Using genetics to identify epigenetic and signal transduction targets in cancer

Joydeep Bhadury

Department of Surgery
Institute of Clinical Sciences
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Men & mice – divided by appearance, united by DNA.

By Joydeep Bhadury

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joydeep.bhadury@gu.se

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The PhD coaster in quotes

- If you are not excited about it, it's not the right path.
- If you fall in love with a storm (read PhD and/or lab here), do you really imagine getting out unscratched?
- Without data, everything you say is just an opinion.
- Whoever is trying to bring you down is already below you.
- Live by chance, love by choice and discover by profession.

This thesis is dedicated to my brother.

ABSTRACT

Cancer arises mostly due to the stepwise acquisition of untamed growth capabilities by various means, ranging from genetic, epigenetic to environmental factors. With the advancement made in molecular biology and associated fields, the complex biological circuits leading to these pathological conditions have now started to be deciphered in-depth. In the present thesis I have shown that mouse exome sequencing may be used to guide targeted therapy in animal models (Paper I). In this study, we for the first time made makeshift genomes of two very popular mouse strains namely BALB/c and DBA/2J.

In a subsequent paper, we could translate the concept of genetics and mouse modeling for guiding patient enrollment into future clinical trials (Paper II). Thereafter, we used RNA sequencing to decipher similarities shared between cell line-derived xenografts (CDXs) and patient-derived xenografts (PDXs) developed in Paper II. Despite similar mutational profiles, CDXs and PDXs were very different irrespective of their genotype. Here, we unravel hypoxia and specifically hsa-miR-210 as a key player orchestrating the differences (paper III). To our dismay, abrogating the regulation dictated by miR-210 using a miR decoy; makes this cells become less sensitive to MEK inhibition *in vivo*, suggesting a possible role of hsa-miRNA-210 in conferring resistance to MEK inhibitors.

Myc proto-oncogene is deregulated in vast majority of cancers types but unfortunately remains to be inhibited by pharmacological means to date. Recently, Bromodomain and extra-terminal (BET) protein inhibitors (like JQ1) have been shown as an indirect means to inhibit Myc. We set out to test the new and orally bio-available BET inhibitor (RVX2135) in a transgenic mouse model of Burkitt Lymphoma (λ-MYC Mouse), where pathogenicity of the disease may be solely attributed to the over-expression of MYC. To our surprise, the data suggested an effect of BET inhibition independent of Myc inhibition using either the prototype JQ1 or the novel compound in our systems (Paper IV). Moreover, we not only show a possible mechanistic insight of BETi but also unravel a synergistic combination of BET and HDAC inhibitors. In a follow up paper, we show lethal synergistic combinations of BET inhibitors and inhibitors of the replication stress kinase ATR in lymphomas (Paper V).

Taken together, this thesis unravels the use of various genetic and epigenetic targets as suitable candidates for therapeutical intervention either as standalone and/or in combination; deciphered using different methods as an effective strategy for combating various cancer types both *in vitro* and *in vivo.*

Sammanfattning på svenska

Cancer uppstår främst på grund av det stegvisa förvärvet av otämjd tillväxtkapacitet på olika sätt, allt från genetiska, epigenetiska till miljöfaktorer. Med framsteg inom har komplexa biologiska kretsar som leder till cancer nu börjat dechiffreras på djupet. I denna avhandling har jag visat att sekvensering av möss arvsmassa kan användas för att styra målinriktad terapi i djurmodeller (Artkel I). I den studien skapade vi provisoriska genom hos två mycket populära musstammar för att spåra arvsmasseförändringar (mutationer) i mustumörer.

I en efterföljande artikel kunde vi använda lärdomarna om genetik och musmodellering för att utveckla en mot för att styra patientrekrytering i framtida kliniska prövningar (Artkel II). Därefter använde vi RNA-sekvensering för att dechiffrera likheter som delas mellan musmodeller (xenografter) som skapats genom transplantation av cellinje (CDXs) eller det patientmaterial (PDXs) som studerats i Artikel II. Trots liknande mutationsprofiler så var CDXs och PDXs mycket olika vad det gällde genavläsningen. Jag intresserade mig för HSA-MIR-210, ett litet RNA som skillde mellan PDX/CDX och som bildas när celler får syrebrist (Artkel III). Till vår förvåning om vi förhindrar effekten av detta RNA så blir cellerna mindre känsliga för MEK hämning in vivo, vilket tyder på en möjlig roll HSA miRNA-210 i resistens mot MEK-hämmare.

Genen MYC är en sk proto-onkogen som är överaktiv i de flesta cancertyper men tyvärr finns i dagläget inga fungerande läkemedel. Nyligen visade sig hämmare av bromodomän och extra-terminala (BET) protein (som JQ1) kunna indirekt hämma avläsningen av MYC-genen. Vi bestämde oss för att testa nya och oralt biotillgängliga BET-hämmare (RVX2135) i en transgen musmodell för Burkitt lymfom (λ-MYC Mouse), där sjukdomen kan tillskrivas överuttryck av MYC. Överaskande nog så antydde mina data att en effekt av JQ1 eller RVX2135 sker oberoende av inhibering av MYC-genavläsningen i vårt system (Artikel IV). Dessutom visade vi att BET och sk HDAC-hämmare kan öka effekten av varandra (sk synergi). I en uppföljande artikel visade vi dödligt synergistiska kombinationer av BET-hämmare och hämmare av replikationsstresskinaset ATR i lymfom (Artikel V).

Sammantaget stärker min avhandling att genetiska och epigenetiska mål som fristående och/eller i kombination kan vara en effektiv strategi för att behandla olika cancertyper både *in vitro* och *in vivo*.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. **Bhadury J**, López MD, Muralidharan SV, Nilsson LM, Nilsson JA*. Identification of tumorigenic and therapeutically actionable mutations in transplantable mouse tumor cells by exome sequencing. Oncogenesis. 2013 Apr 15;2:e44. doi: 10.1038/oncsis.2013.8. PubMed PMID: 23588493; PubMed Central PMCID: PMC3641362.

II. Einarsdottir BO, Bagge RO, **Bhadury J**, Jespersen H, Mattsson J, Nilsson LM, Truvé K, López MD, Naredi P, Nilsson O, Stierner U, Ny L, Nilsson JA*. Melanoma patient-derived xenografts accurately model the disease and develop fast enough to guide treatment decisions. Oncotarget. 2014 Oct 30;5(20):9609-18. PubMed PMID: 25228592; PubMed Central PMCID: PMC4259423.

III. **Bhadury J***, Einarsdottir BO, Podraza A, Olofsson Bagge R, Stierner U, Ny L, Dávila López M, Nilsson JA*. Hypoxia-regulated gene expression explains differences between melanoma cell line-derived xenografts and patient-derived xenografts. Oncotarget. 2016 Mar 18. doi: 10.18632/oncotarget.8181. [Epub ahead of print] PubMed PMID: 27009863.

IV. **Bhadury J**, Nilsson LM, Muralidharan SV, Green LC, Li Z, Gesner EM, Hansen HC, Keller UB, McLure KG, Nilsson JA*. BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma. Proc Natl Acad Sci U S A. 2014 Jul 1;111(26):E2721-30. doi: 10.1073/pnas.1406722111. Epub 2014 Jun 16. PubMed PMID: 24979794; PubMed Central PMCID: PMC4084424.

V. Muralidharan SV, **Bhadury J**, Nilsson LM, Green LC, McLure KG, Nilsson JA*. BET bromodomain inhibitors synergize with ATR inhibitors to induce DNA damage, apoptosis, senescence-associated secretory pathway and ER stress in Myc-induced lymphoma cells. Oncogene. 2016 Jan 25. doi: 10.1038/onc.2015.521. [Epub ahead of print] PubMed PMID: 26804177.

Papers not included in this thesis

I. Lunavat TR, Cheng L, Kim DK, **Bhadury J**, Jang SC, Lässer C, Sharples RA, López MD, Nilsson J, Gho YS, Hill AF, Lötvall J*.Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells--Evidence of unique microRNA cargos. RNA Biol. 2015;12(8):810-23. doi: 10.1080/15476286.2015.1056975. PubMed PMID: 26176991; PubMed Central PMCID: PMC4615768

II. Nilsson LM, Green LC, Veppil Muralidharan S, Demir D, Welin M, **Bhadury J**, Logan D, Walse B, Nilsson JA*. Cancer differentiation agent hexamethylene bisacetamide was likely the first BET bromodomain inhibitor in clinical trials. Cancer Res. 2016 Mar 3. pii: canres.2721.2015. [Epub ahead of print] PubMed PMID: 26941288.

CONTENTS

ABBREVIATIONS

DNA = Deoxyribo Nucleic Acid
RNA = Ribo Nucleic Acid
bp = Base Pair
cDNA = Complimentary Deoxyribo Nucleic Acid
qRT-PCR = Quantitative Real Time Polymerase Chain Reaction
BSA = Bovine Serum Albumin
SDS = Sodium Dodecyl Sulfate
PAGE = Polyacrylamide Gel Electrophoresis
ECL = Enhanced Chemiluminescence
HRP = Horseradish Peroxidase
LB = Lysogeny Broth
TBS = Tris Buffered Saline
TBST = Tris Buffered Saline with Tween 20
PBS = Phosphate Buffered Saline
E.coli = Escherichia Coli
MQ = Milli-Q®
Wt = Weight
Vol = Volume
DAB = Diaminobenzidine
PI= Propidium Iodide
7-AAD =7 Aminoactinomycin D
RNase = Ribonuclease
Nonidet P-40 = Octyl Phenoxypolyethoxylethanol
NGS = Next generation sequencing
SNV= Single Nucleotide Variation
CNV = Copy Number Varriation
SNP = Single Nucleotide Polymorphism
InDel = Insertion and Deletion of nucleotides
EMT = Epithelial to mesenchymal transition
MAPK = Mitogen activated protein kinase
3-MCA = 3-methylcholantherene
PDX = Patient Derived Xenograft
CDX = Cell Line Derived Xenograft
GEMM = Genetically Engineered Mouse Models
NOD = Non-Obese Diabetic Mice

Shi-SCID = Severe Combined Immunodeficiency Mice (Shionogi Pharmaceuticals Inc.)
NOG = NOD/Shi-scid/IL-2Rγnull mice
IHC = Immunohistochemistry
miRNA = Micro RNA
GSEA = Gene Set Enrichment Analysis
FCM = Flow Cytometer
BETi = Bromodomain Inhibitor
HDACi = Histone Deacetylase Inhibitor
IP = Intraperitoneal Injection
b.i.d = biss in die (twice daily in Latin)
FDG = Fluorodeoxyglucose F18
PET = Positron Emission Tomography
FDA = Food and Drug Administration, USA
snRNA = Small Nuclear RNA
snRNP = Small Nuclear Ribonucleoproteins
ChIP-Seq = Chromatin Immunoprecipitation (ChIP) Followed by Sequencing

1. INTRODUCTION

1.1. A Brief History of Cancer

Almost ~5000 years ago, the first description of cancer was documented in the Edwin Smith Papyrus. Hippocrates coined the term carcinos (meaning crab in Greek) and later physician Aulus Celsus translated it to cancer that also meant crab in Latin. The first case report of cancer dates back to the year 1507 by the Roman physician Antonio Benivieni (Hajdu, 2010). Interestingly, surgery has remained the preferred choice of treatment in the clinics ever since Celsus's description.

Every cell within a fully-grown mammal is derived from a totipotent cell made soon after fertilization. It is now a well-accepted fact that almost all tumors tend to go back to their derivative state of embryonic origin during the process of pathogenic transformation, finally forming a tumor. Decades of histopathological observations have divided tumors into the two broad sub-types *viz.* (a) Benign: these tumors do not invade the surrounding basement membrane and grow within a defined/restricted place of origin. (b) Malignant: these tumors are known to invade the basement membrane in the immediate surrounding tissue and most often find their way to distant locations (a process called metastasis)

In general, most tumors are of epithelial origin and are known as carcinomas. A sub-type of carcinomas, called adeno-carcinomas arise from the epithelial cells of glandular origin. The rest of tumors arising from non-epithelial cells are further categorized into: (a) those derived from mesenchymal cells (sarcomas), (b) those derived from hematological origins (leukemia and lymphomas), (c) those derived from central and peripheral nervous system (neuroectodermal tumors). Besides these groups, there are tumors that show characteristics of transdifferentiation (e.g. melanomas) and dedifferentiation (e.g. glioblastoma multiforme).

Cancer is one of the leading cause of death across the globe today. Approximately 8.2 million deaths arising directly from cancer were documented in 2012 alone. Moreover, this number is speculated to increase over the coming decades as 14 million new cases of cancer were documented in 2012 alone

(source: http://www.who.int/mediacentre/factsheets/fs297/en/). Many of the cancer related deaths may be prevented by timely diagnosis and healthier life styles. Alarmingly, tobacco usage, chronic infections and sexually transmitted virus mediated tumorigenesis remains the exclusive causes for life style influenced tumorigenesis (de Martel et al., 2012). Melanoma accounts for the majority of deaths related with skin cancers to date (http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf). In USA, Non-Hodgkin's Lymphoma is among the most alarmingly increasing cause of cancer related deaths in the United States (http://www.lymphomation.org/statistics.htm).

On the positive side, owing to better diagnosis and treatment regimens today, the overall cancer related deaths has started to declined (http://www.cancer.gov/research/progress/annual-report-nation).

1.2. The Coding And Non-coding Genome

In 1800s, Gregor Mendel first suggested that hereditary units called factors determine genetic traits. These 'factors' are now known as 'genes' (coined by Wilhelm Johannsen) and are made up of Deoxyribo Nucleic Acid (DNA). Following the landmark paper discoverying the DNA structure (Watson and Crick, 1953) a new era in the field of molecular biology began. The central dogma of life comprising the unidirectional process of making proteins from DNA, via Ribonucleic Acid (RNA) was established (Figure 1) (Crick, 1970).

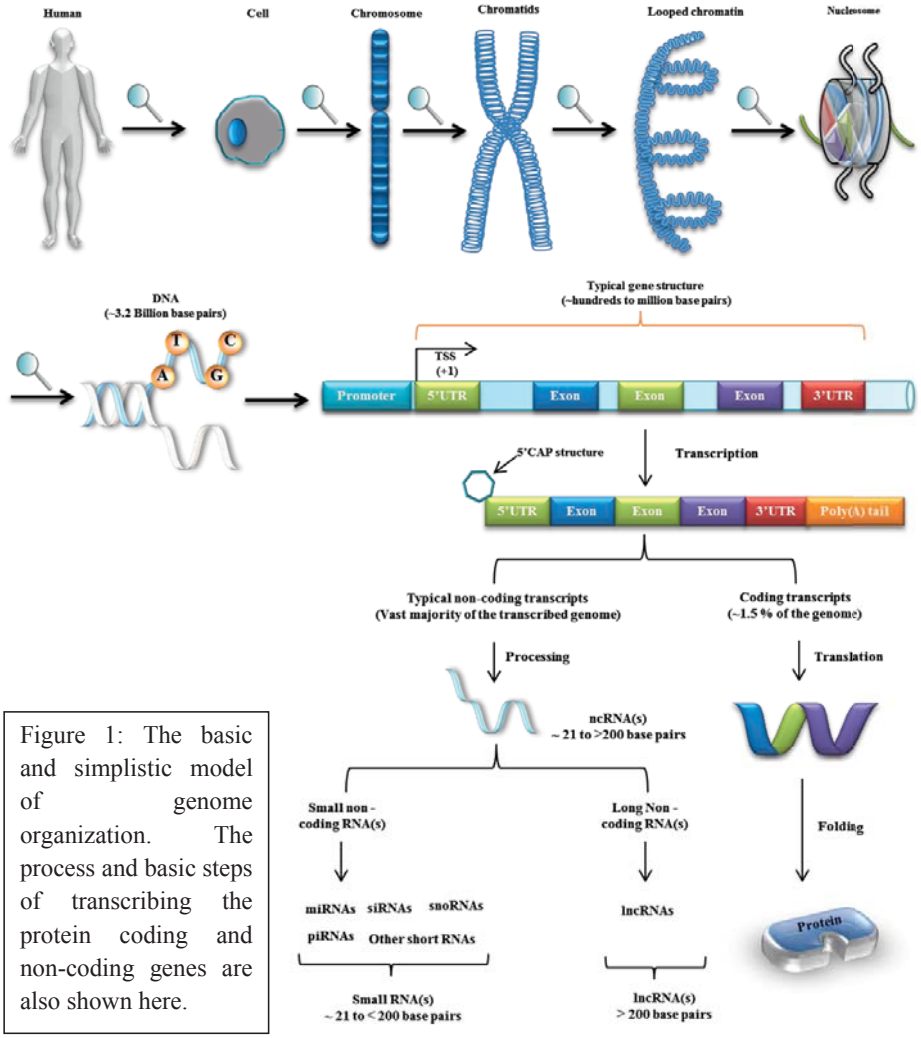


Figure 1: The basic and simplistic model of genome organization. The process and basic steps of transcribing the protein coding and non-coding genes are also shown here.

