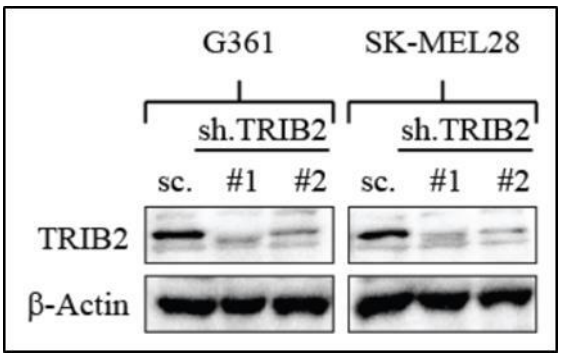

Figure 3.3- The TRIB2 knock down is efficient.
Western blot analysis of TRIB2 Knock Down efficiency. The first lane of each panel represents the scramble transfection of shRNA. Lanes 2 and 3 represent the constructs #1, #2 respectively, that result from the shRNA TRIB2 transfection. 50 µg of total protein lysate was loaded per lane and samples were probed for total TRIB2 and β-actin expression (control).

Having confirmed that our cell lines had matched TRIB2 protein expression levels, it was also necessary to evaluate if the PI3K, mTOR1, mTOR1/2 inhibitors were working properly.

3. Confirmation of PI3K, mTOR1 and mTOR1/2 inhibitor efficiency

To examining the efficiency of the principal targets (PI3K; mTOR1 and mTOR1/2) inhibition, we treated cells with BEZ235 (PI3K inhibitor) and rapamycin (mTOR1/2 inhibitor) and confirmed protein expression for each cell line by Western Blot analysis. We probed for TRIB2, Total-AKT, pSer473-AKT and pThr308-AKT. It was expected that TRIB2 was over expressed in transfected cells (U2OS-TRIB2, treated and non-treated). For U2OS-GFPA TRIB2 should be less concentrated because the endogenous levels of TRIB2 in this type of cells is low. When we use a PI3K inhibitor (BEZ235), the results for TRIB2 confirm the expectations: low levels (endogenous) for U2OS GFP non-treated cells and high expression of TRIB2 is present in the remaining two cell lines, that were transfected to over express TRIB2 (U2OS TRIB2 non-treated and U2OS TRIB2 BEZ235). We also observed that when U2OS-GFP cells are treated with BEZ235, they are stimulated to express TRIB2.

We had an analogous thought when this matched cell line was treated with rapamycin: TRIB2 should be expressed in transfected cells (U2OS-TRIB2, treated and non-treated), and should be less present in U2OS GFP cells (treated and non-treated).

The data shows that TRIB2 is overexpressed in U2OS TRIB2 (treated and non-treated) and is low in U2OS GFP cells (treated and non-treated). To notice that rapamycin produces no effect in the expression of U2OS-GFP treated cells (Fig. 3.5).

AKT is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation. It contains two regulatory phosphorylation sites: Threonine 308 in the activation loop within the kinase domain and Serine 473 in the C-terminal regulatory domain, and the activation of AKT requires the phosphorylation of these two residues. It was expected that at least one of these two residues was activated when cells were treated with BEZ235 or rapamycin.

When we probed for total-AKT (activated and non-activated proteins) the results for protein expression are very similar for all lanes. When we did a more precise analysis to find out which residue of AKT is being phosphorylated in the presence of high TRIB2, we saw that pThr308-AKT is expressed equally in both experiments (rapamycin and BEZ235) with no significant impact. However, a very interesting result emerges when we look to pSer473-AKT: phosphorylated AKT at this residue is overexpressed when TRIB2 is expressed too, independently of the treatment (Non-treatment; BEZ235 or rapamycin) (Fig. 3.4).

These results strongly suggest that the resistance mediated by TRIB2 to PI3K/m TOR1 inhibitors requires the phosphorylation of AKT´s Serine in position 473.

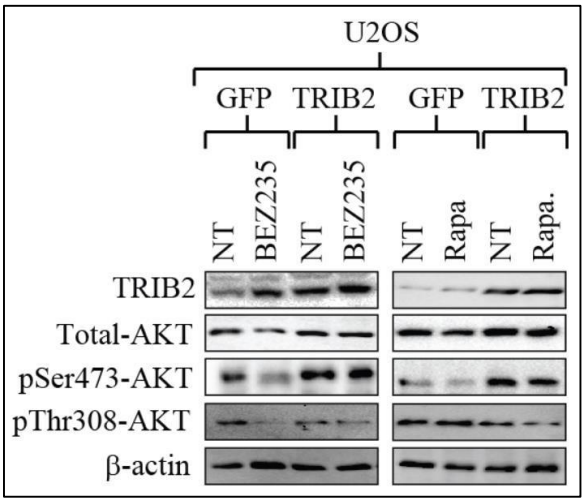

Figure 3.4- TRIB2 resistance occurs due to phosphorylation of Ser473 of AKT.
Western blot analysis of PI3K signalling pathway. TRIB2 positive regulates the phosphorylation of Ser473 of AKT. 50 µg of total protein lysate was loaded per lane and samples were probed for total TRIB2, total AKT, pSer473-AKT, pThr308-AKT and β- actin expression.

Results so far implicate resistance via PI3K and AKT. However, there is also resistance via mTOR1 (but not with mTOR1/2 dual inhibitors). We have seen that this resistance is mediated by TRIB2, which leads to the increase of pSer473-AKT. Another important thing that we know, is that mTORC2 plays a critical role in AKT1 'Ser-473' phosphorylation, facilitating the activation of AKT1´s loop on 'Thr-308' by PDK1.This step is a prerequisite for the full activation of AKT.

These two ideas lead us to investigate what happens if we block mTORC2. One of the principal components of mTORC2 is Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) We transfected two different cell lines (U2OS and 293T) to knock RICTOR down and "turn off" this complex. First, we performed a western blot to analyse the knock down efficiency by measuring the

