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Characterization of TRIB2-mediated resistance to anti-cancer drugs

Mestrado em Oncobiologia:
Mecanismos Moleculares do Cancro

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Declaração de autoria de trabalho

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

ASSINATURA:_____

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"One moment of pain is worth a lifetime of glory."
Louis Zamperini

Abstract

Cancer results from the accumulation of multiple mutations and is characterized by deregulated cell mechanisms including cell growth, tissue invasion, angiogenesis and metastasis, being responsible for a large number of worldwide deaths. Malignant melanoma is the most aggressive type of human skin cancer, being just 5% of all skin cancer cases but accounting for over 80% of all skin deaths. This is primarily the result of melanoma being highly resistant to conventional chemotherapy. The PI3K/AKT signaling pathway is involved in many cell processes like survival, proliferation, growth and is one of the most mutated pathways in all human cancers, particularly in melanomas. FOXO3a, a transcriptional factor, is a crucial component of the PI3K/AKT pathway regulating the transcription of genes that promote apoptosis and cell cycle arrest. Following its activation, AKT negatively regulates FOXO3a, via phosphorylation, leading to FOXO3a export from the nucleus into the cytoplasm where it is ubiquitinated and is degraded. Consequently, if AKT is constitutively active, this enables a cancer cell to avoid apoptosis and to keep proliferating. The TRIB2 protein was discovered to be a FOXO3a repressor and is over expressed in many cancers although how TRIB2 mediates this effect is unknown. This project evaluates TRIB2 drug resistance to a dual PI3K/mTOR inhibitor BEZ235 in a clinically representative in vivo BEZ235 treatment model to confirm if our in vitro data (published and unpublished) correlates in a 3D in vivo model and demonstrates that AKT and TRIB2 interact in both in vitro cell models and in ex vivo clinical samples.

Keywords: Cancer, Melanoma, TRIB2, cancer cell signaling, drug resistance.

Resumo

O cancro é a nível mundial, uma das principais causas de morte. É o resultado da acumulação de várias mutações ao longo do tempo, e caracteriza-se pela desregulação dos mecanismos celulares. Há várias características que uma célula normal necessita para que se tornar cancerígena, sendo designadas como as "*hallmarks of cancer*" e neste momento são conhecidas dez: resistência à morte celular (apoptose), serem capazes de proliferar, serem capazes de escapar à vigilância e destruição por parte do sistema imune, serem imortais através da sua contínua replicação, serem genomicamente instáveis, serem capazes de induzirem a angiogénese (produção de novos vasos sanguíneos), desregularem os mecanismos de produção de energia celular, terem a capacidade de invadir outros tecidos, e por fim serem capazes de metastizar.

Melanoma maligno, é um tipo de cancro da pele que embora não sendo o mais comum, contemplando apenas 5% dos casos totais de cancro da pele, é o tipo mais agressivo, visto que é responsável por 80% das mortes por cancro da pele e a idade média de aparecimento é aos 50 anos, tendo maior incidência em indivíduos caucasianos. Este tipo de cancro é causado pela transformação maligna das células produtoras de melanina, os melanócitos que se encontram situados na epiderme. É também caracterizado por ser dos cancros no ser humano com pior prognóstico, e este facto deve-se à sua rápida metastização e invasão de outros tecidos e ao facto de atualmente não existirem drogas eficazes para o seu tratamento, havendo muitos casos verificados de resistência a quimioterapêuticos convencionais por parte destes pacientes. Este tipo de cancro é inicialmente designado por melanoma *in situ* sendo que nesta primeira fase, o seu desenvolvimento ocorre horizontalmente, dentro da epiderme. Ao longo do tempo, o seu crescimento é em profundidade, penetrando a derme e neste caso passa a ser designado por melanoma invasivo. Caso a doença seja detetada até esta fase, o prognóstico do paciente será bom uma vez que através de intervenção cirúrgica a fim de remover o tumor confere cerca de 95% de taxa de sobrevivência de 5 anos aos pacientes.

A via de sinalização PI3K/AKT está envolvida em múltiplos processos celulares, desde mecanismos relacionados com sobrevivência, crescimento e proliferação celular, e é uma das vias metabólicas mais mutadas em cancros humanos. PI3Ks são enzimas que desencadeiam a cascata de ativação de várias proteínas. A desregulação da via PI3K/AKT está associada a um elevado número de pacientes com melanoma, pelo que é necessário que se encontrem novas drogas de forma a melhorar o prognóstico dos pacientes, através da compreensão desta via de sinalização e dos seus componentes.

Sendo o AKT uma quinase, identificada como estando constantemente ativa nesta via, e estando descrito que esta ativação por parte do PIP3 (um dos substratos das PI3Ks), vai fazer com que o fator de transcrição FOXO3a, responsável pela transcrição de genes que promovem a paragem do ciclo celular (para que sejam remendados erros através dos checkpoints), e a apoptose (necessária para que as células cancerígenas morram) seja exportado do núcleo (onde está ativo), para o citoplasma onde através da fosforilação por parte do AKT, vai ser ubiquitinado e marcado para destruição proteossomal, impedindo assim que os genes com características anticancerígenas sejam transcritos.

Por ser uma das vias mais mutadas, e por não haver terapias eficazes, a via PI3K/AKT está a ser alvo de vários estudos e há várias drogas a ser testadas em ensaios clínicos, sendo BEZ235 principal foco no meu projeto de investigação, tendo já sido descrito por vários autores como tendo um potencial terapêutico anticancerígeno, visto que é um inibidor das PI3Ks.

Atualmente, sabe-se que a grande maioria dos pacientes com melanoma, adquire ao longo do tempo resistência aos tratamentos, pelo que se tem tentado compreender os mecanismos envolvidos nessa resistência, de forma a saber como reverter este problema.

É também conhecido na literatura que o TRIB2 é um gene cuja sobrexpressão está associada a vários tipos de cancro, entre os quais melanomas, estando este gene também associado a mecanismos de resistência a quimioterapêuticos, bem como está descrito o seu envolvimento na regulação negativa do FOXO3a. Não é ainda evidente de que forma é feita esta regulação, pelo que é crucial que haja mais investigação acerca destes mecanismos, uma vez que sabendo de que forma FOXO3a é inativado através do TRIB2, será possível encontrar uma forma de inverter esta situação. A principal hipótese colocada pelo nosso grupo de investigação com base em estudos feitos anteriormente, é a de que embora os inibidores das PI3Ks estejam a atuar, e que estas sejam de facto inibidas, se o TRIB2 estiver constantemente a ativar o AKT hipoteticamente através da fosforilação deste, o inibidor não estará a sortir o efeito desejado, pelo facto de o AKT estar constantemente ativo, visto que consequentemente vai inativar o FOXO3a, impedindo a transcrição dos genes com propriedades anticancerígenas, como por exemplo BIM, FasLG, p27, entre outros.

Neste projeto de investigação, é feita a avaliação do inibidor das PI3Ks BEZ235, de forma a compreender se a inativação do FOXO3a tem ou não relação com a interação entre o AKT e o TRIB2. Para isso fizeram-se vários estudos para não só verificar se há uma co-localização do TRIB2 e do AKT, através de CO-IP e de microscopia de fluorescência, bem como para compreender se realmente se verifica esta resistência a drogas por parte das células tumorais dos melanoma, e se a sobreexpressão de TRIB2 está em concordância um aumento do de AKT no seu estado ativo, ou seja fosforilado (pAKT/PO4-AKT) através de estudos *in vitro*, *in vivo* e *ex vivo* (com amostras clínicas de pacientes).

Atualmente está aprovado apenas um biomarcador para melanoma, LDH. Assim, o ideal seria que o TRIB2 fosse igualmente aprovado como biomarcador para a doença, para que desta forma fosse possível que através de um *"screening"* a pacientes com melanoma, verificar se há sobreexpressão de TRIB2. Verificando ser o caso, evitar-se-ia a administração de inibidores das PI3Ks tal como BEZ235, uma vez que esta droga não terá um efeito benéfico para o paciente e este estará apenas a sofrer efeitos citotóxicos desnecessários.

Devem ser feitos mais estudos de forma a elucidar os mecanismos que levam a que haja esta resistência, para que se descubram alternativas terapêuticas eficazes de forma a melhorar o prognóstico destes pacientes, já que este é dos menos promissores para a sobrevivência destes doentes.

Palavras-chave: cancro, melanoma, FOXO3a, PI3K, AKT, TRIB2, BEZ235.

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ABBREVIATIONS LIST

AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ATCC	American Type Culture collection
ATF4	Activating transcription factor 4
BRAF	B-type Raf kinase
CTLA-4	CTL antigen 4
Co-IP	Co-Imunnoprecipitation
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
FACS	Fluorescent Activated Cell Scanning
FasL	Fas ligand
FDA	Food and Drug Administration
FOXO	Forkhead transcription factor
GPCR	G-protein coupled receptor
IGF-1	Insulin-like growth factor 1
Il-2	Interleukin-2
ING3	Inhibitor of growth family 3
hPTTG1	Human pituitary tumour-transforming gene
HSP90	Heat shock protein 90
KD	Kinase Domain
LDH	Lactate dehydrogenase levels
MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinase kinase
MDM2	Mouse double minute 2 homolog
mTOR	Mechanistic target of rapamycin
NCOA3	Nuclear receptor coactivator-3
p70S6K	Phosphorylation of 40S ribosomal protein S6
PBS	Phosphate buffered saline
PDK1	Pyruvate dehydrogenase lipoamide kinase isozyme 1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3 kinase
PIP2	Phosphatidylinositol-4,5- bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PRAS40	proline-rich AKT substrate of 40 kDa
PTEN	Phosphatase with tensin homology
RGS1	Regulator of G-protein signaling 1
RTK	Receptor tyrosine kinases
S100B	S-100 calcium binding protein B
SPP1	Secreted phosphoprotein-1
TRIB2	Tribbles2
TYR	Tyrosinase
USA	United States of America
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Cancer

The development of organisms involves mechanisms such as proliferation, division and growth of cells. The problem arises when the organism is already developed and the cells keep proliferating and dividing which in case of wound repair is crucial, but when the cells get mutations in their genome, allowing genes to acquire new functions, driving the cells to new phenotypes that will no more follow their normal process and in the worst case, driving our body to the appearance of cancer.[1,2]

Unbalance between cell proliferation and apoptosis as well as the accumulation of several genetic mutations in DNA are needed for a normal cell to become a cancer cell, passing from one mutated cell that goes out of control, to a state of neoplasia, when the cancer spreads to other parts of the body. (Figure 1.1.1).[1,3,4]

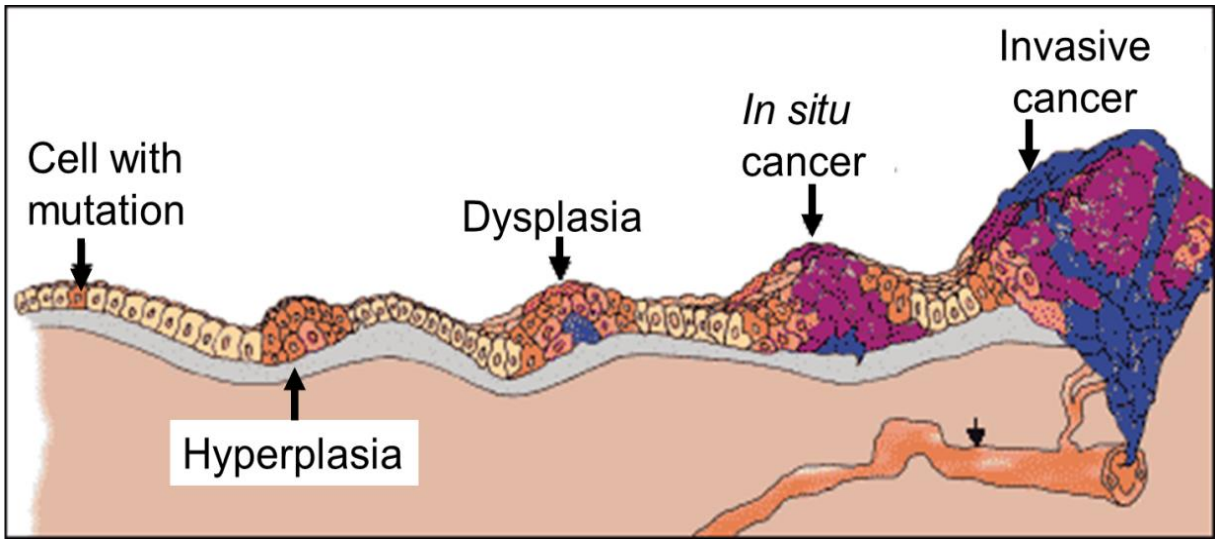

Figure 1.1.1. Cancer development starts with a single mutated cell and progresses with the accumulation of more several mutations followed by changes in gene expression. This genetic alterations are represented in different histopathological stages (Hyperplasia, Dysplasia, In situ cancer and finally invasive cancer).[4]

Long ago, cancer was a concept to describe just one disease, but nowadays incorporates over 100 different diseases, different cancer types wherein each of them consists in a multistage process, consequence of genetic factors like genetic mutations or environmental factors that will have an effect in gene expression (i.e., drugs, diet, UV).[5,6]

For tumor progression, in order to survive and avoid apoptosis, is crucial that a cancer cell acquire particular characteristics which are named *"The hallmarks of cancer"*. In 2000, were defined six leading characteristics[2], however nowadays with the new recent discoveries, are known in total ten (Figure 1.1.2).[1]