As early as 1960s, people started to wonder what the vast majority of the genome coded for, as it seemed that mostly it was not proteins. The "C- Value paradox" theory states that the complexity of any genome is not necessarily correlated to its size (Thomas, 1971). Already in 1972, Susumu Ohno first coined the term "Junk DNA" (Ohno, 1972). The human genome consists of ~3.2 million base pairs of DNA. In 2001, after the release of the first draft of human genome, it became even more evident that only a mere fraction (~1.5%) of genome codes for proteins and a vast majority of the genome was transcribed as non-coding molecules (Lander et al., 2001; Venter et al., 2001). Between 20,000-30,000 genes code for proteins (Pertea and Salzberg, 2010), whereas two thirds of the genome comprises other elements like the repetitive elements, non-coding RNA and other regulators elements (de Koning et al., 2011). Today, it's estimated that almost 70 to 90% of the genome is transcribed at some point or the other during the course of development (Consortium et al., 2007; Djebali et al., 2012; Kapranov et al., 2010) and most likely is deregulated in pathogenic conditions. The noncoding genome may broadly be classified as the short (~20 to < 200 nucleotides) and the long (> 200 nucleotides) non-coding RNAs (Kung et al., 2013; Mattick and Makunin, 2006) (Figure 1). Recent publications have shown that in fact many of the long non-coding RNAs were found to be translated (peptides if not proteins); showing a yet unknown mechanism of translation without having the prerequisite open reading frame (Kim et al., 2014; Wilhelm et al., 2014).

The small non-coding RNAs are evolutionarily more conserved compared to the long non-coding RNAs. Amongst all the non-coding RNAs, microRNAs (miRNA) are the most extensively studied. miRNAs are small ~21 nucleotide sequences that regulate gene expression either post-transcriptionally or post-translationally. Following their discovery in *C.elegans* (Lee et al., 1993), miRNAs have been found to regulate wide variety of molecular pathways across species. Moreover, a third of all protein coding genes are regulated by miRNA in mammals (Filipowicz et al., 2008) and this regulation is indispensable for embryonic development (Bernstein et al., 2003). Moreover, a large number of miRNA's are found to be deregulated in pathological conditions including various malignancies. The role of miRNA's in tumorigenesis and/or resistance to therapy in melanoma (Segura et al., 2012) has been investigated and a large number of miRNAs were found to be (de)regulated. Only a few of these, however, have been studied in detail. e.g. the

hsa-miR-210. This intronic miRNA is transcribed from a non-protein coding transcript as hsa-miR-210-hostgene (HG) which is downstream of a HIF response element (HRE). The hsa-miR-210-HG is subsequently processed to form the mature hsa-miR-210 (Huang et al., 2009; Zhang et al., 2009) (Figure 2). Moreover, hsa-miR-210 is often called the "hypoxamir" or the "hypoxia master regulator" (Chan et al., 2012; Huang et al., 2010) denoting its major role in hypoxia and its regulation among others.

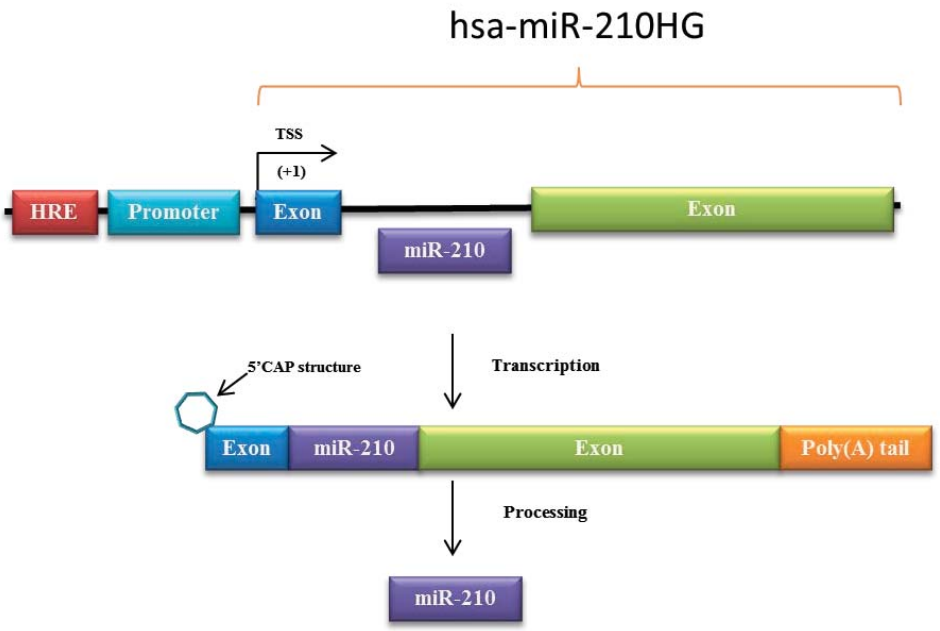


Figure 2: Genomic structure and processing of hsa-miR-210HG to form mature hsa-miR-210.

1.3. Oncogenes

In 1909 Peyton Rous made the seminal discovery of a sarcoma inducing filterable agent that was isolated by him from a chicken having sarcoma in the breast muscle. This filtered agent appeared capable of serial transmission from one chicken to another without losing its capacity of forming tumors and was therefore thought to be an infectious agent (Rous, 1910; Rous, 1911). Later it became evident that this infectious agent was an avian retrovirus, which was called as the Rous Sarcoma Virus (RSV). In late 1950s, the striking discovery of the RSV particles being able to infect chicken fibroblast cells *in vitro* was made (Temin and Rubin, 1958). This *in vitro* infected cells conferred growth advantage like unlimited proliferation, change in morphology and loss of contact inhibition and hinted towards the fact that the cells indeed had transformed into cancer cells (Hanahan and Weinberg, 2000; Weinberg, 2007). In the following years many viruses were discovered which had the capabilities similar to RSV, despite being very different in their genetic makeup. Numerous experiments hinted towards the fact that viral replication and its capabilities to transform a cell were in fact regulated by different components/genes in the viral genome. In 1974, Michael Bishop's and Harold Varmus's laboratory first described that the cloned genomic fragment responsible for the transformation capabilities of RSV virus was in fact also present in the normal chicken cells. This discovery came to the conclusion that the viral sarcoma gene from RSV (v-*src*) and the cellular counterpart found in normal chicken cells (c-*src*) and other related species, shared extensive sequence homology (Stehelin et al., 1976a; Stehelin et al., 1976b). This pointed to the fact that the genes identified in the viral genomes had identical/similar cellular versions, most likely having similar transformation capabilities. The viral genes capable of transforming a normal cell were called oncogenes and their cellular counterparts were termed proto-oncogene, both coding for oncoproteins. During late 70s and early 80s, another avian retrovirus called the myelocytomatosis virus (MC29) was found to have similar transformation capabilities like RSV; and indeed the viral gene (v-*myc*) had a cellular counterpart (c-*myc*) (Sheiness and Bishop, 1979; Sheiness et al., 1980).

A characteristic property of a viral gene bearing transformation capabilities is that it produces enormous amounts of the hijacked oncoproteins. It's not always required that a proto-oncogene be deregulated because of the regulation exerted by the viral genome, instead many proto-oncogenes may be spontaneously

mutated to attain similar transformation capabilities. This is the case of the RAS (a virus first identified as the cause of rat sarcomas) family, wherein *H-RAS* (named after Jennifer Harvey) (Wong-Staal et al., 1981), *K-RAS* (named after Werner Kirsten) (McGrath et al., 1983) and *N-RAS* (first described in human neuroblastoma and hence the name) (Shimizu et al., 1983) proto-oncogenes are found predominantly mutated across cancer types which converts them into potent oncogenes (Bos, 1988; Tabin et al., 1982). Today, the RAS superfamily consists of approximately ~150 distinct cellular proteins. The canonical RAS members (*K/H/N RAS*) combined together is most likely the most mutated gene known to date across human cancer types (Fernandez-Medarde and Santos, 2011).

The RAS family members act as molecular switches, toggling between an active or inactive state, similar to all G- proteins. In the inactive state, RAS is bound to guanosine diphosphate (GDP), whereas it is bound to guanosine triphosphate (GTP) in its active state. The switching of the states is mediated via guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP) respectively (Figure 3A). RAS proteins are known to possess intrinsic GTPase activity, which is stimulated by GAPs. In general, RAS is anchored to the membrane after specific post-translational modifications. Once bound and activated, RAS binds RAF kinases (A/B/RAF-1) which in turn signal via downstream protein kinases namely MEK and ERK (Figure 3B) (Weinberg, 2007). RAS is also known to regulate PI3K and RAL-GEF pathways, besides regulating the RAF/MEK/ERK pathway. Owing to the role of RAS proteins, invariably all Pancreatic Ductal Adenocarcinoma and Colorectal Carcinomas exhibit a mutated canonical *RAS* member and/or its bonafide downstream effectors (Forbes et al., 2011; Vaughn et al., 2011). In melanoma, predominantly either *BRAF*, *N-RAS* or *NF1* (GAP for RAS) are found mutated, pointing towards the indispensable role of the MAPK pathway in melanoma tumorigenesis (Davies et al., 2002; Forbes et al., 2011; Omholt et al., 2002). The most commonly occurring mutant forms of RAS have changes in amino acid residues 12 or 13 or 61 and these hot spots are mutated at varying rates depending on the canonical RAS member (Forbes et al., 2011). Taken together, the MAPK pathway is an appropriate target for therapeutic intervention across cancer types (Anne M. Miermont, 2013; Dong et al., 2011; Gilmartin et al., 2011; Morris et al., 2013; Salama and Kim, 2013; Yang et al., 2010).

To summarize, the process of transformation may be defined as the path taken by normal cells to become malignant due to the accumulation of certain mutations resulting in untamed growth advantages by the virtue of activated or deregulated proto-oncogenes (among other genetic and epigenetic factors).

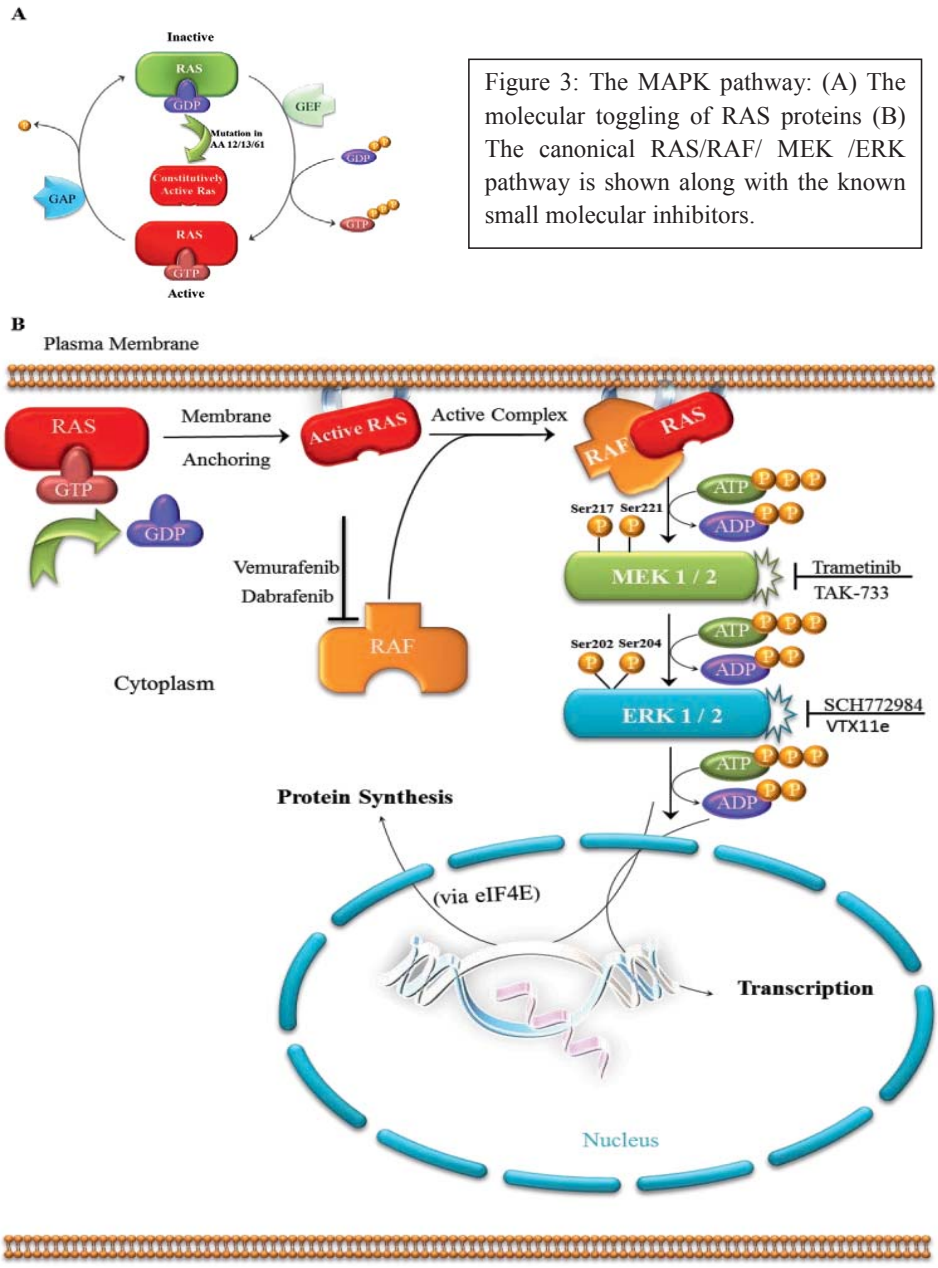


Figure 3: The MAPK pathway: (A) The molecular toggling of RAS proteins (B) The canonical RAS/RAF/ MEK /ERK pathway is shown along with the known small molecular inhibitors.

1.4. Tumor Suppressors

Mere deregulation or mutational activation of proto-oncogenes is not enough to drive tumorigenesis. In fact, this was evident in the first demonstrations concerning RSV in 1900s, where the filtrate (virus) injected into young chicken would develop tumors only after a gap of a couple of weeks. These early experiments hinted towards the fact that something resisted the tumor development (Rous, 1910; Rous, 1911). Today, it is well-accepted fact that tumor suppressors are indeed the resisting forces. By convention, tumor suppressors may be defined as the gene(s) whose mutation either by deletion and/or by loss of expression leads to tumor progression primarily in association with an oncoprotein coupled with other genetic/epigenetic changes. Not surprisingly, compared to the deregulation/activation of oncoproteins, the loss of tumor suppressors is said to have a larger effect in predisposing a cell towards transformation (Weinberg, 2007).

The transcription factor, "tumor protein p53" (TP for human and Trp for mice) of approximately 53kDa (as assessed by SDS page, hence called p53) was the first identified tumor suppressor gene. In 1979, it was co-immunoprecipitated along with SV40 large-T antigen in cells transformed with large T antigen. However, initial experiments performed with multiple cDNA variants of *Trp53* was found to accelerate tumor development in combination with *Ras* oncogenes (Eliyahu et al., 1984). Experiments over the years, have shown that, *TP53* follows the "two-hit hypothesis" (Knudson, 1971), in most tumors. This hypothesis states that unlike oncogenes, loss/mutation of one allele is not sufficient to overcome the regulation exerted by a tumor suppressor. In other words, most if not all tumor suppressors are haplo-sufficient. However, since the p53 protein is a tetramer, it can also operate in a dominant negative manner when mutated. Hence, loss of heterozygosity is not always a pre-requisite for transformation, at variance with e.g. *RB* or *CDKN2A*.

It is now estimated that *TP53* is among the most mutated tumor suppressor gene across human cancer types (Weinberg, 2007). The majority of *TP53* point mutations are missense mutations (resulting in single nucleotide variation) instead of nonsense mutations (which typically results in premature translation termination primarily via stop-gain mutations); giving these dominant negative variants untamed tumorigenic potential (Muller and Vousden, 2013; Weinberg, 2007). Loss of heterozygosity most often results in gaining the dominant

negative mutant forms of TP53, while simultaneously losing the other/only wild type allele.