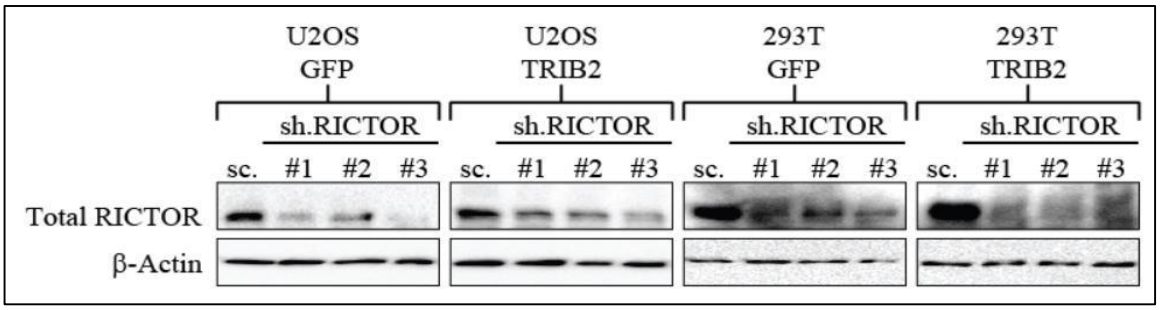

Figure 3.5 The RICTOR knock down is efficient.
Western blot analysis of RICTOR Knock Down efficiency for two different cell line, U2OS and 293T.Scramble lane is the positive control. Lanes 2,3 and 4 correspond to #1, #2 and #3 constructs respectively, obtained after TRIB2-shRNA transfection. 100 µg of total protein lysate was loaded per lane and samples were probed for total Total-RICTOR and β- actin expression.

protein expression of the cells transfected. It is possible to see that RICTOR expression is higher in the scramble lane, compared with the #1, #2, #3 constructs (Fig. 3.5). This result shows that the transfection is efficient, the RICTOR expression levels are low.

4. RICTOR and FOXO3a knock down increases resistance to dual PI3K/mTOR inhibitors

From previous results in our lab, we know that the knockdown of FOXO3a, an important transcription factor and a downstream target of AKT, raises the cell viability in a TRIB2 independent manner. We performed a RNAi knock down assay, to knock down RICTOR, an important component of mTORC2 complex, in order to block AKT activation via mTOR. We expected that this resulted in the molecular shut down of the AKT pathway. As a consequence, cells would resist to mTOR inhibitors.

When we analysed cell samples 72 hours post exposure to BEZ 235, we could observe that empty U2OS RICTOR shRNA (TRIB2 expressing cells) and TRIB2

U2OS RICTOR shRNA (do not express TRIB2) present approximately the same number of sub G1 cells indicating that the number of dead cells is independent from the presence of TRIB2. These results show that when RICTOR expression is silenced, the TRIB2-mediated resistance to BEZ235 is lost. These findings are very similar with FOXO3a knocked down cells results. knockdown of RICTOR expression prevented the activation of AKT and dramatic increase cells survival (Fig. 3.6). It suggests that mTORC2 is crucial in the activation of AKT and sub consequently a key regulatory piece for the full activation of this pathway

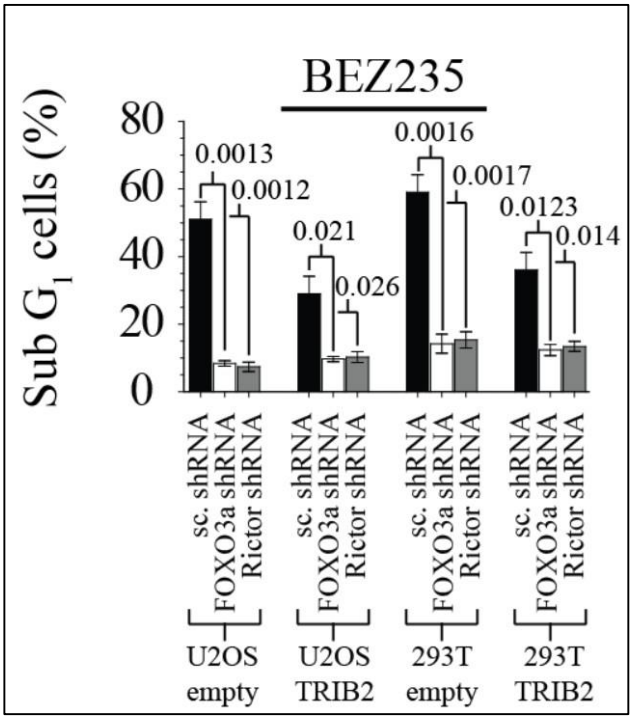

Fig. 3.6 TRIB2 resistance is lost, when RICTOR or FOXO3a are knocked down
Isogenic cell lines, U2OS and 293T, were treated with 100 nM BEZ235 and 72 hours post exposure, SubG1 population was determined by FACS analysis (PI staining). N=6, 50,000 total events counted per samples. Stats are two-tailed ANOVA and P values are shown above each analysis bracket. FOXO3a data kindly provided by Dr Richard Hill.

Several observations made us hypothesize that TRIB2 might be involved in mediating resistance to MEK inhibitors. TRIB2 has been shown to have a prominent role in melanoma and mediates resistance to drugs relevant in the treatment of melanoma.

These compounds are in clinical trials and being proposed to treat metastatic melanoma. One of the most extensively tested agents is the MEK inhibitor BAY766 that we used in our experiments.

To support further this hypothesis, we performed a time drug course in which we treated the cells for 72 hours with BAY 766. The number of dead cells were analysed by FACS for three different isogenic cell lines (U2OS; G361 and SK-MeL28). We the analysed this result and it is possible to observe that cells which contain increased levels of TRIB2 (U2OS TRIB2; G361 scramble shRNA and SK-Mel28 scramble shRNA) presented a higher number of sub G1 cells, when compared with cells that express low levels (Fig. 3.7)

This data suggests that the protein TRIB2 confers resistance to MEK inhibition treatment in different cell lines

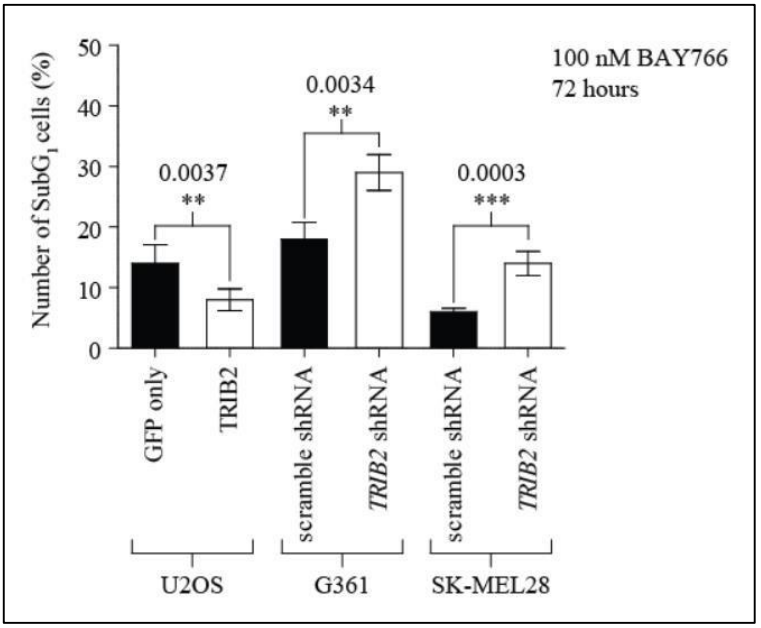

Fig 3.7 TRIB2 is a good candidate for MEK inhibition resistance.
Different isogenic cell lines, U2OS, G361 and SK-MeL28, were treated with 100 nM BAY 766 and 72 hrs post exposure, SubG1 population was determined by FACS analysis (PI staining). N=6, 50,000 total events counted per samples. Stats are two-tailed ANOVA and P values are shown above each analysis bracket.

