

TARGETING MYC-DRIVEN TUMOURS

BETing on ATR

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Cover illustration:
Morphology of A375 Melanoma cells upon BETi-ATRi treatment
by Somsundar Veppil Muralidharan

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ॐ असतोमा सद्गमय ।
तमसोमा ज्योतिर् गमय ।
मृत्योर्मा॒मृतं गमय ॥
ॐ शान्ति शान्ति शान्तिः ॥

From ignorance, lead me to truth;
From darkness, lead me to light;
From death, lead me to immortality;
Let there be peace, peace and peacefulness.

Shanti Mantra (Mantra of peace) from Brihadaranyaka Upanishads (1.3.28)

ABSTRACT

Cancer arises from loss of function of tumour suppressors and/or gain of function mutations in proto-oncogenes that disrupt the delicate balance required for homeostatic cell division, resulting in uncontrolled cell proliferation. Oncogenic transformation of multifaceted proto-oncogene - transcription factor - MYC can give rise to cancers and it is found to be deregulated in more than 70% of the tumours. Being a master regulator of transcription, MYC can transcribe upto 15% of the genome. Targeting MYC directly or identifying the Achille's heel of MYC-driven tumours is thus a promising therapeutic approach to treat these tumours. This thesis, investigates and demonstrates novel therapeutic approaches against MYC-driven tumours. This thesis also investigates and exploits the therapeutic potential of activated DNA Damage Response (DDR) pathway in inhibiting MYC driven tumour cells.

In the first publication (Bhadury et al, 2014), we characterize a novel and orally bio-available BET bromodomain inhibitor (BETi) RVX2135, which could displace BET proteins from acetylated histones. We also identified BET bromodomain proteins as a valuable therapeutic target against MYC driven tumours in vitro and in vivo. Initially dubbed as MYC inhibitors, our study on BETi disproves this notion by attributing these anti-tumoural effects to pleiotropic transcriptional changes caused by BET inhibition. Gene expression profiling to identify these transcriptional changes enabled us to identify subset of genes that are commonly altered by both BETi and HDACi. This study also demonstrates that HDACi and BETi can synergize to hinder Myc-induced lymphoma progression.

The second publication (Muralidharan et al, 2016) in this thesis investigates the role of BET proteins in regulating cell cycle and replication. BETi disable the entry of cells into S-phase of cell-cycle, hamper DNA synthesis and cause DNA damage. A pharmacogenetic screen identified BET inhibitors to synergize with inhibition of PI3K/mTOR family of proteins, to which ATR, an upstream kinase of DDR pathway belongs. Further studies revealed that the thus identified PI3K/mTOR inhibitors indeed affect ATR-Chk1 DDR pathway leading to the discovery of a strong synergy between BETi and ATRi in apoptosing Myc driven tumours in vitro, and in vivo and (by) it induces SASP and ER stress.

The third study translates the above findings into the field of melanoma, a form of skin cancer. We validate the BETi-ATRi synergy in cell lines in vitro and in Patient Derived Xenografts (PDX) in vivo. Using B16F10 in vivo syngenic transplant melanoma model, we also demonstrated that this combination therapy does not affect the therapeutic benefits of Immune Therapy, the front line treatment against melanoma in clinic today.

Taken together, this thesis puts forth a multifaceted approach to treat cancer. It thoroughly describes the effects of BETi and ATRi on cancer cells and how they can be combined to enhance the therapeutic efficacy.

SAMMAFATTNING PÅ SVENSKA

Cancer uppkommer till följd av förlust av funktion av tumörsuppressorer och/eller ökad funktion av proto-onkogener. Detta stör den fina balansen som krävs för homeostatisk celledelning, vilket resulterar i okontrollerad proliferation. Malign transformation av den mångfacetterade proto-onkogen- transkriptionsfaktorn MYC kan ge upphov till cancer och en felaktig reglering av denna är rapporterad för mer än 70 % av alla tumörer. Som generell transkriptionsfaktor kan MYC transkribera upp till 15 % av genomet. Genom att inrikta sig på MYC direkt, eller genom att sikta på MYC-styrda tumörers "Akilles häls" har man således en god möjlighet att behandla dessa tumörer. Denna avhandling undersöker, samt påvisar, nya botande behandlingsstrategier för MYC-styrda tumörer. Ytterligare undersöker denna avhandling den terapeutiska potentialen hos aktiverad DNA-skaderespons (DDR) signalvägen i att inhibera MYC-styrda tumörceller.

I den första publikationen (Bhadury et al, 2014) karaktäriserar vi en ny, oralt biotillgänglig, BET bromodomän inhibitor (BETi) RVX2135, som kan rubba BET-proteiner från acetylerade histoner. Vi visar även att BET-bromodomänproteiner är viktiga terapeutiska behandlingsmål hos MYC-styrda tumörer in vivo and in vitro. Vår studie bestrider den gängse uppfattningen att RVX2135 skulle vara en MYC-inhibitor och visar att dess anti-tumorala egenskaper beror på BET inhibering. Genom genexpressions profilsanalys identifierades ett set av gener som påverkas av BETi samt HDACi. Ytterligare visar denna studie att HDACi och BETi i synergi kan hindra Myc-inducerad lymfomprogression.

Den andra publikationen (Muralidharan et al, 2016) i denna avhandling undersöker BET-proteiners roll i reglering av cellcykeln och replikation. BETi förhindrar cellen att gå in i S-fas i cellcykeln, hindrar DNA-syntes och orsakar DNA-skador. En farmakogenetiskscreening visade att BET inhibitorer samverkar med inhibering av proteiner från PI3K/mTOR familjen. Hit hör bland annat ATR, ett kinas som återfinns uppströms i DDR signalvägen. Ytterligare studier visade att identifierade PI3K/mTOR inhibitorer påverkade ATR-Chkl DDR signalvägen, vilket ledde till upptäkten av en stark synergi mellan BETi och ATRi i apoptotiska Myc-styrda tumörceller in vitro och in vivo. Detta genom induktion av SASP and ER stress.

Det tredje arbetet leder in tidigare fynd i melanomforskningsfältet. Vi validerar BETi-ATRi synergier i cellinjer in vitro samt i Patient Derived Xenografts (PDX) in vivo. Med hjälp av en B16F10 syngen in vivo transplantationsmodell kunde vi visa att kombinationsbehandlingen inte påverkar den botande effekten av immunterapi, som är den ledande behandlingen mot melanom i kliniken idag.

Sammantaget presenterar vi i denna avhandling en komplex strategi för behandling av cancer. Effekterna av BETi och ATRi behandling beskrivs noggrant tillsammans med deras förstärkta terapeutiska effektivitet då de kombineras.

LIST OF PAPERS

This Thesis is based on the following studies:

1. *BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma*
Joydeep Bhadury, Lisa M. Nilsson, Somsundar Veppil Muralidharan, Lydia C. Green, Zhoulei Li, Emily M. Gesner, Henrik C. Hansen, Ulrich B. Keller, Kevin G. McLure, and Jonas A. Nilsson
PNAS, 2014; 111 (26) E2721-E2730;
doi:10.1073/pnas.1406722111 (PMID:24979794)
2. *BET bromodomain inhibitors synergize with ATR inhibitors to induce DNA damage, apoptosis, senescence-associated secretory pathway and ER stress in Myc-induced lymphoma cells*
Somsundar Veppil Muralidharan, Joydeep Bhadury, Lisa M. Nilsson, Lydia C. Green, Kevin G. McLure and Jonas A. Nilsson.
Oncogene, 2016; doi:10.1038/onc.2015.521 (PMID:26804177).
3. *Therapeutic implications for melanoma of combined ATR and BET bromodomain protein inhibition*
Somsundar Veppil Muralidharan, Berglind Einarsdottir, Joydeep Bhadury, Mattias Lindberg, Eric Campeau, Roger Olofsson Bagge, Ulrika Stierner, Lars Ny, Lisa M. Nilsson and Jonas A. Nilsson
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Publications not included in thesis

1. *Cancer differentiating agent hexamethylene bisacetamide inhibits BET bromodomain proteins.*
Lisa M. Nilsson, Lydia C. Green, Somsundar Veppil Muralidharan, Dagsu Demir, Martin Welin, Joydeep Bhadury, Derek Logan, Björn Walse & Jonas A. Nilsson
Cancer Research (in press)
2. *Aberrant Fat Metabolism in Caenorhabditis elegans Mutants with Defects in the Defecation Motor Program.*
Ming Sheng, Ava Hosseinzadeh, Somsundar Veppil Muralidharan, Rahul Gaur, Eva Selstam and Simon Tuck
PLoS One, 2015; 10(4): e0124515. doi: 10.1371/journal.pone.0124515 (PMID: 25849533)
3. *Identification of tumourigenic and therapeutically actionable mutations in transplantable mouse tumour cells by exome sequencing.*
Bhadury J, López MD, Muralidharan SV, Nilsson LM and Nilsson JA
Oncogenesis, 2013 (PMID 23588493)
4. *Therapeutic implications for the induced levels of Chk1 in Myc-expressing cancer cells.*
Höglund A, Nilsson L, Muralidharan SV, Hasvold LA, Merta P, Rudelius M, Nikolova V, Keller U and Nilsson JA
Clinical Cancer Reserch, 2011; (PMID: 21933891)

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ABBREVIATIONS

DDR	DNA Damage Response
ATF4	Activating Transcription Factor 4
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3 related
ATRi	ATR inhibitor
ATRIP	ATR Interacting Protein
AZD	AZD7762
BET	Bromodomain with Extra Terminal domain
BETi	BET inhibitor
ChIP	Chromatin Immuno Precipitation
Chk1i	Checkpoint Kinase 1 inhibitor
Chk1	Checkpoint kinase 1
Chk2	Checkpoint Kinase 2
CHOP	C/EBP-Homologous Protein
cPARP	Cleaved PARP
DDIT3	DNA Damage Inducible Transcript 3
DDR	DNA Damage Response
HDAC	Histone deacetylases
HDACi	HDAC inhibitor
IP	Intraperitoneal
IT	Immune Therapy
PARP	Poly (ADP-Ribose) Polymerase
PDX	Patient Derived Xenograft
RPA	Replication Protein A
RT	Room Temperature
RVX	RVX2135
SQSTM1 (p62)	Sequestosome 1
VE	VE821
HAT	Histone Acetyl Transferase
TAD	Transactivation domain
ROS	Reactive Oxygen Species
CTD	C Terminal Domain
NHEJ	Non Homologous End Joining
DSB	Double Stranded Break
SSB	Single Stranded Break
EMT	Epithelial to Mesenchymal Transition
ECM	Extracellular Matrix
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet Derived Growth Factor
EGF	Epidermal Growth Factor
PD1	Programmed Cell Death 1
PD-L1	Programmed Cell Death Ligand 1
CTLA4	Cytotoxic T-Lymphocyte Associated protein 4

1 INTRODUCTION

1.1 CANCER

Cancer as the pathological condition we know of today, has been in existence since time immemorial, with the earliest description dating back to 1600BC. The term “cancer” was coined by Hippocrates. He described the tumour as *carcinus*, the Greek word for crab. Over the course of time, reports of incidents of cancer have increased manifold, partly due to better documentation and majorly due to an increase in the actual occurrence of cancer caused by the changes in our lifestyle, and environment. Over the past century in the western world, expanses in the medical field, development of antibiotics, eradication of poverty and improvements in living standards have contributed to prolonged life expectancy, whereby, we age old enough to develop cancer.

Cancer is a genetic disease caused by alteration(s) or mutation(s) in DNA, the genetic material of the cell. Our cells have efficient machinery to make faithful copies of the DNA. We also have efficient mechanisms to identify and rectify mistakes caused during replication. However, these machineries lose the ability to do so with age, causing an accumulation of point mutations over the course of time leading to cancer [1, 2]. While this explains why cancer is mostly an old-age related disease, incidence of cancer can occur in all age groups, from new born to young adults to old people. Sometimes cancer can also be a hereditary disease, running into generations. Exposure to radiation, altered diets, habits such as smoking and sunbathing, infections such as Hepatitis B/C or HPV, etc can also cause cancer.

Cancer is a clonogenic disease i.e. it arises from a single cell in our body. To date, evidence is scarce to describe cancer as a disease caused by mutation in a single gene. Instead, cancer is generally believed to be a multistep process involving a series of sequential mutations. In general, cells are continually exposed to various endogenous and exogenous factors that can lead to mutations. In particular, loss of function mutations in tumour suppressor genes and gain of function mutations in proto-oncogenes leads to uncontrolled proliferation of cells. Though there are intrinsic and extrinsic

mechanisms to counter this process, the tilting of balance from cell death to net gain in cell proliferation leads to cancer. These cancer cells proliferate, lose contact inhibition and sail through circulatory and lymphatic system to get lodged in distant organs and tissues, forming metastatic lesions. Besides accumulated mutations, cancer is also characterized by altered metabolism, enhanced genomic instability, ligand independent growth signalling, loss of pro-apoptotic signals, enhanced angiogenesis etc. More about these cancer hallmarks will follow.

According to WHO, approximately 14.1 million new cases of cancer and 32.6 million people living with cancer were documented in 2012. 8.2 million cases of cancer related deaths were reported in 2012, accounting for 13% of all human deaths worldwide. Use of tobacco, exposure to environmental pollutants, unhealthy diet, obesity, alcohol use, radiations, STDs such as HPV, etc are risk factors, which when decreased/avoided can reduce the chances of cancer by 30% (<http://www.who.int/cancer/en/>). Surgical removal of the cancer tissue is the most effective treatment for cancer till date, although targeted small molecule therapy, radiation therapy, chemotherapy and immune therapy are also omnipresent in oncology clinics today.

In recent years governmental and non-governmental spending on cancer research has increased manifold. In fact, several countries including Sweden have identified cancer as an area of strategic importance and research. Several novel treatments and medications for cancer are undergoing rigorous clinical trials today. Recent advancements in research and development in this field have helped in identification of risk factors and biomarkers, enhanced the survival of patients and facilitated development of durable therapies against cancer. However, the complexity of the disease and its increased prevalence calls for continued research to understand the underlying mechanisms better and to develop more effective, and efficient therapies and preventive strategies.

1.1.1 THE HALLMARKS OF CANCER

Cancer is a disease emanating from mutations and consequent dysfunction of the very fundamental building blocks of our body, cells. Exposure to radiations and carcinogenic substances can initiate changes in the DNA and these accumulate faster if the designated repair systems fail or are hampered. However these genetic alterations or mutations occur spontaneously at low rates.

While most of these mutations are harmless, a few can lead to alteration in cellular behaviour. Accumulation of such mutations irreversibly transforms a cell into cancerous cell. This phenomenon worsens as we age, hence cancer incidences increase with age. When they strike somatic cells, mutations lead to sporadic, nonhereditary cancers. Not all such accumulating mutations can lead to cancer, but the ones that attain particular salient features, termed “hallmarks of cancer” can lead to the successful establishment of tumours[3]. On the other hand, mutations in germline cells or sex cells can lead to predisposition of their offspring to cancer. It is noteworthy that only 10% of the cancers are hereditary.