TP53 receives input signal from numerous molecular networks and can respond specifically to each of these signals. Briefly, UV induced damage, hypoxia, replication or oncogene induced stress, transcriptional blocks among others are signals fed by number molecules to TP53; which in turn responds by regulating DNA repair, cell cycle arrest or even apoptosis depending on various other (co)factors. The typical G_1 cell cycle arrest imparted by TP53 is regulated by its interaction with p21 (a cyclin-dependent kinase inhibitor, also known as $p21^{Cip1}$) (Abbas and Dutta, 2009). On the other hand, S/G_2 cell cycle arrest is mediated via regulation of 14-3-3sigma and Cdc25 (Donzelli and Draetta, 2003; Hermeking et al., 1997). Moreover, TP53 is also known to induce *PCBP4* and *GST1* to affect S/G_2 cell cycle arrest, specifically in response to genotoxic stress (Taylor and Stark, 2001). Recently, an array of known and novel *Trp53* targets upon genotoxic stress have been documented (Tonelli et al., 2016). In normal cells, levels of TP53 are very tightly regulated by its interaction with HDM2 (Mdm2 in mice). TP53 regulates *HDM2* and HDM2 tags TP53 for proteasome mediated degradation (by ubiquitin meditated pathways); but phosphorylation of TP53 by certain kinases under specific condition protects it from degradation. In case of ionizing radiations, TP53's interaction with protein kinases from DNA Damage Response (DDR) pathway (ATM, Chek1, RADs, among others), which in turn phosphorylates TP53 preventing it from degradation by HDM2 (Fei and El-Deiry, 2003). Moreover, PI3K pathway is also known to negatively regulate TP53 levels by increasing *HDM2* levels. On the other hand, Arf (another important tumor suppressor) is also known to positively regulate Trp53 by binding Mdm2 and preventing its localization to the nucleus. Owing to this roles of TP53 in maintaining the genomic integrity among others, it's being correctly termed as the "guardian of the genome" (Lane, 1992).

Retinoblastoma protein (pRB or RB) is another major tumor suppressor gene and is mutated or indirectly deregulated across cancer types (Dunn et al., 1988; Liu et al., 2004). It is well established that during G_0 phase of cell cycle pRB is unphosphorylated and it tends to be phosphorylated (hypo-phosphorylated) as the cell prepares to enter the G_1 phase. In order to proceed into S-phase and complete the replication, pRB has to be hyper-phosphorylated at multiple sites (at the R-check point) and remains hyper-phosphorylated until the cell successfully exits M-phase. Thereafter, pRB is dephosphorylated so that it can

again effectively regulate the entry into cell cycle, if required (Figure 4) (Giacinti and Giordano, 2006). Moreover, pRB phosphorylated specifically at serine 780 (S780) is a hallmark of cells actively entering into S-phase of cell cycle (Kitagawa et al., 1996). Furthermore, the precise phase of any cells may be validated by two other indispensable markers of G_1 (CDT1) (Nishitani et al., 2001) and S/G_2M (GEMININ) (McGarry and Kirschner, 1998) phases of cell cycle by either probing with specific antibodies against them or using real time visualization tools like FUCCI factors (Sakaue-Sawano et al., 2008).

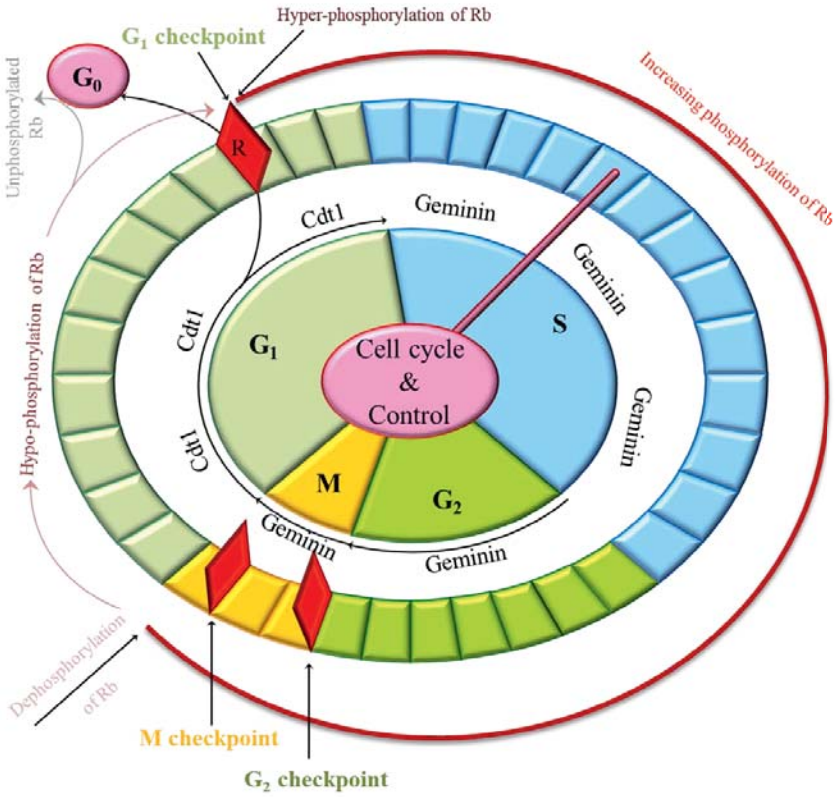


Figure 4: The different phases of cell cycle and the active checkpoints used by cells to tightly regulate this process. The roles of pRb and its phosphorylation status in different phases along with two indispensable marker of G_1 (Cdt1) and S/G_2M (Geminin) phase are also shown.

1.5. Driver And Passenger Mutations

One of the main factors leading the way towards precision medicine is due to the advancements made in Next Generation Sequencing (NGS) technologies, which has seen an enormous development in the last decade (Meldrum et al., 2011). With the unprecedented development, reduced cost and faster turnaround time, researchers can now investigate entire genomic / transcriptomics / epigenomics landscapes of several pathogenic and/or other relevant physiological states (like embryonic development), instead of only a handful of genes and/or proteins.

In 2009, Michal Stratton and colleagues first coined the concept of driver and passenger mutations in cancer (Stratton et al., 2009). Driver mutations are the mutations occurring primarily in proto-oncogenes or its essential control partner, thereby making these genes code for oncoproteins responsible for transformation. Whereas, the other mutations that do not primarily confer growth advantage; but are somehow present during the transformation of the normal cell to a malignant one, are known as passenger mutations.

Among the ~23,000 protein coding genes in the human more, at least 1.6% (~350) are said to be recurrently mutated across various cancer types (like *MYC, RAS, BRAF*, and others). Moreover, many of these driver genes are not important for tumorigenesis but are in fact responsible for development of resistance during the course of treatment (Stratton et al., 2009). Despite the considerable impact of driver mutations, the major bulk of passenger mutations in most cancer type didn't seem very reasonable choice for inheritance by these malignant cells. Recently, it has started to emerge that these passenger mutations are not just bystander but in fact can be very detrimental in the disease pathogenesis. It has also been shown that for the success of immune therapy against melanoma, tumor neoantigens play a very important role and can even influence the treatment response (Snyder et al., 2014). Moreover, a vast majority of the passenger mutations or synonymous variations may be targeted to get anti-tumor response (Castle et al., 2012) and many of the passenger mutations have now been shown to have very significant role in tumorigenesis (Shipman, 2016).

1.6. Intra Tumor Heterogeneity

Despite making seminal advances in understanding disease pathogenicity, the precise cause of resistance to therapy remains to be elucidated. The simple and stepwise evolution of cancer due to mutations and/or deregulations of proto-oncogenes and tumor suppressors appears to explain colorectal tumors (Fearon and Vogelstein, 1990); but unfortunately cannot be extrapolated into other cancer types. In a landmark study, Charles Swanton and colleagues could track multiple heterogeneous driver mutations in the metastatic lesion, which were shown to arise from different regions within the same tumor (Gerlinger et al., 2012). Now, increasing evidence points towards the fact, that in addition to one major driving force behind the initial tumor development, the heterogeneity inside a given tumor may also play a role. Moreover, the involvement of cancer associated fibroblasts and macrophages, and the emergence of cancer stem or initiating cells within a given tumor renders the overall issue of heterogeneity even more complex (Hanahan and Weinberg, 2011).

1.7. MYC: The Untamed Wolf of Cancer

Almost three decades back, the oncogene (v-*myc*) from the myelocytomatosis virus (MC29) was traced back to a conserved cellular counterpart (c-*myc*) (Roussel et al., 1979; Sheiness and Bishop, 1979; Sheiness et al., 1980). In mammals, *MYC* has two other paralogs *MYCN* (neuroblastoma derived homolog) (Schwab et al., 1983) and *MYCL* (lung adenocarcinomas derived homolog) (Nau et al., 1985). *MYC* codes for a basic-Helix-Loop Lelix (bHLHZ) domain containing transcription factor of 439 amino acids and many of the conserved domains are shared with *MYCN* and *MYCL*. As seen in figure 5, MYC protein contains two MYC boxes (MBI and MBII) in the N-terminal and these two domains together are called as transcriptional activator domain (TAD). The PEST (Proline, Glutamic Acid, Threonine and Proline) domain; two other MYC boxes (MBIII and MBIV) and the nuclear localization signal then follow it. The C-terminal domain of MYC contains the bHLHZ domain (Conacci-Sorrell et al., 2014). Like most members of the bHLHZ family, MYC dimerizes with another protein to bind DNA. MYC heterodimerizes with MAX (another bHLHZ protein) to bind specific DNA sequences (5'-CACGTG- 3') typically around promoter regions called canonical E-boxes (Enhancer boxes) and this interaction in turn activates transcription (Amati et al., 1993). On the other hand, binding of this heterodimers to non-canonical E-boxes is believed to result in transcriptional repression (Blackwell et al., 1993). Moreover, both N-Myc and L-Myc heterodimers are also known to bind the canonical and non-canonical E-boxes to interact with specific DNA sequences (Ma et al., 1993). MAX is also known to make self-homodimers but its affinity to bind DNA is extremely weak compared to the MYC-MAX heterodimer, and the self-homodimersation prevents phosphorylation of MAX by Casein Kinase II (CKII) (Berberich and Cole, 1992). Furthermore, MYC is shown to even bind non-canonical E-boxes without MAX (Hopewell and Ziff, 1995) and have probable MAX-independent regulation (Sabo and Amati, 2014). MYC protein has a half-life of less than ~30 minutes in normal physiological condition (Hann and Eisenman, 1984). The typical way of degradation of both MYC and MYCN is by tagging the protein for ubiquitin-mediated degradation. The hotspot residue (Serine 62 and Threonine 58) in MBI is the target for ubiquitin ligase Fbw7 and is subsequently degraded by proteasome mediated pathways. Furthermore, the T58 residue is one of the most frequently found mutations in

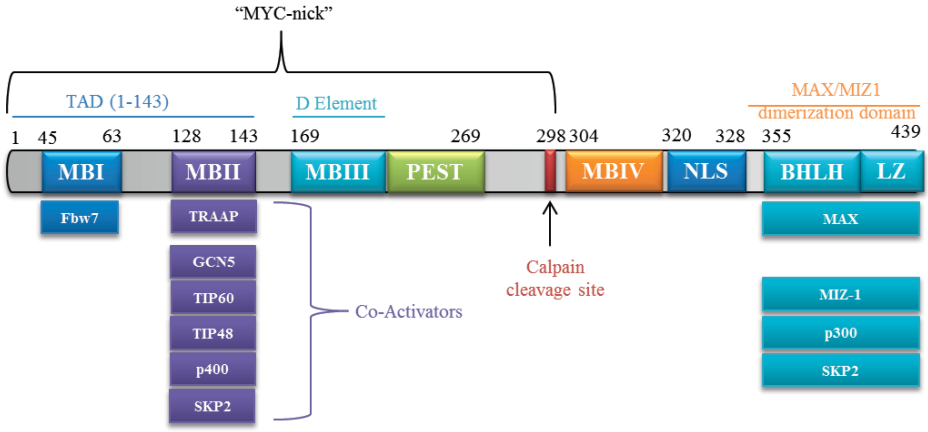


Figure 5: MYC domains along with the associated protein factors. Figure adapted from (Conacci-Sorrell et al., 2014; Farrell and Sears, 2014).

Burkitt lymphomas (Bahram et al., 2000; Welcker et al., 2004a; Welcker et al., 2004b). MBII is the most extensively studied MYC domain because of its indispensable role in transcriptional activation owing to its interaction with a number of histone acetyl transferase and other co-factors (McMahon et al., 1998). Furthermore, the TAD domain is known to interact with the positive transcription elongation complex (P-TEFB) (Gargano et al., 2007; Kanazawa et al., 2003) and Bromodomain containing protein-4 (BRD4) (Wu et al., 2013). MYC is known to give transformation capabilities to cells under deregulated conditions, but on the other hand overexpression of MYC at the same time can lead to extensive apoptosis (Nilsson and Cleveland, 2003). Moreover, MYC overexpressing tumors are known to evade the restrictions imposed by tumor suppressors by either acquiring deletions or point mutations in these genes, e.g. as in lymphomas where TP53 and ARF are inactivated (Eischen et al., 1999; Hemann et al., 2005; Schmitt et al., 1999; Zindy et al., 1998), and immune surveillance (by regulating PD-L1 and CD47) (Casey et al., 2016). Furthermore, MYC regulates a wide range of target genes transcription governed by RNA Pol II, by regulating there transcriptional elongation (Bres et al., 2008). Moreover, MYC also directly interacts with RNA Pol I (Arabi et al., 2005) and III (Gomez-Roman et al., 2003) to stimulate rRNA and tRNA synthesis. MYC not only induces the transcription of target genes but is known to repress a lot them (Herkert and Eilers, 2010). Interestingly, despite having an enormous number of target genes, the regulation of activation and/or repression potential of MYC by the "promoter invasion model" both in the normal and tumorigenesis have been

recently demonstrated (Sabo et al., 2014). Taken together, this shows that MYC is always under tight regulation both at transcriptional and at translational levels in normal cells (Conacci-Sorrell et al., 2014; Farrell and Sears, 2014).

MYC is indispensable in embryonic development and only *Myc* heterozygous mice are born albeit with not completely normal phenotypes (Davis et al., 1993). On the other hand *Myc* null rat fibroblasts remain viable in spite having smaller cell size and slower replication cycles (Mateyak et al., 1997), though the precise molecular mechanism owing to their propagation without Myc is not fully understood to date. Given the indispensable role of MYC in normal and pathogenic states, targeting of MYC was an obvious step. To date not many direct MYC inhibitors are known, primarily owing to its intrinsically disordered protein nature (IDP) (McKeown and Bradner, 2014). In fact, more than a third of the all known proteins are known to be IDPs and are extremely difficult targets for therapeutic interventions (Metallo, 2010; Wright and Dyson, 1999). A typical feature of IDPs is their short half-life primarily owing to either shorter stretch of poly (A) tails; degron regions (ubiquitin dependent or independent); and a PEST motif within the protein (Gsponer et al., 2008; Schrader et al., 2009). On the other hand, proteins of this kind are extremely flexible in nature and are categorized within the "induced fit model", leaving some room for therapeutic intervention (Johnson, 2008) of the complex instead.

In early 2000, Peter Vogt's group (Berg et al., 2002) and Edward Prochownik's (Yin et al., 2003) group provided the first evidence for targeting MYC by targeting the MYC-MAX heterodimer as a possible therapeutic strategy. Among other compounds found in the screen, 10058-F4 was tested quite extensively, showing promising results but could not be used because of its poor pharmacokinetic properties and oral bio-availability. Recently, new generations of compounds like 10074-G5 (Yap et al., 2013) and KJ-Pyr-9 (Hart et al., 2014) are known to possess better potency and *in vivo* efficacy compared to the first generation of inhibitors. Using structure-guided design, a novel 93 amino acid long peptide (bearing four unique amino acid substitutions) comprising the bHLHZ domain called "Omomyc" was discovered (Soucek et al., 1998; Soucek et al., 2002) and is known to inhibit MYC function both *in vitro* and *in vivo* (Savino et al., 2011; Soucek et al., 2008; Soucek et al., 2013). Moreover, Gerard Evan's lab has shown, that compared to continuous induction of Omomyc, no major toxicity in normal tissues were observed when the same was induced only

periodically without compromising its potent anti-tumor effects (Gabay et al., 2014; Soucek et al., 2013).

An indirect targeted inhibition of co-factors and/or target genes of MYC could be a possible choice to block Myc-driven cancers. Towards this end, targeting of ornithine decarboxylase (*Odc*), a bonafide Myc target gene significantly delays formation of lymphomas (Nilsson et al., 2005). On the other hand, abrogation of S-phase kinase-associated protein 2 (*Skp2*; an E3 ubiquitin ligase), which is known to degrade MYC has no significant effect on lymphomagenesis (Old et al., 2010). In fact, many of the MYC target genes are dispensable for its tumorigenic potential (Keller et al., 2005; Nilsson et al., 2004; Nilsson et al., 2007). Moreover, inhibition of PP2A (A tumor suppressor with phosphatase activity) seems effective (Gutierrez et al., 2014), likely since PP2A is crucial for mediating the stability of MYC because of its direct interaction Ser62 phosphorylation. Furthermore, both Aurora Kinases (A and B) are known to be crucial for maintenance of tumorigenic potential primarily regulated via MYC (den Hollander et al., 2010). Indeed, targeting of AURKA in *MYCN* amplified tumors seems effective (Brockmann et al., 2013). Another alternative strategy is to inhibit checkpoint kinases involved in the DNA Damage Response pathways (DDR) like CHEK1/2, ATR as standalone and/or in combination in MCY driven tumors (Campaner and Amati, 2012; Höglund et al., 2011a; Höglund et al., 2011b; Murga et al., 2011). It's been recently shown that inhibition of Bromodomain and extra terminal (BET) proteins can be an effective strategy to inhibit MYC (Alderton, 2011; Dawson et al., 2012; Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010; Herrmann et al., 2012; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011). Couple of decades back, it was shown that by simply restricting RNA Pol II, a potent down regulation of c-myc was achieved leading to the terminal differentiation of promyelocytic leukemia cell primarily due to promoter proximal pausing (Strobl and Eick, 1992). The agent used in this experiment was dimethyl-sulfoxide (DMSO), a molecule later shown to bind BET protein (Lolli and Battistutta, 2013; Philpott et al., 2011). It is logically expected that if one inhibits a member of the complex relieving the promoter proximal pausing, the genes controlled by them should be potently downregulated. Taken together, one can conclude that MYC remains undruggable to date (Soucek and Evan, 2010) and in fact is the untamed wolf of cancer.