5. BAY766 effectively inhibits ERK phosphorylation

The next step was to find if BAY 766 was effective. For that we analysed the protein expression of one of the most important downstream targets of MAPK pathway: ERK kinase. We performed a time drug course in which we treated the cells for 0, 12, 24, 48 and 72 hours with BAY 766, and after probing for two different forms (activated and non-activated) of ERK, we analysed the protein expression by western blot. This compound, as a MEK inhibitor, should decrease the activation of ERK (p-ERK), one of the molecular targets of MEK. The non-phosphorylated ERK (total-ERK) would have no significant changes in both expressing and non-expressing cells.

Analysing the western blot, it is possible to observe in the left panel (SK-MeL28 TRIB2 shRNA cells) and top lane (pTyr204-ERK1) that the shTRIB2 cells (low levels of TRIB2) have significantly lower activated ERK at each time point. On the right panel, TRIB2 over expressing cells have higher pTyr204-ERK that gradually decreases post-BAY766 treatment (12, 24, 48, 72 hours), as expected. (Fig. 3.8).

Analysis at the bottom lane that corresponds to non-active ERK (total-ERK) that function as a control, shows that non-treated TRIB2 expressing cells (right panel) have clearly higher total-ERK compared to TRIB2 shRNA cells (left panel). Surprisingly, it is also possible to see a decrease in total ERK at 48 and 72 hours post-BAY766 treatment.

Another important observation is that, generally, phosphor-ERK increases in cells that over express TRIB2(left and right panel).

These results indicate that BAY 766 produces a decrease in phosphor-ERK in TRIB2 expressing cells. The unexpected result is that BAY766 induces alterations in total-ERK along time, in both cell types.

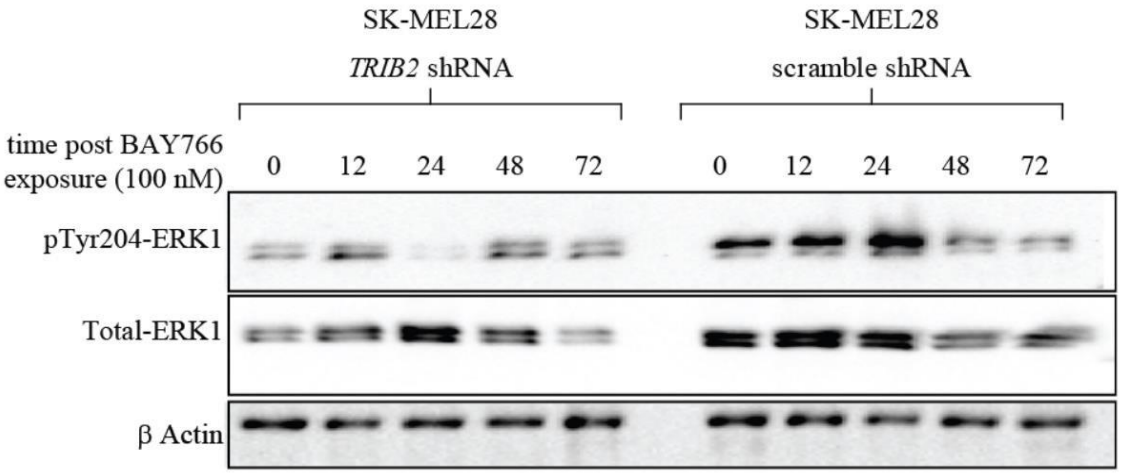

Figure 3.8- Increased pTyr204-ERK in high TRIB2 expressing cells.
Western blot time course analysis for MEK/ERK signalling pathway, following 100 nM BAY766 treatment. 100 µg of total protein lysate was loaded per lane and samples were probed for total ERK, pTyr204-ERK and β- actin expression. The MAPK pathway activity was reflected by the level of phosphorylated ERK detected with specific anti-phosphorylated ERK (pTyr204-ERK) antibody. Total ERK (total-ERK) was used for quantity control of proteins (N=1).

6. Microscopy Analysis

The goal of the next experiment was to evaluate the molecular consequences for the MAPK pathway, when we inhibit MEK. We hypothesised that expressing and non-expressing TRIB2 cells would present significant differences in ERK phosphorylation. Having in mind that ERK is one of the downstream targets of MEK, it was also expected that treated cells in general, would present much less p-ERK than non-treated cells.

After 24 hours of treatment with BAY 766, we observed that SK-Mel28 scramble shRNA_BAY766 cells (high basal levels of TRIB2) are intensive stained for TRIB2 (red) and phospho-ERK1 (green). For non-treated cells (SK-Mel28 scramble shRNA_NT), small differences occur and we clearly can see both signals.

For SK-Mel28 TRIB2 shRNA_NT cells the intensity of p-ERK fluorescent signal is weaker, and this loss of intensity increases for treated cells (SK-Mel28 TRIB2 shRNA_BAY766) (Fig. 3.9).

This results confirm that TRIB2 signal is stronger in expressing TRIB2 cells and p-ERK is weak in treated cells without TRIB2. Surprisingly, TRIB2 treated cells present a higher p-ERK signal than expected.