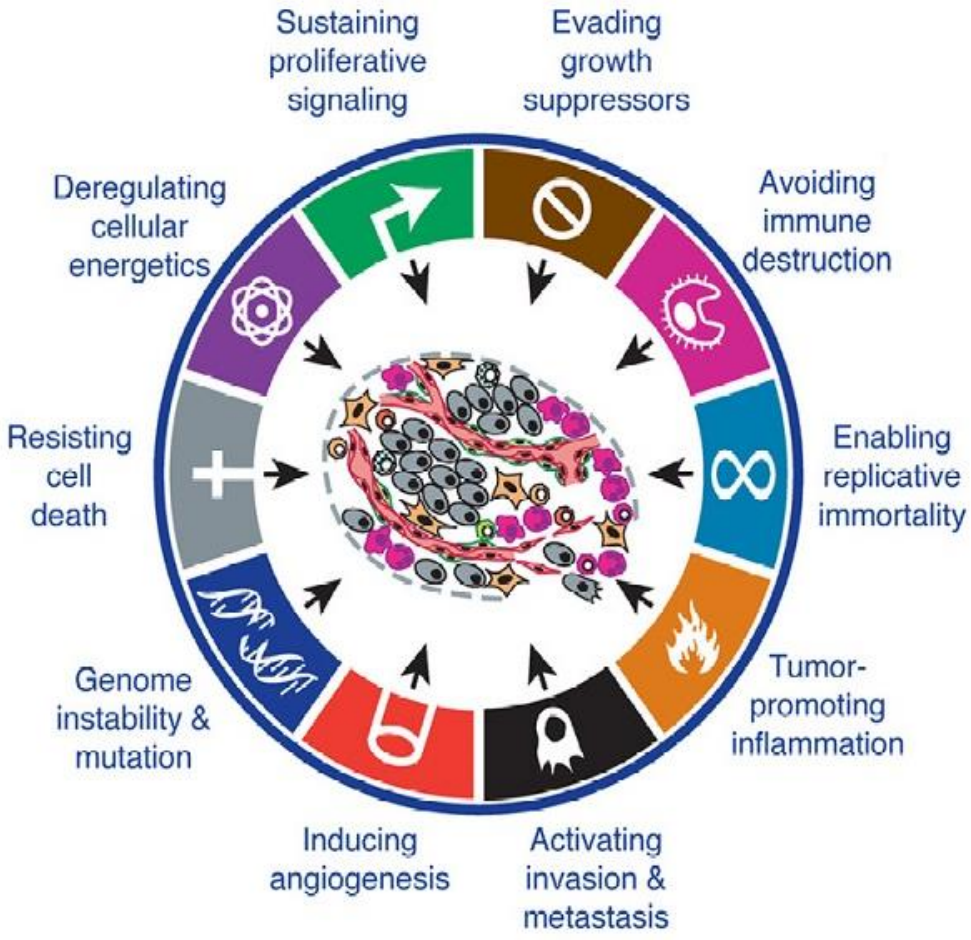

Figure 1.1.2: Hallmarks of cancer in 2011 updated from the six named in 2000.[1]

1.1.1. Epidemiology

Cancer is a worldwide urgent problem since it was responsible for 14.1 million new cases and 8.2 million deaths in 2012, making cancer one of the most deadly diseases in the world whether in non-developed or in developed countries. Worldwide most frequent cancers are breast, colorectal and lung cancer. Is currently called "*the disease of the future"* considering one likely 70% increase of new cancer cases for the following 2 decades, having 22 million cases instead of the 14 million in 2012 due to many factors: the increase of an older population, their lifestyle with associated behavioral changes like a sedentary lifestyle, smoking and a poor diet.[7]

1.1.2. Cancer treatment methodologies

Cancer treatment is chosen according to the cancer type and stage, because each cancer is unique; each cancer will have a different treatment approach. People could have just one kind of treatment or a combination of more than one, for example surgery (procedure made by a surgeon to remove the tumor from your body) is the first approach in case the tumor can be removed, plus radiation therapy or chemotherapy in order to kill cancer cells by damaging DNA and avoid cells to keep diving.[5,8]

1.1.3. Novel cancer therapeutics

Optionally, we have also new therapies to address this problem, like immunotherapy that is used with the aim of stimulating the immune system to fight cancer, through the activation of immunologic cells (i.e. using dendritic cells vaccine).[9]

To avoid tumor progression and induce their regression, targeted therapy is another alternative approach which uses drugs to block the action of mutated proteins in signaling pathways that are deregulated and that are allowing the tumor cells to survive and keep growing.[8,10]

1.2. Melanoma

Skin cancer is classified as non-melanoma (basal or squamous cell) or melanoma (only 5% of all skin cancer cases), according to the cell type that gave rise to cancerous cells. Melanoma arises from the transformation of the melanocytes, cells that are located in the bottom of epidermal layer of the skin and that are responsible for the production of the pigment melanin. This pigment is involved in skin protection mainly against ultraviolet sun rays exposure, which is the major cause of melanocytes transformation. This exposure triggers mutations that will lead to an abnormal cell multiplication and consequently to malignant tumors formation.[11]

Furthermore mutations in *CDKN2A* and *CDK4* genes (involved in cell division control) have been shown to be associated with familial malignant melanoma (10-15% of the cases). However, the most part of the melanoma cases appear sporadically, being named sporadic melanoma. Having blue or green eyes and fair skin, if the person gets easily sunburns, freckles and moles which (could develop to melanoma) are risk factors as well.[12,13]

Melanomas can arise from any part of the skin, appearing commonly in the neck and face, but preferably on the legs in women and on the chest and back in men.[11]

1.2.1. Epidemiology

On average, each year 132,000 melanoma are diagnosed cases worldwide. Caucasian populations have a higher risk to develop melanoma skin cancer comparatively with dark-skinned populations, due to a lower melanin pigmentation protection, for these factor Australia population is the one with highest incidence of melanoma since is predominantly Caucasian.[14,15] The age more susceptible to the appearance of melanoma is the late fifties and are diagnosed more cases in men than in women (1.3/1).[16]

Melanoma although not the most common skin cancer type, contemplating 5% of all skin cancer cases, is the most dangerous one, being responsible for 80% of all skin cancer deaths, making it one of the most aggressive of all human cancers. The American Cancer Society (AJCC) predicts that in 2015, there will be diagnosed around 73,870 new melanoma cases in the US, showing an increase of 200% since 1973, wherein 31,200 in women and 42,670 in males, estimating in total 9,940 deaths. [5,7,8]

Statistical studies show that New Zealand/Australia has the highest melanoma mortality rate in the world with 3.5 deaths per 100 000 habitants, followed by the United States of America (USA) with 1.7/100 000 and the third is Europe with 1.5 deaths/100 000.[7,17]

In Portugal, it was registered in 2007 by the National Cancer Registry, that per 10.000 habitants we have 6 cases of malignant melanoma, corresponding to an increase of 15% since 2001 (with 5.2 cases per 10.000 habitants). Annually, appear about 700-800 new cases being the incidence higher in women than in men (1/1.14) and the average upper the 50.[15,18]

1.2.2. Stages of disease/diagnosis.

In order to know how far the cancer is spread, thereby giving a more appropriate and accurate treatment, melanoma patients need to have a good diagnosis, to consequently have a better prognosis. For that, is used the staging system from the AJCC.[11]

Prior to melanoma staging, ABCDE nomenclature (Figure 1.2.2.1) is performed. This method is used for an early detection and is limited, once the asymmetry, border, color, diameter and evolution parameters are self-skin checked. If there are abnormal changes, next step is to go see an dermatologist.[11]

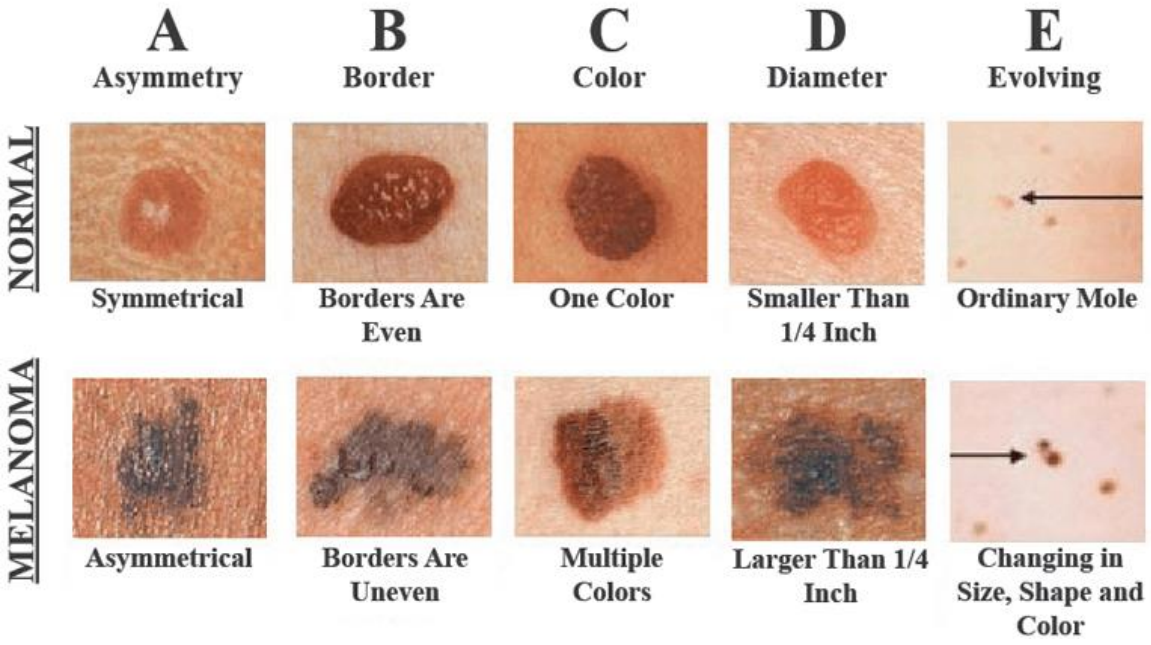

Figure 1.2.2.1: ABCDE system for melanoma diagnosis.[11]

Melanoma staging is usually performed by the American Joint Commission on Cancer (AJCC) TNM system, that evaluates the tumor thickness (T), how many lymph nodes are affected with cancerous cells (N) and finally if there are any metastases, to know how far the cancer spread or not to other organs (M). In combination with these factors, are measure as well the tumor ulceration, mitotic rate and lactate dehydrogenase levels (LDH). (all described in table 1.2.2.1)[19,20]

Nowadays, only LDH is established by the AJCC as a biomarker for melanoma prognostic classification system, over more than 60 years ago and is used because of their high specificity and sensitivity to detect this disease. A good biomarker must obey to some criteria, such as to be measured in an accessible body fluid like blood, must be minimally invasive to the patient and to have a low cost.[20]

T classification	Thickness	Ulceration Status/Mitoses
Tis (*In situ*)	**NA (not applicable)**	**NA**
T1	≤ 1.0 mm	**a:** Without ulceration and mitosis < 1/mm^2
		b: With ulceration or mitoses ≥ 1/mm^2
T2	1.01 - 2.0 mm	**a:** Without ulceration
		b: With ulceration
T3	2.01 - 4.0 mm	**a:** Without ulceration
		b: With ulceration
T4	> 4.0 mm	**a:** Without ulceration
		b: With ulceration
N classification	**Number of metastatic nodes**	**Nodal Metastatic Burden**
N0	0	NA
N1	1	a: Micrometastasis*
		b: Macrometastasis**
N2	2-3	a: Micrometastasis*
		b: Macrometastasis**
		c: In transit metastases/satellites without metastatic nodes
N3	4 or more metastatic nodes, or matted nodes, or in-transit metastases/satellite with metastatic nodes	
M Classification	**Site**	**Serum LDH**
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastases	Elevated

Table 1.2.2.1: TNM classification according to the 7th edition of the Melanoma staging system by the American Joint Commission on Cancer (AJCC). [20,21]

To determine the melanoma patient stage, is made the combination of the TNM parameters in order decide the most appropriate treatment according to the disease stage (table 1.2.2.2).[20]

Stage	Melanoma Characteristics	Classification			5 year survival rate
		T	N	M	
0	Carcinoma in situ (in epidermis and has not spread to dermis)	Tis	N0	M0	99 – 100 %
I A/B	Lesions up to 2 mm but no nodal or distant metastases	T1a	N0	M0	A – 95% B –88 - 92 %
		T1b	N0	M0	
		T2a	N0	M0	
II A/B/C	Lesions greater than 2 mm, no positive nodes or distant metastases	T2b	N0	M0	A – 77 - 79 % B – 61 - 70% C – 43 - 45%
		T3a	N0	M0	
		T3b	N0	M0	
		T4a	N0	M0	
		T4b	N0	M0	
III A/B/C	Lesions of any size with positive lymph nodes	Tx	N1	M0	A – 57 – 73 % B – 41 – 57 % C – 20 – 34 %
		Tx	N2	M0	
		Tx	N3	M0	
IV	Lesions of any size with distant metastases	Tx	Nx	M1	5 – 22 %

Table 1.2.2.2: Melanoma patients staging based in TNM classification according to the 7th edition of the Melanoma staging system by the American Joint Commission on Cancer (AJCC) plus the measurement of lactate dehydrogenase levels (LDH), tumor ulceration and mitotic rate. (Tx-Primary tumor cannot be assessed; NX- Regional lymph nodes cannot be assessed; MX- Presence of distant metastasis cannot be assessed.)

1.2.3. Current treatment options for melanoma. Conventional therapy.

Early melanoma detection makes possible that through surgery (surgical excision of the tumor and the nearby tissue), most part of primary melanomas (about 80%) could be treated and curable. For stage III melanoma patients usually have for option one year treatment with adjuvant immunotherapy with interferon-α. In advanced stages, with the cancer spreading to other organs, making hard to define the best treatment, the prognosis is a real challenge. In these cases, around 45% of patients with metastatic melanoma receive immunotherapy or chemotherapy. For the patients with the worst prognosis, melanoma patients in stage IV, surgery is not a viable option, wherein the suggested treatment is systemic drug therapy. [16,22–24]

Conventional chemotherapy uses drugs with aggressive cytotoxic effects, mostly alkylating agents, to avoid cell replication. Food and Drug Administration (FDA) is the entity that approves these compounds, such as Dacarbazine (DTIC) a drug approved in 1975, Fotemustine (Muphoran), Temozolomide (Temodal). The problem with these treatments is the fact of just 10% triggers an effective response and do not have an overall survival increase. [17,25] FDA recently approved the BRAF inhibitor vemurafenib and the MEK inhibitor trametinib for melanoma patients that harbor *BRAF* mutations.[26,27]

Regarding immunotherapy, in 1998 FDA have approved Interleukin-2 (IL-2), the first immunotherapy for advanced melanoma, however has the same issue of DTIC because even with high-doses of de drug the response is not significant. Nowadays, is used in combination with DTIC (since 2011). [28]

Since 2009, new systemic cancer treatments have been emerging wherein monoclonal human antibody against the CTL antigen 4 (CTLA-4) commercially known as Ipilimumab, was one of the most important discoveries in the research for immunotherapeutic drug crucial in immune system stimulation to respond against tumor antigens. Recently, was found that Ipilimumab in combination with GM-CSF instead Ipilimumab administrated alone is more effective to treat metastatic melanoma patients since there is an overall survival increase and lower toxicity to the patients.[29–33]

1.2.4. Novel therapeutic approaches useful against melanoma

Presently, with the aim to provide more quality of life to melanoma patients, it is important to search novel therapeutic approaches. Currently, several compounds are being tested in clinical trials, including NPV-BEZ235 (BEZ235).[34,35].