1.8. Epigenetic Modulators

Although the exact origins of the term epigenetics is still under debate (Haig, 2004), it is probably derived from the word epigenesis, which is said to be coined by Aristotle (Tarakhovsky, 2010). Back in 1940s, the first evidence of nature and nurture both governing phenotypes were provided by Conrad H. Waddington. He later went on to prove the same, using classical experiments performed in developing chicken embryo (Van Speybroeck, 2002). Notably, all of this was done prior to the discovery of DNA structure (Berger et al., 2009). Today, epigenetic traits may be defined as a potentially inheritable phenotype mainly based on the soft markings on DNA and the chromosome without altering the DNA sequence per se (Berger et al., 2009; Youngson and Whitelaw, 2008). Moreover, the possibility of epigenetic regulation to be not solely orchestrated by DNA sequence and in part be influenced by the immediate surrounding environment is not fully understood yet (Ptashne, 2007).

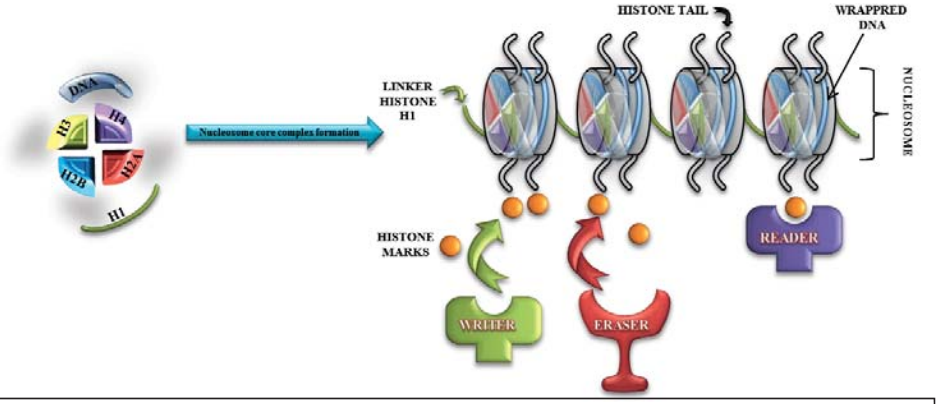


Figure 6: Epigenetic Modulators. "Writers" add the histone marks (orange circles) primarily to histone tails. The "Erasers" remove these marks and the "Readers" recognize and read this specific histone modification to subsequently impart a wide array of regulation processes.

Most epigenetic modulators influence DNA modifications and architecture of chromatin directly or indirectly. The basic unit of chromatin is an octameric nucleosome complex comprising two each of four histone proteins - H2A, H2B, H3 and H4. The octamer is wrapped around by approximately ~147 bp of DNA. Nucleosomes are stabilized and joined by linker DNA bound by histone 1 (H1) (Luger et al., 1997; Segal and Widom, 2009). Epigenetic modulators are divided into three main categories: - Writers, Erasers and Readers (Figure 6). Each of these are explained below

1.8.1. Epigenetic writers classification

Epigenetic writers are the class of enzyme that imprints distinct post-translational modifications (PMTs) across the histones tails (Ruthenburg et al., 2007); among the writers the most widely studied are the Histone

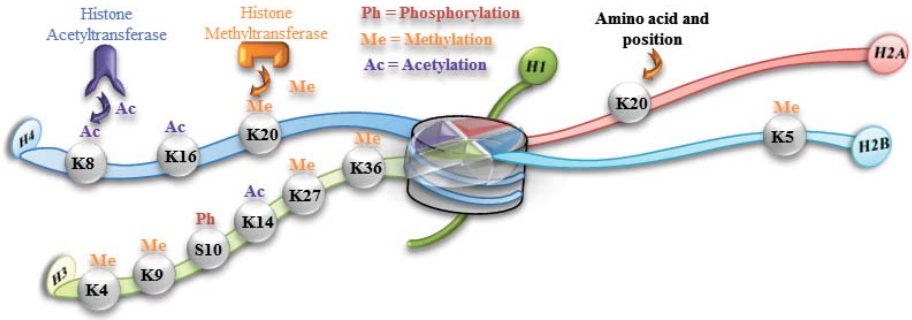


Figure 7: Figure showing most abundant epigenetic writers and their imprinting locations on the specific histone tails.

Acetyltransferase (HAT) and Histone Methyltransferase (HMT) (Figure 7) (Arrowsmith et al., 2012). The first study to show the importance of histone acetylation dates back to 1964 (Allfrey et al., 1964) and thereafter numerous studies discovered the indispensable role of HATs in regulating a wide variety of molecular functions. There are about 18 protein members in the HAT family and approximately 60 members in the extended HMT family (HMT plus the protein methyltransferase). HATs mediate the addition of acetyl group to the ε-amino group of specific lysine residues in the histone tail by using the acetyl of Acetyl-CoA as the donor (Arrowsmith et al., 2012). The acetylation of lysine(s) in the histone tail(s) reduces the overall net negative charge, thereby making the chromatin structure more open and/or accessible to various factors. The acetylation of lysine 16 in histone 4 (H4K16) has been shown to switch the chromatin between heterochromatin (closed/repressive) and euchromatin (open/active) states (Shahbazian and Grunstein, 2007). Moreover, H3K9 and H3K14 are also known to aid active gene transcription (Koch et al., 2007).

On the other hand, HMTs play a crucial role in addition of various methylation marks to specific substrate along the histone tail. As the addition of methyl group doesn't result in change of net charge in this histone, it is assumed that histone methylation doesn't play a prominent role in maintaining the chromatin structure directly. Nevertheless, the addition of these marks (mono, di or tri-

methylation) is known to allow homing of specific protein and factors which can either silence or activate gene expression, dependent on the amount/number of methyl group present in the amino acid (Lee et al., 2007; Nielsen et al., 2001; Trojer et al., 2007). Tri-methylation (me3) of histone 3 at lysine 4 (H3K4me3) is considered as a hallmark for actively transcribing genes, whereas H3K9me3 and H3K27me3 are primarily associated with repressed/silenced genes (Bannister and Kouzarides, 2011; Kouzarides, 2007). Moreover, mono-methylations at H3K4, H3K9, H3K27, H4K20, H3K79, and H2BK5 among others are also marks of active gene transcription (Barski et al., 2007; Benevolenskaya, 2007; Steger et al., 2008). On the other hand, di-methylation (me2) of H3K9, H3K27, H3K79 and tri-methylation of H3K9, H3K27, H2BK5 are shown to associate with suppression of transcription (Barski et al., 2007; Rosenfeld et al., 2009; Steger et al., 2008).

1.8.2. Epigenetic erasers classification

Epigenetic erasers remove the marks imprinted by the writers. Broadly, erasers are classified as Histone Deacetylase (HDAC) and Histone lysine demethylase (HDM) (Figure 8). In general there are around 17 HDACs and 25 HDMs known (Arrowsmith et al., 2012). As this thesis pertains mostly HDACs, I will discuss them further below.

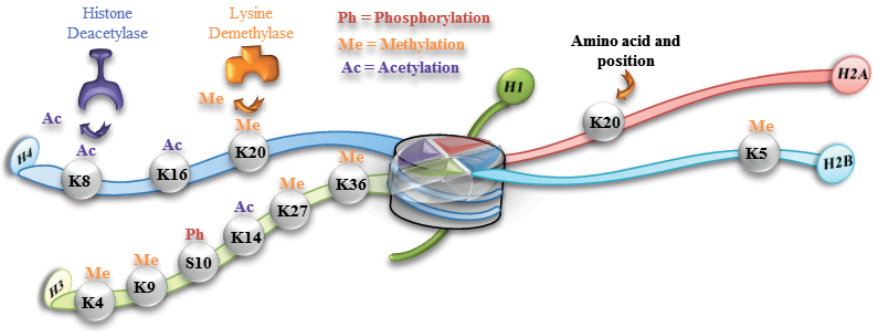


Figure 8: Figure showing the most abundant epigenetic erasers and their imprinting locations on the specific histone tails.

HDACs catalyze the removal of the acetyl group from the ε-amino group in lysine. A delicate and precisely controlled interplay between HDACs and HATs in general is maintained during homeostasis, which is tangled in many pathogenic states. Primarily, HDACs may be further classified as mentioned in table 1.

HDACs and HATs not only regulate the chromatin structure, but are also known to regulate non-histone targets genes, which are involved in tumorigenesis, apoptotic pathways, DNA repair pathways among others (Bolden et al., 2006). HDACs from Class I, II and IV are also known as "Classical HDACs" and require zinc ions as a cofactor in their catalytically active site (Arrowsmith et al., 2012; Atadja, 2009), whereas Class III HDACs are zinc-independent but require Nicotinamide adenine dinucleotide in oxidized form (NAD^+) for their activity (Imai et al., 2000). Not surprisingly, most HDAC inhibitors (HDACi) developed to date somehow or the other mimic and/or compete for this zinc dependent moiety to inhibit the HDACs (Atadja, 2009).

Vorinostat or SAHA is the first non-selective HDACi to be approved by FDA for the treatment of cutaneous-cell lymphoma (O'Connor et al., 2006). Contrary to expectation, only a modest success has been observed in single agent trials using Vorinostat or other HDACi *per se*, especially for solid tumors (Li et al., 2011; Traynor et al., 2009). Owing to the significant alteration of global gene expression patterns and lack of profound activation of apoptosis pathways in response to single agent HDACi, it seems more likely to be used in combination in both pre-clinical and clinical trials setting; specifically against solid tumors (Huffman and Martinez, 2013).

Phylogenetic Class	Name	Drug and their targets	Localization
Class I (Zinc Dependent)	HDAC1 HDAC2 HDAC3 HDAC8	Vorinostat , Panobinostat and Belinostat (HDAC1,3,6) Romidepsin (Class I) Entinostat and Mocetinostat (HDAC 1 & 2) AR-42 (Class I)	Nucleus
Class II a (Zinc Dependent)	HDAC4, HDAC5, HDAC7 HDAC9	AR-42 (Class II) Panobinostat (HDAC 4 & 7 at higher concentrations)	Nucleus and cytoplasm
Class II b (Zinc Dependent)	HDAC6 HDAC10	Vorinostat , Panobinostat and Belinostat (HDAC6) AR-42 (Class II)	Mostly cytoplasm, sometimes in nucleus
Class III (Zinc independent, NAD dependent)	Sirtuin (SIRT1-SIRT7)	Resveratrol (including derivatives) and resveratrol containing formulation in combination.	Nucleus, Cytoplasm and/or Mitochondria
Class IV (Zinc Dependent)	HDAC11	Panobinostat	Nucleus and cytoplasm

Table 1: Defining the phylogenetic classification of HDACs along with their approved/available inhibitors and there cellular localizations. Adapted and modified from (Arrowsmith et al., 2012; Atadja, 2009; Balcerczyk and Pirola, 2010; de Ruijter et al., 2003; Glozak and Seto, 2007).

1.8.3. Epigenetic readers classification

Many of the modifications imprinted by the epigenetic modulators influence the processes of DNA replication, repair and transcription among others, directly or indirectly (Berger et al., 2009; Goldberg et al., 2007; Kouzarides, 2007; Segal and Widom, 2009). The epigenetic readers are the class of the modulators that reads the imprinted marks to finally execute the relevant molecular program. No wonder that these readers are one of the most widely studied proteins. Epigenetic readers are broadly classified as (a) Bromodomain containing proteins that have 61 members (b) Proteins containing Tudor/MBT/PWWP/Chromo domains. These proteins have the methyl lysine and/or methyl arginine-binding domain and consist of approximately 95 protein members (c) PHD domain containing proteins and have approximately 104 protein members identified (Dawson et al., 2012).

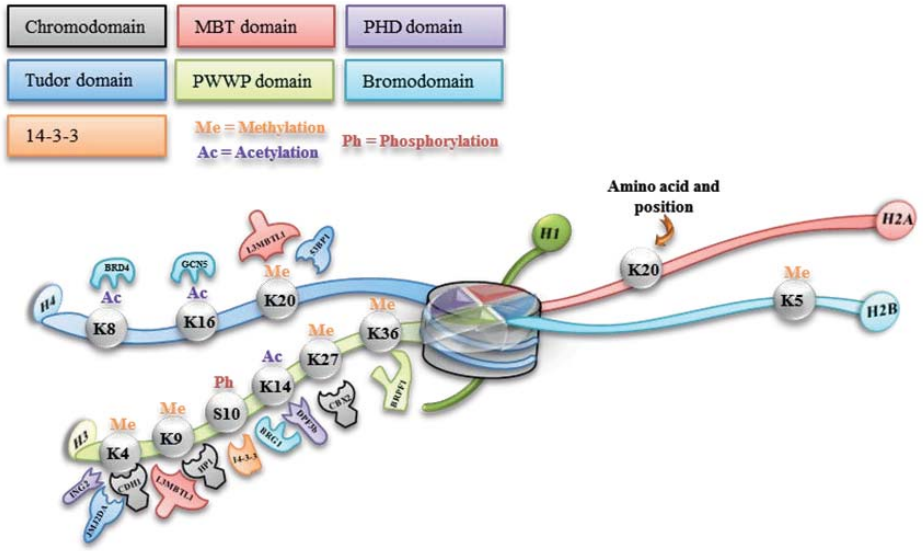


Figure 9: Figure showing the most abundant epigenetic readers and their imprinting locations on the specific histone tails. Figure adapted from (Bannister and Kouzarides, 2011; Dawson et al., 2012)

Moreover, phosphorylated Histone 3 is universally accepted as a hallmark of transcriptionally active region. The recognition of the phosphorylated and/or phosphoacetylated Histone 3 (H3S10Ph) is mediated via the 14-3-3 protein family members (Macdonald et al., 2005). Figure 9 shows the main epigenetic

protein family of epigenetic readers and few proteins from each family binding their respective epigenetic marks.

The protein domains mentioned in figure 9 constitutes the family of epigenetic reader proteins. Many of them are also catalytically active enzymes (Taverna et al., 2007). It's fascinating to imagine how merely a group of three specific domain containing protein members direct and/or react to specific histone modifications. For bromodomian containing proteins, evidence now shows that the specificity to detect explicit substrates is attributed to specific amino acids within the binding pocket of the protein. The amino acids outside of the binding pocket are essential for the recognition of specific histones (Dawson et al., 2012). Furthermore, the Bromodomain family of proteins is also known to interact with proteins other than the histones (Wu and Chiang, 2007).

To date, at least eight different types of histone modifications have been discovered. The complexity is further increased by the fact that every amino acid residues may be in a differential state with respect to the methylation pattern. The first solved structure of epigenetic readers was that of the HAT co-activator protein P/CAF (p300/CBP-associated factor). The P/CAF protein contained a 110 amino acid long Bromodomain module, similar to the ones found in HATs. This was the first proof of the Bromodomain modules playing an active and important role in transcriptional regulation (Dhalluin et al., 1999). Table 2 shows residues in histone tails amiable to acetylation known so far. To date only proteins members containing three particular domains (Bromodomain, Chromodomain and PHD domain) are known to read acetylated histone residues (Filippakopoulos and Knapp, 2014; Yun et al., 2011). Among all the acetylated lysine readers, Bromodomain family is the most widely studied. The name Bromodomain was derived from the name of the Drosophila protein "bramha". Bramha was the first identified reader protein containing the now familiar Bromodomains (Tamkun et al., 1992). As of now, the human proteome is said to code for 61 distinct bromodomains, which are present in forty-six different proteins (localized both in nucleus and cytoplasm) subdivided into eight sub-classes (Figure 10) (Filippakopoulos et al., 2012).

As a matter of fact, all Bromodomains consists of ~110 amino acids which form the four atypical left handed alpha helix (α_Z, α_A, α_B, and α_C) which then loops to form ZA loop (linking α_Z and α_A) and BC loop (linking α_B, and α_C). The ZA and BC loops create the central hydrophobic binding pocket consisting of the

conserved asparagine residue, which makes contact with the acetylated lysine residue in the histone tail (Dhalluin et al., 1999).