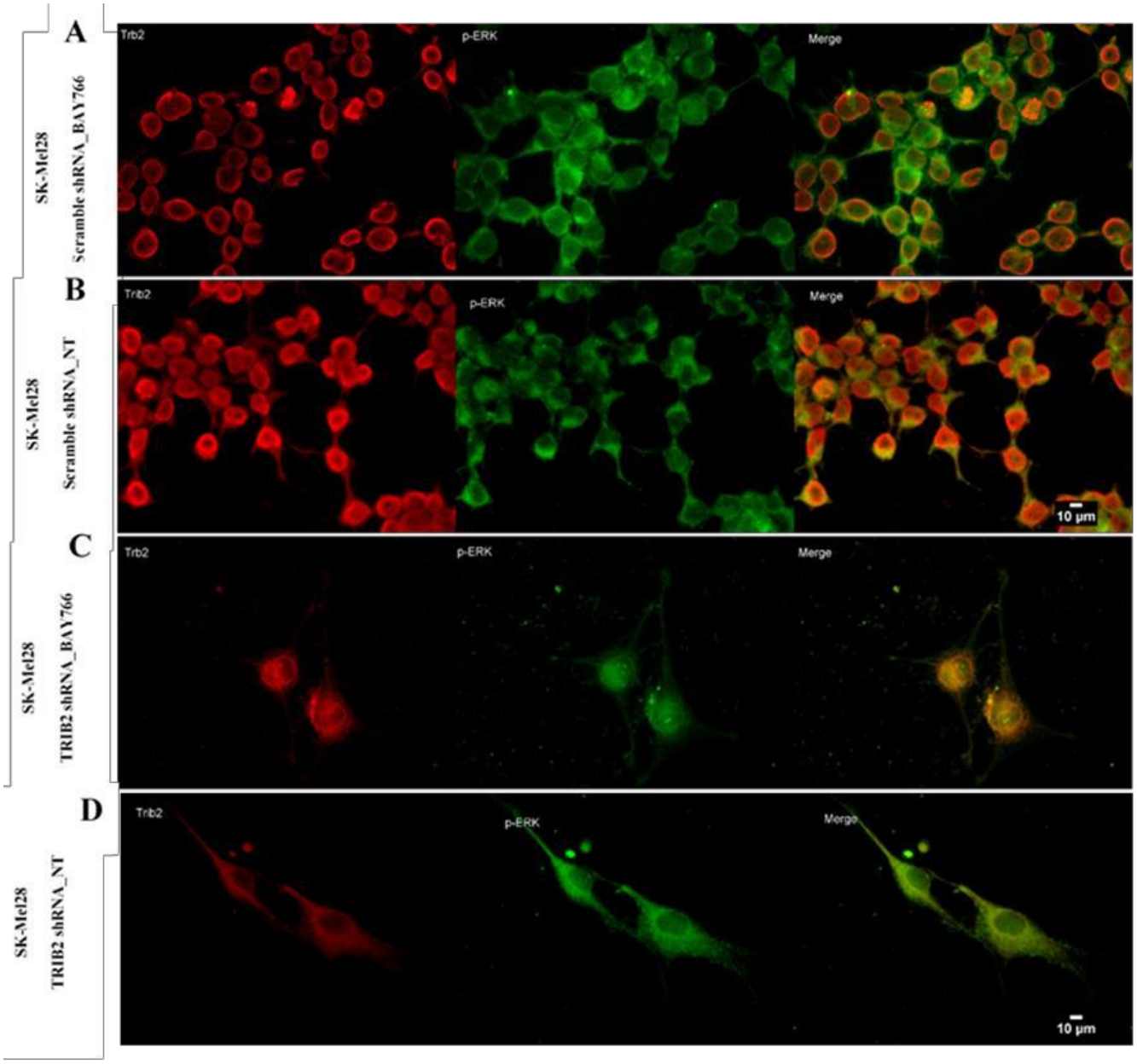

Figure 3.9 MAPK pathway is activated when TRIB2 is overexpressed
Immunofluorescence visualization of p-ERK and TRIB2 after BAY766 100 nM treatment. (A) The signal for activated ERK is intense in cells that also contain high levels of TRIB2.(B) This signal is even more intense in cells that were not treated with BAY766. (C) and (D)The morphology of cells with low levels of TRIB2 is very specific and different from the TRIB2 ones. When this type of cell line is treated the intensity of p-ERK decreases. X63

The strong evidence that TRIB2 is playing an important role in activating the MAPK pathway, when cells are treated with MEK inhibitors, lead us to question the molecular details of this mechanism. As we have seen before, TRIB2 contains a specific motif for MEK1 binding, so we hypothesised that TRIB2 is directly interacting with MEK1.

We used a common technique used to evaluate the interaction between two proteins, the co-immuno- precipitation assay (Co-Ip) described on methods section, to test this hypothesis.

7. TRIB2 and MEK interact and form a protein complex

We performed to different and independent assays (Exp1 and Exp2), in the first one we used a home-made TRIB2 primary antibody, in the second one we used a different antibody detailed described on materials and methods page. After probing with the first antibodies (TRIB2), we immune-precipitated the samples (magnetic beads), and probed in a western blot for Total-MEK protein detection.

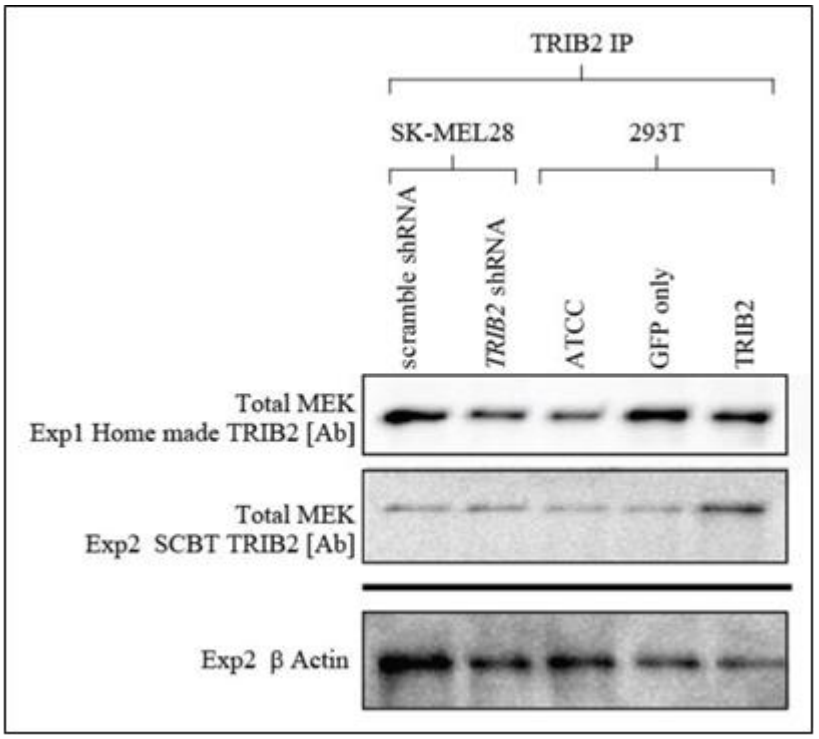

Figure 3.10-TRIB2 and MEK form a protein complex in isogenic matched cell lines. *To analyse the MEK-TRIB2 interaction, Lysates from two different isogenic cell lines, SK-MeL28 and 293T, were subjected to immune-precipitation using a homemade antibody against TRIB2 in experiment 1, and a commercial TRIB2 antibody in experiment 2. Then, lysates were western blotted for MEK and β- actin.*

As expected, there is a signal corresponding for total MEK in all cell types, all cell types contain MEK and TRIB2 proteins. However, in lanes were TRIB2 is over expressed (SK-MeL28 scramble shRNA; 293T TRIB2) this signal is much stronger in both experiments (Exp1-home made and Exp2 SCBT). (Fig. 3.10)

This result demonstrates that TRIB2 interacts with MEK1.