There are intrinsic and extrinsic factors that dictate cell growth and multiplication, but cancer cells do not heed to such signals, they grow unchecked, producing more and more cancer cells. Growing uncontrollably and possessing self-sufficiency in growth signals is thus, one of the first hallmarks of cancer. Normal cells require external growth stimulatory signals (growth factors) to grow and divide. These stimulations are received by receptors on the cell membrane, leading to a cascade of signal transduction, which leads to activation of growth stimulatory genes and deactivation of growth inhibitory genes, eventually leading to cell growth and division.

However, cancer cells have an abundance of such stimulatory signals, as they are able to generate these themselves. For example, glioblastomas can generate their own Platelet-Derived Growth Factor (PDGF), a growth stimulatory molecule[4]. Or sometimes the receptors of such signalling molecules could be over expressed or mutated, latter leading to aberrant and continuous signalling of such pathways. Over-expression of EGFR and HER2 in stomach and breast cancer are well-suited examples [5-7].

Even if cells are proficient in growth stimulatory signals, to develop into a tumour and to establish continuous growth, cancer cells need to turn insensitive to growth inhibitory signals or they have to learn how to divide in the presence of growth inhibitory signals. An example of this is the inactivating mutations in RB, which promotes cell division, as seen in cervical cancer caused by HPV infection[8].

Similar to the signals that regulate cell growth and division, there are signals that lead to programmed cell death or apoptosis. When cells accumulate DNA damage, the cells might be destined to die. Once such signals sets in, cells produce proteases and enzymes that degrade their components and chop the DNA into minute pieces and the cell membrane shrinks, eventually leading to collapse of such cells [9]. This happens when the delicate balance between pro- and anti-apoptotic signals are tilted towards pro-apoptosis. Cancer cells usually over-express anti-apoptotic proteins such as BCL2 or BCLX[10-12]. Inactivating mutations in pro-apoptotic proteins, rendering them inactive are also commonly found in cancer.

A dividing cell has a limited replication potential, termed as the Hayflick limit, a point after which cells undergo senescence. Deactivation of tumour-suppressors such as pRB or p53 makes cells immortal, capable of limitless replication. Also, normal cells lose the ends of their chromosomes during replication, which can trigger senescence or apoptosis[13]. Telomerase, an enzyme that maintain these ends of chromosomes are also found to be over-expressed in various tumour types, which can also confer these cells with unlimited replication potential[14].

A growing tumour also needs enhanced vascularization to supply nutrients and oxygen to meet the growing energy demands and to sustain rapid, unchecked proliferation. Tumours often promote angiogenesis, a process of neo-vascularization of tumour tissue from existing vasculature. Tumours induce the growth of vasculature by secreting various factors such as bFGF and VEGF, which can induce capillary growth, catering to such needs[15, 16].

The ability of tumour cells to lose contact with the adjoining tissue, to invade and to metastasize at distant locations is yet another hallmark of cancer. Nearly 90% of deaths from human cancer are due to metastasis. During metastasis, a series of con-

controls that normally confine a cell to a particular site on the tissue where it normally grows, is usually inactivated – enabling these cells to move to other sites in the body, often leading to disruption of thus evaded tissues. Molecules such as N-CAM, which often makes the cells adhesive, are often deregulated in cancers such as Neuroblastoma and small cell lung cancer [17, 18]. Cancer cells often express matrix-degrading proteases, which facilitates invasion of these cells into stroma, across blood vessels and through epithelial layers, enabling them to metastasize into distant locations [19].

Cancer cells have higher nutrient and energy requirements to foster their faster growth and division. Cancer cells often tweak their metabolic pathways to feed into growing energy and metabolic demands, such as adopting glycolysis. This phenomenon is described as Warburg effect. Often, genes involved in metabolism of all major classes of macromolecules (Carbohydrates, proteins, lipids and nucleic acids), required for building a new cell, are altered in cancer cells[20].

As mentioned earlier, cancer is a multistep-process and accumulation of mutations is an imperative step in the development of a tumour. Cancer cells often have several mutations, large genomic alterations on the chromosomes and on the epigenome, thus altering gene expression and functions[21]. This continuous accumulation of mutations, leads to an evolution of sorts, making cancer cells more and more sturdy and heterogeneous. Thus genomic instability is a key component of tumour formation.

At any given point of time, there can be many mutated cells in our body, but our immune system plays a vital role in weeding out such potential-cancer cells on a daily basis. Cancer cells which are often different in shape and size compared to normal cells have to evade detection from immune surveillance long enough to grow into a tumour. Tumour cells often suppress antigen presentation there by evading immune surveillance. Often, they also express immune suppressive signals such as PD-L1/2, which disable the immune cells from mounting an immune response towards cancer cells [22]. Thus, ability of immune evasion forms a critical step in cancer formation and is an important hallmark of cancer.

1.1.2 THE GENETICS OF CANCER

Cell division is an indispensable part of functioning of our body, which is tightly regulated by signals emanating from within and outside the cell. Growth promoting signals transduce a wave of molecular changes that induce cell division, whereas growth inhibitory signals provide cues to stop proliferation. Cancer cells are usually defiant to such inhibitory signals and divide continuously. Cancer is mostly sporadic and they are caused by somatic mutations.

Loss of function mutations in so-called tumour suppressors and gain of function mutations in growth promoting proto-oncogenes are the starting points for tumour development. Tumour suppressors and proto-oncogenes are mutually opposing forces within a cell, tightly regulating its proliferation (Figure 1). Thus deactivation of tumour suppressor genes and oncogenic activation of proto-oncogenes to oncogenes are essential for development of cancer.

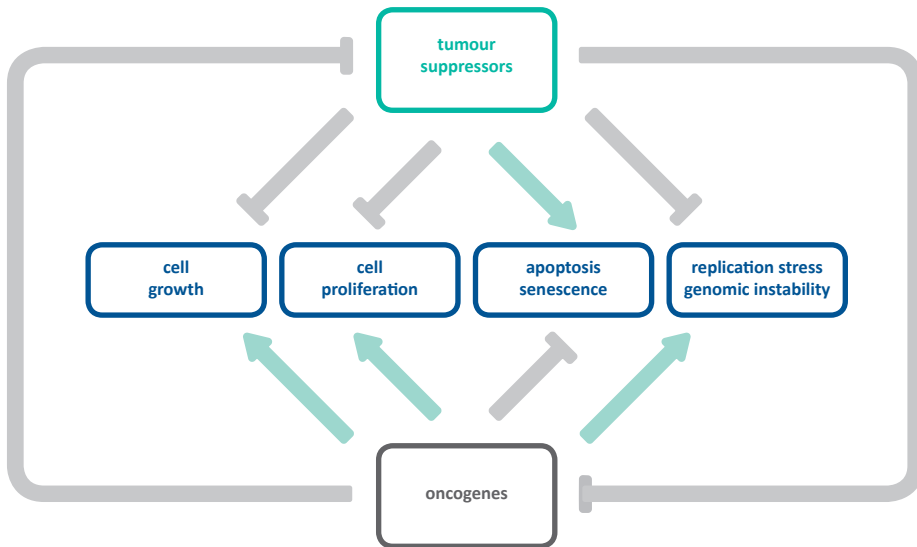


Figure 1: Two counteracting forces: Tumour suppressors & oncogenes.

One of the most noted tumour suppressors is p53, also known as the guardian of the genome. P53 is a transcription factor, that plays a critical role in regulation of cell cycle and in guarding the integrity of our genome. The anti-proliferative role of p53 is vital for its tumour suppressor function, but strong negative regulation is required to allow normal growth and development. In normal unstressed cells, p53 is scanty. In response to stress signals such as DNA damage and illicit oncogenic activation, p53 is modified by upstream sensor proteins, leading to activation of p53. These modifications include phosphorylation, acetylation, methylation, and ribosylation. Once active, depending on the stress and the context of external signals, p53 can induce apoptosis, cell cycle arrest, DNA repair, senescence or protective anti-oxidant activity, by the virtue of its large repertoire of target genes. DNA damage in unperturbed cells often leads to expression of p21, resulting in cell cycle arrest and halt in proliferation, allowing DNA repair. Enhanced ROS, which can cause DNA damage, is countered by the expression of p53 target genes involved in scavenging ROS. The response mounted by p53 in response to stress signals is context dependent and sensitive to severity of stress. Low levels of continuous stress, often leads to p53 mediated temporary cell cycle arrest, removal of ROS and repair of DNA damage, if any. Excessive stress that causes severe damage is dealt with more stringent mechanism of inducing apoptosis via pro-apoptotic target genes such as PUMA and NOXA to eliminate cells that might have acquired irreparable damage and oncogenic alterations. P53 is kept under strict control by E3 ubiquitin ligase called MDM2 (HDM2 in humans), which is capable of promoting ubiquitination and degradation of p53. However, MDM2 mediated p53 degradation is prevented by ARF in response to oncogenic activation. Given these myriads of functions, it is not surprising that p53 is mutated to lose its functions in 50% of tumours in human. Loss of function mutations, or deletion of ARF is also common in various cancers, ultimately leading to a dysfunctional p53 pathway [23-26].

Retinoblastoma protein (RB) is another tumour suppressor, which is mutated in several forms of cancer including retinoblastoma, osteosarcoma and small cell lung cancer. RB can bind and thereby inactivate the E2F family of transcription factors, which are essential for entering cell cycle. However, cyclin- dependent kinases (CDKs) can phosphorylate RB and liberate E2F, thus release cells into cell cycle. pRB can also in-

duce chromatin modifications by recruiting epigenetic modifiers such as HDAC1 and methyltransferases, there by modifying gene expression to promote differentiation and to suppress tumour growth [27-30].

Another major class of tumour suppressors belong to 1) INK4 and 2) CIP/KIP family of CDK inhibitors (CKIs), which regulate the activity of cell cycle promoting CDKs. INK4 (CDKN2A/B/C/D) family of proteins bind to CDKs (CDK4/6), there by preventing phosphorylation of RB, which often leads to G1 arrest. On the other hand CIP/KIP family of CKIs (p21/p27) binds to CDK-cyclin complexes there by rendering them inactive, such as in p27-cyclin-D-CDK2 complex. During cell cycle progression, cyclin-D-CDK4 holoenzyme phosphorylates p27, there by releasing it from the multi-protein complex and marking it for degradation, leading to cell cycle progression. This class of CKIs play vital role in cell cycle regulation by preventing aberrant S-phase entry in response to growth inhibitory and cellular stress signals. Not surprisingly, these genes are mutated in various types of cancers [31-36].

PTEN, a protein with low phosphatase activity is another well-studied tumour suppressor that is deactivated or reduced in expression in various tumour types. PTEN negatively regulates PIP3 by dephosphorylating it and thus preventing the activation of AKT, which can promote proliferation, growth and survival of cells. PTEN is also a transcriptional target of p53 and PTEN is required for p53-mediated apoptosis. PTEN can also negatively regulate cell migration [37-39]. Besides p53, pRB and PTEN, a few other tumour suppressors include APC, members of DDR pathway like ATM, BRACA1, CHEK2 and pro-apoptotic proteins such as BIM and BAX.

Besides deactivation of tumour suppressors, oncogenic mutations leading to activation of proto-oncogenes is also a pre-requisite for transformation. Oncogenes are essential for regular functioning of the cell and to promote cell division and proliferation. However, mutated oncogenes negate the restrictions imposed by tumour suppressors to promote uncontrolled cell proliferation, survival and transformation. Examples of oncogenes are cell surface receptors such as EGFR, VEGFR2, cell cycle regulators such as CDK4, MDM2(HDM2), transcription factors such as members of

MYC family, E2F1, anti-apoptotic proteins such as BCL2 and BCLX, and regulators of cell signalling such as KRAS and AKT.

RAS is an oncogene mutated in 25% of cancers. A wide spectrum of mitogenic stimuli emanating from external environment is transduced to RAS, a GTPase, which function as a molecular switch. Activated RAS utilizes a myriad of downstream effectors, including RAF-MEK-ERK pathway to cause diverse biological functions. Point mutations render RAS constitutively active, which leads to transformation. Besides RAF, RAS can also activate PI3K, yet another oncogene, which is required for PIP3 production, resulting in AKT activation, leading to proliferation and survival. Activated RAS pathway can also promote transcriptional functions of other transcription factors such as MYC, JUN, and NFκB, and also promote genomic instability, all of which contributes to transformation and cancer [40-43].

To summarize, tumour suppressors sense cellular stress and restricts cell proliferation, guards the stability of the genome and checks illicit activation of oncogenic stimuli to check transformation. These functions are negated by growth promoting oncogenes, which promote cell proliferation and genomic instability and negates apoptosis leading to transformation. For normal cellular functions, a delicate balance between growth promoting signals from oncogenes and suppressive functions of tumour suppressors are required, which ensures regulated cell division. When this delicate balance is lost, either by deactivation of tumour suppressors and/or by activation of oncogenes, the result is transformation and cancer.

1.2 MYC

C-MYC is a multifaceted proto-oncogenic transcription factor found to be deregulated in more than 70% of the cancers [44]. It is one of the first oncogenes to be described in the context of virus induced avian tumours. MYC gained its name from myelocytomatosis, a form of diffused growth of myeloid cells arising due to the infection of MC29, an avian virus, which dates back to 1964 [45]. However, it was in 1982 that the cellular version of MYC, c-MYC was discovered, cloned and characterized in chicken embryos [46]. Since then MYC has been one of the most well studied genes in the field of cancer.

1.2.1 STRUCTURE & FUNCTIONS

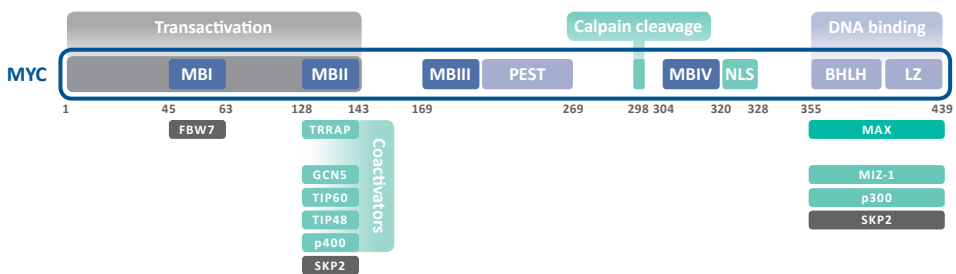


Fig.2: Structural Organization of MYC Protein.