Histone	Amino Acid Residues
H2A	K5 and K9
H2B	K5, K12, K15, K16, K20 and K120
H3	K4, K9, K14, K18, K23, K27, K36 and K56
H4	K5, K8, K12, K16, K20 and K91

Table 2: Amino acid residues known to be amiable to acetylation. Adapted from (Musselman et al., 2012)

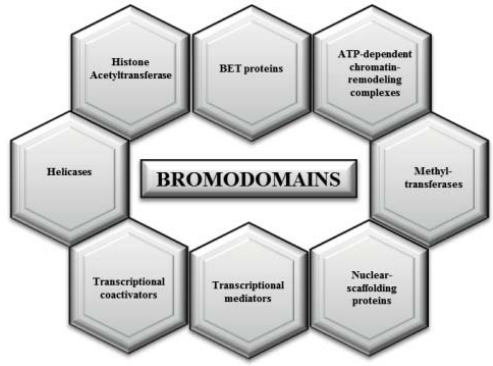


Figure 10: The classification of Bromodomain containing proteins families. Adapted from (Chen et al., 2014; Filippakopoulos et al., 2012)

1.8.3.1. BET Inhibitors: Is it all about MYC?

Bromodomain and Extra Terminal (BET) protein are the sub-class II of the Bromodomain containing family of epigenetic readers. In vertebrates, Bromodomain containing protein-4 (BRD4), BRD3, BRD2 and BRDT (Testis specific) proteins constitute the BET sub-family. In most cell types *Brd2/3/4* are expressed ubiquitously and *Brdt* is expressed only in the testis and ovaries (Paillisson et al., 2007; Shang et al., 2004). Moreover, the BET proteins are evolutionarily conserved (Wu and Chiang, 2007). Basically BD1, BD2 and extra terminal (ET) domains are found specifically in BET sub-family whereas domain B and SEED (Ser/Glu/Asp-rich region) regions are also found to an extent in other members of the Bromodomain family. Domain A and C-terminal domain (CTD) are not absolute pre-requisite in the Bromodomain family (Paillisson et al., 2007). Moreover, as in the case of all Bromodomain containing proteins, BET proteins share more similarities (>75%) between Bromodomain 1 or 2 (BD1/BD2) of two BET proteins compared to BD1 and BD2 of the same protein (~44%) (Figure 11) (McLure et al., 2013; Nakamura et al., 2007). It's now very well documented that most BET proteins express a short and long isoform.

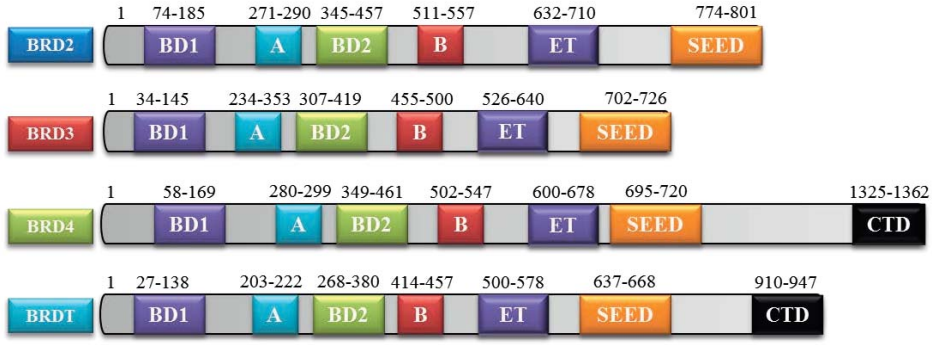


Figure 11: The human BET protein family members. The domains are indicated with their respective amino acid numbers. Adapted from (Wu and Chiang, 2007).

In case of BRD4 in humans, the long and the short isoforms differ in their 3' region due to differential splicing (Wu and Chiang, 2007). Predominantly, the BRD4/Brd4 long isoform accounts for most of the activity (Dey et al., 2000; Shang et al., 2004) and role of the shorter isoform still remains unexplored (French et al., 2003).

Really Interesting New Gene 3 protein (RING3), now known as BRD2 was the first BET protein identified (Beck et al., 1992). It is believed by some to function as a nuclear kinase having preferential chromatin binding capacities especially for H4K12 and H2A.Z (Draker et al., 2012; Kanno et al., 2004). Moreover, nuclear BRD2 is also known to interact with transcription factor E2F and the chromatin-remodeling complex (SWI/SNF) (Denis et al., 2006; Denis et al., 2000). NF-κB is specifically transcriptionally regulated by BRD2 (Dawson et al., 2011) and selective abrogation of BRD2 results in drastic reduction of p105/p50 (NFKB1) protein, a NF-κB subunit (Gallagher et al., 2014).

BRDT and BRD3 are not that well characterized as compared to the remaining two BET protein members. BRDT has been shown to regulate chromatin structural reorganization in an ATP dependent manner by specifically binding the acetylated residues in H4 (Pivot-Pajot et al., 2003). Moreover, BRD3 has been shown to interact with acetylated GATA1, undermining its probable role in hematopoiesis (Gamsjaeger et al., 2011).

BRD4 is the most widely studied protein in the BET sub-family. BRD4 was previously known as the Mitotic Chromosome-Associated Protein (MCAP), due to the fact that it was found to be associated with chromatin (especially euchromatin) even during mitosis (Dey et al., 2000). Importantly, BET proteins (shown for Brd2 and Brd4) have a characteristic of being attached to the mitotic chromatin (Wu and Chiang, 2007), when most nuclear factors (including other bromodomain family members) are excluded from the nucleus and in fact found to be floating in the cytoplasm (Muchardt et al., 1996). As expected, inhibition of BRD4 using anti-BRD4 antibody resulted in potent arrest of cell in S/G_2M phase of cell cycle (Dey et al., 2000). On the other hand, overexpression of Brd4 resulted in strong G_1 arrest of cells (Maruyama et al., 2002). It's now being shown that the depletion of BRD4 abrogated the BRD4 and SPA-1 axis, which might have led to the S/G_2M arrest (Farina et al., 2004). Whereas, the G_1 arrest seen due to the over expression of BRD4 is attributed to its direct interaction with Replication Factor C (RFC) complex specifically with RCF-140 (the large sub-unit of RFC complex) (Maruyama et al., 2002). Furthermore, the previously thought association of BRD2 with the mediator complex is now shown to be mediated probably via a specific isoform of BRD4 (Wu et al., 2003) interacting with the mediator complex (Wu and Chiang, 2007).

Almost a decade back, it was found that BRD4 was directly associated with CyclinT1 and CDK9; the "Positive Transcription Elongation Factor B" complex (P-TEFb) and this association can positively regulate full length transcriptional elongation in cells (Jang et al., 2005). The positive regulation on transcription imparted by BRD4 and P-TEFb complex was also verified using ChIP-Seq (Loven et al., 2012). In general P-TEFb can be found in an active or repressive form. In the repressive form, P-TEFb is found to be in complex with 7SK small nuclear RNA and HEXIM1 protein (7SK/HEXIM1 snRNP) where HEXIM1 is known to inhibit the kinase activity of CDK9 with the help of 7SK snRNA. On the other hand, the active form of P-TEFb complex is the one that is bound to BRD4. It is estimated that during homeostasis, P-TEFb is found approximately in equal ratio in either the active or repressive complex form (Yang et al., 2005).

Generally, as in the case of genes regulated by transcriptional pausing a specific set of events takes place during transcription initiation for the transcription machinery to make full-length transcripts. Mostly, after the assembly of transcription pre-initiation complex at the promoter, a small length of RNA is transcribed and the complex is halted. For the process of transcribing this small RNA segment (known as transcription initiation), the C-terminal domain (CTD) of RNA Polymerase II (RNA Pol II) is phosphorylated primarily at Serine 5 (p-S5-RNA Pol II) (Hsin and Manley, 2012; Komarnitsky et al., 2000). It is known that at least a third of the total genes in the human genome at any given time have a paused RNA Pol II (p-S5-RNA Pol II) around the transcription start site (referred to as promoter proximal pausing) (Core et al., 2008). These promoter proximal pausing is in fact a checkpoint regulated by two factors namely: the DRB Sensitivity Inducing Factor (DSIF) and the Negative elongation factor (NELF). These checkpoints not only allows the addition of CAP structure to the 5' end of the paused/nascent RNA transcripts, it also simultaneously adds an extra layer of regulation (Core and Lis, 2008) governing the production of full length transcripts. On getting proper signals, P-TEFb complexed with BRD4 (Active P-TEFb complex) phosphorylates Serine 2 in the CTD of RNA Pol II and also the NELF, marking RNA Pol II to extend for full length transcript production (Zhou and Yik, 2006). On the other hand, BRD4 is known to interact with transcriptional silencing complexes. The E2 viral protein encoded by bovine papillomavirus (BPVs) and human papillomaviruses (HPVs) are shown to interact with chromatin bound BRD4. When E2 protein interacts with cellular BRD4, the viral protein E2 prohibits the recruitment of RNA Poll II and other

associated transcription factors to the viral promoter, thereby facilitating the transcriptional repression capabilities of viral protein E2 (Wu et al., 2006; You et al., 2004).

Given these indispensable roles of BRD4 in transcriptional regulation, it's not unexpected that BRD4 might orchestrate the regulation of very many and important genes across cell types. Indeed, the important role of BET proteins is document by the fact that both Brd2 (E11.5) and Brd4 (post implantation) knockout mice were embryonically lethal (Houzelstein et al., 2002; Shang et al., 2009). Hypomorphic *Brd2* mice, however, have been shown to survive embryonic development albeit showing distinct characteristics of obesity, underlining the role of Brd2 in metabolic reprogramming (Wang et al., 2010a). Furthermore, given the interaction of Brd3 with GATA1, it was not unexpected to see defects phenocopying erythroid maturation upon abrogation on Brd3 in cells (Lamonica et al., 2011); no knockout mouse of Brd3 exists to date. Surprisingly, targeted deletion of BD1 in Brdt resulted in viable but sterile male offspring's in Brdt::ΔBD1$^{-/-}$ genotype (Shang et al., 2007).

Given the important role of BET proteins in development, it is not unexpected to have these proteins deregulated in malignant conditions. Indeed, ectopic expression of Brd2 restricted to the lymphoid origin makes B-Cell lymphoma and leukemia in mice (Greenwald et al., 2004). Also, BRD4 (BRD4-NUT fusion) is known to be the driver in lethal NUT midline carcinoma (French et al., 2003). Recently, many publications have underlined the indispensable role of BRD4 across malignancies, thereby making BET sub-family proteins and/or BRD4 specifically an apt target for therapeutic intervention (Arrowsmith et al., 2012; Chen et al., 2014; Dawson et al., 2012; Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos and Knapp, 2014; Herrmann et al., 2012; Lockwood et al., 2012; Loven et al., 2012; McLure et al., 2013; Mertz et al., 2011; Musselman et al., 2012; Ott et al., 2012; Picaud et al., 2013; Zuber et al., 2011). Indeed, an even large body of scientific literature is now available which shows the promising avenue of BET protein inhibition using small molecule inhibitor.

Among the number of BET inhibitors (BETi) available today, JQ1 is one of the most widely used. The scaffold for JQ1 is inspired from a compound first made by Mitsubishi Tanabe Pharma included in the patent document "WO2009084693" (Filippakopoulos et al., 2010). Many, if not most BETi are derived from benzodiazepine (BZD) based scaffolds (Evans et al., 1986). BZDs

have been in clinical practice for long and are generally known as modulators of central nervous system (CNS), as they selectively target $GABA_A$ receptors (Olkkola and Ahonen, 2008). The Mitsubishi compound (thienotriazolodiazepines) was the first known BZD compound derivative to specifically inhibit BET proteins (Smith et al., 2014). The functional groups attached to the BZD derived structure make specific contact with the BC and ZA loops of BD1 and/or BD2 (Figure 12), giving them the required specificity. The target selectivity of BET proteins is maintained by the presence of the WPF shelf (an amino acid motif) and the "gatekeeper" residues within the BD1 (isoleucine) and BD2 (valine) specific for BET proteins (Figure 12) (Smith et al., 2014). On the other hand, the addition of the bulky tetra-butyl ester group in JQ1 compared to the methyl ester in the Mitsubishi compound is said to be one of reasons for its specificity for targeting BET proteins (Smith et al., 2014). Many BZD based BETi (I-BET, MS417, etc) are known today (Nicodeme et al., 2010; Zhang et al., 2012).

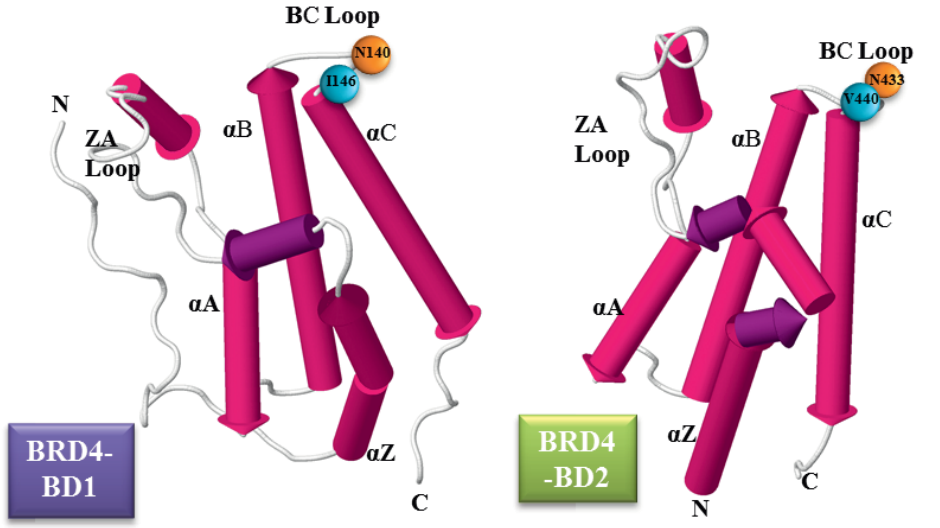


Figure 12: The crystal structure of BD1 (PDB ID 3MXF) and BD2 (PDB ID 2OUO) of human BRD4 is visualized using JMol (http://www.jmol.org/) (Filippakopoulos et al., 2010). The conserved Asn (N) residue in BD1/BD2 is shown with the orange circle; the "gatekeeper" residues are shown in blue. All BETi directly or indirectly make contact with the Asn residue among others to show its potency and specificity of inhibiting the BET proteins.

Moreover, many well-known kinase inhibitors have also been found to be potent BETi (Dittmann et al., 2014; Ember et al., 2014; Martin et al., 2013). As of now, many BETi inhibitors are in different phases of clinical trials across malignancies (Chaidos et al., 2015; Jung et al., 2015).

It is a well-accepted fact that many oncogenes and/or target specific proteins/factors are not amenable to be targeted via pharmaceutical intervention (Darnell, 2002). The targeting of epigenetic modulators or any indirect effector proteins, therefore, seems to be the method of choice (Arrowsmith et al., 2012). Owing to this analogy among others, targeting of BET proteins seems logical across cancer types. Despite making great advances, one aspect that still remains to be molecularly understood is how inhibition of these ubiquitously expressed BET proteins specifically down regulate a particular gene network instead of the expected global transcriptional down regulation?

Recently, most BETi (starting with JQ1) have been dubbed as MYC inhibitors (Alderton, 2011; Dawson et al., 2012; Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010; Herrmann et al., 2012; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011). The MYC proto-oncogene from its discovery almost three decades back is one of the most studied genes and yet remains directly un-druggable to date (McKeown and Bradner, 2014; Meyer and Penn, 2008; Soucek and Evan, 2010). One possible explanation for MYC being down regulated at transcript and/or protein level because of BETi might be attributed to the fact that MYC is one of the most deregulated genes in cancer (Beroukhim et al., 2010) and is a transcriptional hub of multiple converging /feeding pathways. So when the transcription machinery is targeted by BETi, MYC being one of the most actively transcribed genes appears downregulated and consequently the entire network regulated by MYC also appears downregulated. If targeting of MYC, because of its association with BRD4 was the primary reason for the potent effect of BETi; the failed rescue experiments with ectopic expression of any of the concerned molecules should have rescued the phenotypes, but the results say otherwise (Delmore et al., 2011; Lockwood et al., 2012).

1.9. Mouse Models

Traditional 2-D cell culture has served as a longstanding and effective model used in life science research including cancer. In early 70's the use of cell lines to facilitate drug discovery was pioneered by the National Cancer Institute, USA (Venditti et al., 1984). NCI over the years collected and distributed several cell lines as a panel of widely used cancer cell lines called NCI-60. This was thoroughly characterized to interrogate its genomic makeup and sensitivity to various approved or potential small molecule inhibitors (Abaan et al., 2013). However, the way cell lines are setup and grown in continuous culture forces them to adapt to the culture conditions. These adaptations may not necessarily require the mutation of the DNA but might instead rewire the entire transcriptome. These adaptations among various other factors indeed contribute to the false positive responses to small molecule inhibition both *in vitro* and *in vivo* (Gillet et al., 2011; Hausser and Brenner, 2005; Johnson et al., 2001).