For this project, BEZ235 (Novartis) was our particular research focus. BEZ235 is a selective PI3K and mTOR dual inhibitor, classified as an imidazoquinoline and tested already in many clinical trials that have showed anti-cancerous properties, such as the induction of apoptose, suppression of cell proliferation, induction of G1 cell cycle arrest and inhibiting the function of many proteins such as AKT, S6, S6K. This drug is given orally to the patients, having side effects like nausea, headache diarrhea, with a stable response demonstrated in clinical trials.[36–40]

1.3. PI3K/AKT pathway

PI3K/AKT signaling pathway (showed in Figure 1.3.1) has been a crucial target for research not only because it is involved in many regulatory cell mechanisms (differentiation, migration, growth, proliferation and survival)[41,42] , but also for being one of the most mutated pathways in human cancers.[43]

Phosphoinositide 3-kinase (PI3K) is a lipid kinase. It is a heterodimer since it is constituted of two subunits: catalytic (p100) and regulatory (p85). [40,44] PI3K is one of the most important component in this pathway, having a central role in the regulation of angiogenesis (formation of new blood vessels), apoptosis (programmed cell death), cellular metabolism, and Deoxyribonucleic acid (DNA) repair.[45,46]

PI3K activation could be made through many signals, such as the interaction between transmembrane tyrosine kinase-linked receptors (RTKs), G-protein coupled receptor (GPCR), or simply by cell-to-cell interaction.[47] This activation will trigger the phosphorylation of PI3K substrate phosphatidylinositol-4,5-bisphosphate (PIP2, present in the cellular membrane) into phosphatidylinositol-3,4,5- triphosphate (PIP3), a fast action that takes just seconds.[42,45,47] After this conversion, PIP_3 will recruit to the cell membrane proteins that contain a pleckstrin homology (PH) domain, like the protein kinase B (PKB or more known as AKT). AKT named as the first downstream mediator of PI3K signaling pathway, belongs to serine-threonine kinase family, and in mammals exists in three isoforms (AKT1/ AKT2/AKT3) encoded by three different genes, been expressed in variable levels, according to the tissue in question.[48] In order to achieve total activity, AKT must have both their conserved residues (threonine 308 and serine 473) phosphorylated, being threonine 308 residue targeted by the kinase phosphoinositide-dependent kinase 1 (PDK1) and serine 473 residue by Mechanistic Target of Rapamycin Complex 2 (mTORC2). At this moment, p-AKT is ready to phosphorylate other downstream substrates that are involved in cell regulation. One negative regulator of this pathway is PTEN (tumour suppressor), that will through their phosphatase function, dephosphorylate PIP3 into PIP2 avoiding that AKT becomes activated.[40,45]

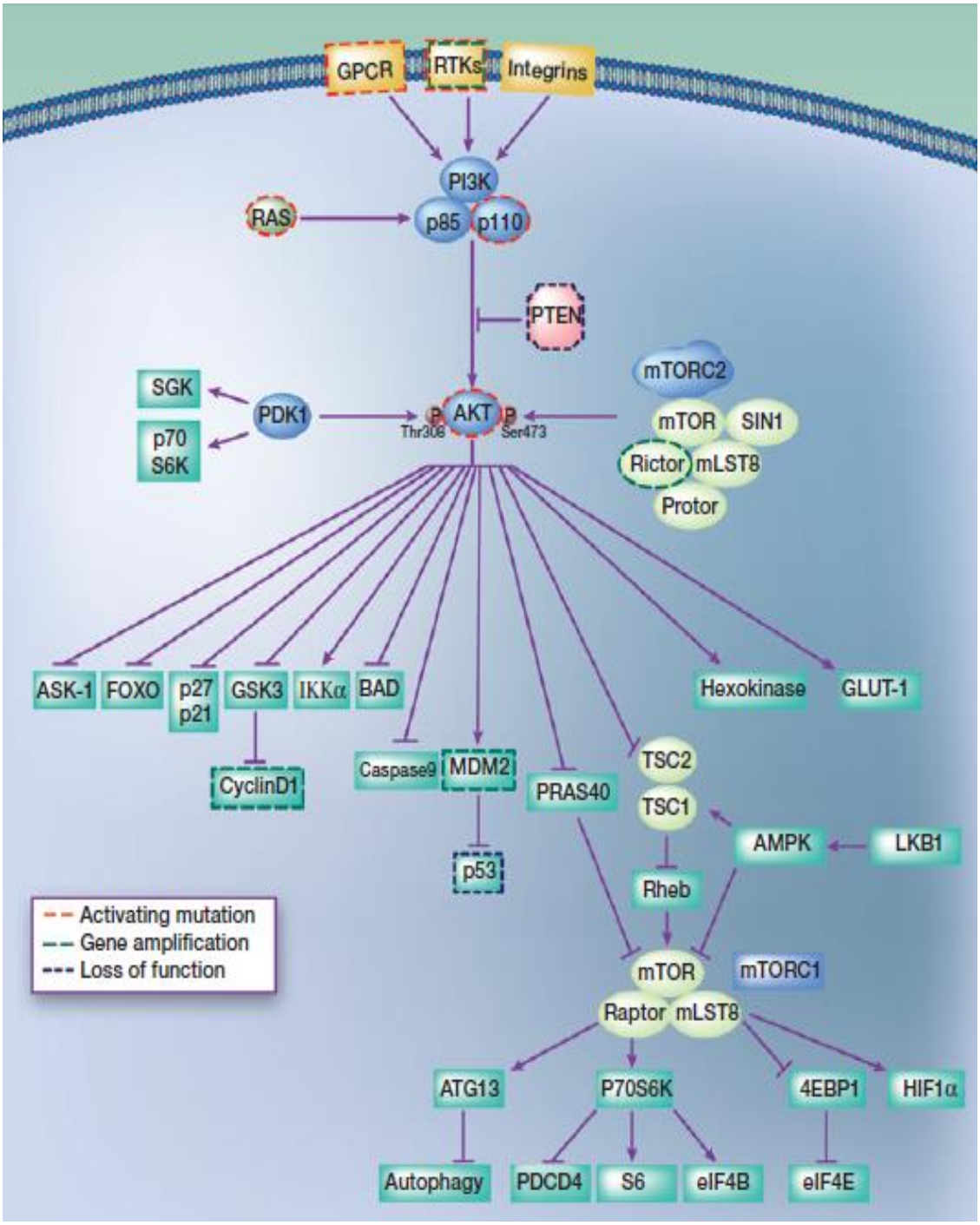

Figure 1.3.1: Regulators, effectors, and somatic alterations in the PI3K–AKT pathway in melanoma.[40]

1.3.1. The role of the PI3K network in Melanoma

PI3K/AKT signaling pathway in our particular field of research, is deregulated in a high proportion of melanomas, so by understanding how the molecules of this pathway intact and the mechanisms involved, will make possible the discovery of molecules in order to avoid melanoma progression. [43,44,46]

In Melanoma cells, AKT3 isoform has a higher expression than AKT1 and AKT2, since their activation is identified in about 60% of sporadic Melanomas.[26,49] AKT promotes a perfect environment to these cancerous cells, since it allows these cells to avoid apoptosis, to keep growing, proliferating, to resist to hypoxia (low levels of oxygen in the cells/tissues) and increasing cell metabolism, due to the fact of AKT is constitutively active. When AKT is activated it translocates from cell membrane to the cytoplasm (figure 1.3.1.1) and then to different cell places to target substrates.[47,50,51]

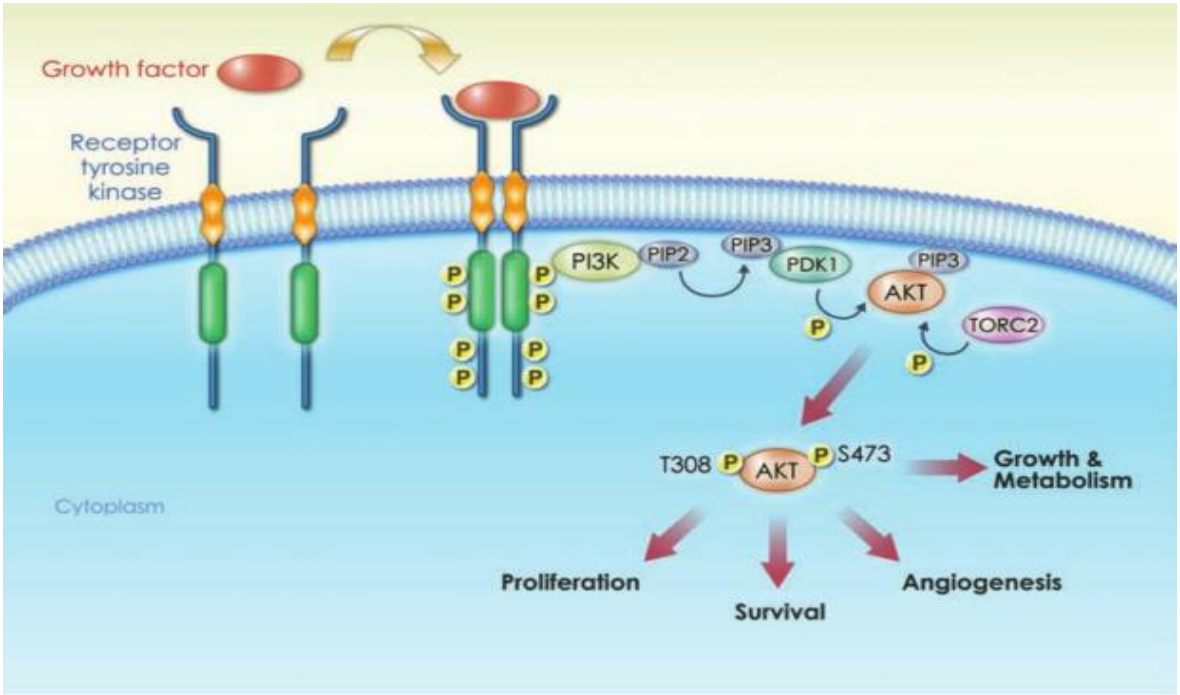

Figure 1.3.1.1: AKT activation through PDK1 and mTORC2 phosphorylation.[46]

1.3.2. Forkhead transcription factors

As mentioned previously, many effector proteins and substrates are phosphorylated by pAKT (active form of AKT) and Forkhead box O3a (FOXO3a) is one of them. FOXO3a is a member of the family FOXO which is a subgroup of Forkhead transcription factor family.[52] FOXO3a is a transcriptional factor located and active in the nucleus, responsible for the regulation of the expression of genes that are involved in many mechanisms such as *Cyclin-dependent kinase inhibitor p27* (*p27*), a cell cycle inhibitor; Fas ligand (*FasL)*, a ligand responsible for Fas-dependent cell-death; *Bcl-2 Like protein 11* (*Bcl-2L11*, also known as *BIM*), a Bcl-2 family member (which possess conserved Bcl-2 homology [BH] domains 34) responsible for pro-apoptotic signaling.[53]

FOXO3a is negatively regulated by AKT phosphorylation, leading FOXO3a to translocate from the nucleus to cytoplasm, becoming inactive and consequently represses transcription anti-apoptotic genes. In the cytoplasm FOXO3a will be polyubiquitinated and marked for proteosomal destruction (Figure 1.3.2.1).[53]

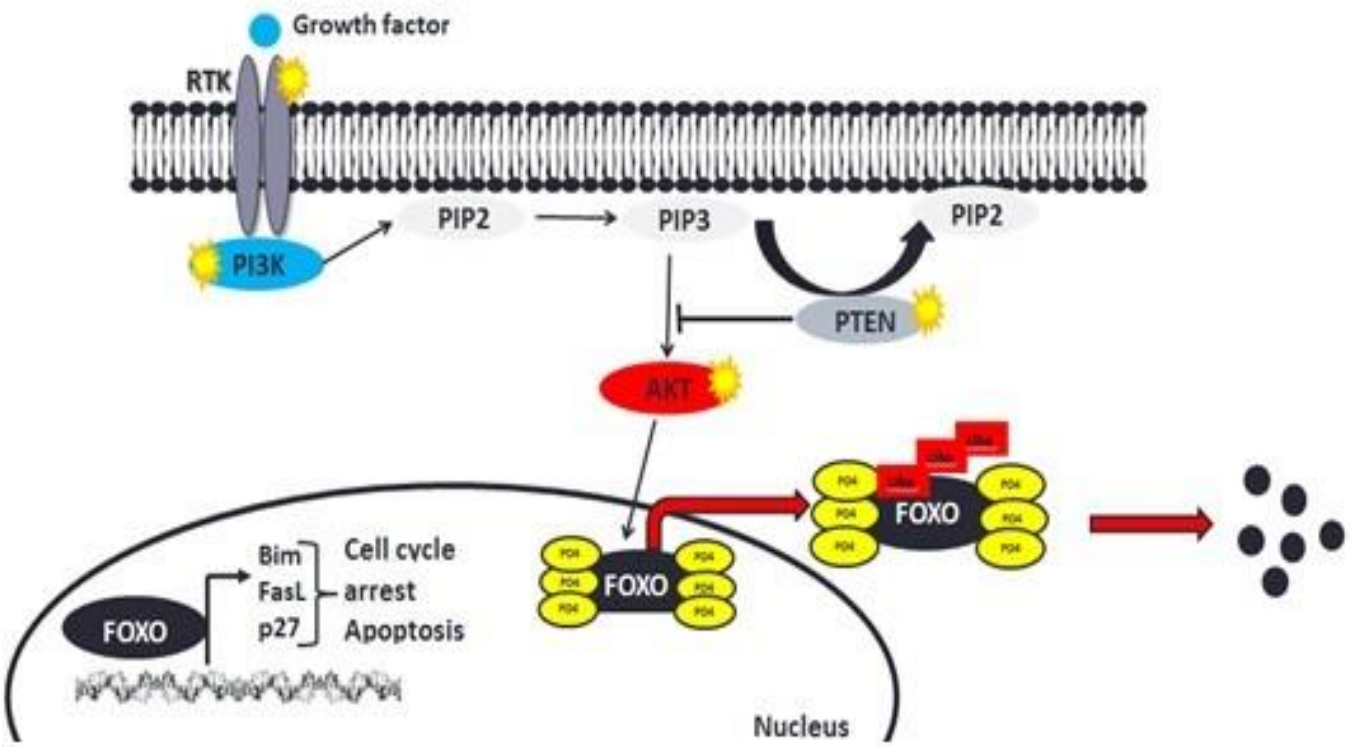

Figure 1.3.2.1: A) FOXO active in the nucleus, transcribing genes involved in cell cycle arrest and apoptosis (p27, FasL and Bim); **B)** FOXO regulation through AKT leads to their exportation from the nucleous to the cytoplasm.