C-MYC (MYC) belongs to the MYC family of transcription factors that also includes L-MYC and N-MYC. They share similar structure and functions but vary in their expression and role in various cellular processes, however MYCL largely lacks transcriptional and tumorigenic activities [47, 48]. Mutation, stable translocation or copy number variation of MYC can lead to cancer. Translocations involving MYC have been observed in Burkitt's lymphoma, Multiple myeloma, diffused large cell B-cell lymphoma and T-cell acute lymphoblastic leukaemia [49-52]. MYC is capable of binding and regulating several thousand genomic loci comprising nearly 15% of the mammalian genome and promoting RNA Pol I / II & III mediated transcription [53-55]. This widespread effect of MYC can be attributed to salient structural features of MYC, which enable it to bind to DNA, and allows it to have a large interactome. MYC is a 439 amino acid long protein with a well characterized Basic Helix Loop Helix – Leucine Zipper (bHLHZ) DNA binding domain at the carboxyl terminal (~100 aa long), which is preceded by a NLS (Figure 2). The N-terminal of MYC is uncharacterized, and highly flexible, enabling MYC to have a large interactome. The N- terminal also houses various conserved domains termed as MYC boxes (MBI – MBIV), which are essential for MYC functions [56]. The Transactivation Domain (TAD) of MYC houses MBI and MBII and act as a hub for binding of various binding partners. MBI is a site for phosphorylation, required for ubiquitination and proteasomal degradation of MYC, a highly unstable protein with a half-life of 20-30 minutes [57, 58]. RAS activated RAF/ERK pathway phosphorylates MYC at serine 62 (S62) via ERK, giving it stability, on the other hand RAS pathway governed by PI3K phosphorylates MYC at Threonine 58 (T58) via Glycogen Synthase Kinase (GSK), tagging it for degradation by ubiquitination [59]. The T58A mutation in MYC thus makes MYC highly stable and it is more tumorigenic than the wildtype form [60]. MBII is a critical component of TAD, a docking site for various interacting partners including components of HAT like TRAPP-GCN5, TIP60, and TIP48, involved in histone acetylation and gene activation. MBII also acts as a site for FBW7, leading to ubiquitination and proteasome mediated degradation of MYC[61-63]. The TAD domain in complex with P-TEFb is involved in transcriptional pause release of RNA POL II [64, 65]. Other functional units of MYC protein including NLS, MBIII and MBIV, are implicated in transformation, transcription and apoptotic functions of MYC [66-69]. A calpain cleavage site, proximal to C-terminal of MYC, is involved

in generation of MYC-Nick, a short fragment of MYC devoid of NLS and DNA binding. This cleavage product of MYC is involved in cytoplasmic reorganization and differentiation [70].

MYC is capable of transcriptional activation and repression depending on the transcriptional and epigenetic binding partners. MYC and its binding partners bind to canonical E-boxes (CACGTG) or MYC E-Boxes to induce transcription, while binding to non-canonical (non-E-boxes) sites usually causes transcriptional repression [71].

1.2.2 BINDING PARTNERS OF MYC

MAX, a small bHLH-LZ protein is the obligate binding partner of MYC, capable of forming stable heterodimers with bHLH-LZ region of MYC – leading to DNA binding and transcriptional activation at E-boxes proximal to promoter regions. MYC-MAX dimerization can stimulate activity of all three major RNA Polymerase – I, II and III – in all most all tissues, promoting cell growth and proliferation [54, 55, 72]. MAX-MAX homodimerization occurs, albeit weakly, relative to MYC-MAX dimerization. However, MAX can also bind to other bHLHZ proteins like MAD proteins (MXD1-4) and MNT and the binding of the resulting heterodimer to proximal E-boxes leads to transcriptional repression[73, 74]. MYC-MAX complexes are observed in highly proliferating cells, where as MAX-MNT and MAX-MXD proteins are seen in resting or differentiating cells [75, 76]. MYC and MXD-MNT proteins vary during cell cycle but MAX is present at a constant level [77, 78]. Upon mitogen stimuli or MYC overexpression, MYC out competes MXD proteins to bind to MAX, leading to gene activation. A constant level of MXD proteins is seen in every phase of cell cycle. Though MXD proteins are quite redundant, specialized functions of these proteins have also been reported. MNT however, acts as a more direct antagonist of MYC as loss of MNT phenocopies MYC overexpression [77, 79].

Besides these binding partners, MYC can also bind to HATs, GCN5 and TIP60. MYC can alter global acetylation via induction of GCN5 target genes or directly by re-

cruiting GCN5 or TIP60 to target promoters [80, 81]. These factors are recruited to MYC via scaffolding protein TRRAP5 [82, 83]. MYC is also known to repress genes. Recruitment of MYC to transcriptionally active sites of MIZ-1 along with HDACs and DNA methyl transferase (DNMT), results in suppression of such genes. In short, binding of MYC/MAX/HAT and MAX/MXD/HDAC complexes results in epigenetic modulation via histone acetylation and deacetylation respectively, thus acts as molecular switch [83, 84].

1.2.3 ONCOGENE COLLABORATION

MYC has been implicated in several types of cancers, however, genetic abrasion in MYC or its over-expression alone cannot cause cancer. Though MYC can also interact with components of pre-replication complex and promote replication, overexpression of MYC in normal cells can often have destructive outcomes, such as proliferative arrest, senescence and/ apoptosis [85-89]. MYC overexpression alone can induce DNA replication and G2 arrest, but not mitotic entry [90]. This can lead to polyploidy and DNA damage, owing to enhanced replication stress or ROS [91]. Besides these, MYC can induce apoptosis via a variety of mechanisms including up-regulation of ARF (that inhibits MDM2 thereby activating p53) and down-regulation of anti-apoptotic BCL2 family members [92, 93]. Also, gene dosage of MYC also plays a pivotal role in outcome [94]. Thus, besides MYC over-expression, for neoplastic transformation to occur, other permissive mutations/genetic lesions are essential.

MYC induced tumours are often an outcome of collaboration between other genetic lesions. MYC can synergize with over-expression of BCL2 or loss of tumour suppressors like p53, and ARF [92, 95-97]. MYC can also collaborate with other oncogenes such as RAS [98, 99]. RAS induced senescence can be suppressed by MYC while RAS can mitigate MYC induced apoptosis [100, 101]. Most of the tumours arising from deregulation of MYC are dependent on MYC and its suppression (via oligonucleotides or by omoMYC) can lead to tumour regression [102]. This has been shown in hepatocellular carcinoma, osteosarcoma, gliomas, lung cancer, etc [102-

106]. However, activation of other oncogenes or loss of tumour suppressors in such tumours could lead to tumour growth. Such resurgent tumours often have reactivation of MYC [106].

Alteration of transcriptional network due to MYC overexpression phenocopies all hallmarks of cancer (Figure 3). The following section exemplifies a few Myc target genes and their contribution to Myc-induced tumorigenesis and hallmarks of cancer.

1.2.4 MYC & HALLMARKS OF CANCER

1.2.4.1 MYC & CELL CYCLE REGULATION

MYC induced expression of cyclin D2 and CDK4, subsequent degradation of p27 and progression of cells into S-phase depicts self-sufficiency of MYC-driven tumours in growth signals [85, 107-109]. This is furthered by suppression of p53 target genes, such as p21 and p27, thereby promoting G1/S transition. As MYC overexpression alone can often lead to p53 mediated apoptosis, MYC-driven tumours often have mutations in p53 and ARF of p53-MDM2-ARF pathway[109-111].

1.2.4.2 APOPTOSIS & LIMITLESS REPLICATION

MYC over expression leads to p53 dependent and p53 independent activation of apoptosis. MYC promotes ARF expression, thus stabilizing p53, which can activate apoptosis. MYC can also engage BAX and BIM, two pro-apoptotic proteins by inducing their expression while pro-survival protein, BCLX, is repressed [112, 113]. Furthermore, repression of NFκB and FLIP sensitizes these cells to extrinsic apoptotic signals [114, 115]. While assessing these functions of MYC, it is intriguing how MYC can promote tumorigenesis when it engages these safety mechanisms to prevent aberrant growth. There exists a delicate balance between apoptotic and anti-apoptotic signals and several mechanisms to override the apoptotic pressure upon MYC over-expression have been established. As mentioned above, MYC driven tumours often have mutations in p53-ARF pathway rendering them inactive. There exists a selection pressure to lose these tumour suppressors. T58A mutation in MYC, found in BL,

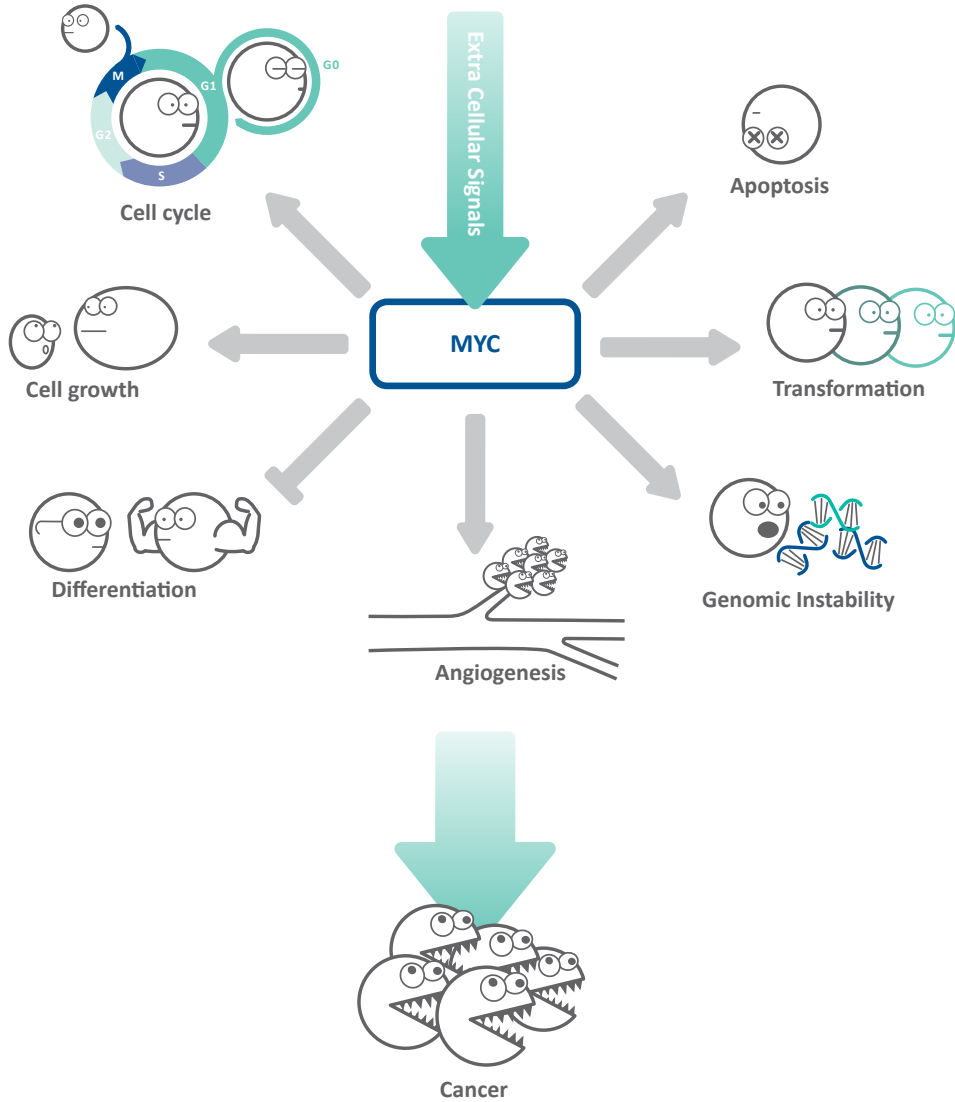


Figure 3: MYC & hallmarks of cancer.

makes it incapable of BIM activation [60]. Oncogenic MYC induction can also lead to illicit expression of BCL2 and BCLX, a collaboration that can lead to pro-survival stimulation [93]. Epigenetic deactivation of pro-apoptotic protein, PUMA, in MYC induced B-cell lymphoma has also been documented [116]. MYC-induced senescence is genetically dependent on the ARF-p53-p21Cip1 and p16INK4a-pRb pathways in Mouse Embryonic Fibroblasts (MEFs). However, CDK2 expression liberates cells from MYC induced senescence [107]. Also, oncogenic collaboration between MYC and RAS that causes suppression of apoptotic and senescence pathways can lead to robust growth of tumours as documented in mouse models of breast cancer [84]. The development of these tumours is facilitated by MYC's ability to promote limitless replication. Interestingly, TERT, the gene encoding the enzyme telomerase that regulate the telomere length (a pre-requisite for maintaining telomere and genomic integrity), is found to be a transcriptional target of MYC. Association of MYC to TERT promoter and its ability to induce TERT expression makes MYC driven cells capable of limitless replication [117].

1.2.4.3 ANGIOGENESIS & IMMUNE EVASION

Angiogenesis is an inevitable part of tumour progression. MYC is essential for angiogenesis and vascularization during tumour progression as it is involved in positive regulation of VEGF and Angiopoetin-2, essential factors for angiogenesis [118, 119]. Studies in colorectal cancer have also demonstrated a MYC dependent regulation of HIF-1 α and VEGF, two angiogenesis promoting factors, enabling angiogenesis and tumour progression [120]. Angiogenesis not only promotes tumour progression and metastasis, it also brings into contact - the immune system, which plays a vital role in eradication of tumour cells. MYC can suppress immune system and thus help in tumour progression as exemplified by MYC inhibition studies using the omoMYC mouse model. Deactivation of MYC by the expression of omoMYC lead to stable disease in the absence of functional immune system. Reduced kinetics of tumour regression, increased minimal residual disease, and inevitable tumour recurrence was observed besides MYC inactivation in mice lacking functional T and B lymphocytes [121, 122]. Moreover, MYC down-regulates HLA 1 in melanoma, there by reducing presentation of tumour antigens, leading to escape from immune surveillance [123].

1.2.4.4 EMT & METASTASIS

The ability of cancer cells to lose contact inhibition and migrate to distant locations to form tumours is an important part of tumour progression, called metastasis. Loss of cell adhesion and invasion are prerequisites for the same. MYC can regulate LGALS1 and OPN, two proteins widely implicated in cell invasion and migration [124, 125]. MYC-MIZ1 mediated RhoA expression is essential for migration, invasion and metastasis in vivo [126, 127]. Studies in breast cancer models demonstrated that inactivation of MYC could prevent invasion and distant metastasis. The same study identified 13 different “poor outcome” gene expression signatures are co-ordinately regulated by the MYC oncogene [128]. Selective amplification and overexpression of MYC is seen in both high-grade pre-malignancy and invasive tumours and is associated with poor outcome in different human tumour types [129-131]. Additionally, several oncogenes ultimately drive MYC expression either directly or indirectly [132]. However, MYC mediated suppression of α v and β 3 integrin subunits leading to suppression of metastasis has also been reported in breast cancer [133]. MYC can also regulate miR-9, which directly targets and suppresses E-cadherin, leading to loss of cell-to-cell contact, promoting metastasis [134]. As described earlier, MYC can promote proliferation, cell survival, genetic instability, and angiogenesis, all of which may contribute to metastasis. EMT is essential for invasion and migration in some contexts. Over-expression of MYC promotes EMT in mammary epithelial cells via ERK dependent GSK3- β inactivation and subsequent SNAIL activation [135]. MYC can also promote EMT by promoting TGF- β mediated activation of transcription factor SNAIL directly and indirectly through microRNA network involving LIN28B/let-7/HMGA2 cascade [132, 136]. Beyond transcriptional regulation of genes involved in invasion, migration and metastasis, MYC plays a global role in regulation of metastatic phenotype. MYC’s role in differentiation and its ability to regulate stem cell associated transcriptional profile gives cancer cells more undifferentiated phenotype, which could also contribute to migration and metastasis.

1.2.4.5 MYC & DIFFERENTIATION

Induction of differentiation to promote cancer regression has been widely studied. Cancer cells are often poorly differentiated and might share transcriptional profiles similar to stem cells, which makes them aggressive and refractory [81, 128, 137]. Retinoic acid based differentiation of neuroblastoma and teratocarcinoma are citable examples. It is documented that N-MYC and C-MYC down regulation precedes retinoic acid induced differentiation of these respective cancer cells [138-140]. In fact inhibition of N-MYC by 10058-F4, a MYC inhibitor, could induce differentiation phenotype in NB, depicting the role of MYC in promoting poor differentiation [141, 142]. Furthermore, inactivation of MYC could induce differentiation of hepatocellular carcinoma into hepatocytes and biliary cells, resulting in tumour regression [143, 144]. Besides these, MYC regulates polycomb-related genes involved in differentiation [145, 146]. Moreover, MYC being one of the Yamanaka factors, used in iPSC technology also demonstrates MYC's ability to promote potency, to maintain stemness, and to induce dedifferentiation [147-149].