8. Confirmation of co-localization using confocal microscopy

Previous results from CO-IP demonstrate that TRIB2 and MEK interact and form a protein-protein complex. We hypothesised that these two proteins would also co-localise.

The cells when submitted to dual staining for MEK and TRIB2, reveal that there is a clear overlapping between the two colours green (TRIB2 staining) and red (MEK staining). As expected, this overlap (co-localization) happens in cells that over express TRIB2 (SK-Mel28 scramble shRNA_NT or SK-Mel28 scramble shRNA_BAY766).

As it is not possible to completely knock down the total amount of protein, it is also possible to see the same overlapping in the cells with low levels of TRIB2 (SK- Mel28 TRIB2 shRNA_NT and SK-Mel28 scramble shRNA_BAY766). However, this signal is weaker (Fig. 3.11)

This result confirms that MEK and TRIB2 co- localize within the cell.

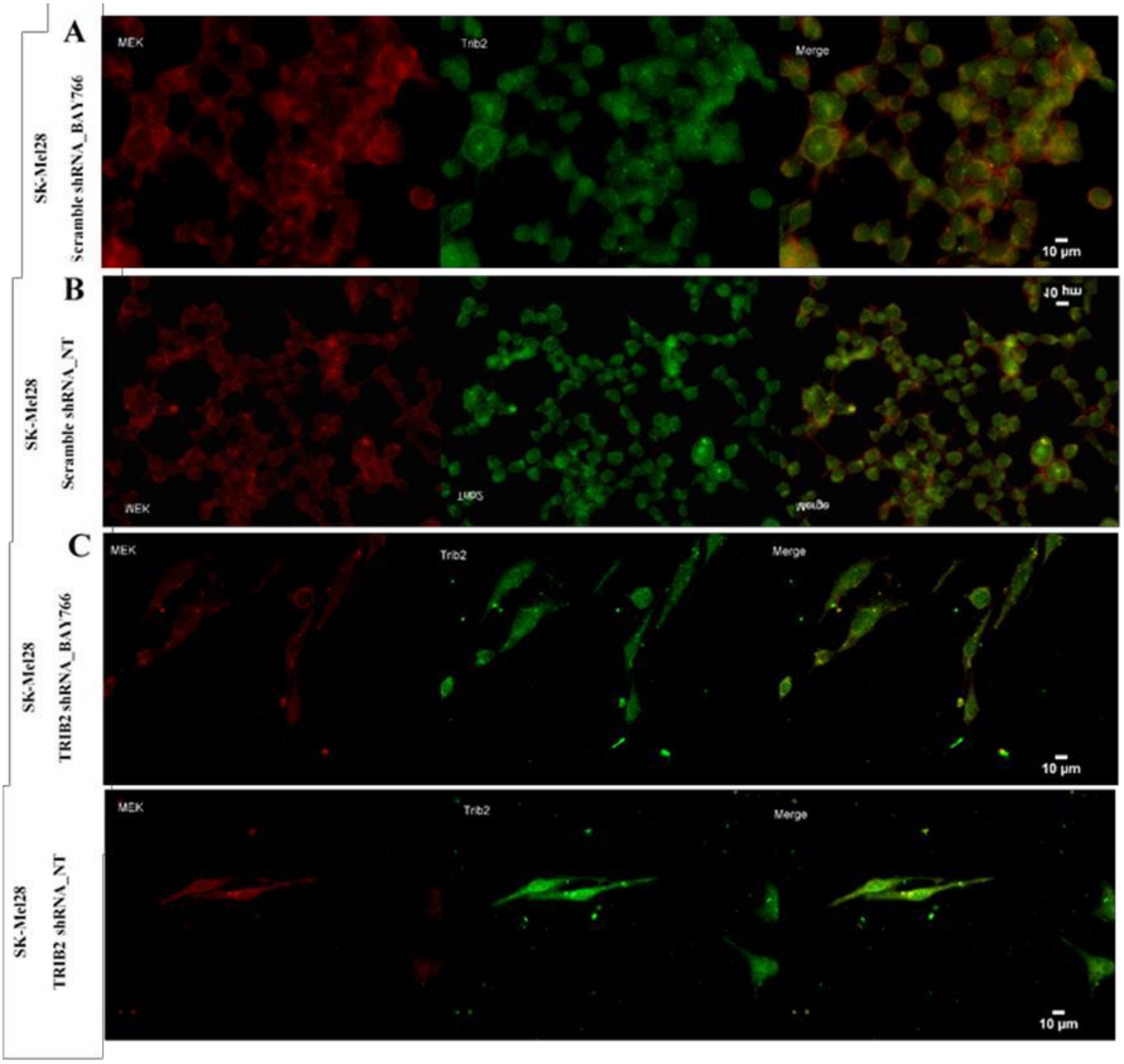

Figure 3.11 - MEK and TRIB2 co-localize.
Imunofluorescence visualization of total-MEK and TRIB2 after BAY766 100 nM treatment. The signal for both proteins is overlapped, as demonstrated by the yellow colour in the merge panel. This phenomenon is more evident in cells that contain high levels of TRIB2. The strength of both signals between treated and non-treated cells in insignificant (A) and (B). Although the levels of TRIB2 are low, it is possible to see that small amounts of protein are sufficient to overlap with MEK(C) and (D). X63

9. Cells that over express TRIB2 repress the transcription factor FOXO3a

So far, we have observed that at the protein level, the increase of TRIB2 within the cell is critical to confer resistance to the MEK inhibitor BAY766. At this point, we also wanted to investigate if high TRIB2 expressing cells repress two of the most important transcription factors present in this pathway: FOXO3a and NF- Kappa B. DNA replication and recombination, DNA repair, chromosome segregation, chromosomal stability, cell cycle progression, epigenetic silencing, and regulation of gene expression are mediated through protein–DNA interactions. Thus, it is of principal importance to understand the significance of these interactions in driving a biological response.

The first step consisted in choosing a promotor that FOXO3a is known to bind and activate: p27 promoter. Then we hypothesised if FOXO3a lacks the ability to bind the promoter, in the presence of an inhibitor.

Input samples (left panel) were submitted exactly to the same procedure that FOXO3a ChIP samples, but these samples were not probed with FOXO3a antibody. These samples can function as a negative control. By comparing the FOXO3a ChIP with Input samples, we can clearly note that there is an increase in the signal for lanes 293T GFP BEZ235 and 293T GFP BAY766 (TRIB2 non-expressing cells). On the other hand, in over expressing cell lines (293T TRIB2 BEZ235 and 293T TRIB2 BAY766), the intensity of bands is similar to the control (input samples).