1.4. Tribble2 homolog.

Being LDH the only biomarker approved by FDA for melanoma, there is a need to find new melanoma biomarkers and actually many are been tested in clinical trials, such as S-100 calcium binding protein B (S100B); tyrosinase (*TYR)*; human pituitary tumour-transforming gene 1(*hPTTG1); RGS1* (Regulator of G-protein signaling 1); heat shock protein 90 (HSP90); Secreted phosphoprotein-1 (*SPP1*); Inhibitor of growth family 3 (ING3); Nuclear receptor coactivator-3 (NCOA3). [54–60]

In addition to the discovery of these biomarkers, it was discovered Tribbles2 (TRIB2) correlates with melanoma. TRIB2 is the mammalian homolog of the drosophila gene *tribbles2* (was described firstly in this specie). In drosophila, this protein is involved in cell cycle control during morphogenesis and oogenesis, being involved as well as in regulation of cell proliferation, metabolism, motility and cancer development.[52]

TRIB2 is a kinase-like protein or a pseudokinase as it belongs to the TRIB pseudokinases family, composed for three members (TRIB1/TRIB2/TRIB3) whose catalytic residuesare not conserved, conditioning their catalytic function. Structurally, is divided in three distinct regions: N-terminal region, a serine-threonine kinase-like domain (KD, that has evident homology with serine-threonine kinases) and a C-terminal domain, a binding site for E3 ubiquitin ligases (Figure 1.4.1.).[52]

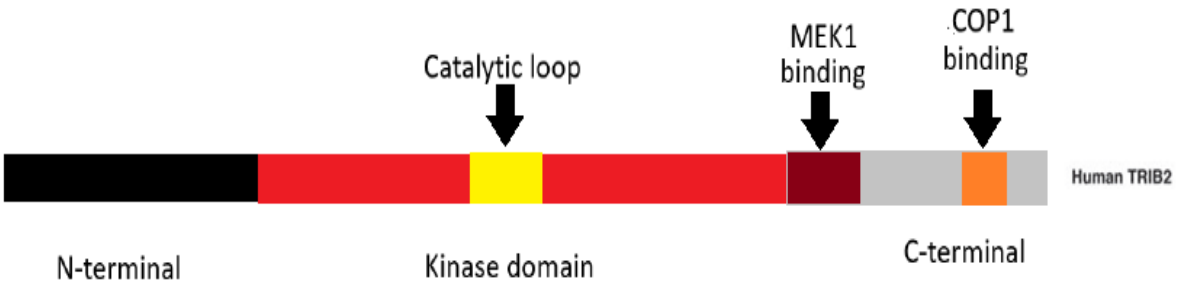

Figure 1.4.1: TRIB2 domains: N-terminal, kinase domain and C-terminal (two binding motifs, one for MEK1 and one for COP1).

The three TRIB members are all related with human cancers, having roles in signaling pathways such as MAPK and PI3K/AKT, interacting with many transcription factors and signaling molecules: MAPKK, ATF4, p65, COP1, CtIP, and AKT, being implicated in regulation of gene expression, apoptosis, cell activation and mitosis.[52,61,62]

TRIB2 is known to be overexpressed in human malignant melanoma and it is related with FOXO3a export from the nucleus to the cytoplasm, leading to the inhibition of FOXO3a tumor suppressor function. Although it is not known exactly how this mechanism works, it is believed that TRIB2 is a good research target to avoid melanoma cells growth and increase patients overall survival since is implicated in the maintenance of melanoma cells properties. [22,52]

TRIB2 is is involved in cell division and has been reported to be up-regulated in a subset of acute myeloid leukemia's and members of tribbles family have been also reported to interact and modulate the activity of signal transduction pathways, including the PI3K/AKT and the MAPK, our pathways of interest. It is important to say that TRIB2 has been implicated in the negative regulation of FOXO3a and this knowledge is important to understand the mechanism(s) of action of how TRIB2 mediates PI3K inhibitor resistance and the role of FOXO3a in this response. Thus, if we knew how this pathway works and their mechanisms are related to tumor progression and melanomas appearance, we could design strategies to treat cancer in earlier stages through target therapy.[35,61–63]

1.5. Hypothesis

It is known that PI3K/AKT is one of the most mutated pathways in human cancer, is also known that FOXO mediates the action of many anticancer drugs and that TRIB2 negatively regulates FOXO. Our research group has demonstrated that TRIB2 is not only a biomarker for melanoma diagnosis, but is also involved in this cancer type progression being our biggest aim to understand how is TRIB2 allowing melanoma patients to acquire resistance to chemotherapeutic drugs, in particular to PI3K inhibitors, like BEZ235. Through *in vivo* models with an oral administration to be a representative model to the patients in the clinic, and *ex vivo* samples from melanoma patients to see if there is a drug resistance caused by TRIB2 and knowing that AKT negatively regulates FOXO, to understand this mechanism of resistance, was hypothesized if does this happen because TRIB2 is in AKT network and if they interact. Since preliminary results from our group indicate that TRIB2 confers chemotherapeutic resistance, the central aim is to elucidate the mechanism(s) of this resistance and in particular how the TRIB2 protein responds following PI3K inhibition.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1. *In vitro* studies

2.1.1. Cell culture

For this project were used isogenic cell lines, SK-MEL-28 derived from a range of melanoma cell lines settled from skin primary tumor samples from a 51-year-old male patient (unknown ethnicity), acquired from American Type Culture collection (ATCC) and SK-MEL-28 TRIB2 shRNA; G-361 also acquired from ATCC was established from a malignant melanoma of a 31 year old male Caucasian. U2OS cell line (Human Bone Osteosarcoma Epithelial Cells) derived from a fifteen-year-old human female suffering from osteosarcoma; 293T shrna TRIB2 cell line originated from human renal cancer cells. All cell lines were defrosted from -80ºC and cultured within 35mm plates in 3ml DMEM (Dulbecco's Modified Eagle Medium Sigma, USA) medium with 10% heat inactivated FCS (Sigma) supplemented with Pen/Strep (Gibco) to be used for BEZ235 (10nM) drug treatment whereby were added 3ul of the drug to get a 1:1000 ratio. After the drug addition, all the cell cultures were kept overnight in a humidified incubator in 5% CO2 at 37ºC.First of all, the medium was aspirated and to detach the cells from the culture plates was used 1ml of Trypsin-EDTA 1x solution (SAFC Biosciences, UK). After trypsinization, the trypsin with cells was transferred to a falcon to centrifuge at 11rpm during 5 minutes.

The pellet was washed with 800ul of *Phosphate buffered* saline (PBS) 1x and shifted to an eppendorf for protein extraction.

2.1.2. Transfection

We used JetPrime transfection Protocol to transfect our cell lines with a dilution of 2 µg of our DNA into 200 µl jetPRIME® buffer, both posteriorly mixed by vortexing. After, was added 4 µl of jetPRIME®, vortexed for 10 seconds and then spun down briefly. 10 minutes room temperature incubation and thereafter it was added 200 µl transfection mix per plate. Lastly, the plates were slowly shaken and placed into the humidified incubator in 5% CO2 at 37ºC for 24h.

2.1.3. Protein fragment complementation assays (PCA)

5 µg of our each plasmid construct (ZIPV1, ZIPV2, AKT1, AKT2, TRIB2, TRIB3 or JIP1) were transfected (JetPRIME following the manufacturers protocol) into 293 cells. 24 hours post transfection, cells were stained for YFP imaging and visualized on a Leica TCS SP5 II confocal microscope. Duplicate studies were conducted and fluorescent signal intensity was measured 24 hours post dual construct transfection using a BD FACS Calibur scanner. The leucine zipper constructs ZIPV1 and ZIPV2 serve as positive control for these studies.

2.1.4. Co-Imunnoprecipitation (Co-IP)

After protein sample quantification using the Bradford assay followed by Nanodrop measure, were added PBS up to 500ul depending of the protein value to be loaded, plus 5ul of primary antibody and stayed overnight in the rotator at 4ºC. For each sample were needed 20ul of protein A/G-agarose beads (Sigma) which were first washed 2x with PBS. 1300 spin for 2min and only keep the pellet to be mixed with 20ul of PBS and the resuspended. Spin 1300 rpm for 2min and waste the PBS. Were added 20ul of beads to each sample, then resuspended and placed 1h on the rotator. Another 1300 rpm spin for 1min and after that were made one wash with 200ul of PBS. Spin 1300rpm 1min. Once again the pellet was kept and were added 40ul of laemmli, boiled for 5min at 98ºC and 20ul of each sample were used to be loaded in SDS-PAGE gel.

2.1.5. Immunofluorescence

In order to have samples to see in the Microscope, we need to prepare them previously after cell growing in the desired conditions (Table 2.4.1.1). First of all we put each lamella with respective cell line condition in a six well plate with PBS solution (10x).

Cell lines for Immunofluorescence	
293T	**Skmel-28**
293T EMPTY NT	Skmel-28 EMPTY NT
293T EMPTY BEZ	Skmel-28 EMPTY BEZ
239T TRIB2 NT	Skmel-28 TRIB2 NT
293T TRIB2 BEZ	Skmel-28 TRIB2 BEZ

Table 2.4.1.1: Cell lines for Immunofluorescence.

Then, we transferred the lamellas with the cells side face up to a new six well plate wash 3x with PBS (1x) and after that see in the Microscope if there is a good cell confluence. If the cells look fine, we could proceed with the dual staining protocol. We cover a western blot glass with parafilm paper and put four equal drops (more or less 200ul) of 1:30 block solution (900ul PBS (1x) + 30ul block solution (stock))and after that lay down the lamellas with the cells side face down to be in contact with the solution, one per drop. Incubate in the Humified Chamber for 1h, 37ºC. (Cell culture incubater). We took the lamellas from the Humified Chamber and put them with the cells face up in the parafilm paper, cleaned the block solution from the glass with parafilm and prepared 1:500 1ºAB for 1ul p-AKT (Santa Cruz) and 1ul TRIB2 (Santa Cruz) in PBS (1x) and put four drops of that mix in the glass with parafilm. Then, put the lamellas face down in contact with the 1ºAB and incubate in the Humified Chamber for 1h, 37ºC.

Took lamellas from the Humified Chamber and put them with the cells face up in the six plate wells and wash them 3x with PBS (1x). Clean the 1ºAB from the glass with parafilm. We prepared 1:500 2ºAB for 1ul α-mouse (against TRIB2) (1ul) and 1ul α-rabbit (against p-AKT) in PBS (1x) and put four drops of that mix in the glass with parafilm. Then, put the lamellas face down in contact with the 2ºAB. Incubate in the Humified Chamber for 1h, 37ºC. Took lamellas from the Humified Chamber and put them with the cells face up in the six plate wells and wash them 3x with PBS (1x). Clean the 2ºAB from the glass with parafilm. For each condition/lamella, one lamina where was placed one drop of ClearMount™ Mounting Solution, INVITROGEN™. Then we put the cells face down and incubate in a 60ºC chamber for 30 minutes. After the 30 minutes, we've saved them there in the dark, very important to not leave them in the light! The Microscope used for image visualization were Axio Imagen Z2.

2.2. *In vivo* studies

2.2.1. BEZ235 treatment of Xenografted mice.

This work was performed with 21 animals (table 2.2.1.1), which were divided into two initial groups to be injected with different isogenic cell lines, in order to form tumors: 14 mice injected with $2x10^6$ 293T-empty cells into the right flank of matched NOD/SCID mice and at the same time, the other 7 were injected with $2x10^6$ 293T-TRIB2 cells into the right flank of matched NOD/SCID mice. These animals were monitored daily for tumour development, and once tumors were visible, they were measured prior to *in vivo* treatment. The 14 mice containing 293T-empty tumors were then separated into two groups of 7 mice prior to the BEZ235 *in vivo* study commencing, having after one group with endogenous levels of TRIB2 and no drug treatment, 293T Empty Vehicle (n=7); other group with endogenous levels of TRIB2 and BEZ235 drug treatment, 293T Empty BEZ235 (n=7) and finally the last group with overexpression of TRIB2 plus BEZ235 drug treatment, 293T TRIB2 BEZ235 (n=7). Drug aliquots were prepared for one or two days (since the compound is stable for only two days), being the average mice weight 25g, and the concentration to be administrated to the mice 30mg/Kg, the calculation is 10,5mg of BEZ235 dissolved in 2,1ml of 20% NMP/80% PEG 300 for one day. This compound must be protected from the light and be stored at 4ºC, the NMP is added to BEZ235 and must stir for 30 min at 40ºC (protected from light), PEG300 as well must stir but for 60 min at 40ºC (protected from light) and then keep at RT to be used in the drug administration.

The animals had a daily tumor measurement and daily drug treatment with BEZ235: 30mg/kg, orally administrated (syringes provided by Prof. Inês Araújo) but with the mice anaesthetized/sedated with isoflurane (Cell line injection to the mice, tumour measures, and drug administration was made by Dra. Patrícia Madureira).

Xenografted mice (n = 21)		
Groups	**Number of animals**	**Number of injected cells**
293T Empty Vehicle	n=7	$2x10^6$
293T Empty BEZ235	n=7	$2x10^6$
293T TRIB2 BEZ235	n=7	$2x10^6$

Table 2.2.1.1. Xenografted mice groups.

In the end of the study, the animals were sacrificed after been exposed with CO2 (by Dr. Richard Hill), and the organs (spleen and tumors) were surgically removed (by Dra. Patrícia Madureira) for protein and RNA extraction.

2.2.2. Western Blotting

We've collected the following organs for protein extraction and quantification: the liver, the spleen and the tumors. The organs homogenization was made in a manual potter homogenizer (Sigma) with 200ul-500ul (depending on the organ size) of RIPA Buffer (Tris-HCL ph 7.4, NaCl, 10% Nonidet P-40, 10% sodium deoxycholate, 100 mM EDTA, PIC, 200 mM NA-F, 100mM Na3VO4 and protease inhibitors cocktail) for the tumors and livers or 300ul for the spleens. Then, the homogeneized samples incubated for 30 minutes on ice. The lysed samples were spun at 13000 rpm and the supernatant transferred to a new eppendorf for posterior protein quantification. Extracted proteins were stored at -80ºC until use. For protein quantification to determine their concentrations we used the BCA assay (Thermus Scientific Protein Assay Kit). Then, to be quantified we used 2ul of that mix to be read in the NanoDrop 2000/2000c (ThermoScientific) UV-Vis Spectrophotometer.