1.2.4.6 MYC & ALTERED METABOLISM

Altered metabolism to support rapid proliferation and preference for anaerobic glycolysis even in the presence of oxygen (the Warburg effect) is yet another characteristic of tumour cells. Altered metabolism is also an effect of MYC induced altered transcriptional changes, of which many are metabolic genes. Altered metabolism feeds the growing demand of highly proliferating cancer cells, leading to cell growth and proliferation [150, 151]. MYC Core Signature (MCS), a set of 50 MYC target genes, has been identified to regulate ribosomal biogenesis, which plays a critical role in cell mass accumulation and also in protein synthesis [152]. Studies show that T-cells devoid of MYC were unable to mount a growth response [153]. MYC-transformed cells have increased glutamine and glucose utilization, facilitated by MYC induced enhanced expression of glycolytic and glutaminolytic genes [154-156]. These processes form toxic by-products such as lactic acid, which is further processed/regulated by MYC induced MCT1 or LDHA [157-159]. Intermediate products of these pathways are used as carbon and nitrogen source for catering nucleotide and amino acid biosynthesis [160]. MYC regulated ODC and SRM regulates polyamine biosynthesis, making MYC-driven cells self-reliant in polyamines [161, 162]. MYC binds to and regulates

several genes involved in purine and pyrimidine synthesis, thus fuelling nucleotide production [163, 164]. MYC knock down reduced the nucleotide pool in several cell lines, demonstrating a direct role of MYC in their production. PPAT, PAIC, PAFS, IMPDH2, etc are a few MYC regulated genes that regulates nucleotide metabolism [163-165]. MYC also coordinates the increase in PPP activity, glycine and folate synthesis, and glutamine uptake to fuel nucleotide production. Role of MYC in driving lipid metabolism such as fatty acid and cholesterol synthesis has been well documented [166-168]. Besides these, MYC's role in regulation of organelle (Mitochondria and Ribosomes) and protein biosynthesis have also been documented [169].

1.2.4.7 MYC & GENOMIC INSTABILITY

Tumour progression is often accompanied by accumulation of newer mutations, leading to heterogeneity, making these cells genomically unstable, which is a hallmark of cancer. MYC induced genomic instability and DNA damage, was first evidenced by increase in copy number of DFHR gene in transient MYC over-expressing cells selected for methotrexate resistance [170].

Increased copy number variation of various other genes, such as ODC, R2, Cyclin B1, Cyclin D2, etc, were experimentally validated (by FISH, Southern Blot) in cells with transient or constitutive over-expression of MYC [171, 172]. It is noteworthy that genes involved in cell cycle and DNA synthesis give growth advantage for these cells, where as DFHR gene conferred metastatic potential [173].

Later studies revealed that MYC not only causes lesions in single genetic locus, but in multiple loci leading to karyotypic changes, termed as karyotypic instability, as assessed by karyotyping. Formation of telomere and centromere fusions, extra chromosomal elements, translocations, chromosome and chromatid breaks, aneuploidy, etc was observed in MYC deregulated cell [174-177]. Irreversible chromosomal aberrations were observed in Rat1A cells following activation of MYC-ER [175], but not in mouse embryo fibroblasts [178]. This could be attributed to functional elements of MYC, excluding MBII as MBII mutants were able to cause genomic instability but not tumorigenesis in mouse [179]. Also, over-expression of MYC and genomic instability appear to be correlated in cancers of liver, breast, and colon and in colorectal cancer [180-182]. However, it has not been convincingly demonstrated that MYC

can cause DNA damage and genetic instability, in a direct manner *in vivo*, or if it is secondary to checkpoint overrides.

Over-expression of MYC in human fibroblast lead to DNA breaks caused by accumulation of ROS in the absence of apoptosis, suggesting a defunct DNA damage response [91]. Recent studies to this end also shows that MYC can induce DNA breaks independent of ROS production as assessed by IF staining of APE-1 (SSB) and γ H2AX (DSB) in normal human foreskin fibroblasts [183]. MYC plays an important role in regular replication directly and indirectly via expression of several down stream target genes. However, oncogenic MYC activation or overexpression leads to illegitimate replication of the DNA, by acting as illegitimate replication licensing factor, inducing a spurt of random replication fork firing [184]. Random replication fork firing can cause DNA damage during S-phase, but MYC's ability to over-ride check points nullifies the cells ability to cause cell cycle arrest and DNA repair, thus furthering such damage [88]. Although these damages can limit life span of the cells, impaired DDR pathway in MYC over-expressing cells instead leads to genomic instability and tumour progression [184]. ATR-CHEK1 pathway, which is constitutively active in MYC driven cells allows cellular proliferation, unlike DSBs induced ATM pathway which halts replication and prevents transformation [184, 185]. Furthermore, MYC regulates several genes involved in DSB repair (APEX, BRCA1, BRCA2, DNA-PKc, RAD50, etc), and miss match repair (MSH2, MLH1) [53, 186-188]. Taken together, MYC promotes DNA damage and genomic instability while promoting cell proliferation and tumour progression.

1.2.5 TARGETING MYC

Though MYC family of transcription factors are deregulated in over 70% of the tumours and presents a poor prognosis, MYC-targeted therapies are scarce. Genetic inhibition of MYC exemplified by dominant negative omoMYC in mouse model, suggests effective tumour regression and enhanced apoptosis by hampering transcriptional ability of MYC, while repressive functions were unaltered [189]. Pharmacological inhibition of MYC by a small molecule inhibitor, 10058-F4, capable of binding to monomeric MYC and disrupting the association between MYC and its obligate bind-

ing partner MAX could inhibit cell proliferation in C-MYC and MYCN driven tumour cells [141, 142, 190]. Similar effects were observed with oligopeptides that inhibit MYC-MAX dimerization [191]. A recently described MYC inhibitor identified from Krönke pyridine library could block the growth of xenotransplants of MYC driven tumour cells [192, 193].

Besides targeting MYC, several MYC target genes have also been investigated for their therapeutic potential. Although MYC stimulates transcription of many metabolic genes, they are not always suitable drug targets as assessed genetically in mouse models of MYC-induced lymphomagenesis [194-196]. Only Spermidine synthase (SRM) and Ornithine decarboxylase (ODC), enzymes of polyamine bio-synthetic pathway have thus far been shown to be suitable therapeutic targets to hinder MYC-induced lymphomagenesis and MYC driven tumour growth in APC^{min} mouse [162, 196]. Besides these, MYC-driven cells are also sensitive to inhibition of ribosome biogenesis and protein synthesis. WRN deficiency is also known to hamper MYC driven lymphomagenesis [197]. Inhibition of PIM3, a direct target of MYC, with a pan-pim kinase inhibitor could induce cell death independent of caspase activation [198]. Inhibition of Aurora A and B kinases could induce apoptosis independent of p53 function in MYC induced lymphomas [199]. Also, genetic or pharmacological inhibition of DNA Damage Response pathway proteins such as ATR and CHK1, which is constitutively active in MYC induced tumours, is also proven to cause apoptosis [185, 200, 201]. MYC over-expressing cells are also sensitive to several cytotoxic drugs [202]. RNAi based synthetic lethal screen identified chromatin and transcriptional process, besides DNA repair and checkpoints to be synthetic lethal with MYC. Analysis of datasets from two such screens identified 3 functional hubs: 1) genes involved in transcription initiation and elongation complex, 2) both positive and negative regulators associated with MYC-MAX network, and 3) ubiquitination and sumoylation functions related to cell cycle checkpoint and DNA repair, and kinases involved in these processes [203, 204]. Recently, targeting of HUWE1 by small molecule inhibitor was reported to cause global suppression of MYC target genes in cancer selective manner in colorectal cancer [205]. Besides these, targeting epigenetic modulators regulating transcription of MYC gene has been shown to have potential therapeutic benefits including cell death and cell cycle arrest [206].

1.3 BET PROTEINS

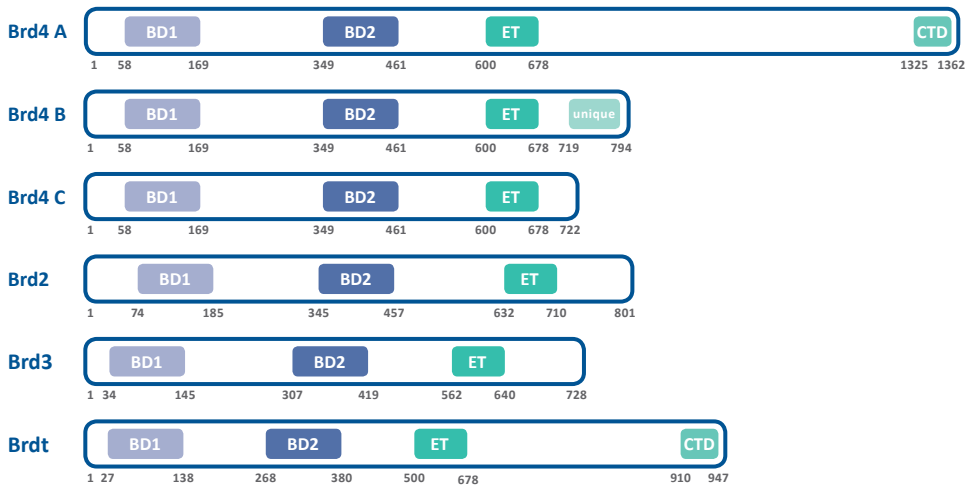


Figure 4: Structural organization of BET proteins.

BET proteins are Bromodomain and Extraterminal domain adaptor proteins that consist of BRD2, BRD3, BRD4 and BRDT, the latter being exclusive to testis (which will not be discussed). Human genome codes for 61 bromodomains contained in 46 different proteins, which act as epigenetic modulators/readers [207]. BET family of proteins bind to poly-acetylated lysine residues on histone tails and form a part of transcription regulatory complexes [208]. These proteins play a pivotal role in regulation of transcription by RNA pol II and in replication. All the four BET proteins have two bromodomains in the N-terminal end, namely BD1 and BD2, which are crucial for binding to acetylated lysines on histones and other nuclear proteins, and an ET domain towards the C-terminus. BRD4 and BRDT also contain a CTD towards

the end of C-terminus (Figure 4). Acetylated lysines are recognized by a hydrophobic cavity formed by 4 alpha helices separated by a variable loop, together forming the bromodomain [208, 209]. A single bromodomain pocket can recognize two acetyl-lysines. This could be a reason why these proteins have greater affinity towards poly-acetylated lysines than mono acetyl-lysines. Recognition of these acetylated residues on chromatin leads to binding and localization of BET proteins, which then recruits other enzymes and modulates gene expression at those loci [208, 210-212].

BRD4 is the most well studied of BET proteins and it is essential for embryogenesis [213]. It is capable of binding to regulatory proteins of transcriptional complexes consisting of mediators, pTEFb and RNA POL II and releasing the transcriptionally paused RNA POL II (Figure 5). While bromodomains on BRD4 binds to histone H4 at its di- (K5, K12) or tetraacetylated (K5, K8, K12, K16) lysines, the CTD of BRD4 can directly bind to pTEFb components, Cyclin T1 and CDK9. The BD2 domain can also bind to Cyclin T1 [214]. These interactions recruit pTEFb proximal to the paused RNA POL II [214-217]. CDK9 then phosphorylates DISF and NEFL, resulting in removal of their inhibitory interaction with RNA POL II. This is followed by phosphorylation of RNA POL II on S2 by CDK9, which ensures pause release and unperturbed elongation [217]. These interactions between BRD4 and pTEFb prevent the association of the latter from an inhibitory complex consisting of HEXIM1 and 7SK [218, 219]. Besides these effects, BRD4 is also proven to be a serine kinase *in vitro* for its ability to phosphorylate RNA POL II, however, lack of specific kinase domain and lack of *in vivo* assessment of the same makes it a disputed finding [220]. Besides these interactions, BRD4 is also known to be associated with mediator and it is known to stabilize each other's organization in varied genomic loci; but the domains of interaction are yet to be identified [217, 221, 222]. The ET domain of BRD4 can also regulate transcription in BD1/2 independent fashion. These independent interaction involves other chromatin and nucleosome modifying enzymes such as NSD3, SWI/SNF, and CHD4 [223, 224]. BRD4 also interacts with RFC1 to inhibit S-Phase progression, thus playing a direct role in replication [224]. Besides binding to acetylated histones, BRD4 is also known to bind to various other acetylated proteins/TFs such as TWIST specifically via BD2 domain [225]. Acetylated RELA, a component of NFκB, can also be bound by BRD4, facilitated by the interaction of both BD1

and BD2 [226]. A protein-protein interaction screen also identified BRD4 to interact with various transcription factors including p53, c-JUN, MYC/MAX dimers and AP2, in acetylation independent fashion [227]. Of these interactions, phosphorylation of BRD4 by CK2 is essential for productive interaction with p53 to induce DNA binding and transcriptional activation [227].

BRD2 and BRD3 are other members of BET family of proteins of which, the former is essential for embryogenesis and cell proliferation. BRD2^{-/-} mice show abnormalities in neural tube development, abnormal brain structure and die during embryogenesis [228, 229]. BRD2 and BRD3 bind to acetylated histones at varied marks, such as H3K14, H4K5, and H4K12. These signatures are found in the transcribed portion of active genes and are thought to contribute to the recruitment of BRD2/3. BRD2 and BRD3 can facilitate the histone acetylation dependent passage of pol II through nucleosomal templates [230]. The ability to bind to varied number of acetylation marks

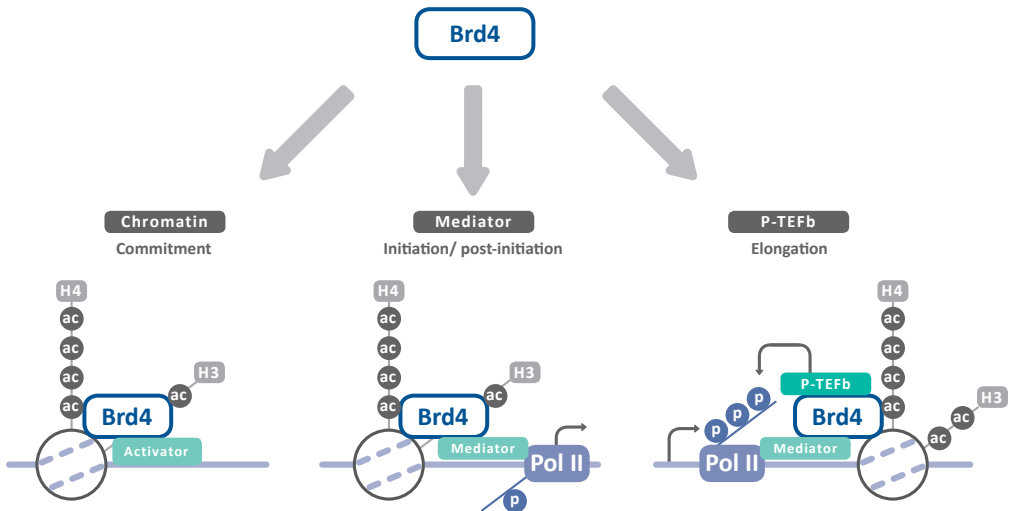


Figure 5: Role of Brd4 in transcriptional regulation.

expands the way acetylated lysine marks are interpreted while providing an additional layer of specificity. BRD2 associates with E2F transcription factor and acts as a transcriptional adapter and mediate recruitment of TBP, transcriptional complex and chromatin-remodelling activity to the cyclin A promoter, promoting its transcription and cell cycle progression [231, 232]. The CTD of BRD2 is essential for chromatin interaction, regulation of transcription and gene expression, and alternate splicing [233]. BRD3 can also bind to acetylated GATA1 via BD1 to promote chromatin binding and gene expression [234].