This result indicates that in cells with low levels of TRIB2, FOXO3a has the ability to bind DNA (p27 promoter). When TRIB2 is highly expressed, FOXO3a is repressed (Fig. 3.12).

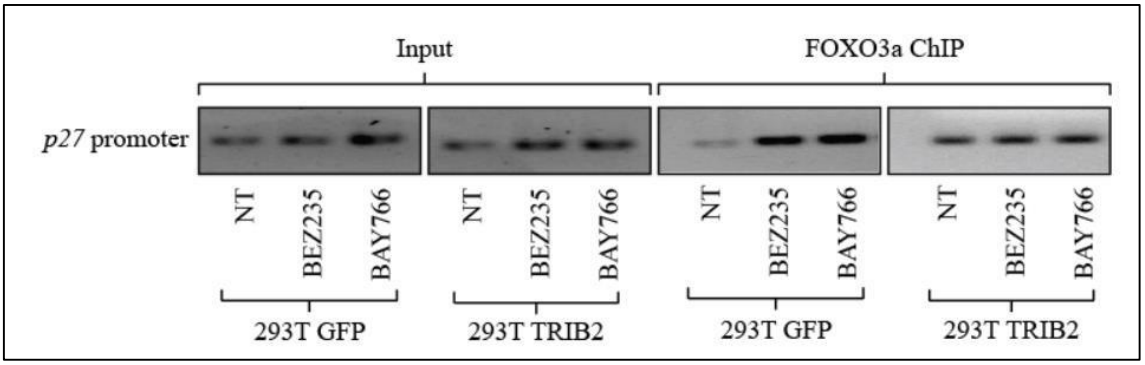

Figure -3.12- FOXO3a is repressed by over expression of TRIB2.
Representative DNA gel following FOXO3a ChIP assay, after 24 hours post drug treatment. Protein-DNA complexes from 293T isogenic cell line were immune-precipitated with FOXO3a antibody, as indicated. Input control has no antibody. After cross-link reversal, the co-immunoprecipitated DNA fragment corresponding to the FOXO3a promoter region was amplified by quantitative RT-PCRPCR using the appropriated primers and resolved in 2% agarose gels. N=3.
TRIB2 cell lines present darker bands in comparison with input control

Qualitatively, we saw that TRIB2 over expressing represses the transcription factor FOXO3a, and subsequently the transcription of the gene p27 via its promoter. Next, we decided to quantify the FOXO3a ChIP assay

Calculating the fold change/difference between input (control) and the FOXO3a samples, allows to quantify the repression of FOXO3a.

In cell lines exposed to BEZ 235 and BAY 766, it is possible to see a great difference in terms of fold change. This difference is not so significant in the cells that were not treated. This fact validates the theory that FOXO3a is being repressed (Fig.3.13).

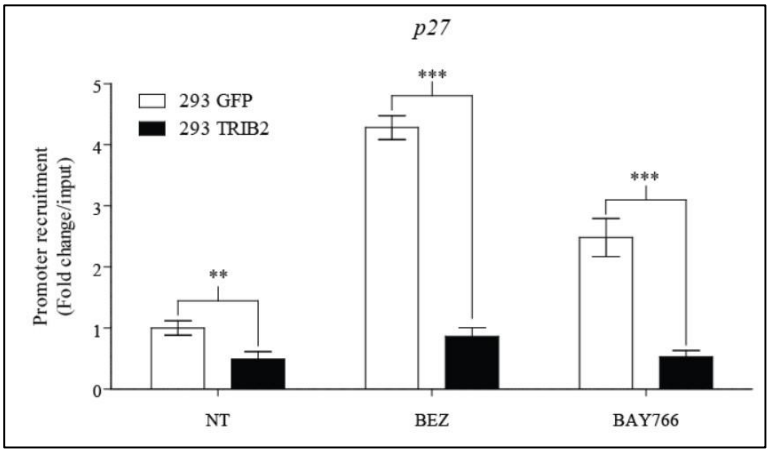

Figure 3.13-FOXO3a has more affinity to promoter with high levels of TRIB2
293T GFP (White bars) and 293T TRIB2 (black bars) cells were treated with BEZ 235, BAY 766 or non- treated or 24 hours. Quantitative Real Time RT-PCR analysis for our p27 ChIP gels were normalised to inputs from each respective cell line endogenous control. All samples were normalized with GAPDH. N=4. Cells treated with BEZ 235 are recruited to the promoter efficiently

10. TRIB2 over expressing cells have higher levels of NF-κB in their nucleus

The next approach was to figure out if the TRIB2 over expression leads to the increase of NF-κB in the nucleus, because deregulation of NF-κB pathway is frequently observed in cancer cells and is associated with tumorigenesis and tumor cell resistance to cancer therapies, especially in melanoma. We suspected that NF-κB efficiency to bind the promotor would increase, in the presence of higher levels of TRIB2.

To address this hypothesis, we performed a NF-κB ChIP assay in the same way that we did for FOXO3a

DNA gel was made 24 hours post drug treatment (BEZ 235 and BAY766), NF-κB ChIP assay reveals that the bands are more intense in the presence of high TRIB2 expression (293T TRIB2:NT, BEZ 235 and BAY 766). The same is not valid for cells with low TRIB2 (293T GFP:NT, BEZ 235 and BAY 766), as these corresponding bands are much less intense in comparison with the Input control. NF-κB has more affinity to bind the SESTRIN 1 promoter, when TRIB2 is highly present in the cell.

This data suggests that NF-κB is present in higher levels in the nucleus of TRIB2 over expressing cells (Fig. 3.14).

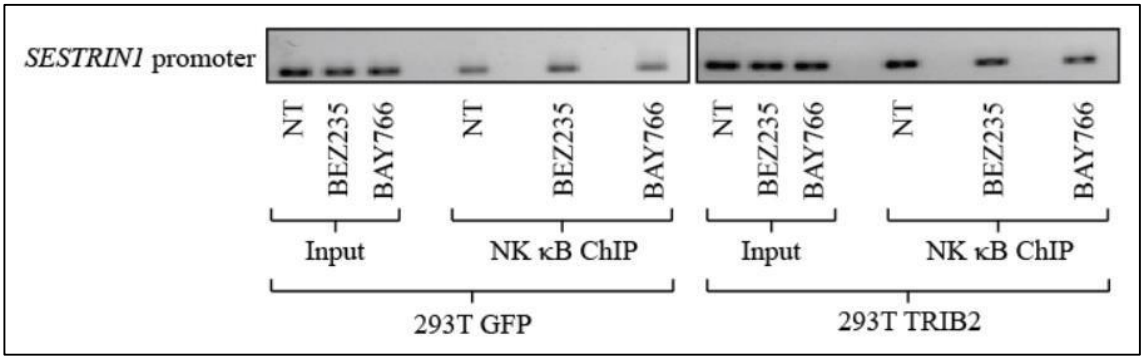

Figure 3.14- NF-κB has more affinity to bind the promoter, when TRIB2 is present
Representative DNA gel following 24 hours post drug treatment. Protein-DNA complexes from 293T isogenic cell line were immune-precipitated with NF-κB antibody, as indicated. Input control has no antibody. After reversal cross-link, the coimmunoprecipitated DNA fragment corresponding to the NF-κB promoter region was amplified by quantitative RT-PCR using the appropriated primers and resolved in 2% agarose gels. N=3.TRIB2 cell lines present darker bands in comparison with input control.