Our extracted protein samples were diluted into 2x lammeli loading buffer (containing β-mercaptoethanol) and heat for 5 minutes at 95ºC to be loaded into 10% SDS-PAGE gels with the NZYBlue Protein marker. Once the proteins were separated, the gel was transferred to a nitrocellulose membrane (Amersham Hybond ECL) Nitrocellulose Membrane through TRANS-BLOT® SEMI-DRY TRANFER CELL (BioRad) system for 1h10min. The membranes were blocked with skimmed milk powder (exception when we used p-MDM2 Antibody, in which case BSA was used) and placed on a shaker for 1 hour to prevent the binding of non-specific antibodies and probed with many primary antibodies (table 2.2.2.1) overnight at 4ºC.

Primary antibodies	Supplementary information
Total AKT	C-20; sc-1618; Goat; Santa Cruz Biotechnology
p-AKT	Ser 473; sc-7985; Rabbit; Santa Cruz Biotechnology
Actin	I-19; sc-1616; Goat; Santa Cruz Biotechnology
p53	DO-1; sc-126; Mouse; Santa Cruz Biotechnology
Total MDM2	C-18; sc-812; Rabbit;Santa Cruz Biotechnology
p-MDM2	S166; 3521S; Rabbit; Cell Signalling Technology
TRIB2	Custom, Rabbit, CNIO Madrid
Total FOXO	N-16; sc-9813; Goat; Santa Cruz Biotechnology
p-FOXO	Ser253; sc-101683; Rabbit; Santa Cruz Biotechnology
BIM	H-191; sc-11425; Rabbit; Santa Cruz Biotechnology
p21	C-19; sc-817; Mouse; Santa Cruz Biotechnology
p70 S6 Kinase α	C-18; sc-230; Goat; Santa Cruz Biotechnology
Fas-L	C-178; sc-6237; Rabbit; Santa Cruz Biotechnology
Cleaved Caspase-3	h176; sc-22171; Rabbit; Santa Cruz Biotechnology

Table 2.2.2.1: Primary antibodies.

After incubation, were washed for 10 minutes with TBS 0.1% tween$_{20}$ (3x) and then, probed it with matched secondary antibodies for 1 hour at room temperature in the shaker.

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

Table 2.2.2.1: Secondary antibodies.

Membranes were once again washed for 10 minutes with TBS 0.1% tween$_{20}$ (3x) and perform an image visualization, the membranes were placed in a ECL solution which is a mix of a Buffer 1 (5ml 1M Tris pH 8.5 100nM, 0.5ml luminol 2.5nM, 222.3ul p-coumaric-acid 360uM, 45.5ml H_2O) and Buffer 2 (4ml 1M Tris pH 8.5 100nM, 46ml H2O, 31ul 30% H_2O_2) and then achieved through a Molecular Imager® ChemiDoc™ *XRS* System (BioRad).

2.2.3. RNA Extraction/cDNA Synthesis

RNA extraction and isolation was made from the tissue samples of the animals and to each were added 500ul of TRI Reagent® (Sigma). After 5 minutes of room temperature incubation, was added 100ul of chloroform for each sample and we've mixed them manually up and down and then centrifuged at 12000xg for 15 min at 4ºC, resulting in 3 visually and distinct phases: in the bottom of the Eppendorf the organic phase which contains protein, the interphase with the DNA and finally our phase of interest in the top, containing RNA, the aqueous phase. We just collected the aqueous phase, without taking any interphase to avoid contamination with DNA, transferred to a fresh Eppendorf adding 250ul of isopropanol to each sample, incubated for 10 minutes at room temperature, and centrifuged at 12000xg for 10 minutes at 4ºC, obtaining a white pellet of RNA. The supernatant was discarded and the pellet washed with 500ul of 75% ethanol. Next step was a centrifugation at 7500xg for 5 minutes at 4ºC, thereon the ethanol was discarded and the pellets air dry around 5 minutes. Finally we ressuspended the RNA pellets in RNase-free water (DEPC-treated water) aiming to be used for cDNA synthesis. To obtain cDNA we used NZY First-strand cDNA Synthesis Kit (NZYTech, Portugal), wherein the protocol is described the following steps: on ice, add for each sample 10ul NZYRT 2x MasterMix, 2ul EnzymeMix, 6ul of DEPC-treated water and 2ul of RNA. After slightly mixed, were used the BioRad C1000 Therm machine with the Programe BioRad manager where the samples incubated for 10 minutes at 25ºC and then at 50ºC for 30 minutes. It was made the inactivation of the synthesis reaction through samples heating at 85ºC for 5 minutes and then placed on ice to add 1ul of NZY RNAse H (E.coli) for the last step at 37ºC for 20 minutes.

2.2.4. Statistical studies with two-tailed ANOVA analysis

ANOVA analysis could be either one-way/one-tailed if we want to evaluate just one categorical factor, or could be made a two-way/two tailed analysis if we want to evaluate two categorical factors.

Statistical analysis were done using two-tailed ANOVA analysis, once the goal were to see if there was a significant difference in the weight and length of the spleens among the different animals groups (293T Empty Vehicle, 293T Empty BEZ235, 293T TRIB2 BEZ235) and if that could be related to the drug treatment. For that, graphs were made based on the average of the values obtained with the spleens measures. Statistical analysis results were treated using unpaired two-tailed student t-test (Graph pad PRISM). The *P value* generated for each comparison is used to show if the results are or not significant. The symbols used in order to see if there is or not a significant difference, were based in the following values: * P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤ 0.0001. When P≥0,05 there is no significant difference, where N/S is indicated and in this case *P value* is not significant. For example, if the value is P ≤ 0,01, the result is more statically significant comparatively with a P≤0.05.

2.3. *Ex Vivo*: Patient Tissue Samples

Dr Selma Ugurel (Julius-Maximilians- Universität Würzburg, Germany) provided to our group normal and melanoma tissue samples (represented in the table 2.3.1) being cryo-preserved until processing. These samples were sectioned for Immunohistochemistry (at Faro Hospital) and part of the tissue was used for protein and RNA analysis using TRI-Reagent (Sigma). This tissue samples were surgically excised prior to first-line treatment from patients with stage IV metastatic lesions.

Melanoma samples			Normal samples
Progressive Response	**Partial Response**	**Complete response**	
CSM027	CSM002	CSM006	1440
CSM094	CSM099	CSM066	1425
CSM038	CSM178	CSM068	1274
CSM143	CSM200	CSM203	1408
CSM111	CSM214	CSM209	1474
CSM213			1489
CSM105			1428
CSM060			1508
CSM057			
CSM108			

Table 2.3.1: Melanoma and Normal tissue samples used in this work.

CHAPTER 3

RESULTS

3. Results

3.1. Increased TRIB2 protein expression confers significant resistance to PI3K/mTORC1 inhibitors *in vivo*.

Previous data from our group (published and unpublished) indicated that TRIB2 is a negative regulator of the forkhead transcription factor FOXO3a.[52] As a critical cellular protein that mediates the transcription of several pro-apoptotic and cell cycle arrest genes, we hypothesized that TRIB2 could confer resistance to many standard front line chemotherapeutics as well as a number of novel PI3K inhibitors, including BEZ235. A large *in vitro* screen using isogenic cell lines generated within our group confirmed this hypothesis. Critically, it was unknown if high TRIB2 expression could confer resistance to the PI3K/mTORC1 inhibitor BEZ235 *in vivo*. This is a crucial as patient tumors do not present as a flat monolayer and rather, are large, tumour masses that contain leaky blood vasculature, hypoxic regions and are in general, highly inaccessible for direct exposure to these therapeutics.

To address this question, we first grew low passage isogenic 293T cells with stable TRIB2 over expression. Using an immunoblotting approach, we confirmed that our cell lines had matched TRIB2 protein expression (Figure 3.1.1). We collected and ran 100 µg of total cell lysate (RIPA lysis buffer) on a 10% SDS-PAGE gel.

This immunoblot analysis also confirmed that our shRNA constructs against TRIB2 were effective (although opted against using these in our *in vivo* study) and rather, for our *in vivo* studies, compared the endogenous 293-GFP and 293T-TRIB2 cell lines.

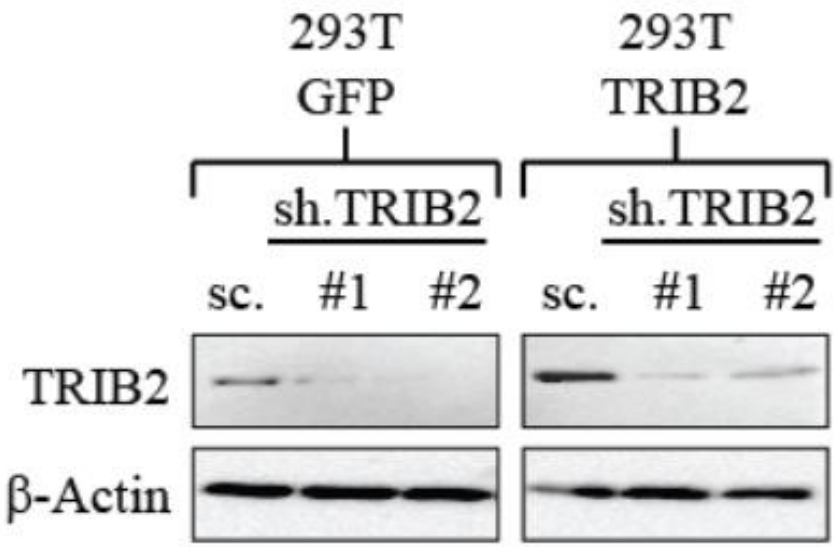

Figure 3.1.1: Representative immunoblot analysis of TRIB2 status in our 293T isogenic cell lines. Stable scramble shRNA sequences sc.) in the lanes indicated with no knock down of target. Our two shRNA constructs (#1 and #2) knock TRIB2 down over 90%. β-Actin is used as a loading control for immunoblot analysis.

Having confirmed the status of our 293T cell lines, we questioned if 1), TRIB2 status affected the rate of *in vivo* tumour growth and 2). If TRIB2 status correlated with *in vivo* tumour response following BEZ235 treatment. To address question 1, we injected $2x10^6$ 293T-TRIB2 cells into the right flank of 7 age and sex matched *NOD/SCID* mice. At the same time we injected $2x10^6$ 293T-empty cells into the right flank of 14 matched NOD/SCID mice. These mice were monitored daily for tumour development. Once tumors were noted they were measured prior to *in vivo* treatment. The 14 mice containing 293T-empty tumors were separated into two groups of 7 mice prior to the BEZ235 *in vivo* study commencing.

Palpable tumors were present in each mouse (100% tumour take) and at the time the study as initiated, there was no difference between any of the test groups (Figure 3.1.2, time post-treatment day 1). When the average tumour volumes were calculated at day 1 and the averages compared, we note a *P* value of 0.915 indicating that there is no significant difference at all between the *in vivo* tumour growth rates prior to BEZ235 treatment. Strikingly, we note that following the daily oral administration of BEZ235, 293T-empty cells start to show reduced tumour burden as early as day 5 post-treatment.

In contrast, 293T-TRIB2 tumour bearing mice treated with an identical dose of BEZ235 (30mg/kg) do not display any delay or change in tumour growth. The 293T-TRIB2 growth plot was very similar to the profile noted for the 293T-empty-vehicle only treatment group.

At the completion of the *in vivo* study, we noted that there was a highly significant difference between the treated 293T-empty BEZ235 treated group versus the matched 293T-TRIB2 BEZ235 treated group (*P* value = 0.0299) and the 293T-Empty BEZ235 treated group versus the 293T-empty vehicle treatment only group (*P* value = 0.0062) (Figure 3.1.2).

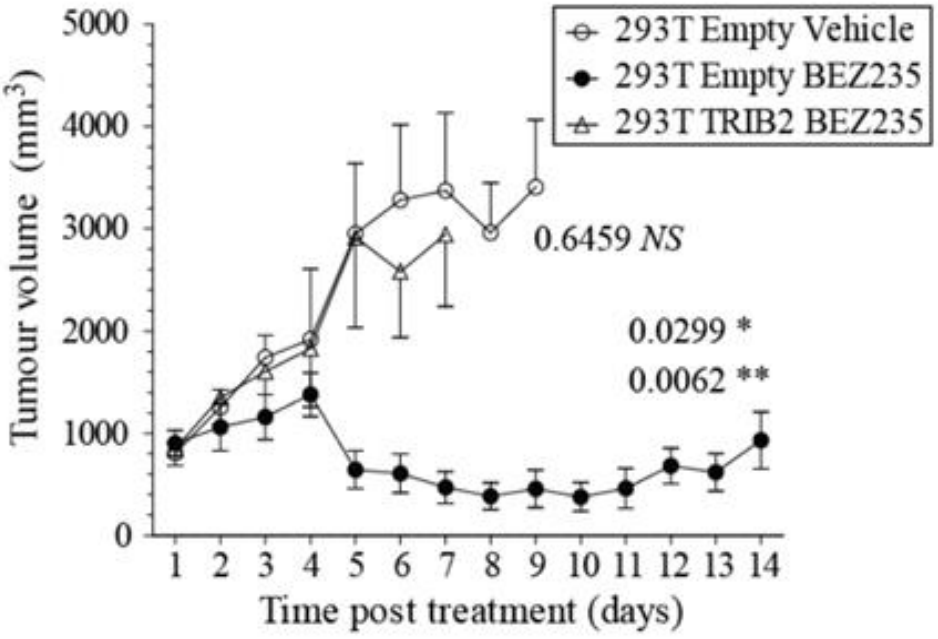

Figure 3.1.2. TRIB2 conferred resistance to PI3K inhibitors *in vivo*. TRIB2 overexpressing 293T tumors (n = 7) show little to no response to daily administration of BEZ235 compared to isogenic lines with endogenous (low, n =7) TRIB2 expression. (Tumors measures done by Dra. Patrícia Madureira)

In contrast, the 293T-TRIB2 BEZ235 treated group versus the 293T-empty vehicle only treatment, had no statistically significant difference (*P* value = 0.6459). From this study, we can conclude that BEZ235 treatment drives significant tumour reduction only in 293T tumors with endogenous (low) TRIB2 expression. When TRIB2 protein level is significantly increased, there is little to no effect following daily BEZ235 administration.

Based on this data, we questioned if the significantly reduced tumour burden correlated with an equivalent difference in survival. From this study, Kaplan-Meier survival plots were generated and each treatment group was evaluated (Figure 3.1.3).