BET proteins are known to regulate transcription of several genes involved in cell cycle regulation and apoptosis. These proteins are deregulated in several types of tumours and hence it has been a subject of intense investigation for therapeutic inhibition. JQ1 and I-BET were the first structurally similar BETi (BET inhibitor) to be described, having high affinity towards BD1 and BD2 of BET family of proteins [235-237]. JQ1 could induce tumour regression and prolong disease free survival in various blood and solid malignancies [235, 238-243]. Several of these publications also attribute the anti-tumoural effects observed upon BET inhibition to down-regulation of MYC [239, 240, 243]. BET inhibition causes global alteration in gene expression and attribution of these effects to MYC has been disputed [242, 244]. Irrespective of this, BETi has retained its profile in the field of cancer. Several novel BETi have been described and many of them are under clinical investigation (ClinicalTrials.gov). However, implication of BET proteins in inflammation, obesity, cardiovascular diseases and in viral replication warrants for further investigation as to how BETi can affect other organs and tissues in an adult body [219, 245-247]. Its role in stem cell renewal and division has to be ascertained as well. Given its anti-inflammatory/anti-immune capacities, a scenario where BETi act as adjuvant therapy for Immune Therapy (IT) needs to be investigated further to unearth plausible effects of BETi on anti-tumoural activities of the immune system.

1.4 DDR PATHWAY

Cell division is an essential part metazoan biology. Right from development of a zygote into an organism, to wound healing, and regeneration of skin and intestinal lining, cell division is a key process. It is essential that the integrity of our genome is maintained and that the initial copy of the original DNA is copied exactly the same way into every new cell. For this, our replication machinery has diverse fool proof mechanisms, 3'-5' exonuclease activity of DNA polymerase, for instance [248]. However, intrinsic factors such as replication stress, accumulation of ROS and extrinsic factors such as exposure to UV, tobacco smoke, etc. can cause DNA damage [249, 250]. It is estimated that a cell receives around 100000 such lesions everyday. These lesions, if undetected lead to oncogenic mutations, ultimately causing cancer [251]. Also, DNA lesions can occur due to excessive replication stress and replication fork firing as a result of oncogene activation. These could enhance the mutational diversity of cancer, making it heterogeneous and uncontrollable [252].

Our cells have evolved diverse mechanisms to ameliorate these genotoxic stress, collectively called DDR (Figure 7). This involves, identifying the lesions, halting the cell cycle progression (cell cycle checkpoints), to initiate the suitable repair mechanisms and to destroy the cells with damage beyond repair. Mutations in components of this pathway often predisposes individuals to cancer and other diseases, underlying the importance of this interactome. DNA lesions or replication stress can cause formation of aberrant DNA structures, which are sensed by sensor molecules like RPA2, MRN complex etc. These activate the three most upstream serine/threonine kinases belonging to PI3KK family - ATM, ATR and DNAPKc. These kinases phosphorylate thousands of downstream transducer molecules, which further activates effector molecules - setting into play a well orchestrated signalling cascade that leads to corrective mechanisms to rid cells of DNA damage. ATM, ATR and DNAPKc activates several proteins and contribute to a wide variety of cellular processes acting in concert to safeguard the genomic integrity, but they all differ in their functions with some redundancy [253].

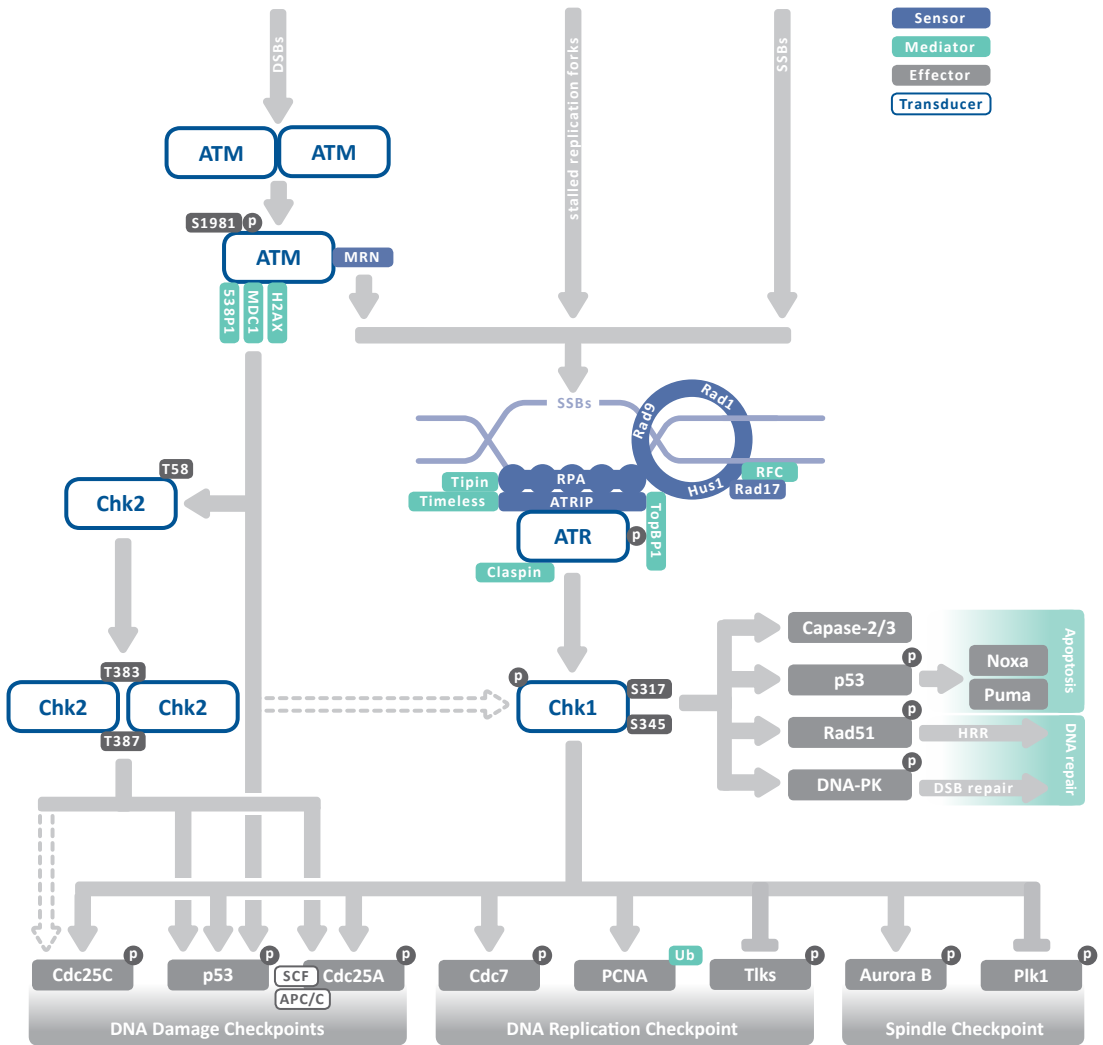


Figure 6: The DDR Pathway

Unattended ssDNA breaks and irradiation can lead to formation of deleterious DSBs which can activate ATM. Defects in ATM leads to A-T syndrome, an inherent disorder and these patients are 2-4 times more sensitive to radiation. ATM deficient cells are also defective in DSB repairs and have defective G1/S, intra-S, and G2/M checkpoints [254-261]. DSBs lead to localization and activation of ATM to these sites by MRN (MRE11-RAD50-NBS1) complex [262, 263]. Activation of ATM by MRN complex or by auto-phosphorylation leads to phosphorylation of H2AX at the sites of DNA damage [262]. This activation leads to the phosphorylation of a number of substrates by ATM, such as BRCA1, CHK2, and p53, to mediate DNA repair, cell-cycle arrest, apoptosis, and other downstream processes [264-269]. ATM can also phosphorylate and activate CHK1, mediated via NBS1 in response to radiation [270]. DSBs not only activate ATM but also ATR kinase and their respective activities are essential for DSB induced checkpoint activations. In response to DSBs caused by radiation, ATM can regulate the localization and activation of ATR onto chromatin. These responses of ATR to DSBs, is ATM-MRE11 mediated [271-273]. Furthermore, proteomic analysis of target proteins upon DNA damage identified ATM and ATR to regulate nearly 700 proteins functioning in cell cycle regulation, replication and DNA repair [274]. However, the third member of DDR pathway, DNA-PKc is primarily involved in NHEJ [275].

1.4.1 ATR – THE GUARDIAN OF GENOMIC STABILITY

SsDNA is prone to reactive agents and must be protected to conserve the integrity of the genome. This is primarily done by RPA proteins, which have high affinity towards ssDNA. SsDNA coated by RPA is essential for ATR localization and activation, a key structure that also elicits ATR response to DNA breaks. Upon generation of ssDNA, RPA covers the DNA and this is sensed by ATRIP. ATRIP recruits ATR to these locations [276, 277]. Activity of this ATR-ATRIP complex is regulated by evolutionarily conserved RAD9-RAD1-HUS1(9-1-1) complex which is loaded onto nicked DNA by RAD17-Replication Factor C complex (RFC). Occupancy of RAD17-9-1-1 complex and ATR-ATRIP complex at the lesions is independent of but essential for ATR acti-

vation and the downstream signalling. This also prevents random signal transduction and checkpoint activation. Activation of ATR-ATRIP by RAD17-9-1-1 also requires TOPBP1. 9-1-1 complex recruits TOPBP1 and activates ATR-ATRIP complex via the recruited protein [278-283]. NEK1 kinase is required for maintaining levels of ATRIP and its association with ATR, enhancing the stability of ATR, priming ATR-ATRIP complex for a robust DNA damage response [284]. Activating auto-phosphorylation of ATR at T1989 up on DNA damage is recognized by TOPBP1 and it is also essential for ATR mediated responses [285, 286]. Recruitment of these interacting complexes to the sites of DNA lesions and binding of CLASPIN leads to activation (by phosphorylation) of CHK1 (a S/T kinase) on S317 and S345 by ATR [285, 287, 288]. ATR can also activate p53 by phosphorylation, leading to activation of cell cycle checkpoints [289]. ATR is also involved in replication fork stabilization and activation through phosphorylation of annealing helicase, SMARCAL1, and in prevention of replication fork firing. It is also essential for reactivation of stalled replication forks after resection of DNA damage [290, 291]. ATR regulates levels of RRM2, a cell cycle dependent component of ribonucleotide reductase, thus dampening DNA damage and limiting origin firing [292-294]. Also, increasing amounts of RPA-ssDNA is generated at or behind replication forks when the coordination between helicase and DNA polymerases is compromised by DNA damage and other impediments. In addition to this, activated ATR in response to RPA-ssDNA is also involved in various other mechanisms such as nucleotide excision repair, HR, mismatch repair, long-patch base excision repair, post-replication repair, inter-strand cross-link repair, and replication fork restart. To summarize, the ability to respond to ssDNA gives ATR its versatility to respond to replication stress and DNA damage [276, 277, 295-299].

1.4.2 CHK1 – ACTIVATION & FUNCTIONS

CHK1 is an essential kinase and a haplo-insufficient tumour suppressor, but an extra allele can paradoxically promote transformation [295, 300-302]. Inactive CHK1 activity is similar to that of null allele and most of inactive CHK1 is confined to nucleus [303]. CHK1 can be activated by both ATR and ATM in response to DNA damage caused by IR, UV and HU [270, 301]. Phosphorylation of CHK1 at S345 leads to “opening up” of kinase domain of CHK1 by setting apart the auto inhibitory C-domain from binding to kinase domain at the N-terminal of CHK1. This phosphorylation of CHK1 is essential for its activity and this leads to release of CHK1 from chromatin, a prerequisite for its functioning [304, 305]. CHK1 can regulate all the cell cycle checkpoints - G1/S, intra S phase, G2/M and intra-mitotic checkpoints [306-313]. Phosphorylation of p53 by CHK1 leads to transcription of p21, an inhibitor of cell cycle, leading to G1 arrest. Besides direct activation of p53, CHK1 can phosphorylate MDM2 and promote p53 stability [314]. CHK1 can regulate S- phase and replication stress by regulating origin firing via two pathways: 1) by phosphorylation of its activating phosphatase CDC25A and marking it for degradation thus inhibiting CDK1/2 (in complex with Cyclin A or E) 2) by phosphorylation and inhibition of the initiation kinase CDC7/DBF4 (DBF4-dependent kinase, DDK) which, phosphorylate the preRC (ORC, CDC6, CDT1, and MCM2-7) to facilitate loading of the replicative helicase cofactor CDC45 and thus origin activation [306, 310, 313]. In accordance with the latter observation, inhibition or depletion of ATR and/or CHK1 can increase origin firing in unperturbed cells but the rate of replication (fork progression) is dampened [315, 316]. In addition to altered kinetics of origin firing and fork progression, cells inhibited or depleted of CHK1 display stretches of ssDNA at new replication forks as well as an induction of the DNA damage response, increased γ H2AX phosphorylation, and double strand breaks [317, 318].

Besides regulating S-phase, CHK1 plays a critical role in regulation of mitotic entry following G2 phase to prevent untoward incidences of premature chromatin condensation and mitotic catastrophe, which is observed up on CHK1 inhibition [319]. During DNA damage, mitotic entry is prevented by CHK1 mediated phosphorylation and inactivation of CDC25C phosphatase and WEE1 [309]. Checkpoint activation

also occurs when spindle fibers are unattached to kinetochores, which is essential for proper chromosomal segregation. CHK1 is also essential for functioning of Aurora kinase B during G2/M progression and separation of sister chromatids [311].

Among other functions, CHK1 mediated suppression of Casp-2-apoptic pathway is documented, however, Caspase mediated cleavage of CHK1 leads to its activation [320, 321]. A short splice variant of CHK1, called CHK1-S acts as an endogenous CHK1 inhibitor, promotes pre-mature mitotic entry and mitotic catastrophe, thus negating CHK1 functions [322]. Besides these ATR-CHK1-RAD51 signalling cascade is essential for induction of HR [323]. Thus CHK1 is an essential governor of checkpoints that ensures genomic integrity and stability, indicating that CHK1 inhibition could lead to genotoxic stress and cell death [324-326].