1.9.1. Xenograft models

To overcome the pitfalls of *in vitro* cell culture, various genetically engineered mouse models (GEMM) and Patient derived xenografts (PDX) models have been established. The use of PDX models is not new (Fiebig et al., 1985) but recently the PDX models have become the method of choice in both academia and industrial settings (Calles et al., 2013; Gao et al., 2015; Khaled and Liu, 2014; Malaney et al., 2014; Tentler et al., 2012). As expected, PDX models not only better recapitulate the disease but may also used in parallel or co-clinical trials to predict treatment responses in patients (Hidalgo et al., 2011; Malaney et al., 2014; Siolas and Hannon, 2013). Moreover, these models can also be used for biomarker discovery possibly prior to clinical trials (Gao et al., 2015; Hidalgo et al., 2014). But as with all methods, there are limitations of using PDXs namely (a) they are not as tractable for genetic and pharmacological high-throughput screens as cultured cells and (b) the tumor take rate is not always very appropriate across malignancies. Hence, cultured cells will continue to contribute to cancer discoveries (Barretina et al., 2012).

A few intriguing questions remain to be answered for the use of PDX models *viz.* (1) Which type of tumors to be used, primary or metastatic lesion? It has been shown that for some cases the metastatic lesion have a growth advantage (Nemati et al., 2010; Sivanand et al., 2012). (2) Which mouse model should be

used for engraftment? The most severely immunocompromised model doesn't offer significant benefit over the older models in certain PDX models (Zhang et al., 2013b). (3) Should the orthotropic xenograft models be preferred over sub-cutaneous xenografts as the orthotropic model despite being time consuming yields higher uptake rate compared to the sub-cutaneous model (Hidalgo et al., 2014). The above mentioned variables are an active area of research to adapt PDX models for cancer research.

1.9.2. GEMMS of MYC Induced Lymphomas

In 1958, the first case of Burkitt Lymphoma (BL) was reported by Dennis Burkitt in Africa and hence the name of the disease (Burkitt, 1958). The disease had a high penetrance rate ranging from 30% to 50% of childhood cancer reported then. Three forms of BL are now recognized: Endemic BL is found primarily in Africa and the patients are invariably infected with Epstein-Barr virus (EBV), while Sporadic BL tumors arise in patients elsewhere across the globe excluding Africa. There is no correlation with EBV infections in these patients. Immunodeficiency-related BL is the third recently developed category of BL, where patients previously infected with human immunodeficiency virus (HIV) are seen to be more prone to be diagnosed with BL (Schmitz et al., 2014).

The cause of BL was first linked to a chromosomal (Ch) translocation involving the long arm of both chromosome 14 and 8 (Zech et al., 1976). Just in a time of four years, the oncogenic potential of this translocation was narrowed down to the MYC proto-oncogene. Due to this translocation, MYC comes under the strong and ubiquitously expressed immunoglobulin (Ig) heavy-chains (H) locus; thereby making abnormally high levels of MYC (Dalla-Favera et al., 1982; Taub et al., 1982). Having pinpointed the probable reason for the pathogenic condition, it was obvious to make models that could mimic the disease in laboratory conditions and model organism. Below mentioned are the two most widely used transgenic mouse models of BL, where the disease pathogenicity is primarily driven by MYC proto-oncogene.

1.9.2.1. Eμ-Myc

The first transgenic mouse model bearing the *Myc* translocation was made in 1985. This transgenic mouse expressed Myc under the control of the IG heavy chain intronic enhancer (Eμ) and developed a disease state closely resembling B-Cell lymphomas (Figure 12) (Adams et al., 1985). There is an absolute disease penetrance in this mouse model and mice invariably develop tumors around 100-150 days post birth. Despite this advance it's now known that Eμ-Myc mouse model doesn't recapitulate the human BL to a great extent. It's a well-accepted fact that human BLs has an expression profile matching with that of the normal germinal center B-Cells (because of their origin) and also expresses Adenosine deaminase (AID) (Dave et al., 2006). Contrary to these,

the lymphomas arising in this model are in fact from pre or naïve B cells and not from the germinal center B-Cells (Schmitz et al., 2014). Moreover, tumors arising in these mice were more similar to lymphoblastic lymphoma than BL; as inferred from the histopathological observations (Kovalchuk et al., 2000).

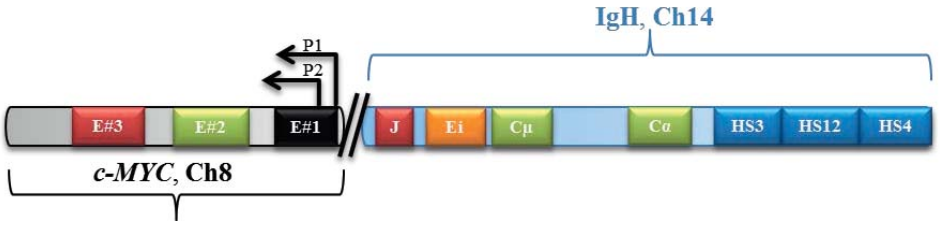


Figure 12: Chromosomal (Ch) translocation involving Ch14 & 8. This is the most frequent translocation in human BL. P1 and P2 are the two most used promoters for *MYC* transcription. Here E# is exon. Figure adapted from (Boxer and Dang, 2001).

1.9.2.2. λ-MYC

Even though most translocations observed in BL is between chromosome 8:14; some variants in both endemic and sporadic BL shows translocation of *MYC* to the Ig light chain gene locus on chromosome 2 (Kappa) (Igκ) or 22 (Lambda) (Igλ) (Dalla-Favera et al., 1982; Taub et al., 1982). In order to overcome the shortcomings of Eμ-Myc mouse model, the development of a new mouse model was absolutely required, thus, the λ-MYC mouse model was developed (Kovalchuk et al., 2000). In the λ-MYC model, human *MYC* from a BL patient was used to make the transgenic construct. Moreover, experiments were performed to ensure high levels of c-MYC were present in the tested system and expression was driven primarily from the P1 promoter instead of P2. The switching of promoter usage from P2 (generally used in non-malignant cells) to P1 is indeed a hallmark of BL, as the P2 promoter is not regulated by promoter proximal pausing (Figure 13) (Strobl and Eick, 1992; Strobl et al., 1993; Taub et al., 1982). The exact mechanism governing the switching of promoter is yet unknown but it seems that the involvement of the enhancer (data shown for IgH enhancer (Boxer and Dang, 2001)) from the IgH locus is indispensable. Therefore, the usage of the 12 kb long Igλ locus fragment for the transgenic construct of λ-*MYC* mice seems logical. Moreover, this locus also contains the λ enhancer (Eλ) (Figure 13) and it might be rational to speculate this enhancer is

one of the reasons aiding the shift in promoter usage among other observed phenotypes in the λ-MYC models (Kovalchuk et al., 2000). As one might expect, this model does recapitulate the disease resembling more closely to human BL. Furthermore, compared to the BL arising from the pre or naïve B cells in Eμ-Myc model, the BL developed in λ-MYC model bears more resemblance to that of mature B-cells (Kovalchuk et al., 2000).

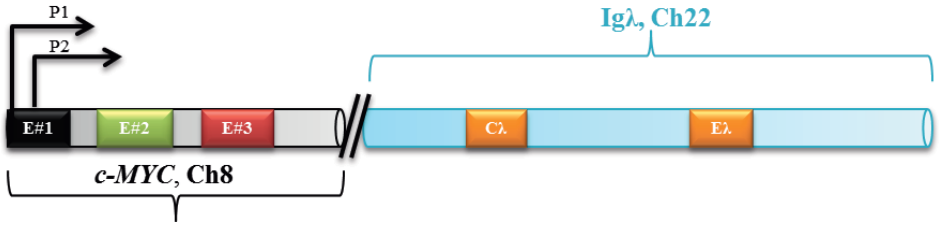


Figure 13: Chromosomal (Ch) translocation involving the less frequent 22:8 as documented in some human BL. P1 and P2 are the two most used promoters for *MYC* transcription. Here E# is exon. Figure adapted from (Boxer and Dang, 2001).

With all these advances, it was clear that the time required for developing BL in either of the model points toward the fact that MYC alone is not enough for driving tumorigenesis in these settings (Eischen et al., 1999; Schmitt et al., 1999). With the recent advancements in genome wide studies, the role of various gene networks became clear and in fact various sub-types of human BL were identified (Dave et al., 2006). Recently, another mouse model of BL was developed where Myc and PI 3-kinase (Pik3) are activated specifically in the germinal center B-cells (Sander et al., 2012). It is now believed that this new mouse model having both Myc and Pik3 recapitulates human BL more faithfully compared to all the existing models of BL (Schmitz et al., 2014).

1.10. Massive Parallel or Next generation Sequencing (NGS) Techniques

The first literature describing any sort of sequencing method dates back to 1960s, where the sequence of 5S-ribosomal RNA (rRNA) of *E.coli* was reported (Brownlee et al., 1967). Soon after, Frederick Sanger described a method of DNA sequencing (Sanger et al., 1977) which is commonly referred to as Sanger sequencing and was in fact used to sequence the first version of the human genome (Collins et al., 2003). In the same year another similar method of DNA sequencing was developed but is not used so widely as Sanger sequencing because of the use of certain toxic chemicals (Maxam and Gilbert, 1977). Briefly, in Sanger sequencing the DNA polymerase incorporates a modified nucleotide (one nucleotide per reaction) which contains a hydrogen group instead of the 3' hydroxyl group, thereby terminating the further incorporation of nucleotides. Despite the labor intensiveness of this method, many if not all model organisms had their whole genome sequenced using the automated machines using Sanger sequencing.

Almost a decade back, the first system deploying the massive parallel or Next generation sequencing (NGS) techniques were introduced. Now there are many commercial ventures which uses similar methods and more are under development (van Dijk et al., 2014). Briefly, in NGS first a library (of a certain nucleotide size fragment) is prepared from the samples to be sequenced. Specific adapters and sequencing primers (instrument specific) are annealed and amplified using PCR based methods. This whole process takes place in a flow cell and in millions of spatially isolated fragments. Contrary to Sanger sequencing, in NGS all four bases are added parallelly (all bases are labelled with different fluorophore) and thereafter the detection takes place. Then a washing or unmasking step is performed and the process is repeated. Because of the scale and parallel processing of enormous number of reactions, this method is called massive parallel or next generation sequencing (Mardis, 2013). The Roche 454, Solexa/Illumina GA and ABI SoLID systems are among the most widely used NGS platforms today (Liu et al., 2012; Mardis, 2013; van Dijk et al., 2014). Here only the Illumina platform will be further elaborated as it was used in the projects concerned

1.10.1. Illumina platform

In 2007, Solexa developed a method based on reversible terminator chemistry for DNA sequencing and was later acquired by Illumina Inc., here after referred to as Illumina platform (Bentley et al., 2008). Briefly, a library of defined nucleotide fragment size is prepared. The library ends are repaired using an enzyme mix that fills in the ends (making blunt ends), phosphorylates the 5' ends and also adds an Adenosine deoxy ribonucleotide. Subsequently, instrument specific sequencing adapters (numbers vary by choice of sequencing method) are ligated. The proprietary Illumina flow cell allows the amplification of the generated library in its surface using bridge amplification method *in situ*, generating millions of DNA clusters. Primers complementing the adapter sequences are used for initiating the sequencing reaction for millions of bases in each cluster parallelly. Here, all the four modified nucleotides (labelled with different fluorophore containing a cleavage site and a blocked 3' hydroxyl group) are added at the same time. The addition of this nucleotide is processed by a DNA polymerase and because of the altered 3' hydroxyl group the reaction never extends more than a single base per cycle. Thereafter, the free/unattached nucleotides are washed off and the flow cell is imaged to determine the specific nucleotide added across the clusters. This step is followed by demasking of the 3'hydroxyl group and finally the cleavage of the attached fluorophore. This process is repeated to get the desired read length from the cluster (known as single end reads). The generated cluster may be read from the other end (known as paired end sequencing). This is done by removal of the newly synthesized strand (by denaturation) post first round of sequencing, and is followed by minimal bridge amplification of these clusters. Then, another round of chemical treatment is performed such that the adapter sequence corresponding to the other end is released from the flow cell. Sequencing is performed as mentioned above (Liu et al., 2012; Mardis, 2013; van Dijk et al., 2014).

2. AIM

The aims of my thesis were:

- To investigate if mutations identified by exome sequencing can be used to predict therapeutically actionable targets *in vivo* (Paper I).

- To further investigate and develop a melanoma patient derived xenograft (PDX) platform to guide treatment regimens and to allow biomarker discovery (Paper II).

- To define the similarities and difference between PDX models and cell line-derived xenografts (Paper III).

- To characterize the therapeutic efficacy of a novel BET inhibitor in mouse models of cancer (Paper IV).

- To identify possible combination therapies to synergistically enhance the anti-cancer effects of low dose of BET inhibitors (Paper V).

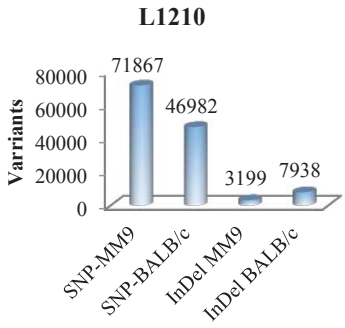
3. RESULTS AND DISCUSSION

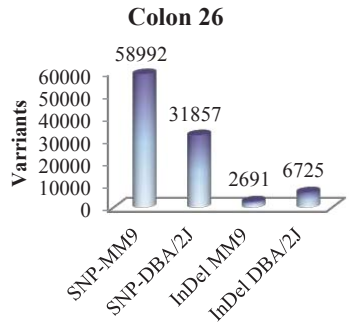
3.1. Targeting The Translational Genome (Paper I)

Here we set out to ask if the mutations identified by whole exome sequencing be used to design and execute treatment regimens at least in pre-clinical setting. To accomplish this, we subjected three established murine syngeneic transplantable cancer cell lines namely Colon 26 (Balb/c strain), L1210 (DBA/2J strain) and Panc 02 (C57BL/6 strain) were subjected to whole exome sequencing.

We observed around ~20 folds higher SNV's in L1210 and Colon 26 mapped sequences as compared to Panc 02. On further scrutiny, the increase in SNVs was attributed to the misalignment of L1210 and Colon 26 genomes to the then available MM9 (C57BL/6) genome. To circumvent the problem of genome (mis)alignment, we made in-house makeshift genomes by replacing the SNV's and InDel's in the MM9 genome with either BALB/c or DBA/2J SNP's. As seen in figure 14, this method indeed reduced numbers of SNV's in the makeshift genomes and resulted in better detection of genomic alterations.

A list comparing the top most commonly mutated genes in cancer from the COSMIC database (Forbes et al., 2015) has been compiled in table 3. Panc 02 cells were derived from one of the serially transplanted ductal adenocarcinomas, which developed in this mice post exposure to 3-methylcholantherene (3-MCA) in there pancreatic tissue (Corbett et al., 1984). As seen in table 3, Panc 02 cells harbor a homozygous *Smad4* stopgain mutation and heterozygous missense mutation in *Nkx2.1* and *Braf*. In mammals, a group of eight SMAD proteins comprising three distinct classes (Shi and Massague, 2003) play an important role in mediating signals received from transforming growth factor β (TGF-β) (Yang and Yang, 2010). SMAD4 orchestrates an important role of relaying TGF-β mediated signaling for EMT transitions, thus uncoupling the antitumor or migratory effect of TGF-β (Levy and Hill, 2005). Moreover 50% of pancreatic tumors harbor either a mutation or deletion in *SMAD4* (Akhurst and Derynck, 2001). Taken together, the important role of SMAD4 in tumor progression especially in pancreatic cancer may not be stressed any further (Yang and Yang, 2010) but unfortunately no small molecule inhibitor of SMADs exists to date. Beside these, a yet unknown *Braf* (I1313T heterozygous)





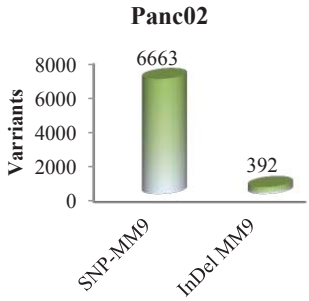


Figure 14: Effect of polymorphism on genome alignment. Exome sequences being aligned to either MM9 or to their respective makeshift genomes (BALB/c and DBA/2J).