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After NF-κB ChIP assay, we used the same layout to the FOXO3a ChIP assay to quantify this result (Fig.3.15).

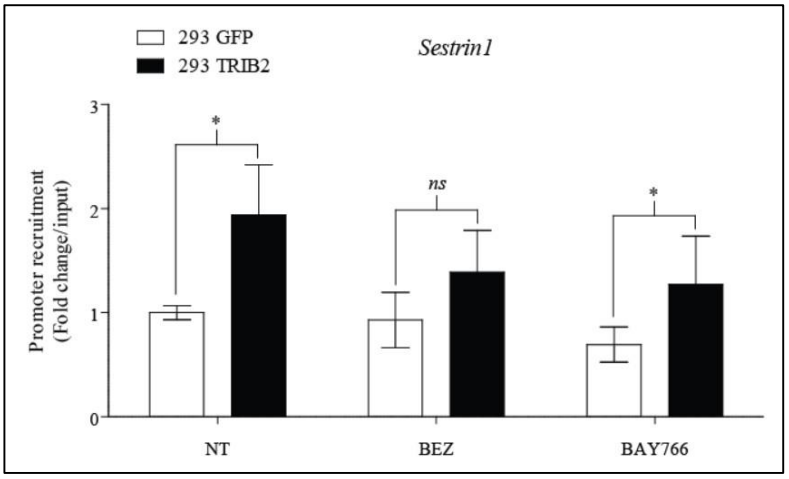

Figure 3.15 NF-κB has more affinity to promoter with high levels of TRIB2 *293T GFP (White bars) and 293T TRIB2 (black bars) cells were treated with BEZ 235, BAY 766 or non-treated for 24 hours. Quantitative Real Time RT-PCR analysis for our Sestrin1 ChIP gels were normalised to inputs from each respective cell line endogenous control. All samples were normalized with GAPDH. N=3. The number of nuclear NF-κB molecules is higher in non-treated cells that have high levels of TRIB2*

CHAPTER 4. *DISCUSSION*

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1. Discussion

Melanoma is one of the deadliest types of cancer. Although it is very easy to detect in early stages, melanoma in stage IV of progression can spread to other organs such as lungs and brain. These melanoma patients (stage IV), also resist to the traditional anti-cancer treatments. It is very important to develop new strategies to fight against chemical resistance. This capability is due to the genetic background of melanocytes: this type of cells is prevenient from the neural crest during the embryonic development. The neural crest cells migrate extensively to generate a prodigious number of differentiated cell types[78].

In our Laboratory, we have investigated in which way tumour suppressors such as FOXO3a and P53 or oncogenes like RAS influences cancer formation and progression with particular attention directed towards resistance to anticancer treatment.

In Previous studies, we have seen that the protein TRIB2 modulates resistance to traditional drugs like DTIC or new target inhibitors such as PI3K. TRIB2 is an oncogene overexpressed in melanoma, that acts through the negative regulation of FOXO. All these findings and the structural information that TRIB2 contains a specific MEK1 binding motif, lead us to hypothesised that TRIB2 could be a good candidate for mTOR and MEK inhibitory resistance, and play an important role in two most affected signalling networks in melanoma: the MAPK and PI3K/AKT pathways.

It is currently unclear whether TRIB2 regulates mTOR, an important activator of AKT, in melanoma. Here, different isogenic cell lines were created and shRNA constructs were used to silence TRIB2 in cells with high endogenous levels of this protein. Rapamycin (mTORC1 inhibitor) treatment provides a simple and straightforward method to inhibit the mTOR signaling pathway and to study the influence of mTORC1 on the TRIB2-dependent mechanism of resistance. We found that expression of TRIB2 in the presence of rapamycin enhances cell viability. We concluded that TRIB2 confers resistance to mTOR inhibition via complex1 but not to mTORcomplex2. We observed that TRIB2 is not determinant to affect viability when cells are treated with a mTORC2 inhibitor (TORIN1). This evidence is reinforced,

when we knock down RICTOR a key structural protein from mTOR complex2. Since mTORC2 positive regulates AKT phosphorylation, we expected that loss of RICTOR results in mTORC1 blockage and AKT pathway disruption, raising cell death. This shows that TORIN is effective and TRIB2 is not participating in the mechanism of resistance through mTORC2

AKT is a molecular target of mTOR and one of the most studied genes in melanoma research. We wanted to know what is the molecular mechanism, regulated by TRIB2, which confers resistance to mTOR inhibition via AKT. Although TRIB2 does not affect the phosphorylation of Thr308, we conclude that TRIB2 functions as an adaptor protein that facilitates the phosphorylation of the Serine 473, recruiting mTOR and sub consequently the activation of AKT. Velasco et al. reported that TRIB3 possesses a tumour inhibitory activity through the regulation of AKT pathway in breast cancer cells.[50] Inhibition of TRIB3 increases tumorigenesis due to enhanced phosphorylation of AKT by the mTORC2 complex and subsequent inactivation of FOXO3. Homology between TRIB family members is high, so it is possible to speculate that TRIB2 and TRIB3 have opposite molecular functions and compete for the same substrate (mTORC1)

High and low expression levels of TRIB2 correlate with immature and mature subtypes of human T-ALL, respectively, and associate with MAPK pathway.[51] Here, we identified TRIB2, a pseudokinase protein, as an important MAPK downstream effector in melanoma: we observed that MEK and TRIB2 co-localize and also confirmed that TRIB2 has the capability to interact with MEK and form a protein-protein complex, suggesting that this interaction is important to cells resist treatment. Our results, highlight what has been described in literature. TRIB proteins have previously been reported to bind to MEK in leukemia cells: TRIB1 and MEK1 interaction is required for Trib1-induced leukemogenesis[52].