1.4.3 TARGETING CANCER BY CHECKPOINT INHIBITION

Genotoxic agents have been one of the first classes of compounds to be used against cancer, the underlying principle being their ability to cause DNA damage. A healthy DDR response could lower the impact of these treatments. Oncogenes such as MYC and RAS can cause excessive replication stress owing to their ability to ignite random replication fork firing and initiation of cells into uninhibited cycling, a phenomenon termed as oncogene induced replication stress. Enhanced susceptibility of MYC driven tumours to genotoxic compounds such as topoisomerase inhibitors and alkylating agents indicates this as a targetable weak spot [202, 327]. Therefore targeting proteins involved in DNA damage and repair involving ATR-CHK1 pathway must be deleterious as well. In fact, others and we have demonstrated that CHK1 is highly expressed in MYC driven tumours and its pharmacological/genetic inhibition can cause cell death. CHK1 (pharmacological or genetic) inhibition has been demonstrated to cause tumour regression and apoptosis in Burkitt's lymphoma, NSCLC and NB [185, 201, 328]. Anti-tumoural effects of CHK1i has also been demonstrated in melanoma and pancreatic adenocarcinoma with excessive replication stress. ATR has also been targeted pharmacologically in pancreatic cancer cells and colorectal tumours

in vivo [329-333]. Several novel, potent and selective ATR and CHK1 inhibitors are described and undergoing clinical trials. Inactivation of these components upon DNA damage in SCs and high dependence of tumours with excessive replication stress on this pathway makes them a very valuable target for therapy. Combining these drugs with genotoxic compounds is an obvious choice and has been tried in a few of the referred papers and in a few clinical trials [334, 335]. However, novel combinations are to be discovered to reduce side effects and to attain maximum remission from cancer.

1.5 BURKITT'S LYMPHOMA

Burkitt's lymphoma is one of the most aggressive and rapidly fatal malignant diseases of the immune system, arising from B-cells. It is a non-Hodgkin's lymphoma caused by translocation of the c-MYC gene to immunoglobulin loci, leading to abnormal MYC expression. Three distinct translocations have been documented, 1) translocation to IgH loci t(8;14), 2) translocation to Igk loci t(2;8), and 3) translocation to IgL loci t(8;22). Translocation involving IgH loci accounts for nearly 85% of the cases, while the other translocations are rare and accounts for the rest [49, 336-338]. BL develops from clonal expansion of B-cells carrying these translocations. They also carry B-cell markers such as B220, CD22, CD19, etc [339, 340]. Besides these, BL cells also express AID, an enzyme which mediates both Ig somatic hyper mutation and Ig class switching in V(D)J recombination, which could cause translocation of MYC to Ig loci and mutation of the translocated MYC oncogene [341, 342].

BL is predominantly found in young children in equatorial Africa and accounts for 30-50% of all childhood cancers there. It is found to be associated with malaria and EBV infection, by unclear pathologic mechanisms, albeit influence of EBV in hindering the immune response to BL has been postulated [343, 344]. Besides this type of endemic BL, sporadic and immunodeficiency associated BL are also found. Of all haematological malignancies, BL account for only 2.3%.

Though BL is a fatal disease when left untreated, outcome can be improved with aggressive chemotherapy in children. BL today is a potentially curable disease. Surgical removal of the tumour, immune therapy, radiation therapy, chemotherapy, bone marrow transplant and autologous stem cell transplant are used in clinics to fight BL. Harsh chemotherapy such as Magrath regime (cyclophosphamide, vincristine, doxorubicin, high-dose methotrexate / ifosfamide, etoposide, high-dose cytarabine) is dose adjusted to treat the patients, which results in a close to 90% complete remission. Moreover, a recent study shows an increase of overall event free survival upon intensive short-term therapies [345]. Rituximab, a monoclonal antibody against B220

surface marker is combined with chemotherapy for durable response. Although there is a 70-90 % chances of prolonged progression-free and overall survival under these treatments, toxicity and tumour lysis syndrome are major concerns [346].

1.6 MALIGNANT MELANOMA

Malignant melanoma is a cancer arising from pigment producing cells called melanocytes, present primarily in the skin but also in other parts of the body. Skin melanoma is the tumour type with the highest mutational load, making it highly heterogeneous and sturdy. Exposure to ultraviolet radiation (UVR), mostly UVB and to a lesser extent UV-A, and pigmentation traits are the biggest risk factors in melanoma. It is noteworthy that melanoma is most common among light skinned people with lighter or red hair colour and blue or green eyes than other populations with darker skin tones [347, 348]. Early detection and removal of melanocytic lesions can reduce the chances of the disease from disseminating and causing malignant lesions. But late stage malignant melanoma (stage IV) with distant metastasis shows poor prognosis and survival. Historically, the 5-year survival rate is about 15% to 20% and the 10-year survival 10% to 15% (Cancer.org, [349]).

1.6.1 MELANOMA GENETICS

Genetically, BRAF (V600E) and NRAS (at 12, 13 or 61) mutations are the most common mutations in skin melanoma, accounting for 50% and 20% of all assessed cases respectively, and are mutually exclusive mutations [350, 351]. Activating RAS mutation can activate RAS-RAF-MEK-ERK and PTEN-PI3-AKT pathways, the two most active pathways in melanoma. Oncogenic activation of these genes leads to the activation of MAPK pathway with Cyclin D1 expression, which promotes cell proliferation [352]. Furthermore, ERK can phosphorylate and stabilize MYC [59]. PTEN, a tumour suppressor regulating downstream kinases PI3K and AKT can also regulate cell survival by modulating pro-apoptotic signalling through AKT and BAD [353]. Inactivating PTEN mutations are the next most common mutations found in melanoma after BRAF and NRAS mutations [354-356]. Exome sequencing has also validated mutational biases for RB and p53 pathway deregulation [356]. Somatic CDKN2A

mutations are also well documented and germline mutations in this gene accounts for 5-25% of all familial melanomas [357-361]. Mutations in CDK4 is found in rare familial melanoma [362]. Highly penetrant mutation has also been documented in the TERT promoter in hereditary and spontaneous melanoma [363]. Also, germline mutations in PTEN can also enhance the chances of developing melanoma [364].

Light skin, red or light hair colour, high density of freckles and photosensitivity are associated with significantly increased risk of melanoma [347]. SNPs in pigmentation genes such as MC1R, TYR, AISP and TYRP are low risk genes associated with increased chances for melanoma. Aneuploidy, CDKN2A deletion, C-Kit mutations, etc. exhibits poor prognosis [365-367].

Melanoma is mostly a disease of light skinned people with Australia, New Zealand, and the Nordic countries bearing the highest incidence rates of melanoma in the world (<http://globocan.iarc.fr/pages/map.aspx>). Incidence of this cancer has increased over the last years owing to attitude changes towards tanning and sunbathing. Melanoma accounts for 5.5% of all diagnosed cancers in Sweden and it is the 5th and 6th most common cancer among women and men respectively [368]. Though the incidence of this cancer has increased, mortality rates does not follow the same trend owing to preventive measures, early detection and improved management of the disease.

1.6.2 TREATMENT OPTIONS FOR MELANOMA

Therapeutic interventions via chemotherapy made inroads into melanoma clinics in 1970s with the FDA approval for alkylating agent, DTIC (Dimethyl Traozene Imidazole Carboxamide) that showed 30% response rates. Several other agents tested in clinical trials failed to show additional efficacy (Figure 7). High doses of interferon and bolus IL-2 was given approval for treating late stage melanoma in late 1990s, however, these drugs do not form a part of standard therapies in several countries including Sweden. Frequency of BRAF and NRAS mutation and thus activated MEK-ERK pathway has been investigated for small molecule therapeutic interventions. NRAS inhibitors have been difficult to develop, while BRAF inhibitors vemurafinib and dabrafenib gained FDA approval in 2011 and 2013 respectively. MEK inhibitor trametinib was approved for clinical use in 2013. These inhibitors give short-term remission and tumour regression, however durable responses are scarce and relapse have been common.

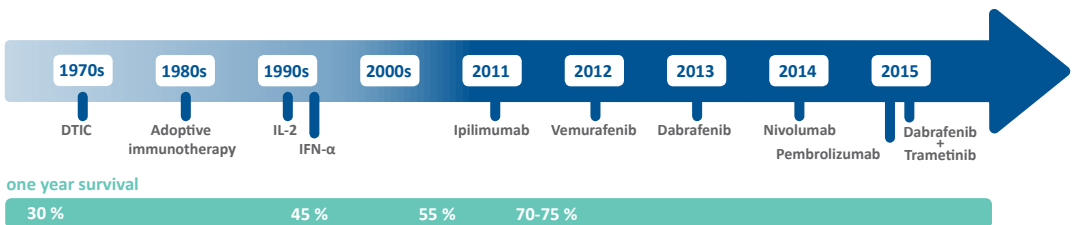


Figure 7: A time line of therapeutics against malignant melanoma and its benefits.

Following the failure of durable responses by small molecule inhibitors against melanoma in oncology clinics, inhibitors against immune checkpoints were introduced following sound pre-clinical evaluation.

Inhibitors of immune checkpoints demonstrated superior outcomes and durable responses in some cases compared to systemic chemotherapy in randomized clinical study, leading to FDA approval for ipilimumab (anti-CTLA4) in 2011, and for pembrolizumab and nivolumab (Anti-PD-1) in 2014. Today these form the front line treatment strategy against melanoma in several countries besides the use of BRAF and MEK inhibitors. Therapeutic benefits of various small molecule inhibitors and immune checkpoint inhibitors in treatment of malignant melanoma are demonstrated in Figure 7 [369-373]. Several small molecule inhibitors and immune modulating antibodies are undergoing intensive clinical trials today. However, lower durable response and relapse in cases of malignant melanoma makes it one of the sturdiest cancers with highest mortality rates and this calls for development of novel therapeutic strategies and combinatorial treatments.

2 AIM

The overarching aim of this thesis is to identify novel pathways and molecules that can be targeted to curtail MYC-induced cancers.

Specifically, this thesis aims at:

- Characterizing a novel BET inhibitor with respect to its anti-tumoural effects against MYC induced cancer.
- Identifying treatment combinations that can enhance the activity of BET inhibitors against MYC-induced cancers

3 MATERIALS AND METHODS

3.1 INHIBITORS

ATR inhibitors, AZ20 and VE821 were purchased from AXON Medchem and MedChem express respectively. RVX2135 was kindly provided by Zenith Epigenetics corp., Canada. JQ1 was purchased from Cayman chemicals while pancaspase inhibitor q-VD-Oph was purchased from Sigma. CHK1 inhibitor AZD7762 was purchased from Selleck chemicals. Pharmacogenetic library was procured from Selleck chemicals and have been described before. All the inhibitors were dissolved in DMSO and stored at -20 °C or -80 °C.

3.2 CELL CULTURE

Mouse Burkitts lymphoma cell lines, λ 820 and λ 663 were established from tumours derived from λ -MYC mouse, where as E μ 239 and E μ 580 were established by serial culturing of tumour that arose in E μ -MYC mouse. Lymphoma cell lines both mouse and human were cultured in regular B-Cell medium comprising of RPMI media supplemented with 10% heat inactivated FBS, stable glutamine and 50 μ M of β -Mercaptoethanol. P493-6 (a kind gift from G. Bornkamm, Munich, Germany) cells were cultured in regular B-cell media or in B-cell media supplemented with 0.1 μ g/ml of tetracycline (Sigma) to turn off MYC.

Melanoma cell lines, MeWo, A375 and B16F10:Luc cells were cultured in EMEM, DMEM and RPMI respectively, supplemented with 10% heat inactivated FBS and stable glutamine. All cell lines were routinely tested and confirmed free of mycoplasma by regular PCR.

3.3 CELL VIABILITY & CELL CYCLE ANALYSIS

Cells were lysed and stained in modified Vindelov's solution (20mM Tris, 100mM NaCl, 1 μ g/ml 7-AAD, 20 mg/ml RNase, and 0.1% NP40 adjusted to pH 8.0) for 30 minutes at 37°C. DNA content was analysed on BD Accuri C6 on linear mode of FL3 channel for S-Phase and sub-G1 (1 log) population on logarithmic scale on FL3 channel was considered apoptotic. For measurement of S-phase progression, cells were plated into 96-well plates and cultured in the presence of vehicle (DMSO), JQ1, or RVX-2315-2135. Cells were incubated with 3H-thymidine for the last 4 hours of treatment and were subsequently harvested onto glass fiber filters and counted in a TopCount scintillation counter.

Cell viability (drug screening and synergy experiments) was measured using the ATP-based Cell Titer Glo assay (Promega Inc) in a VICTOR plate luminometer (Perkin-Elmer).

3.4 CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

ChIP assay was carried out as per the manufacturer's protocol (SimpleChIP Plus Enzymatic Chromatin IP Kit, Cell Signaling Technology). In short, cells were fixed and lysed after being treated with RVX2135 or Vorinostat. The chromatin was partially digested with micrococcal nuclease and then sonicated. The chromatin preparation was quantified and also resolved on an agarose gel to assess the former step. Approximately 7.5µg of chromatin preparation was immunoprecipitated using 2µg of anti-Brd2, anti-Brd4 and anti-pRNA Pol II (S2) while anti-Rabbit IgG served as the isotype control. Thus immunoprecipitated chromatin was reverse cross-linked and purified. Quantitative PCR was carried out using primers flanking various regions of Egr1, Puma and Cd74. The relative binding was calculated as the product of Input % and difference between the Ct values of Input and ChIP (Relative binding = Input % (Ct input – Ct ChIP)).

3.5 RNA ANALYSIS

RNA from cells treated with JQ1 or RX was isolated using NucleoSpin® RNA II kit (Macherey-Nagel, Germany). After quantification, 500ng of RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qRT-PCR was performed using KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix (Kapa Biosystems, Inc, Woburn, MA, USA). Data analyses were performed by comparing Ct values with a control sample set as 1.

Expression profiling was carried out using Illumina Mouse RefSeq bead array as per manufacturer's instructions and the data has been deposited at NCBI Gene Expression Omnibus (GEO accession# GSE74873). PCA and GSEA were performed using the Qlucore software and clustering analyses were performed using Qlucore or GENE-E. Additional pathway analyses were done using the Ingenuity pathway analyzer.

3.6 IMMUNOBLOTING

Cell pellets were lysed in Arf lysis buffer. Approximately 20-50 µg of protein was resolved on 4-20% ClearPAGE™ gels and transferred to nitrocellulose membrane (Protran, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), after which they were blotted for specific antibodies. Antibodies against the following proteins were used: MYC, p-ATR, p-CHK1, p-4EBP1, p-AKT, p-S6 (Cell Signaling Technology), Geminin, c-Rel, Rel-B, CHK1, CHOP, ATR, ATF4 (Santa Cruz Biotechnology), p62 (Progen Biotechnik), LC3 (Novus), Actin (Sigma-Aldrich).

3.7 IMMUNOFLORESCE (IF)

Cells were cultured on 8well chamber slides and treated with desired drugs. The media was removed and cells were fixed in fixing solution (0.1% triton X100 in 4% PFA) for 15-20 minutes, after which the slides were washed in PBS. Fixed cells were permeabilized using permeabilization solution (PBS+0.5% Triton X100) at RT, after which slides were washed in PBS and the blocked using blocking solution (3%BSA, 0.1% Tween20 in PBS) for 1 hour at RT. Blocking solution was removed, slides were incubated with primary antibody diluted (2µg/ml of anti-phospho-γH2x antibody from Millipore) in 3% BSA overnight at 4°C. Slides were then washed thrice with PBST and then incubated with secondary antibody diluted in 3% BSA for 1 hour at RT. The slide was washed thrice with PBST and then mounted with Prolong Gold anti-fading mounting media with DAPI.