Cell line	Gene name	Change	AA	Allele
Panc02	Braf	missense	I313T	Heterozygous
	Gnas	missense	R51H	Heterozygous
	Smad4	stop gain	E174X	Homozygous
L1210	Brca1	missense	L1145P	Heterozygous
	Cdkn2a/b	deletion	whole	Homozygous
	Ezh2	missense	L328P	Heterozygous
	Gnas	missense	D426N	Heterozygous
	Kdr	missense	V1175F	Heterozygous
	Kras	missense	G13R	Heterozygous
	Nf1	missense	S1525P	Heterozygous
	Notch1	frameshift	V453fs	Heterozygous
	Notch1	missense	L2209F, S1597R	Heterozygous
	Tet2	missense	S120R	Heterozygous
	Trp53	deletion	M240fs	Homozygous
	Tsc1	missense	L912H	Heterozygous
	Wt1	missense	A346S, K491N	Heterozygous
Colon 26	Cdkn2a/b	deletion	all	Homozygous
	Gnas	missense	A309T	Heterozygous
	Kras	missense	G12D	Heterozygous
	Pdgfra	missense	V88I	Heterozygous

Table 3: Mutation status of the top most frequently mutated genes in human cancer from COSMIC database in these samples.

mutation and amplification of *Usp15* gene were seen. Either of the genes is known to regulate and influence the MAPK signaling pathway (Dhillon et al., 2007; Eichhorn et al., 2012; Hayes et al., 2012).

L1210 cells were derived from tumor that originated by painting the skin of DBA/2J mouse with 3-MCA (Law et al., 1949). The strain of this mouse (DBA/2J) could be verified here, as many of SNP's initially identified were due to the misalignment of these sequences with the MM9 genome and were annotated as SNP's for DBA/2J genome. In L1210 cells, we observed heterozygous inactivating mutation in *Trp53* (Table 3) and absolutely no reads for Cdkn2a/b (see Suppl. figure S2A (Bhadury et al., 2013)). Moreover, many genes known to be commonly mutated in hematological malignancies (Braggio et al., 2013) were also mutated in this cells (Table 3), including *Kras* (G13R) mutation.

Colon 26 cells were derived from undifferentiated carcinoma's arising in BALB/c mice challenged with Nitrosomethyl urethane (Corbett et al., 1984). As seen in table 3, it harbors a *Kras* (G12D) activating mutation and complete deletion of *Cdkn2a/b* locus (no reads) (see Suppl. figure S2A (Bhadury et al., 2013)) among others.

We handpicked a small molecule inhibitor library comprising of 148 molecules which were in various stages of pre-clinical or clinical testing. We hypothesized that Colon 26 cells and to some extent Panc 02 cells would be affected by MAPK pathway perturbation using a small molecule inhibitor, whereas L1210 cells would rather be affected by cell cycle checkpoint or p53 pathway inhibitors. Indeed as expected, Colon 26 cells were highly sensitive to a MAPK pathway inhibitor (Multiple MEK1/2 inhibitors) while Panc 02 cells were affected to a lower extent. Surprisingly, Panc 02 cells despite bearing *Braf* (I313T) mutation were completely insensitive to BRAF inhibitors present in the library, suggesting that either the above-mentioned mutation does not activate and/or depend on Braf mediated signaling. In case of L1210 cells, inhibitors of Wee1, Chk1, Polo like kinase and a few others were seen to severely affect the cells. It is tempting to speculate the probable role of *Trp53* mutation, in making cells sensitive to either check point kinase inhibition or inhibitors against mitotic progression (for data see Suppl. tables (Bhadury et al., 2013)).

It is a known fact that activating or gain-of-function mutations in either RAS or BRAF proto-oncogenes results in hyper-activation of the MAPK pathway

leading to tumorigenesis (Sebolt-Leopold and Herrera, 2004). Moreover, almost a third of all human cancers, half of colon cancers, approximately 90% of pancreatic cancers and 36% of lung adenocarcinoma among others harbor a mutation in KRAS proto-oncogene (Adjei, 2001; Cerami et al., 2012; Gao et al., 2013). Taken together, targeting of KRAS or its bonafide downstream targets like MEK or ERK seems logical next.

We hypothesized that *Kras* mutations can predict sensitivity to targeted therapies; and to this end we procured two allosteric bio-available MEK1/2 inhibitors JTP-74057/GSK-1120212 (Gilmartin et al., 2011) and TAK-733 (Dong et al., 2011) that are currently in different phases of clinical trials. To check the efficacy of these molecules, we treated Colon 26 cells with varying concentration of the inhibitors and performed western blot analysis. As seen in figure 15A, GSK-1120212 is approximately 10 fold more potent compared to TAK-733. To gain better insight into the molecular mechanism, we challenged different cell lines with varying concentration of GSK-1120212 inhibitor for 48 hours and counted thereafter. As seen in figure 15B, Colon 26 cells were the most sensitive to the treatment. HCT-116 (Human colorectal carcinoma) cell line bears an activating *KRAS* mutation (Schroy et al., 1995) and was indeed sensitive to MEK inhibition albeit to a lesser extent compared to Colon 26. Interestingly, even though Panc 02 cells do not carry a *Kras* mutation, they are still seem to be sensitive to MEK inhibitors at concentrations that showed no effect in non-transformed BALB/c 3T3 cell line. To get further molecular insights, we treated L1210 and Panc 02 with varying concentration of GSK 1120212 for 24h. From the western blot analysis shown in figure 15C, L1210 cells despite having a *Kras* (G13R) mutation do not seem to rely on MAPK pathway signaling, as ERK1/2 was not phosphorylated. This data is in accordance with previous finding, which predicts *Kras* (G13R) mutation to be non-oncogenic (Spoerner et al., 2010). Moreover, given the afore mentioned reasons, modest sensitivity of Panc 02 cells to MEK inhibitors is in accordance with previously published data (Yamaguchi et al., 2011). Further experiments are required to find the relevance of the novel *Braf* (I313T) mutation, as well as elucidating the driver pathway in the Panc 02 cells

We next treated Colon 26 cells with varying concentrations of GKS1120212 to better characterize them and analyzed by FCM and WB (Figure 15D-F). As seen in the figure, we could see a dosage dependent effect of MEKi. To further validate our findings *in vivo*, we injected ten BALB/c mice, each with $5x10^5$

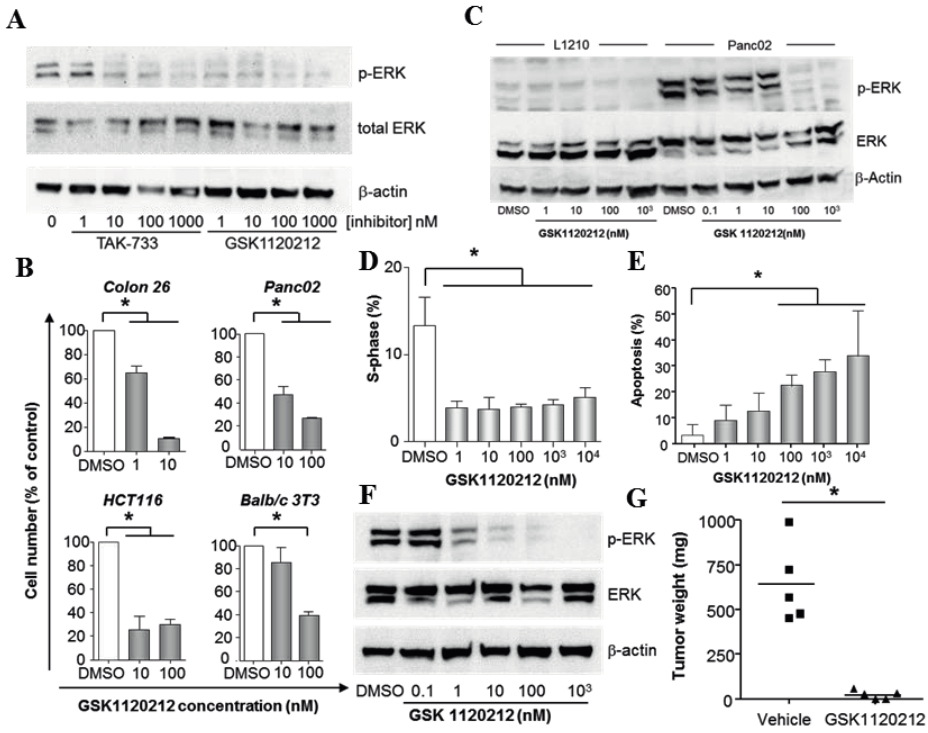


Figure 15: MEK inhibitors sensitize *Kras* mutated cells (A) Colon 26 cells treated for 24h with varying concentration of MEK1/2 inhibitors and analyzed by western blot. (B) Colon 26, Panc 02, HCT-116 and Balb/c 3T3 cell lines treated with vehicle (DMSO) or the indicated concentration of GSK1120212. Cells were counted after were counted 48h post treatment. (C) Western blot analysis of Panc 02 and L1210 cells treated with varying concentrations of GSK1120212 for 24 h (D) Colon26 cells were treated for 16h with indicated concentrations of GSK1120212 and FCM analysis showing cells in S-phase. (E) Colon26 cells were treated for 40h with indicated concentrations of GSK1120212. FCM analysis showing cells in sub-G1 phase. (F) Western blot analysis of Colon26 cells treated with varying concentration of GSK1120212 for 24 h. (G) Colon 26 cells were injected subcutaneously into BALB/c mouse. One week after injection, mice bearing tumors were randomly divided and treated with 1 mg/kg GSK1120212 or vehicle substance once daily for 4 days by intraperitoneal injections. Six hours after the last injection, tumors were excised and weighed.

Colon 26 cells into the flank. Seven days post transplantation, all mice developed palpable tumors. Mice were randomized into two groups, wherein five mice received four daily injections with GSK1120212, whereas the rest were treated with vehicle. By the fourth injection, all mice treated with GSK 1120212 had complete regression of tumor. In fact, post-euthanizing the animals, hardly any remnants were visible in three of the treated mice; whereas two showed no evidence of tumors whatsoever (Figure 15G).

Taken together, these data demonstrate that exome sequencing can reveal mutations that predict treatment responses *in vivo*. We also propose the use of next-generation sequencing in the standard clinical setting to facilitate precision treatment regimens and development of potential biomarkers. Moreover, use of exome sequencing would provide a better view of the genomic mutational status and aid treatment decision; which is now primarily based solely on pathological observations alone.

3.2. Mouse Avatars Guiding Treatment: it does not get more personalized than this (Paper II)

Melanomas arise via stepwise transformation of melanocytes and are highly aggressive when metastatic. If detected early, melanomas are almost always curable by resection, but prognosis and overall survival for patients with advanced-stage melanomas still remain very poor (Balch et al., 2009; Siegel et al., 2015). Here, we set to ask if a platform similar to the one used in Paper I may be used as a pre-selection/screening tool for inclusion of patients with metastatic melanoma into appropriate clinical trials. Figure 16 illustrates the schematic representation of the proposed platform.

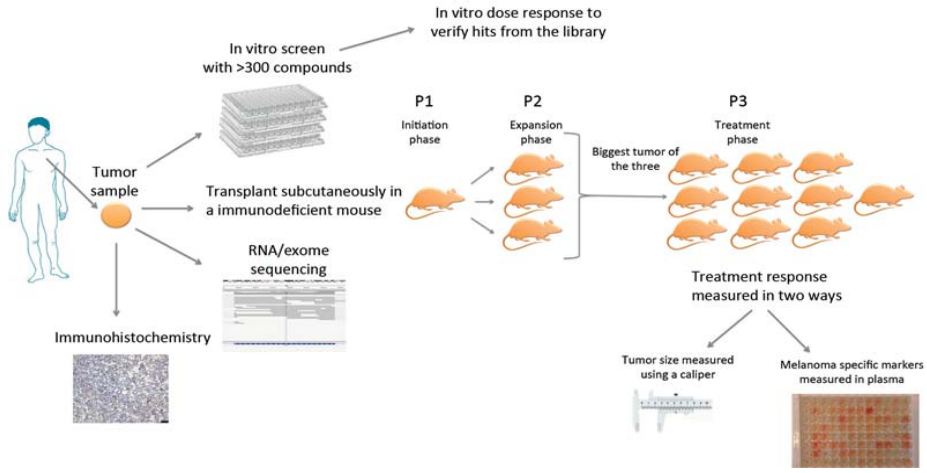


Figure 16: Schematic depiction of the proposed PDX platform

In general, owing to the extensive shared similarities between the tumor micro-environment among other factors, the uptake rate of PDXs from melanomas in NOG mice (Ito et al., 2002) is very apt for conducting clinical trials for patients in this category (Quintana et al., 2008). This type of xenografts in fact become ortho-xenograft. Here, tumor samples from the patients recruited in the trial were injected sub-cutaneoulsy into NOG mice to develop individual mouse avatars. It is now accepted that PDX models better recapitulates the disease (Malaney et al., 2014; Tentler et al., 2012) and we could test the same here. As seen in figure 17A, indeed, the different histological staining on sections from tumors in the PDX models and its corresponding counterpart from the patient does show a very high degree of similarity.

We next used NGS techniques to perform whole exome and/or transcriptome sequencing (RNA sequencing) of the samples. As expected, most of the tumors did carry SNVs resulting in either BRAF V600 or NRAS Q61 mutations (Figure 17B) along with the known driver mutations in melanoma (Hodis et al., 2012). Reassuringly, sample distance matrix analysis from the RNA sequencing data did cluster the tumor samples from patients and its corresponding PDXs (even from different passage wherever applicable) into same clusters (Figure 17C). Moreover, known tumor suppressors like *TP53* or *CDKN2A* were mutated or silenced and/or deleted (as inferred by no reads from RNA sequencing) (see Suppl Table S1 (Einarsdottir et al., 2014)).

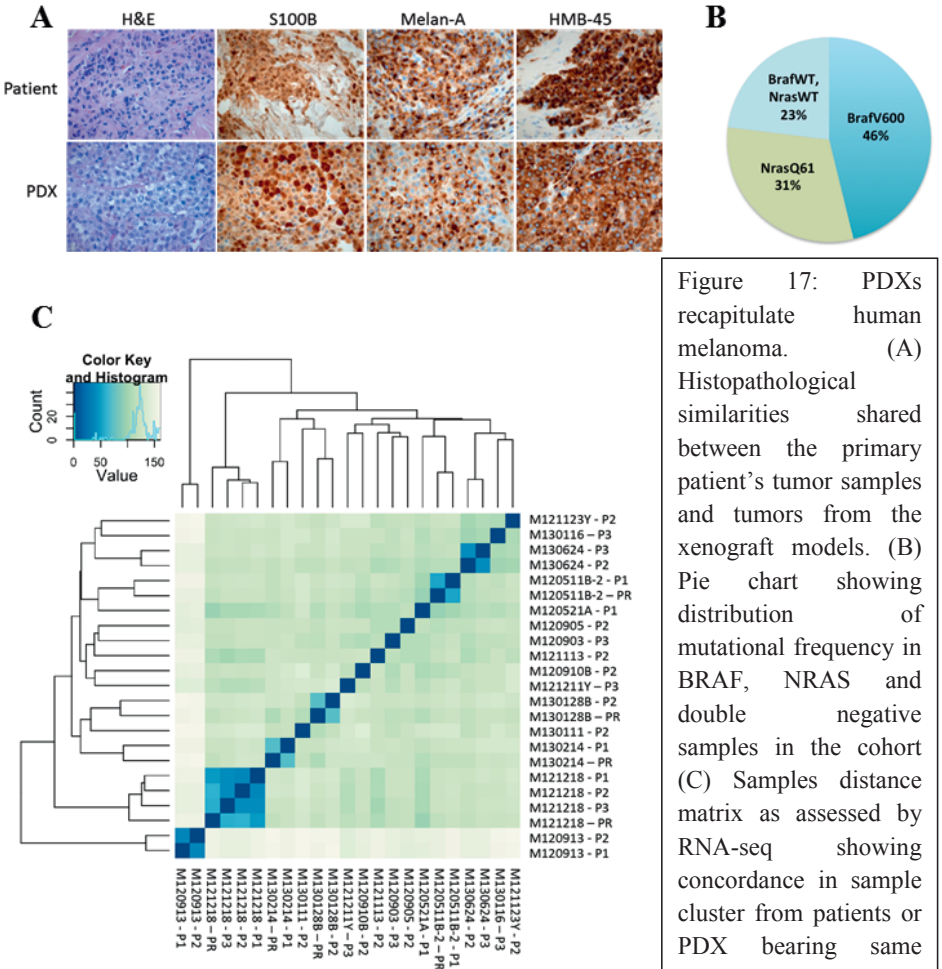


Figure 17: PDXs recapitulate human melanoma. (A) Histopathological similarities shared between the primary patient's tumor samples and tumors from the xenograft models. (B) Pie chart showing distribution of mutational frequency in BRAF, NRAS and double negative samples in the cohort (C) Samples distance matrix as assessed by RNA-seq showing concordance in sample cluster from patients or PDX bearing same tumors.

We could perfectly consolidate mutational signature for select genes from samples (wherever applicable) used for RNA sequencing using Sanger sequencing and/or allele-specific PCR's of the primary samples.