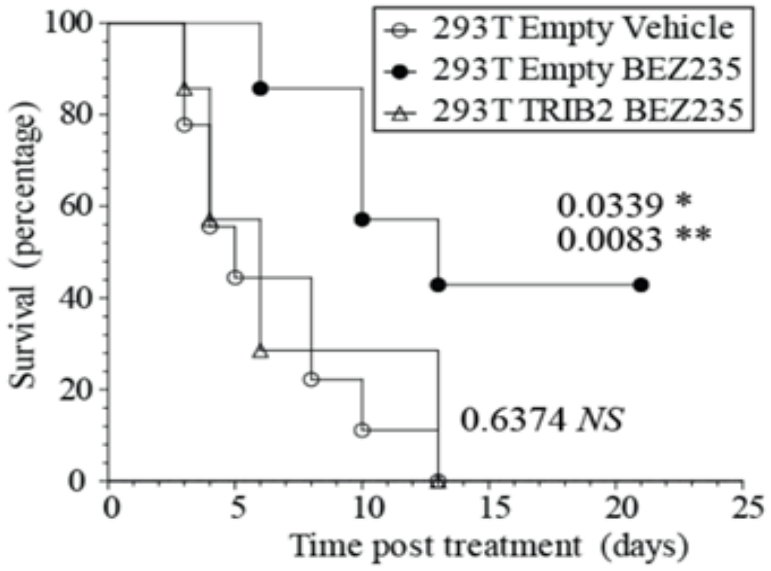

Figure 3.1.3. Elevated TRIB2 expression significantly reduced the survival of 293T tumour bearing mice. Kaplan-Meier analysis of isogenic TRIB2 cell lines grow subcutaneously in *NOD/Scid* mice treated with vehicle (n = 7), or BEZ235 (n = 7). The presence of TRIB2 significantly reduced survival (log rank *P* = 0.033) when treated daily with BEZ235.

As we would have predicted based on our *in vivo* tumour measurement data, we note that there is a highly significant difference in overall survival. The 293T-empty treated mice compared to 293T-TRIB2 BEZ235 treated mice show a highly significant survival difference (*P* value = 0.0339) and when the 293T-empty BEZ235 group is compared to the 293T-empty vehicle only group, this difference is even more pronounced (*P* = 0.0083). In contrast, when the 293T-TRIB2 BEZ235 group is compared to the 293T-empty vehicle only group, there is no statistically significant difference (*P* = 0.6374). Based on these findings, we conclude that the over expression of TRIB2 significantly reduces the effectiveness of BEZ235 following daily oral administration. This was not related to the delivery agent at all (as 293T-empty vehicle only treated tumors grew at the same rate as the 293T-TRIB2 BEZ235 treated tumors) and was not a result of the oral treatment procedure itself as every mouse underwent the same procedure (including volume of drug/vehicle directly administered).

3.2. *In vitro* cell characteristics are retained in our *in vivo* model.

Having noted that high TRIB2 expression significantly increased *in vivo* resistance to daily BEZ235 treatment, we questioned if the *in vitro* differences between our isogenic cell lines were retained within the 3-dimensional architecture of a solid tumour. As each mouse was sacrificed (either due to tumour burden or the study being concluded [as established in our ethics documentation]) we surgically extracted the tumour (if present) and examined all of the internal organs (discussed in 4.3).

Tumors were extracted from the mice in each group and from this we extracted RNA (TRI-Reagent, SIGMA) and total protein (RIPA lysis buffer) as described in materials and methods. Following the extraction of the total protein, we conducted immunoblot analysis (Figure 3.2.1).

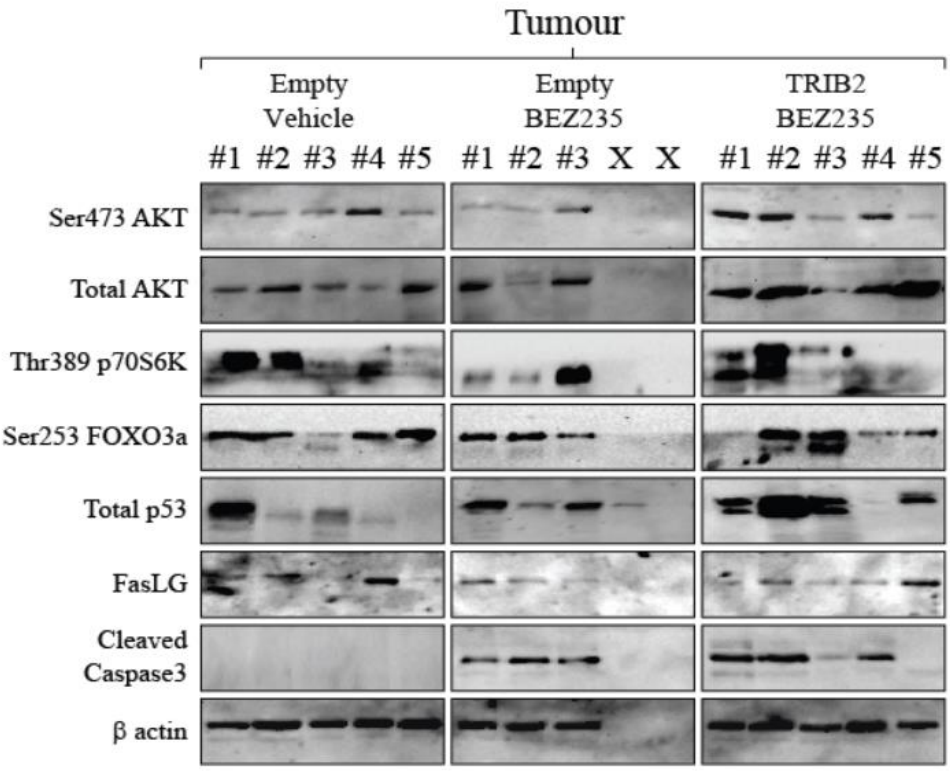

Figure 3.2.1: Representative immunoblot analysis from *in vivo* treated/mock treated isogenic 293T-Empty/TRIB2 tumors. Individual tumors are labelled (1-5), X indicates that the mouse was alive at the end of the study however there was no detectable tumour. 50-150 µg total protein lysate was loaded per lane (depending on target protein). β-Actin is used as a loading control for immunoblot analysis.

We note that there was a significant difference in the level of activated AKT, specifically pSer473-AKT within the 293T-TRIB2 BEZ235 treated tumors compared to the 293T-empty BEZ235 treated tumors. This was also observed for total AKT where 293T-TRIB2 tumors had extremely high levels of this protein compared to either 293T-empty BEZ235 or 293T-empty vehicle only treatment groups.

Consistent with this observation we note that BEZ235 treated 293T-TRIB2 tumors show higher pThr389 p70S6K compared to BEZ235 treated 293T-empty tumors and interestingly, 293T-empty vehicle only treated tumors retained very high levels of pThr389 p70S6K and pSer473-AKT compared to the 293T-empty cells treated with BEZ235 indicating that this drug was reaching the tumors and inhibiting PI3K/mTORC. We examined the downstream signalling of the PI3K pathway and evaluated the expression of the known AKT target FOXO3a. We were unsuccessful in our immunoblotting attempts to visualize total FOXO3a however, we did detect the critical pSer253-FOXO3a. In 293T-empty BEZ235 treated tumors, we see a significantly lower level of pSer253-FOXO3a compared to either 293T-TRIB2 BEZ235 or 293T-empty vehicle only treated tumors suggesting again that in 293T-empty BEZ235 tumors, there is significant inhibition of PI3K/mTORC signalling.

Next we questioned if there was a difference regarding FOXO3a-dependent target gene expression. As the pro-apoptotic *FasLG* is regulated by FOXO3a, we investigated the protein level of FasLG. Interestingly, our immunoblot analysis showed that in general there was little difference between any of our treatment groups. This does not exclude the possibility that while the total level of FasLG was similar, that the activity of this death receptor was the same (this is currently under investigation in the laboratory). A critical aspect to consider is that in the 293T-empty BEZ235 treated mice, there was a number of mice that displayed complete tumour regression (indicated in the blots as X where no protein could be loaded). Our *in vitro* studies indicated that TRIB2 could confer resistance by significantly reducing the percentage of Sub-G_1 cells following BEZ235 exposure (currently under review). Based on this data, we questioned if, *in vivo*, there was a difference in a critical apoptosis marker, cleaved caspase3.

We note that in both BEZ235 treatment groups, irrespective of TRIB2 status, that there was significant cleavage of caspase3 that was not detectable in 293T-empty vehicle only treated tumors. Interestingly, there seems to be more caspase3 cleavage in 293T-TRIB2 BEZ235 treated tumors, however one must consider a number of caveats from this observation.

First, the actual tumour mass for each tumour was significantly higher in the 293T-TRIB2 BEZ235 group suggesting that while there is apoptosis occurring, it is insufficient within this background (high TRIB2) to mediate sufficient tumour destruction. In contrast, within the 293T-empty genetic background, the level of apoptosis is sufficient to drive potent tumour-reduction.

We also evaluated the protein expression of the tumour suppressor p53 and note that in 293T-TRIB2 BEZ treated tumors that there was a very high level of total p53 compared to either 293T-empty vehicle treated tumors or 293T-empty BEZ235 treated tumors. A possible reason for this could be that the large tumour mass is generating large hypoxic regions where p53 can accumulate while there is also clearly detectable apoptosis and this is capable of activating p53, driving more apoptosis in a feedback-loop.[64]

Our immunoblot data indicates that after the oral administration of BEZ235, the drug was reaching both 293T-empty and 293T-TRIB2 tumour. However BEZ235 was only mediating an effect in 293T-empty tumors. This raised the question as to whether there was any noted side-effects following treatment in the tumour bearing mice or if any non-tumour organs were effected post-BEZ235 treatment. When the tumors were excised, we carefully examined all the organs within the mice. We noted no adverse effect in the oesophagus, stomach, liver, heart, lung or kidneys of BEZ235 treated mice (data not shown). Strikingly, we noted a highly adverse effect on the spleen in treated mice. We carefully excised the spleens (at the same time we removed the tumour) and measured them (Figure 3.2.2).

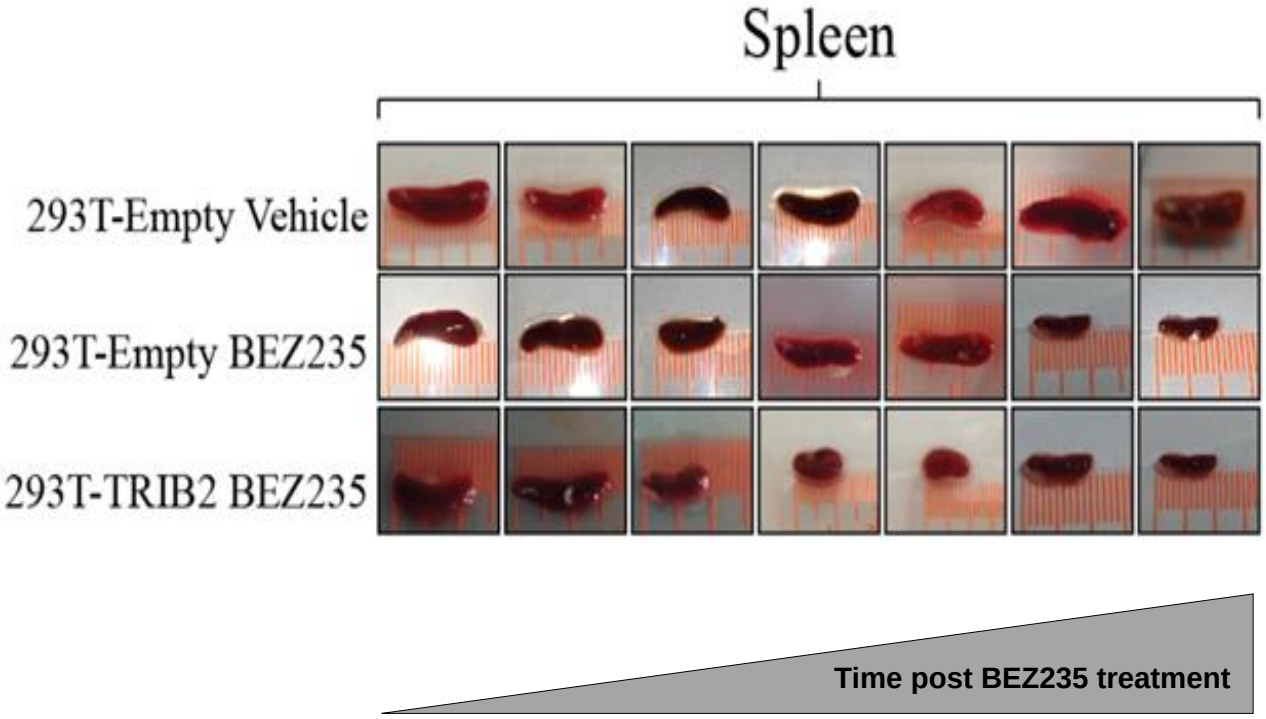

Figure 3.2.2. Following the daily administration of BEZ235, in the end of the study the animals were sacrificed and when the tumors were surgically removed, it was found a significant and an unexpected change in spleen morphology which aroused interest and for that reason surgically removed as well and measured. This effect was more pronounced the longer the mice were exposed to BEZ235.

Irrespective of the tumour background (endogenous or exogenous TRIB2 protein expression), BEZ235 treatment induced a very significant morphology change. The BEZ235 treated mice show a highly significant reduction in spleen length that was dependent on the length of time that the animals were treated with BEZ235 (Figure 3.2.3).