3.8 PATIENT SAMPLE PROCESSING

Tumour samples were collected from consenting patients who were undergoing tumour resection at Sahlgrenska University Hospital with approval from regional human ethics committee (regiona Västra Götland, Sweden #288-12). Tumour was mechanically dissociated and filtered through cell strainer. Live cell freezdowns were prepared in RPMI supplemented with 10% DMSO and 50% FBS.

3.9 IN VIVO MOUSE EXPERIMENTS

All animal experiments were carried out in accordance with approval from regional/local ethical committees (approval numbers 287/2011, 288/2011 and 36/14).

Lymphoma models: λ -MYC mouse with human MYC is placed under the λ -light chain enhancer has been described previously. Cdkn2a null mice were procured from Jackson laboratories. In tumour transplant models of lymphoma, approximately 200000 Lymphoma (λ 820 or #2749) cells per 100 μ l was transplanted to syngenic C57BL/6-Tyr (albino) mouse via tail vein injection. Blood was routinely harvested by puncturing saphenous vein and nucleated cell count was determined by NucleoCounter (NC-100TM, Chemometec). When the count rose above 15000 cells/ μ l, the animals were randomly divided into various treatment groups and the treatment commenced.

Melanoma & PDXs: For PDXs, frozen down live cells were thawed, re-suspended in RPMI and then enumerated. Approximately 400000 cells/ml was diluted 1:1 in matrigel and 100 μ l of this mixture was subcutaneously injected under the skin of SCID, IL2 chain receptor - γ knockout mice (NOG mice, Taconic, Denmark). Tumour growth was measured by vernier caliper. S100B in the blood drawn from saphenous vein of mice carrying PDXs were measured using ELISA kit (Abnova, Taiwan). B16F10:Luc cells, diluted in 1:1 of RPMI:Matrigel were syngeneically transplanted under the skin of C57BL/6-Tyr (albino) mouse. Mice were administered Luciferin by IP injections and imaged using IVIS imager (PerkinElmer) to measure tumour growth, twice a week.

Treatments: RVX-2315 at 70mg/kg was administered bi-daily by oral gavage or AZ20 at 50mg/kg via IP injections. Mice receiving combination treatment received both. Control mice received vehicle (10 % PEG300, 2.5 % Tween-80, pH 4).

3.10 STATISTICAL ANALYSIS

The bars depict mean \pm STD. Combination indices (CI) between drug A and B was calculated using the formula $CI = \text{Expected additive} / \text{Observed}$ where $\text{Expected additive} = 1 - (\text{value drug A/vehicle} * \text{value drug B/vehicle})$. Value less than 1 is considered synergistic, Value equal to 1 is additive and value greater than 1 is antagonistic. All cell culture experiments were repeated thrice or more, the microarray was performed on two biological replicates and the animal studies had a minimum of four animals

per group. The two-tailed student's t-test or tumour-free survival (log-rank) analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

*denotes p-value< 0.05, **means p<0.01, ***means p<0.001 and **** means p<0.0001

4 RESULTS

4.1 BET AND HDAC INHIBITORS INDUCE SIMILAR GENES AND BIOLOGICAL EFFECTS AND SYNERGIZE TO KILL IN MYC-INDUCED MURINE LYMPHOMA

BET proteins bind to acetylated proteins including histones to regulate transcription. Inhibitors against these proteins or BETi have been shown to have anti-tumoural effects in a wide range of tumours ranging from blood malignancies to solid tumours including melanoma. In this article we introduce and characterize RVX2135, a novel and bio-available BET inhibitor, to be capable of displacing BET proteins from their natural harbouring docks – acetylated-lysines on histones in cell free assay systems (Figure 1A). We also demonstrated its anti-tumoural effects in mouse lymphoma cell lines, λ 820, λ 663, E μ 580 and E μ 239 by affecting their cell proliferation (Figure 1B). RVX2135 also induced apoptosis by cleavage of Casp-3 and PARP (Figure 1C). These encouraging results lead us to investigate the in vivo efficacy and anti-tumoural potential of this drug. We could demonstrate this in several syngenic tumour transplant models of lymphoma and in λ -MYC:Cdkn2A^{-/-} mice where lympholeukemia and tumour free survival was significantly enhanced by the administration of RVX2135 (#2749 - Figure 1D).

Many reported studies have used BET inhibitors as ‘MYC inhibitors’, since this had been the conclusion drawn in several studies as JQ1 could suppress MYC transcription[239, 240, 243]. Unexpectedly, our studies revealed pleotropic transcriptional alterations upon BET inhibition and the observed tumour regression did not correlate with suppression of MYC transcription. Intrigued by this exciting finding, transcriptome analysis of BET inhibited samples were scrutinised to find possible mechanistic insight. That analysis revealed that one third of the genes induced by BETi were also induced by HDACi in multiple myeloma. To verify this finding in our model systems, we treated λ 820 cells with HDACi (vorinostat) or BETi, which were later subjected to transcriptome analysis and flow cytometric analysis. HDACi and BETi indeed shared a huge 25% of genes that were induced, besides sharing similar effects on cell cycle (Figure 1E), however, genes that were suppressed did not show high similarity. This observation lead us to hypothesis that BETi and HDACi could synergize owing to stronger induction of commonly induced genes, of which many were stress re-

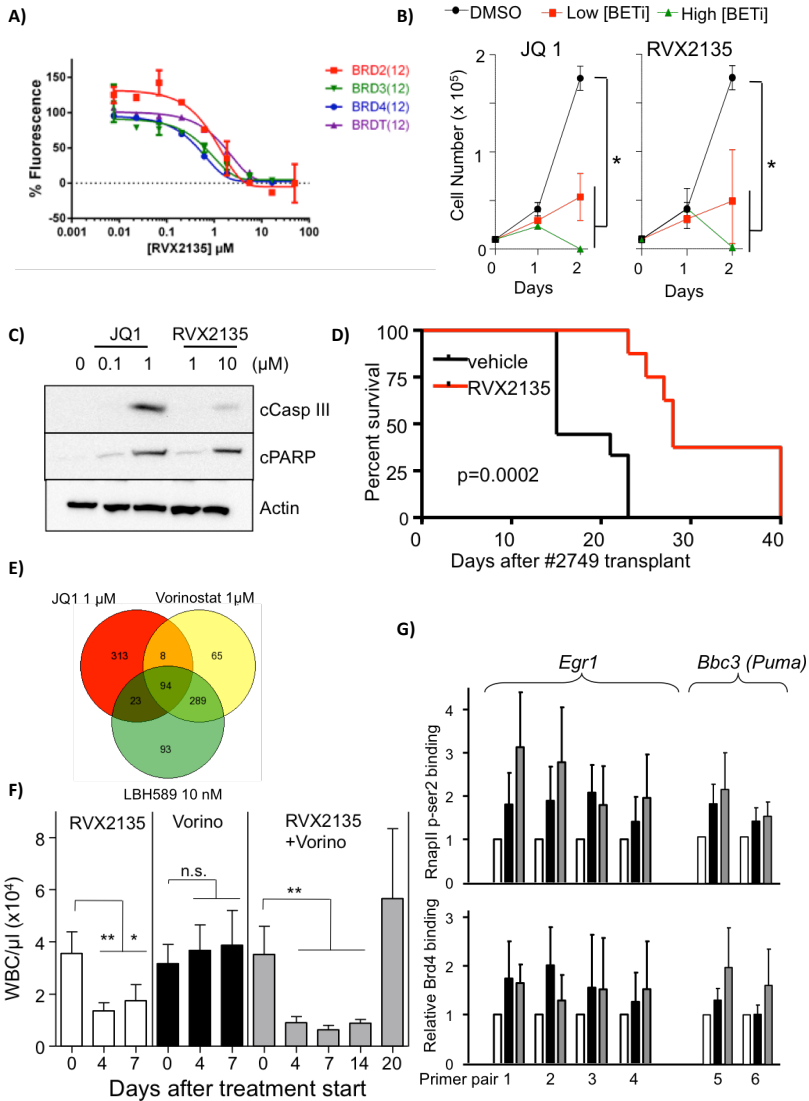


Figure 1: RVX2135 is an effective BETi capable of displacing BET proteins from acetylated lysines and can suppress Myc induced lymphomas. BETi & HDACi can induce similar genes and can therapeutically synergize.

response-related and pro apoptotic; for example *Egr1*, *Gadd45a*, *Bbc3*, etc. To test our hypothesis, λ 820 cells were treated either with BETi or HDACi or a combination of both. Individual treatments induced G1 arrest while the combination treatment lead to massive cell death, demonstrating a synergy between the two inhibitors. This was furthered in vivo using syngeneic transplant of λ 820 cells in mice. Administration of RVX2135 or Vorinostat or both showed synergistic suppression of lympho-leukemia in HDACi/BETi double treatment group, thus validating our hypothesis on synergy in vivo(Figure 1F).

To delineate the molecular mechanism behind this, I performed chromatin immunoprecipitation assays with antibodies against BRD4 and phospho-RNA POL II (S2) on RVX2135 and vorinostat treated samples. QPCR on the precipitated DNA using primer walking revealed enhanced binding of BRD4 and phospho-RNA POL II (S2) on gene loci of *Puma* and *Egr1*, two commonly up-regulated genes (Figure 1F). The *Cd74* locus, however, had enhanced and diminished binding of BRD4 when treated with Vorinostat and RVX2135 respectively, which correlated with the changes in expression as shown on the microarray and validated by qPCR. Based on publically available ChIP seq. data and on our findings, we put forth a model of BRD4 mediated transcription of genes that are differentially pause-regulated (refer the original article).

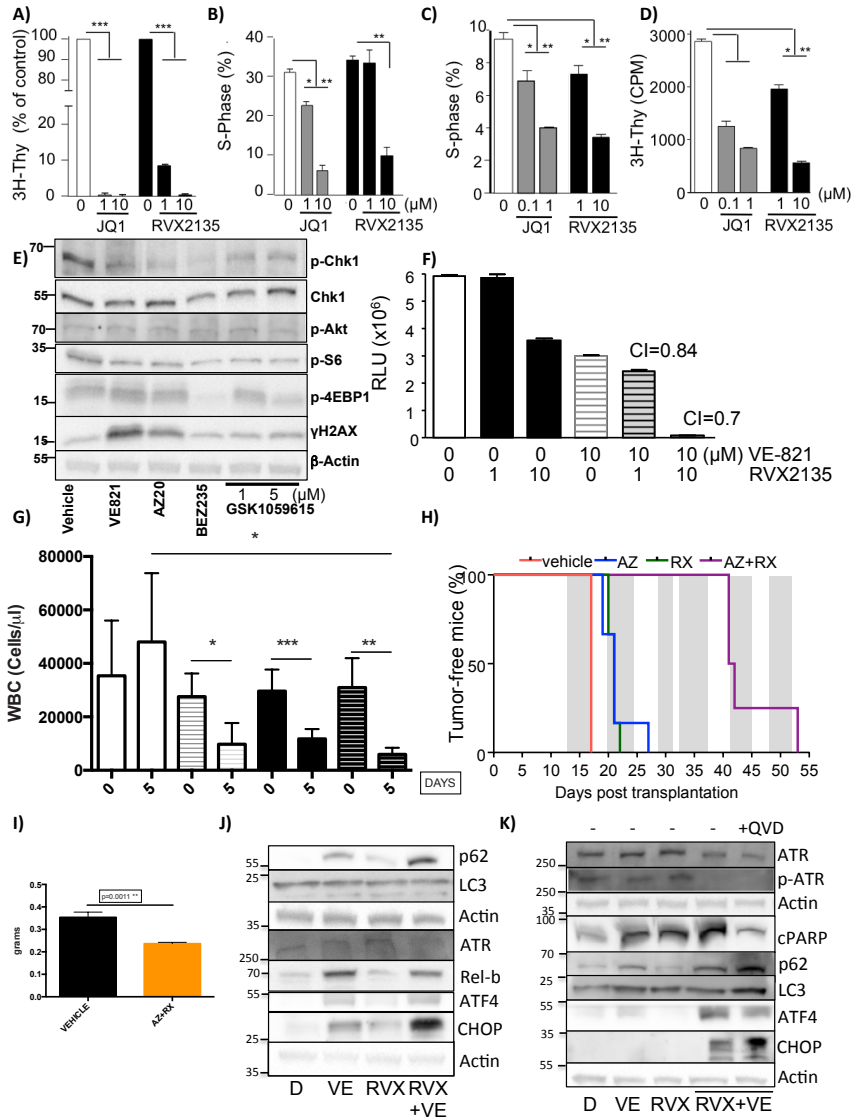


Figure 2: BETi can affect cell cycle regulation and synergize with ATRi to suppress Myc driven tumours by induction of apoptosis, SASP activation, DNA damage and ER stress.

4.2 BET Bromodomain Inhibitors Synergize with ATR Inhibitors to Induce DNA Damage, Apoptosis, Senescence-Associated Secretory Pathway and ER Stress in MYC-Induced Lymphoma Cells

One of our primary observations in Paper 1 was that BETi could lead to G1 phase arrest and apoptosis at higher concentrations but at lower concentrations cells were growth impaired. Tritiated thymidine incorporation assay on murine lymphoma cell lines, λ 820 and E μ 239 exhibited an enhanced reduction of DNA synthesis, as measured by lower 3H-Ty incorporation, up on BETi treatment at as low as 100nM of JQ1 or 1 μ M of RVX2135 where transcriptome remains largely unaltered (Figure 2A-B). To address this in a synchronous cell population, we used P493-6 cells, which is equipped with tetracycline-regulated MYC allele. In this system, addition of tetracycline lead to G1 arrest by suppression of MYC expression. P493-6 cells, following 72 hours of tet repression of MYC (MYC-OFF), followed by releasing this suppression (MYC-ON), were treated with BETi in increasing concentrations. Flow cytometry analysis of DNA histogram and 3H-ty incorporation assays on these samples could demonstrate that lower doses of BETi restricted the entry of cells into S-phase (Figure 2C, D), while higher doses prevented the same even though MYC levels remained unaltered. Using an in vitro cell free system we could also demonstrate that BETi does not affect DNA polymerases and these effects observed were indeed an effect of inhibited BET proteins.