Besides the directly interaction with MEK, we also wanted to know how does TRIB2 confers resistance to MEK inhibitors and unveil its mechanism. We examined the functional importance of MEK-dependent TRIB2 expression. We submitted cells to drug time courses with BAY 766, a potent MEK inhibitor and analysed cell viability and protein expression. TRIB2 regulates the proliferation of developing thymocytes[51] Jiayi Wang and Junhao Mao reported that TRIB2 is critical for liver cancer cell survival[53] and transformation. These ideas converge with our first conclusion, TRIB2 is up regulated in melanoma cells (SKMl28 and G361) and inhibition of TRIB2 expression (knock down)

decreased cell numbers and cell proliferation, in the presence of a MEK inhibitor. One of the TRIB2 expression consequences is that phospho-ERK increases in cells, keeping the pathway activated. When cells are treated with a MEK inhibitor, BAY766, MEK function is blocked and consequently ERK phosphorylation decreases, as expected. However, when we looked to the control (total-ERK) we did not expect that some variations occur post BAY766 treatment. We conclude this is a consequence of experiment limitations. We should perform this assay with a proper number of repetitions (N=3) to confirm these results, as well as study other different markers and key regulators of MAPK pathway must be evaluated, since ERK is not an exclusive marker of this pathway being active.

Qualitatively, we saw that TRIB2 over expressing represses the transcription factor FOXO3a, and subsequently the transcription of genes via pP27 promoter in the presence of inhibitors.

A NF-κB increase is associated with inflammatory responses that favours the tumour development and progression. Kloster et al shows that the ability of cAMP to potentiate the induced activation of NF-κB requires the activity of MEK for the inhibitory effect of cAMP on DNA damage-induced cell death[54]. We demonstrate that TRIB2 over expressing cells have more affinity to recruit NF-κB, suggesting that this transcription factors concentration is higher in the nucleus of resistant cells

Altogether, our study uncovers a novel regulatory mechanism underlying inhibitory resistance output for melanoma, and suggests that TRIB2 functions as a signalling nexus to integrate the MAPK and PI3Kpathways in cancer cells (figure 4.1).

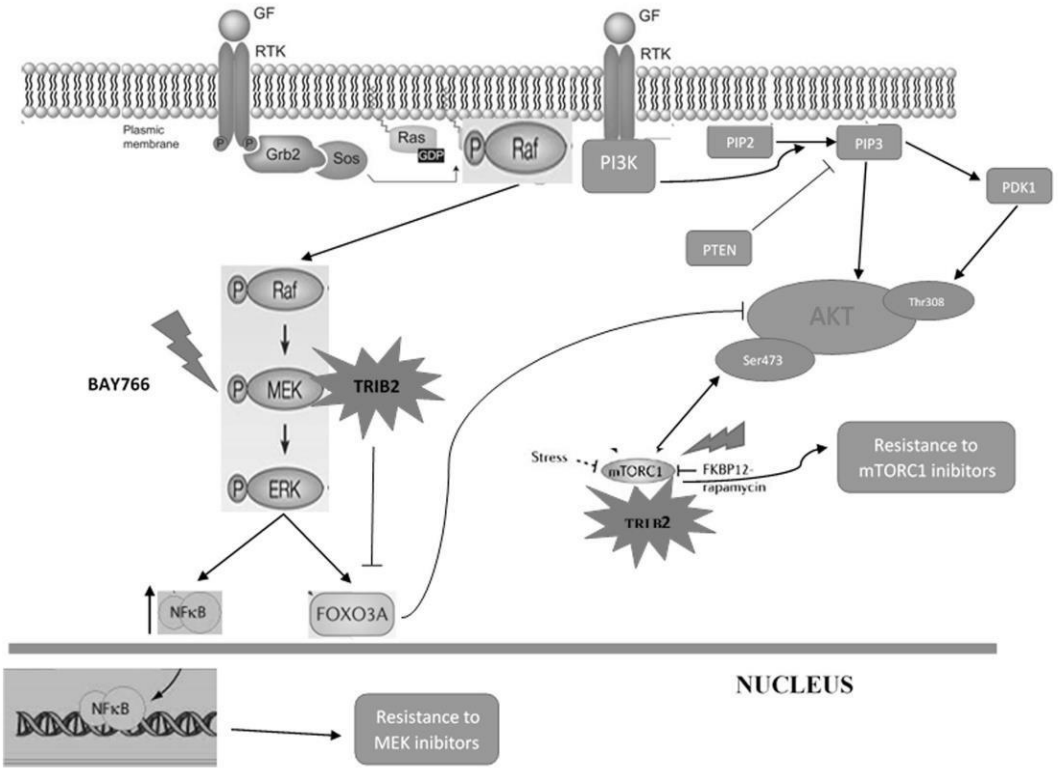

Figure 4.1-Mechanism in which TRIB2 regulates the resistance for cells treated with MEKor mTOR inhibitors.

We propose that post mTOR inhibition, TRIB2 has the capacity to function as an adaptor protein. It binds AKT and recruits mTORC1 that phosphorylates AKT in residue Ser473, a requesit for full activation.

TRIB2 is also essential in the mechanism of MEK inhibition resistance. TRIB2 forms a protein-protein complex with MEK activating the MAPK pathway. NF-KB has high affinity for P27 promotor in TRIB2 expressing cells. FOXO3a is repressed in this type of cells after drug inhibition

2. Future Directions

Over the last decade, knowledge of the two most important molecular signalling networks in melanoma, MEK and AKT pathways, has greatly progressed. This is a project that contributes with new findings which will be useful in future basic and translational research leading to new therapeutics insights on metastatic melanoma resistant treatment.

In the future is possible to reinforce the findings of this project. We could start to study the impact of TRIB2 expression in different cell lines and repeat the expression analysis for ERK and for different markers (MEK, downstream targets of ERK, etc.)

Cellular abundance of transcription factors is an important determinant of their regulatory activities. It is crucial to understand how these proteins control gene expression. To complete the analysis of transcription factors modulated by TRIB2 in MAPK and PI3K pathways, it would be interesting to measure the affinity to bind promotors or track expression of different genes. It is possible to do such analysis repeating Chip, use a gene reporter assay (Luciferase) or EMSA. We also can track when these transcription factors are in the nucleus or cytoplasm performing immunostaining assays.

We should expand our studies to in vivo and clinical samples. We have very interesting conclusions about what happens in vitro, that must be emphasised with the information prevenient from protein expression analysis, RNA and DNA in clinical samples. A Knock out animal model would also be useful to evaluate drug treatment resistance phenotypes.

Nowadays, there are in clinical trials, melanoma patients being submitted to combined therapy with MEK and RAF inhibitors. Some of these patients´ tumours bypass cell death, so it would be interesting to test *in vitro* if TRIB2 participates in the molecular network that origins this resistance

TRIB2 can be established as a predictive biomarker capable and help to stratifying cancer patients into responders and non-responders to melanoma therapeutics. New insights will establish TRIB2 as a molecular target in personalized medicine. This way, new inhibitory compounds will be design against this protein to treat melanoma.

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