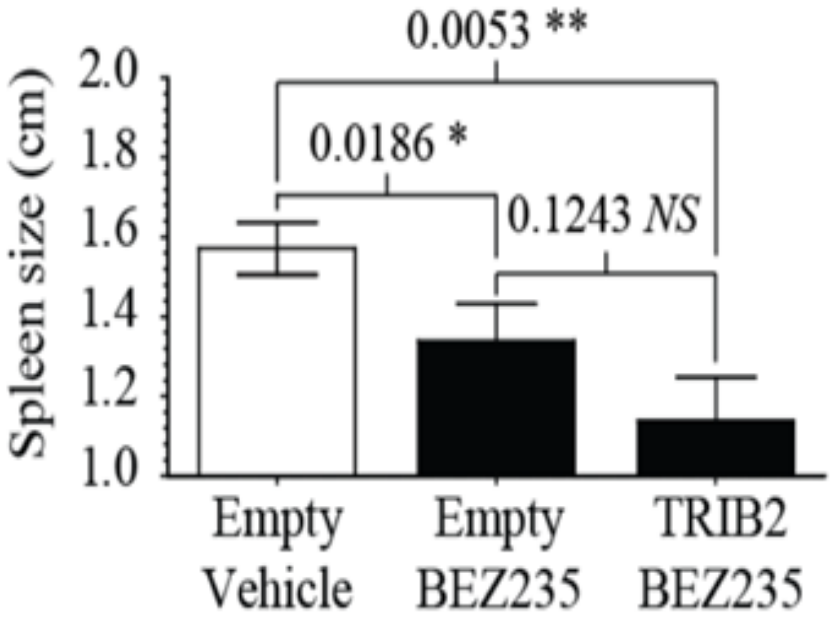

Figure 3.2.3: Following the daily administration of BEZ235, there was a significant change in spleen size. This effect was more pronounced the longer the mice were exposed to BEZ235. *P* values indicated were generated by two-tailed ANOVA analysis. (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

In addition to the noticeable length change, we also measured the weight of each spleen from the respective treatment groups (Figure 3.2.4). Consistent with significant length reducition following BEZ235 treatment, we note a concomitant reduction in spleen weight in 239T-empty BEZ235 treated mice. This significant difference was not observed in 293T-TRIB2 BEZ235 treated mice. The high *P* value (0.7058) is not due to a different dose of BEZ235 but rather the fact that 293T-TRIB2 tumors did not respond to BEZ235 and as a consequence, the mice were sacrificed due to tumour burden, thus the did not have sustained daily exposure to BEZ235 unlike the majority of the 293T-empty tumour bearing mice who showed a noticeable response in almost all treated animals.

This can be noted in the 293T-TRIB2 BEZ treated spleens towards the right hand side of figure 3.2.2 where the size difference is noticeable compared to those on the left of the same figure.

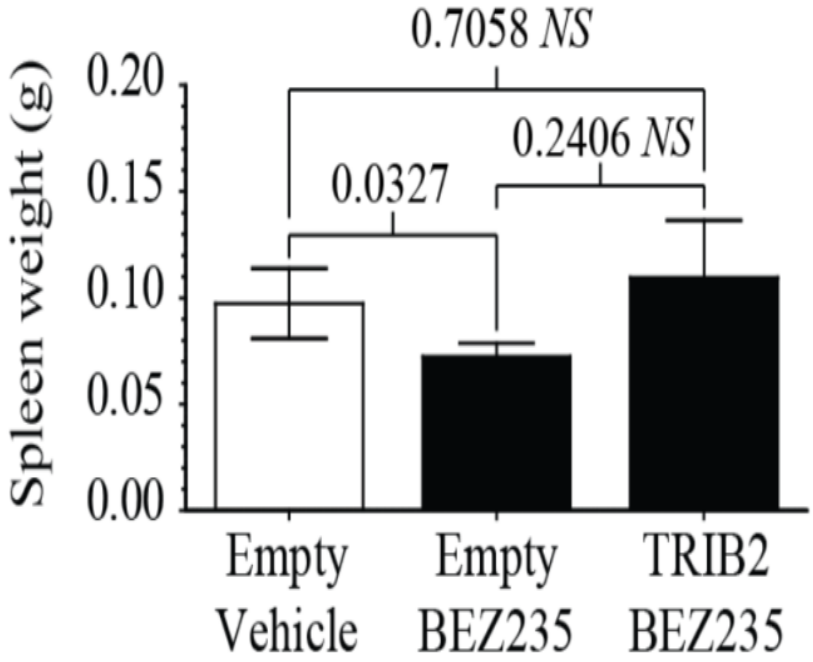

Figure 3.2.4.: Following the daily administration of BEZ235, there was a significant change in spleen weight in 239T-empty treated mice. This effect was more pronounced the longer the mice were exposed to BEZ235. *P* values indicated were generated by two-tailed ANOVA analysis.

3.3. The TRIB2 and AKT proteins interact both *in vitro* and in *ex vivo* clinical samples.

Our previous *in vitro* and our novel *in vivo* data indicate that TRIB2 status correlates with AKT activation. High TRIB2 cell lines display significantly elevated levels of pSer473-AKT and as a consequence of this have repressed FOXO3a activity (manuscript under review). This raised the interesting question as to whether the TRIB2 protein and AKT could interact. To address this question, we applied a number of methodologies including confocal microscopy, co-immunoprecipitation and in collaboration with Dr E. Kiss-Toth conducted protein fragment complementation assays (PCA)[65–67] Our PCA studies showed a strong interaction between TRIB2 and AKT1 (Figure 3.3.1).

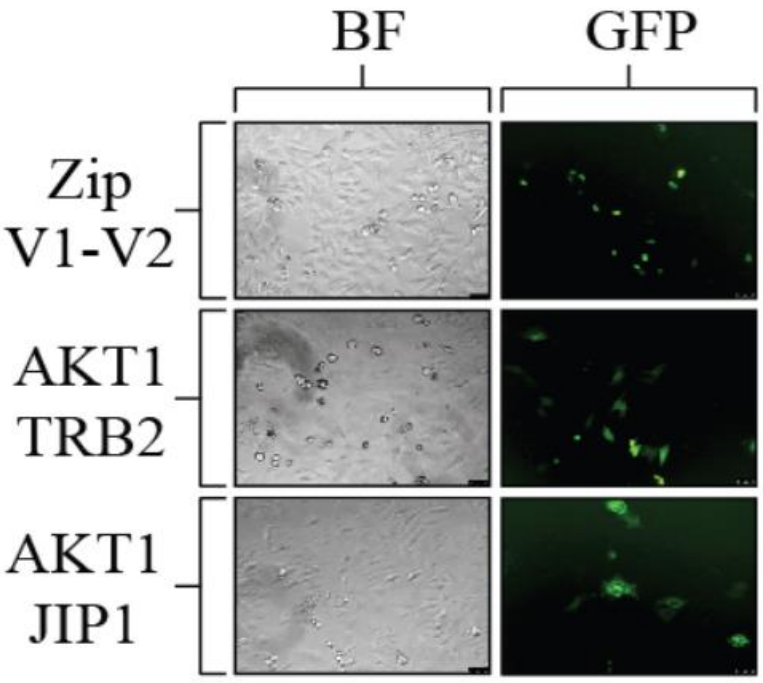

Figure 3.3.1. Representative yellow fluorescent protein (YFP) complementation microscopy analysis assay for the assessment of the interaction between AKT1/2 and TRIB2. The leucine zipper Venus1 and Venus2 (ZIPV1-V2) constructs were used as a positive control. BF indicates bright field, GFP indicates green fluorescent protein (with a very similar excitation spectrum as YFP for image acquisition). (generated in Sheffield by Prof. Kiss-Toth)

As a positive control, we included the protein JIP1 in this study as this is an established, known AKT interacting protein.[68] Having observed this interaction, we next questioned the intensity of this interaction, asking if the interaction between TRIB2/AKT was higher, lower or similar to JIP1. Using FACS analysis (for yellow fluorescent protein [YFP]), the intensity of the complemented protein complex was evaluated (Figure 3.3.2). Strikingly, we observe that the interaction between AKT and TRIB2 was as intense as AKT/JIP1.

Having noted that TRIB2 and AKT interact and that this interaction was as strong as the known AKT/JIP1 interaction, we questioned if we could detect this interaction in our *in vitro* cell lines without the requirement of conducting PCA.

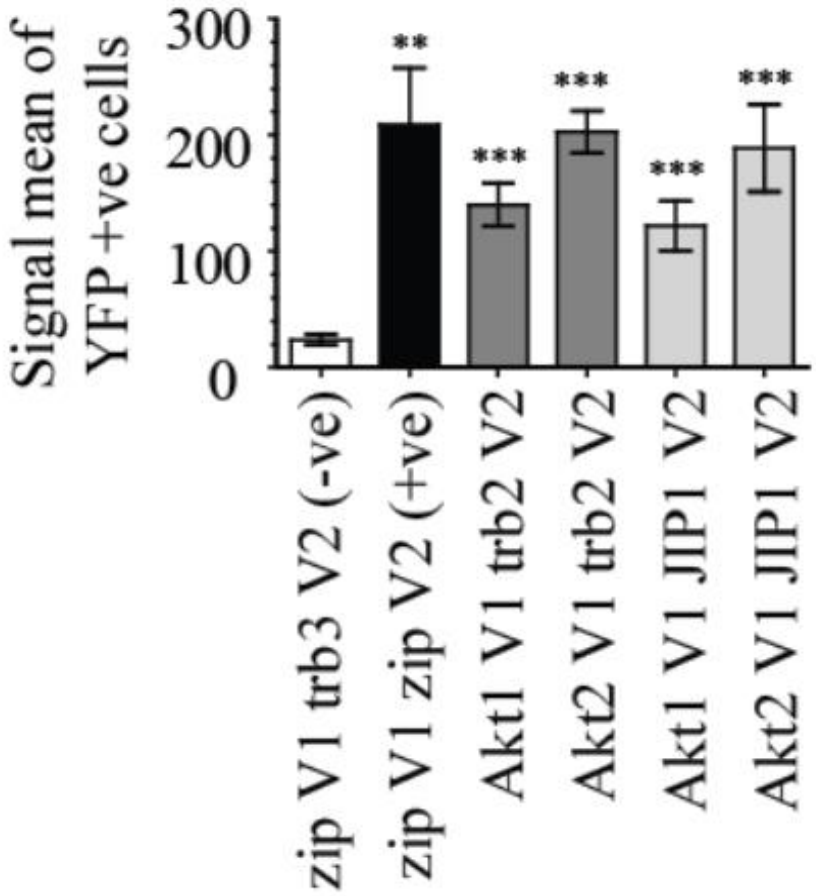

Figure 3.3.2. Mean fluorescent intensity of YFP positive cells. One-way ANOVA with Dunnett's multiple comparison test, n = 4 (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). All analysis was conducted compared to zipV1trib3V2 (lane 1).

Using our cell lines with high TRIB2 protein expression (exogenous [293T, U2OS], endogenous [SK-MEL28]) we conducted TRIB2 co-IP) CNIO antibody) and probed for either TRIB2 (SCBT antibody) or total-AKT. Strikingly, we note that there was a clearly detectable protein complex under normal cell growth conditions (i.e. in the absence of any inhibitors or chemotherapeutic).

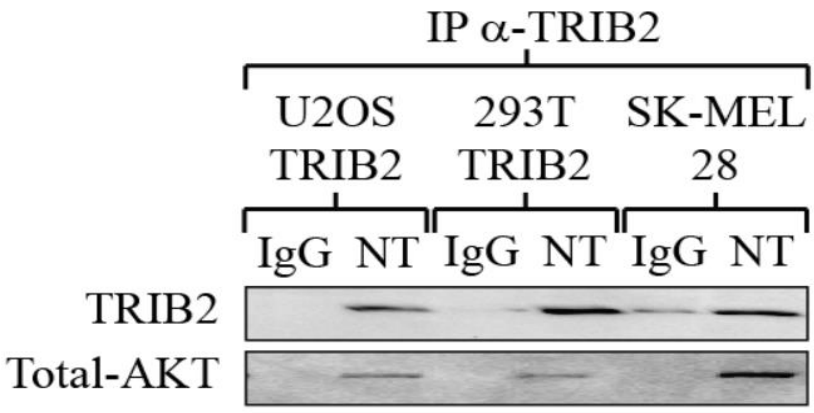

Figure 3.3.3. Co-immunoprecipitation of TRIB2 or total AKT1 from indicated cell lines, (500 µg total protein lysate immunoprecipitated per lane) and separated by 8-12% SDS-PAGE.

We confirmed the equivalent protein load for each Co-IP (Figure 3.3.4) and that TRIB2 and AKT interact.

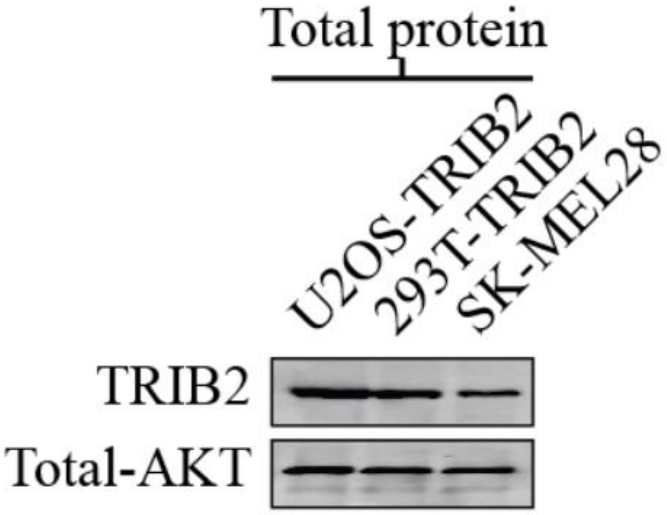

Figure 3.3.4. Total protein levels in each indicated cell line for co-IP targets (100 µg total protein loaded per lane and separated by 8-12% SDS-PAGE)

Having seen that TRIB2 and AKT interact in both an artificial assay (PCA) and in our *in vitro* cell line models, we questioned how clinically relevant this interaction could be. To this end, we obtained a number of stage IV metastatic melanoma samples and had these sectioned on a cryostat at Faro Hospital. We stained these sections for pSer473-AKT and TRIB2 addressing if in clinical patient samples there was co-localisation (Figure 3.3.5).

Consistent with the PCA and *in vitro* co-IPs that we previously conducted, we note that there was significant co-localisation of AKT and TRIB2. Interestingly, whereas the previous studies examined TRIB2 interaction with total AKT, this co-localization study demonstrated that not only does AKT and TRIB2 interact, that it is pSer473-AKT and TRIB2 that form a protein complex. This is under active investigation within our group (data unpublished/manuscript under review).