To investigate what maintained lymphoma cells alive at low doses of BETi we performed a pharmaco-genetic screen in the presence or absence of sub-lethal doses of BETi. The screen revealed 3 classes of compounds to synergize with BETi, namely – 1) Aurora kinase inhibitors, 2) JAK inhibitors and 3) inhibitors of PI3K/mTOR pathway. One of the PI3K/mTOR inhibitors that synergized was NVP-BEZ235, which was also previously identified as an ATR inhibitor [374]. Furthermore, having observed that BETi could cause histone H2AX phosphorylation and such profound effects on cell cycle, we hypothesized that BETi could synergize with ATRi and that these PI3K/mTOR inhibitors could be affecting ATR, leading to this synergy. Immunoblot analysis of λ 820 cells treated with 1 μ M of two different PI3K/mTOR

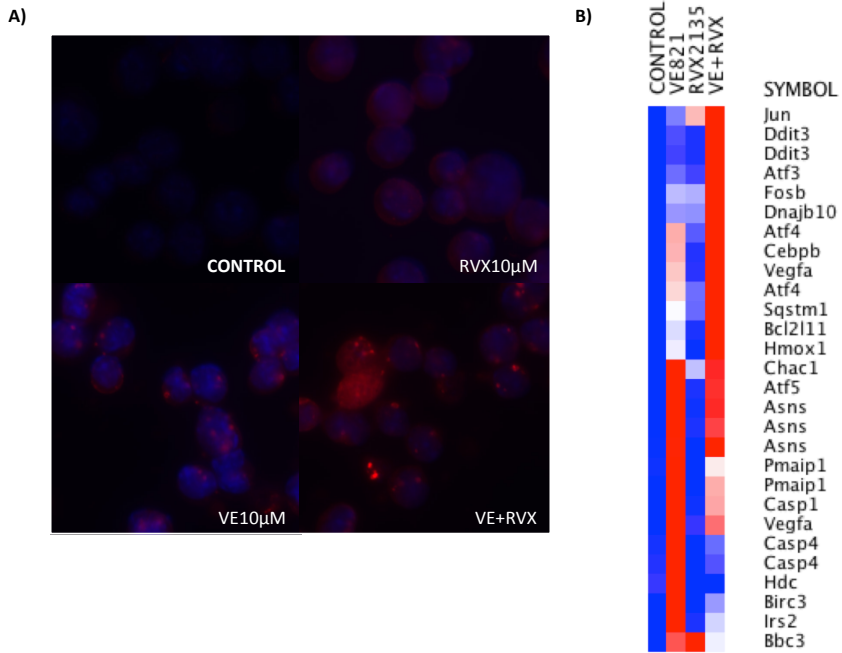


Figure 3: RVX2135/VE821 combination induces excessive DNA damage and ER stress.

inhibitors or ATR inhibitors revealed that PI3K/mTOR inhibitors indeed reduced the phosphorylation of CHK1 at S345, an ATR target, besides lowering the levels of p4EBP1 and pS6. ATRi however only reduced the phosphorylation of CHK1, but not of 4EBP1 or S6, validating our hypothesis (Figure 2E). Treatment of λ 820 cells with BETi in the presence of ATRi, AZ20 or VE821 showed synergistic reduction in cell viability and enhanced γ H2AX staining with respect to individual treatments and the control (Figure 2F & 3A). To further validate our hypothesis, we could also show that CHK1i, AZD7762, could synergize with BETi, indicating that it is indeed the ATR-CHK1 pathway inhibition that could synergize with BETi. Having found this synergy, we extended these studies into human Burkitt's cell lines namely, Akata, BJAB and Daudi, successfully recapitulating the synergy. We also investigated the efficacy of this combination therapy on two very aggressive syngenic *in vivo* models of lymphoma, λ 820 and #2749. In both the cases, not only did BETi-ATRi combination significantly extend the tumour-free survival of the mice with respect to individual treatments and vehicle, but also lowered the lympho-leukeamic burden more efficiently than the individual treatments (#2749 - Figure 2G & 2H). Acute treatment of lymphoma-bearing mice with BETi-ATRi duo lead to significant reduction in spleen size with respect to vehicle treated mice, a sign of reduced lymphoma burden (Figure 2i).

To understand the molecular mechanism behind the synergy, we analyzed the transcriptome of mono-therapies (ATRi or BETi), vehicle treatment and combination treatment. GSEA and IPA pointed towards exacerbation of SASP, ER stress (Figure 3B) and NF κ B pathway components including RELA. Western blot analysis (Figure 2J & 2K) and qPCR analysis verified these findings. Taken together, we demonstrated a novel role of BET proteins in cell cycle regulation, which is independent of MYC. We also describe a novel synergy between ATRi and BETi.

4.3 THERAPEUTIC IMPLICATIONS FOR MELANOMA OF COMBINED ATR AND BET BROMODOMAIN PROTEIN INHIBITION

Malignant melanoma is one of the most deadly forms of cancers with very high mortality rate. To identify new modes of treatment against melanoma has been a focal point of our research group. Having shown promising results in lymphoma model, the BETi-ATRi synergy was tested on several melanoma cell lines. Though ATRi, VE821, and BETi, RVX2135, were growth inhibitory, the combination of these inhibitors had profound effects on cell viability and proliferation, as shown by trypan blue exclusion assay and cell titer glow analysis. Microscopic phenotyping of these cells treated with BETi-ATRi duo showed large vacuole formation in the cytoplasm of these cells, indicative of disrupted autophagy. Immunoblotting revealed enhanced p62 expression and PARP cleavage in cells treated with ATRi-BETi combination. Elevated levels of p62 (SQSTM1), Cxcl1, DDIT3 and ATF4 transcripts were observed upon analysing the RNA of cells treated with these drugs individually or in combination.

To further validate the anti-tumoural effects of this combination therapy, tumour material from melanoma patients were xenografted under the skin of NOG mice. When tumours exhibited exponential growth, mice either received vehicle or the combination treatment (bi-daily doses of RVX2135 at 75mg/kg, single IP injection of AZ20 at 25mg/kg). Three out of the 4 PDXs responded to the treatment with reduced tumour growth. Protein analysis of resected tumours from two of the PDXs showed enhanced PARP cleavage in combination treatment group with respect to the vehicle group. CHOP and Phosphorylated γ H2AX were also present in elevated amounts in tumours that received the combination therapy. This study was also extended to, the B16F10 syngenic transplant model of mouse melanoma, which were equipped with luciferase (B16F10:Luc). In vitro assessment of individual inhibitors showed slight growth inhibitory effect, where as combination treatment had cytotoxic effect as measured by cell count. Live imaging of animals transplanted with B16F10:Luc cells showed significant tumour regression upon AZ20/RVX2135 combination treatment (5 days of treatment), while tumours in vehicle group continued to grow. Individual drugs were not tested in vivo, as they were ineffective as suggested by cell culture data.

Immune Therapy (IT), aimed at abrogating the immune checkpoints via administration of anti-CTLA4 and or anti-PD1 have shown durable but variable response rates in melanoma patient cohorts. Therefore, IT has been established as a frontline therapy against melanoma in several countries. The obvious question at hand was, does ATRi-BETi combination therapy strengthen immune response elicited against the tumours or if it would diminish the effects of IT when combined with it. To test this hypothesis, B16F10:Luc cells were orthotopically transplanted under the skin of C57BL/6-Tyr (albino) mouse. Live imaging was carried out to ascertain exponential growth of the tumour and the mice were randomly divided into two treatment groups, akin to clinical trials, receiving either IT alone or IT in combination with AZ20/RVX2135 treatment. All the mice were administered a starting cycle of IT, followed by which IT group received regular doses of IT for a week, where as combination group received AZ20/RVX2135 during that time. This was followed by regular doses of IT in both the groups. IT group showed complete durable response, where as the second group had mixed response, while 7/9 tumours grew, the rest continued to shrink as assessed by IVIS imaging. This could be due to the lack of IT treatment during the one week when combination group received only AZ20/RVX2135 treatment. Taken together, our data does not rule out that BETi-ATRi could be combined with IT.

5 DISCUSSION

BET inhibitors and ATR inhibitors are emerging as strong anti-tumoural agents and are undergoing various phases of clinical trials. An understanding of the molecular mechanisms behind their action is vital in gaining insights into how they function, what could be the possible resistance mechanisms, how to identifying the subsets of patient population that might benefit better from these treatments and also how to understand and develop modalities for handling adverse outcomes.

The first publication shows that RVX2135, like JQ1 is capable of displacing BET proteins from chromatin, can cause apoptosis and suppress tumour growth in transplantable and genetic models of MYC induced lymphoma. It is to be noted, however, that the two inhibitors are structurally distinct and has different properties. Though these inhibitors could alter the transcriptome widely and share a large subset of altered genes upon treatment, there does exist a difference. This could be attributed to the varied selectivity of these inhibitors towards BD1 and BD2, RVX2135 being more selective against the latter. It is not well documented yet, how these two domains of BET proteins vary in function, what their essential roles are and inhibition of which one of these two would be therapeutically more viable. One must also remember that the ET and CTD domain of BET proteins are very flexible protein structures and have been attributed various functions [223]. How these inhibitors can alter the functions of ET domain and the effects of these inhibitors on binding of BET proteins to other acetylated proteins also needs to be carefully addressed.

Literature on BET proteins and their anti-tumoural role in blood and solid malignancies mostly attribute these effects to down-regulation of MYC and thus BETi have been misrepresented as a MYC inhibitor. Murine lymphoma cell treated with BETi retained MYC mRNA expression, besides being sensitive to these inhibitors, resulting in apoptosis. In this account, one must remember that epigenetic cues vary between cell types, disease conditions and the state of cell division. Thus logically, alteration of MYC transcription upon BETi treatment can only occur in those cell types where MYC transcription is under the control of BET proteins. In our study, all the experiments were carried out in models where MYC is driven by transgenes, making them optimal for studying MYC independent effect.

On the other hand, many studies on BETi and MYC have failed to demonstrate that overexpression of MYC could rescue all the effects of BET inhibition, which challenge their own inference that BETi is a 'MYC inhibitor' [240, 375]. Also, MYC is known to have various promoters and altered promoter usage in MYC transcription, which could also be a decisive factor on the outcome of MYC transcription during BET inhibition [376].

Another focal point of this study is that BETi and HDACi induced similar gene expression. We speculate that it is rather gene elongation that is affected rather than transcription initiation. Positive Transcriptional Elongation Factor b or pTEFb, and its component CDK9 is essential for pause release of stalled RNA POL II by phosphorylation (at Ser 2) and to facilitate transcriptional elongation. BRD4 mediated recruitment of pTEFb to the stalled RNA pol II is a probable cause of proximal interaction between the latter two, leading to the catalysis of S2 phosphorylation. During genotoxic stress, and several treatments including BETi and HDACi, pTEFb is released from inactivating HEXIM1-pTEFb complex, aiding the recruitment of pTEFb to various genomic locations [214, 377-379].

The synergy arising from HDACi and BETi combination have a great therapeutic potential and could be applied to wider range of tumours. If the synergy is due to the altered gene sets and its consequences or an additive effect of the genes commonly induced by these drugs is not clear. Nevertheless, our mouse experiments clearly indicated the possibility of lowering the drug concentrations, which can help in reducing the side-effects.

We also discovered the synergy between BETi and ATRi. Both these classes of drugs are being evaluated in clinical trials and its noteworthy that this study put forth an option to lower the concentrations of the drugs used and also that ATRi need not be combined with DNA damaging agents to attain greater efficacy. The underlying mechanism behind this synergy, however, needs to be investigated to know 1) if ATRi potentiates BETi effects or 2) if BETi potentiates the effect of ATRi or 3) if synthetic lethality is at play. Enhanced γ H2AX phosphorylation observed during BETi and ATRi individual treatments and its synergistic enhancement up on combination ther-

apy can be due to the activation of other members of DDR pathway, namely DNA-PK or ATM kinase. It has been proposed that a short isoform of BRD4 can insulate the chromatin, protecting it from “pseudo-DNA damage” elicited by ATM mediated phosphorylation of H2AX [380]. In this situation, it may not be far-fetched to speculate that the ATR-CHK1 pathway is essential in preventing death signals transcending from activated “pseudo-DNA damage” signals due to BET inhibition. It also needs to be verified if, H2AX phosphorylation caused by BETi is indeed due to DNA double stranded breaks or not. A possible scenario of replication-transcription collision arising due to the blockage of transcription and slower progression of replication, leading to massive double stranded breaks could also be the reason for this synergy. Also, factors like RFC1, DNA-PK, CAF1, which are regulated by ATR during replication stress is also known to interact with BRD2 and BRD4 [224, 231].

One of the other interesting findings of this paper is the reactivation of NFκB pathway upon combination therapy, which is usually dormant in MYC induced lymphomas [381, 382]. It is plausible that constitutively active ATR-CHK1 pathway is involved in phosphorylating and inactivating the transcription factor involved in transcription of NFκB, which is released from suppression upon ATR inhibition. This released factor could function in tandem with released BET proteins in activation of NFκB pathway.

Another discovery reported in this paper pertains to the off-target effects observed with PI3K/mTOR inhibitors. Having shown that these inhibitors affect ATR-CHK1 pathway, many of the published data might have to be re-evaluated. Perhaps the effects observed are a sum total of PI3K/mTOR and DDR pathway inhibition, rather than mere PI3K/mTOR inhibition. Such off-targets can be at times beneficial for tumours. For instance, tumours with low replication stress, when treated with these drugs (with off-target effects) might be benefited from inhibition of tumour suppressive functions of ATR-CHK1/DDR pathway. If such tumours are treated with PI3/mTOR inhibitors at concentrations, which can affect ATR-CHK1/DDR pathway, adverse effects and tumour growth might be observed. Also, this study warrants further evaluation of blood level concentration of these drugs to evaluate possible off-target effects.

Our results also demonstrate efficiency of ATRi/BETi combination therapy in hindering cancer progression in vitro and in vivo in PDXs and B16F10 syngenic transplant model of melanoma. A few of the effects of the combination therapy was commonly shared between lymphoma and melanoma, such as activation of SASP and p62 accumulation. This indicates a common underlying mechanism leading to cell death and tumour regression. However the effect of these inhibitors, and the combination on immune system and other tissues have to be scrutinized further. A recent publication shows that disruption of BRD4 in ESCs severely disrupted the hemato-endothelial potential. Strong suppression of Brd4 has deleterious effects on healthy tissues, including severe organ stress, depletion of stem cells, etc. [383, 384]. Thus the effect of these drugs on proliferation and activation of immune cells and undifferentiated stem cell populations must therefore be assessed carefully. Though the combination was well tolerated in mice carrying low tumour burden, we did observe drug-related casualties in mice with high tumour burden. Taken together, it is essential to develop novel combination therapies, which allows for lowering the drug dosage, thus reducing the side effects and also to identify the optimal therapeutic window for using these inhibitors to achieve maximal benefits and cancer remission.

6 CONCLUSION

Data published in the articles and the attached manuscript, has scientifically established the following:

1. RVX2135 is a very potent and bioavailable BET inhibitor, capable of displacing BET proteins from its natural docking ports. RVX2135 induces a massive alteration in the transcriptome leading to anti-tumoural effects in vitro and in vivo, significantly enhancing the tumour free survival in mice transplanted with syngenic MYC driven tumours. These drugs can synergise with HDACi to further these anti-tumoural effects.
2. BETi (RVX2135 and JQ1) has a profound effect on cell cycle regulation and DNA synthesis; these effects are independent of MYC. BETi can synergize with ATR-CHEK1 pathway inhibition to induce apoptosis in vitro and in vivo. Combination treatment with RVX2135/AZ20 treatment can significantly enhance the tumour-free survival in syngenic transplant models of lymphoma. ATRi/BETi were found to elicit massive transcriptional changes, including reactivation of suppressed NFκB signalling. The activation of SASP and ER stress, induction of DNA damage and apoptosis forms the basis of this synergy. This publication also demonstrates and warns about off-target effects of so-called “specific inhibitors”.
3. BETi/ATRi combination therapy is effective against aggressive melanoma cancer cells both in vitro and in vivo. Tumour regression was observed in PDXs and syngenic transplant modal of B16F10 mouse melanomas. Our data also indicates that AZ20/RVX2135 dual therapy can be combined with IT and the former does not hinder the effectiveness and durability of Immune Therapy.

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