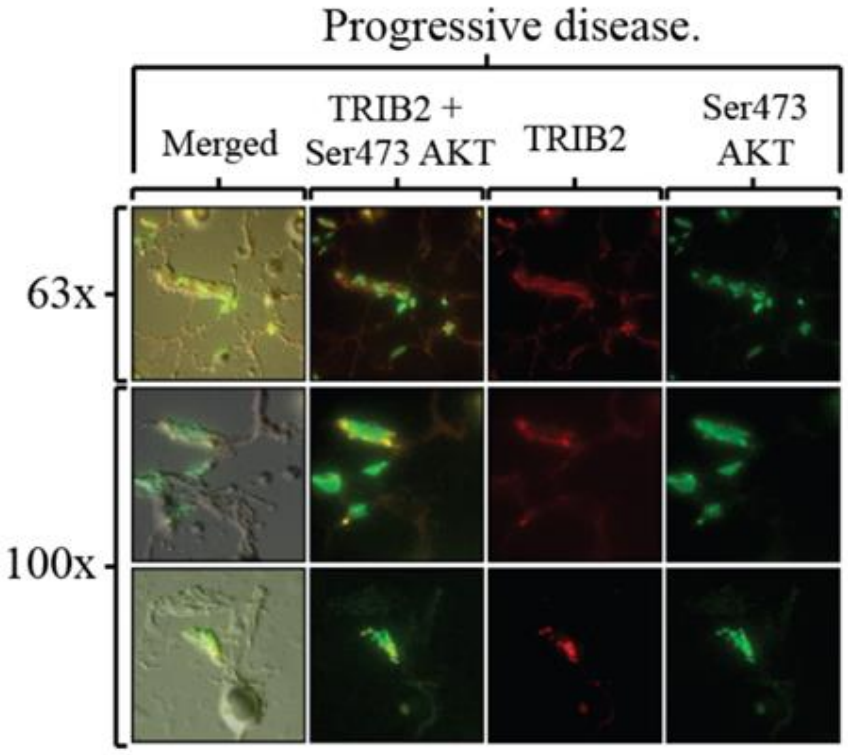

Figure 3.3.5. :Representative immunofluorecent images of stage IV metastatic melanoma stained for pSer473-AKT1 (green) and TRIB2 (red) showing co-staining and interaction. The left row includes bright-field image for the sample. Yellow region indicate areas of co-localisation.

CHAPTER 4

CONCLUSION

4. Conclusion

Melanoma although not the most frequent skin cancer type, is the most fatal, aggressive, is the one with poorest prognosis, with low levels of effective response, due the fact that metastasize very fast, the tumors heterogeneity, and that the patients acquire drug resistance to almost all treatments. For these reasons, researchers have been looking for melanoma biomarkers, with only one, LDH approved by the FDA.

Previous data from our group showed that in malignant melanoma there is an over expression of TRIB2 at both the mRNA and protein level, suggesting both an oncogenic role in melanoma and a potential biomarker.

Before being used in clinical trials, drugs must be tested in cell lines, in this case preferably melanoma cell lines (*in vitro* model) and then in animal models (*in vivo* studies) and my project allowed me to test not only in cell lines, but also in *in vivo* with xenograft mice and *ex vivo* with clinical samples from patients with the disease. The obtained results show over expression of TRIB2 in different melanoma cell lines, once again correlating previous lab results. This over expression, seen mainly in melanoma, suggests that TRIB2 may be a good prognostic biomarker for malignant melanoma. Published and unpublished data from our group already pointed to an association between TRIB2 and drug resistance and this project allowed me to see that TRIB2 confers resistance to PI3K/mTOR inhibitor drug BEZ235. One of the biggest questions of my research was about the mechanism behind drug resistance due to TRIB2 present in PI3K/AKT signaling pathway and the answer could be the fact of TRIB2 is having an action downstream of PI3K, and once the drugs act upstream, TRIB2 could be activating constantly AKT through its interaction. However much the drug is administered to the patients will have no effect (figure 4.1.1)

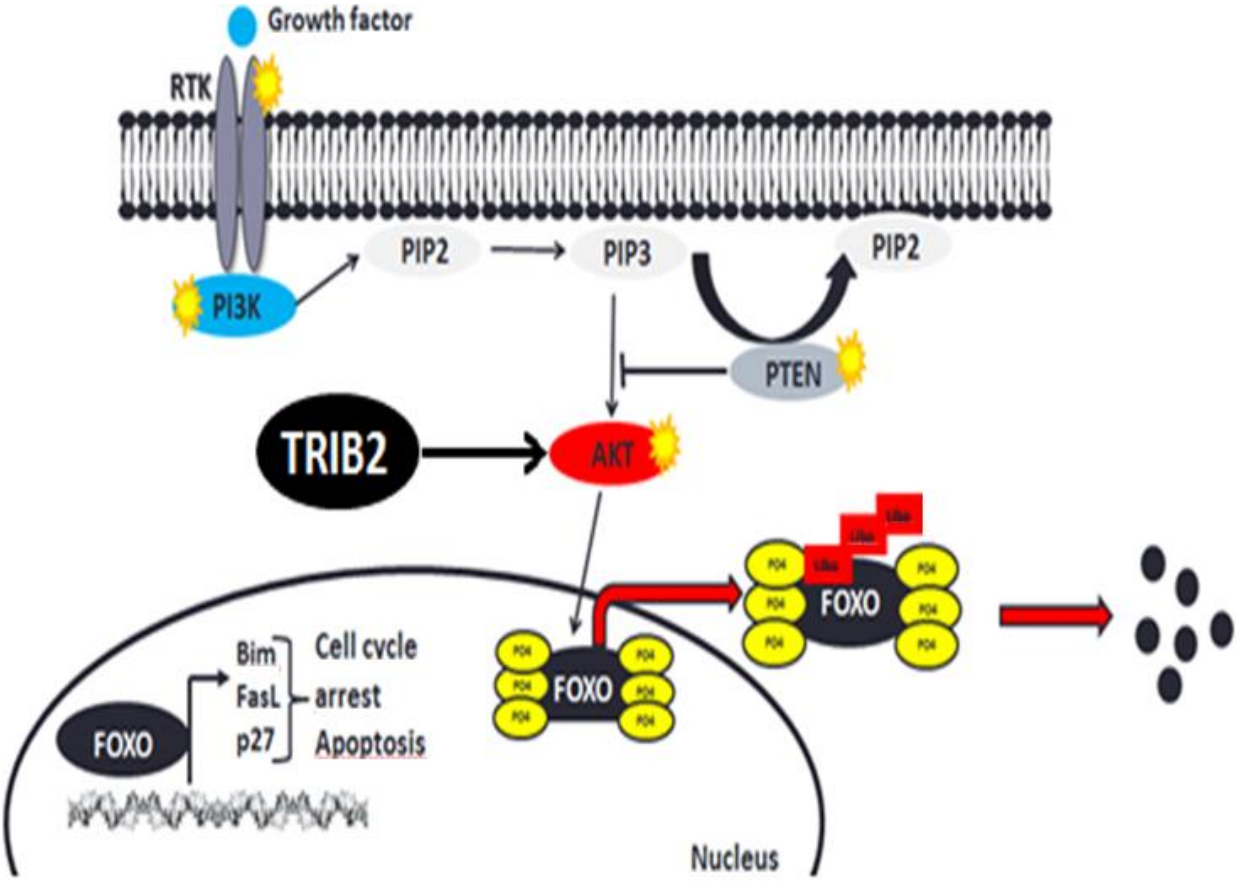

Figure 4.1.1: AKT/TRIB2 interaction and FOXO3a inactivation.

Other interesting result that corroborates this hypothesis, is the fact that when we see an overexpression of TRIB2, there is an increase of the active form of AKT (pSer473-AKT) both in clinical, *in vitro* and in *in vivo* samples. To turn this story more interesting, Co-IP demonstrated that TRIB2 and AKT interact, so there really is a relation between higher levels of TRIB2 and AKT activation.

To understand how this interaction is done, our lab is seeing if TRIB2 interacts with AKT through COP1 domain (unpublished data). Our results show as well a correlation between TRIB2 and AKT interaction and a decrease of FOXO3a expression, once AKT phosphorylates FOXO3a, causing FOXO3a exportation from the nucleus to the cytoplasm, resulting in lower levels of pro-apoptotic genes like BIM and genes involved in cell cycle arrest such as p27.

So if there is none expression of this genes, cancerous cells will keep proliferating, growing, avoiding cell death allowing tumor progression.

In case of BEZ235, will be more effective if the patient doesn't have an overexpression of TRIB2, in contrary there is no advantage in administering this drug, as it will have no beneficial effect and the patient will be subject to unnecessary cytotoxic effects. Ideally, a patient screening to identify if there is an overexpression of TRIB2 will avoid giving unnecessary, ineffective and aggressive treatments to the patients, promoting a personalizes diagnosis and treatment.

4.1. Future directions

These results highlighted how TRIB2 interacts with AKT in PI3K/AKT pathway, and for future directions it would be crucial to keep evaluating TRIB2 interactions through Co-IP, optimizing this process. ´

Would be important as well to clarify the mechanism of drug resistance by TRIB2 to somehow reverse this process avoiding cancer progression and promoting an increase of life quality to the patients, perhaps an increase of patients overall survival. Are needed more clinical samples analyses, a larger number of them to elucidate the characterization of PI3K/AKT/ TRIB2 to know how even in the present of PI3K inhibitors, how TRIB2 acts in this pathway.

CHAPTER 5

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CHAPTER 6

APPENDIX

6. Appendix

invitrogen™ CE IVD

00-8010 18 mL
ClearMount™ Mounting Solution
Lot No. See product label

ClearMount™ Mounting Solution

INTENDED USE
For In Vitro Diagnostic Use. CAUTION: Not for human or animal therapeutic use. Uses other than the labeled intended use may be a violation of local law.

INTRODUCTION
This product is an aqueous-based mounting medium for immunohistochemical procedures. It can be used as for permanent staining of alcohol soluble and non-alcohol soluble substrates. Synthetic resins, such as Permount cannot be used with some immunostaining techniques because the organic solvent present in the media will dissolve alcohol-soluble chromogens such as AEC or Fast Red. An aqueous mounting media is thus needed for coverslipping. In the past, certain problems occurred using these media: Immunostaining faded over time and resolution was poor. ClearMount™ eliminates those problems. Just place drops of ClearMount™ over the tissue sample, and heat-dry. Hence, a liquid coverslip is formed. The hardened ClearMount™ has a superior refractive index when compared to other aqueous mounting media. Thus, slides are easier to read. However, resolution for high magnification or photography can be improved by applying a permanent coverslip. The hardened ClearMount™ forms an imperious barrier to organic solvents and a permanent coverslip can be placed on the hardened polymer.

REAGENTS PROVIDED
18 mL. Reagent contains 0.1% sodium azide.

REAGENT PREPARATION
This reagent is ready-to-use.

STORAGE
Store at room temperature.

RECOMMENDED GUIDELINES FOR USE
1. Cover stained tissue section with a few (2-4) drops of this mounting medium.
2. Place slides in a drying oven set at 60°C for 30 minutes or until slides are thoroughly dried. Slides are now ready for microscopic evaluation.
3. If a permanent coverslip is desired, dip slides in xylene and apply mounting medium (Invitrogen product HistoMount™, Cat No. 00-8030) over the hardened ClearMount™, and coverslip.

   Note: Hardened ClearMount™ can be removed from the slide by simply soaking the slide in warm water for approximately 10-30 minutes.

Figure A.1: Mounting solution protocol for immunofluorescence.

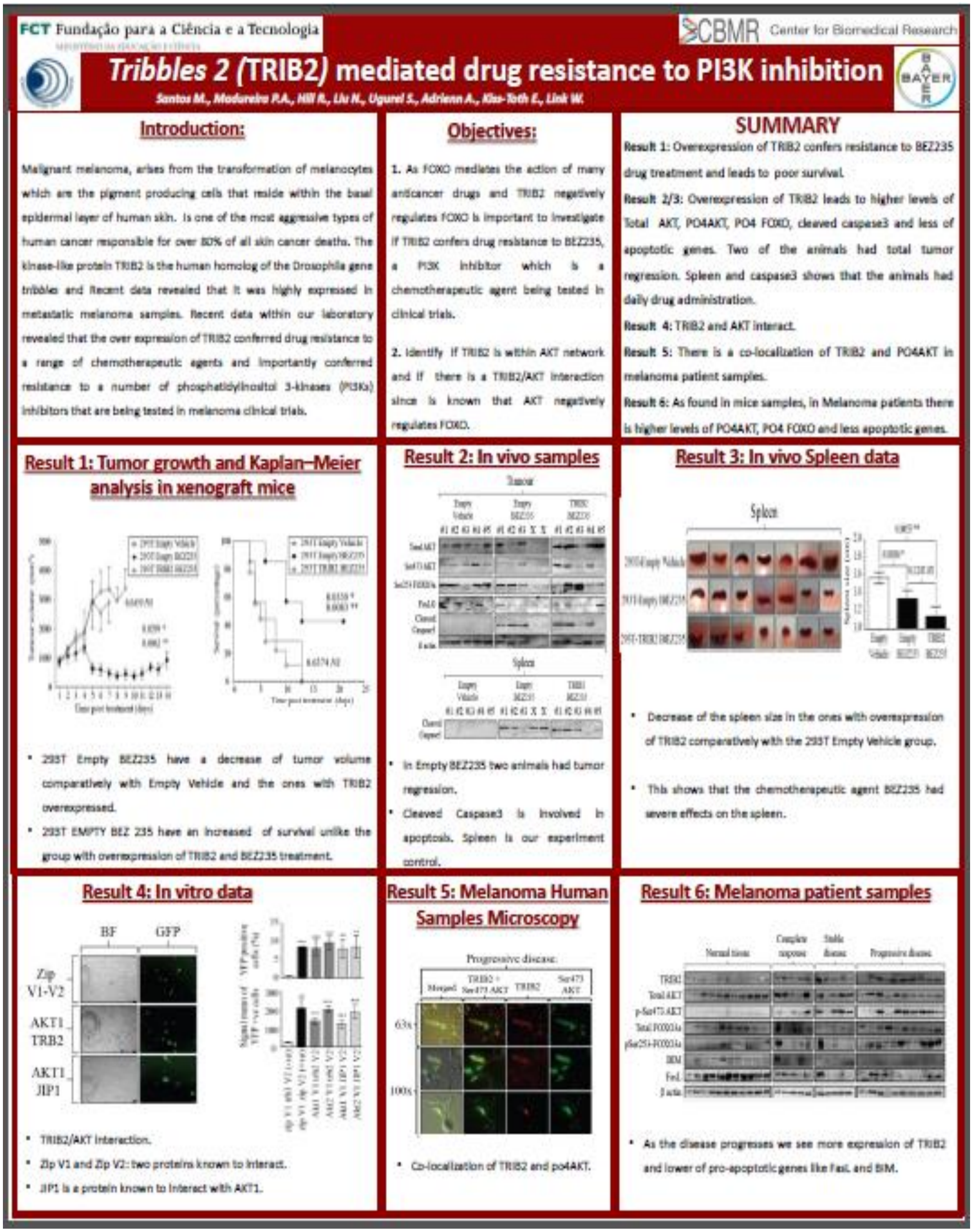

Figure A.2: Poster done by me for the Oncobiology Retreat Session and Poster presentation.