From the assessment of RNA sequencing and histology data of the patients and PDXs, we succeeded in setting up a platform that not only recapitulates the tumor states to a very high extent but also maintains the integrity for multiple passages. We next wanted to investigate if the platform may be used in predicting clinical response and/or to find novel biomarkers. We present two case studies from the patients M120903 and M120521A that were subsequently used to develop xenograft models and/or cell lines (for *in vitro* screening).

The M120903 model arose from a patient bearing lymph node metastasis diagnosed with grade IIIC melanoma at the time of inclusion to the clinical trial. The resected tumor piece was finely chopped to make a cell suspension, from which a part was used to make a cell line for *in vitro* use; another part was injected sub-cutaneously into NOG mice and the rest was frozen down as live cells. *In vitro* drug screening was performed on this cell line using a custom library consisting of around 300 plus small molecules. As shown in figure 18A, this cells were indeed sensitive to MAPK pathway perturbation using MEK inhibitors among others. To further strengthen our assumption, we performed a dose dependent study using two third generation allosteric MEK inhibitors (Dong et al., 2011; Gilmartin et al., 2011). In line with our previous data (Bhadury et al., 2013), here trametinib seems to be more efficient when tested in this newly established cell line (Figure 18B). Bearing in mind that M120903 cells are *NRAS* mutant, we wanted to check the efficacy of trametinib *in vivo*. As seen in figure 18C-E, not only the treatment resulted in significantly prolonged survival and reduction in tumor burden, it also resulted in reduced plasma levels of well-known melanoma markers S100B (Gogas et al., 2009). Moreover, comparing the IHC staining of tumor explants harvested from the control or trametinib treated mice reveled that tumor cells were cleared by apoptosis mediated pathway; as assessed by cleaved caspase-III staining (Figure 18E) (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995). Unfortunately, despite having significant pre-clinical data, the patient couldn't be treated with MEK inhibitor as the same was not a treatment option for *NRAS* mutated tumors at the local University hospital.

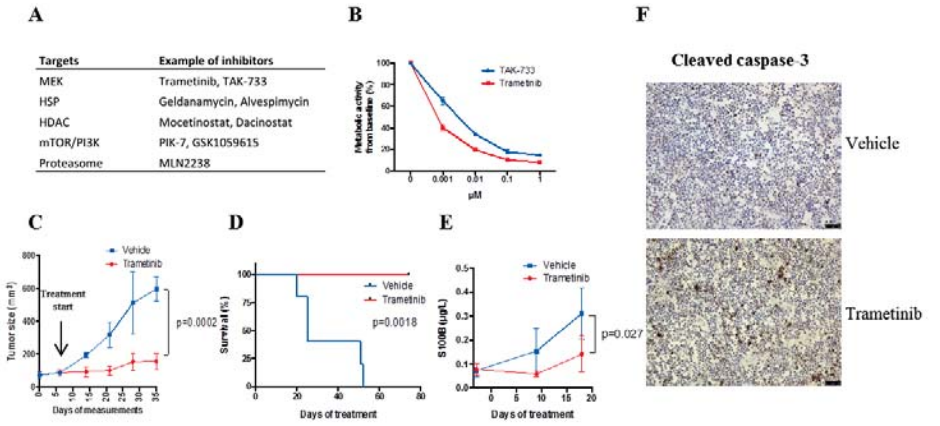


Figure 18: PDX and cell line derived from *NRAS* mutated patient (M120903) shows sensitivity to MEK inhibitors. (A) Small molecule inhibitors showing potent effect. (B) Dosage dependent decrease in cell viability with both MEKi. (C & D) MEKi showing potent anti-tumor effect, as assessed by tumor size and disease free survival respectively. (E) In line with the anti-tumor effect of MEKi in this model, reduced level of human specific S100B was observed. (F) IHC staining confirms apoptosis mediated via Caspase-III mediated pathway in MEKi treated PDXs.

To test our platform further, we next went on to perform a parallel PDX trial alongside the clinic. In this double-blinded clinical trial, the patient was to be treated with either a BRAF inhibitor or a BRAF+MEK inhibitor combination. Briefly, this patient (M120521A) diagnosed with a BRAFV600E mutation developed an acral malignant melanoma on his foot, which was removed by surgery. Rather soon, metastatic lesions were detected and the patient was treated with limb perfusion. Unfortunately, the disease couldn't be contained and promptly progressed to stage IV, with multiple metastatic lesions across the body. One of the surgically removed metastatic lesions was used to create the M120521A PDX model. The mice bearing the tumor from this patient were randomly divided into three groups, which either received a BRAF inhibitor (Vemurafenib) (Yang et al., 2010) or MEK inhibitor (Trametinib) or a combination of both. As expected the patient showed good response with reduction of tumor burden (see figure 3A (Einarsdottir et al., 2014)) and decrease in serum plasma levels (Figure 19A), so did the PDXs (Figure 19B & C). To our surprise, as seen in figure 19B & C there seemed to be no significant difference between the response in PDXs treated with either single agent or the combination.

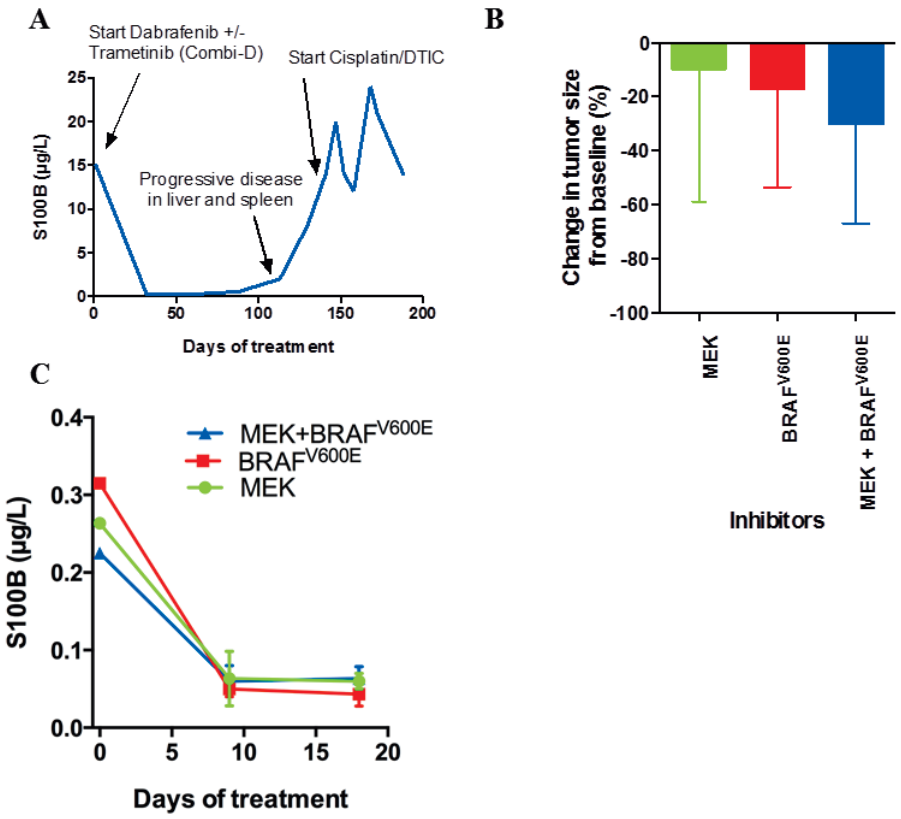


Figure 19: PDX and cell line derived from *BRAF* mutated patient (M120521A) shows sensitivity to MEK inhibitors. (A) S100B levels during the course of treatment and disease progression (B) Effect of MEK or BRAF or combined inhibition on PDXs as by tumor size post treatment. (C) S100B levels during the treatment period in PDXs.

We next wanted to see if this whole procedure from including patient to treating the PDX may be done in a reasonable time before it's too late for the patient. As seen in figure 20, we do in fact convincingly show that there is enough time for co-clinical trials like this to be used to guide clinical regimes.

Taken together, to the best of our knowledge we for the first time show in a systematic way that the transcriptome-wide similarity is maintained between biopsies from patient and PDXs (serially transplanted, wherever applicable) as assessed by RNA sequencing. Moreover, we also convincingly show that there is indeed time to use platforms like this to aid or even guide clinical treatment

regimens without any bias towards the age, disease stage or gender of the patients recruited.

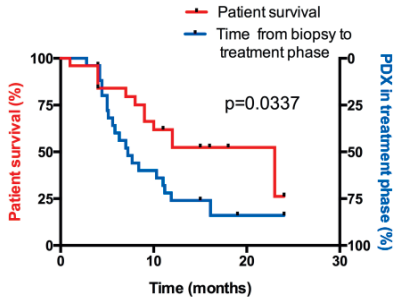


Figure 20: Kaplan-Meier graph showing time to death post autopsy (Y-axis) compared to time taken to establish PDXs models for treatment regime. Indeed for most patients, sufficient time may be there for generation of these models for personalized/experimental treatment regimes.

3.3. PDXs And CDXs Are Inherently Different (Paper III)

For decades, cell lines have been used to obtain various mechanistic and pathological insights into cellular phenomenon. We have previously shown that despite prolonged culture *in-vitro* not much genomic instability occurs in the cell lines tested (Bhadury et al., 2013). These observations were based only on exome sequencing and might miss out on the transcriptome wide changes acquired due to the artificial culture conditions. Nevertheless, cell lines are an indispensable part of cancer discovery despite these shortcomings (Barretina et al., 2012).

Here, we set out to decipher the molecular insights at transcriptome level in metastatic melanoma by comparing gene expression profiles (GEPs) of the cell line derived xenografts (CDXs) to our PDXs platform (Einarsdottir et al., 2014). The use CDXs instead of cell lines was attributed to the hypothesis that growing the cells sub-cutanouesly in mice might reduce the *in vitro* culture related artifacts. As seen in figure 20A there is an even distribution of the frequently mutated genes in melanoma between the PDX's and CDX's (Cancer Genome Atlas, 2015) and for the list of genes mutated here see Suppl. Table S1 (Bhadury et al., 2016). Having seen the uniformity in mutational load across the CDXs and PDXs, we went on to analyze if the same was true at the transcriptome level. Contrary to our expectations, CDXs and PDXs did not cluster together despite bearing similar mutational signatures. As seen in the principle component analysis (PCA) plot (Figure 20B), PDX's and CDX's are not intermixed; highlighting a major difference in their transcriptome. From the samples distance matrix analysis, the biological duplicates (for CDXs) or the serially transplanted PDXs and their primary biopsy from the patient (PR) does cluster appropriately (Figure 20C). The observed difference were not due to variation in samples but rather may be attributed to the inherent differences between PDXs and CDXs in general. Moreover, the transcriptome wide difference between the CDXs and PDXs/PR were still vividly prevalent when the top 8000 expressed genes were analyzed by unsupervised hierarchal clustering (see Suppl. figure S1A, (Bhadury et al., 2016)).

Finding no obvious gene expression signature for pathways primarily regulated in melanoma, we wanted to investigate if microRNA's (miRNA) were orchestrating the observed differences. Owing to the relatively low expression

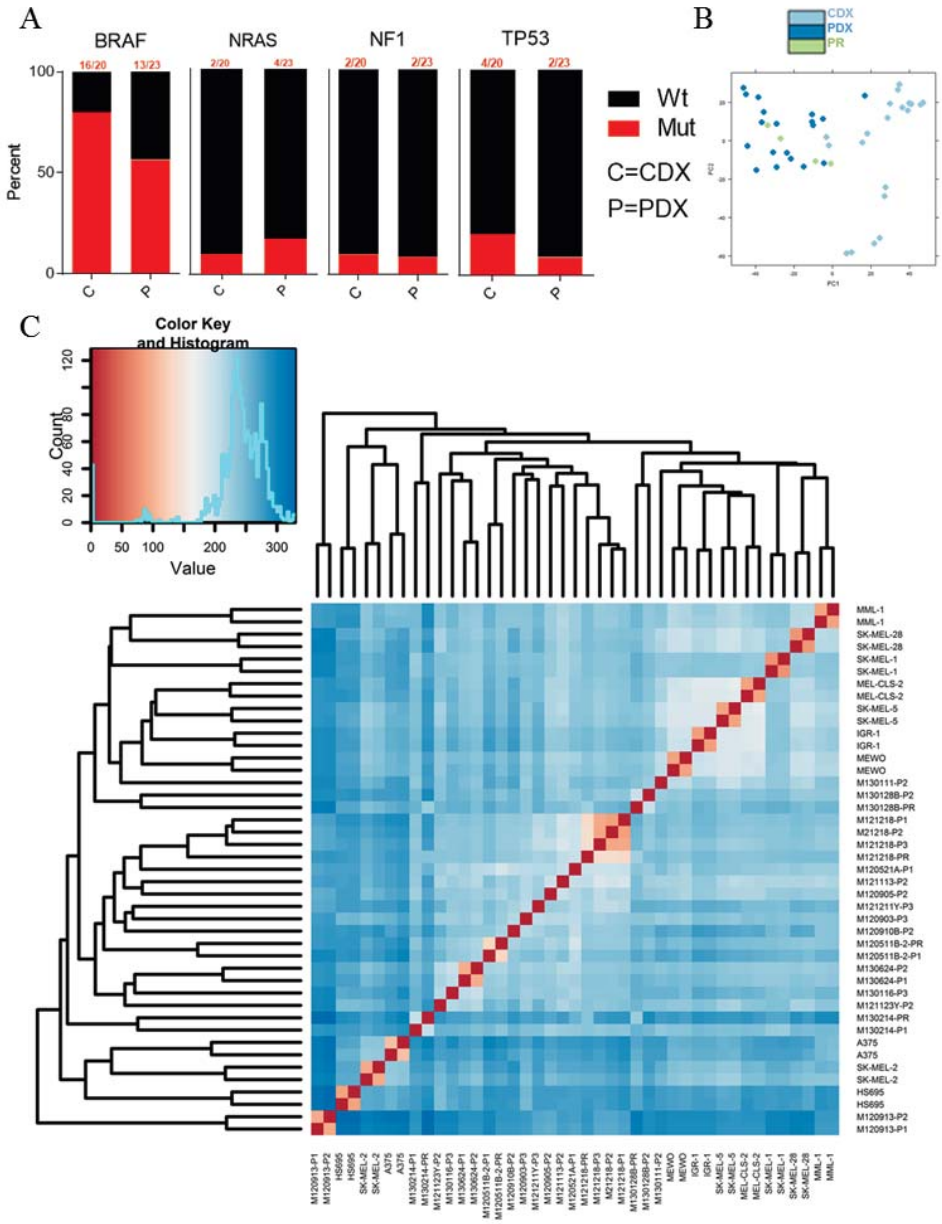


Figure 20: PDXs and CDXs don't share similar transcriptome signatures (A) Uniform mutational load across PDXs and CDXs for commonly mutated genes in melanoma (B) PCA plot showing marked transcriptome wide differences separating PDXs and CDXs. (C) Sample distance analysis separate PDXs and CDXs into separate and distinct groups irrespective of the mutational similarities shared.

levels of miRNA's, all annotated raw read counts were extracted from the dataset using "MIR" as an identifier and analyzed for differential expression. Similar to the whole transcriptome data, there were marked differences between PDXs and CDXs (Figure 21A and see Suppl. Table S3 (Bhadury et al., 2016). As seen in figure 21 A-B, hsa-miR-210HG and hsa-miR-600HG were the two most differentially expressed miRNAs among the groups. Moreover, PCA plots from miRNA differential analysis show trends similar to whole transcriptome PCA plots), albeit less pronounced compared to entire transcriptome PCA plot (compare figure 20B and figure 21C).

For further experimentation, we narrowed down to hsa-miR-210, as no experimental evidence was publicly available for hsa-miR-600. hsa-miR-210 is an intronic miRNA, which is spliced out from the non-protein coding transcript hsa-miR-210HG. Interestingly, the promoter region of hsa-miR-210HG contains a hypoxia inducible factor (HIF) response element (HRE) and is widely known as the "hypoxiamir" or "hypoxia master regulator" (Huang et al., 2009; Huang et al., 2010; Kulshreshtha et al., 2007). Taking into consideration the important role of hsa-miR-210 in hypoxia, we performed Pearson correlation analysis to find genes correlating with hsa-miR-210HG expression in our dataset. As seen in figure 21D, many of the top 40 genes (marked with an arrow) were either involved in glycolysis and/or are HIF target genes (as assessed by GSEA analysis; see Suppl. Table S5 (Bhadury et al., 2016)). There may be many underlying reasons for the differences observed between PDXs and CDXs. Firstly; in traditional 2D cultures the cells don't get the support of the natural extra cellular matrix, which has now become evident with the superiority of 3D cultures in better predicting treatment response *in vivo* (Holle et al., 2015). Secondly; there is no regulated distribution kinetics of growth factors received from fetal bovine serum in cell culture (Ince et al., 2015). Thirdly, cancer cells are routinely cultured *in vitro* in up to 4.5g/l glucose, which is extremely hyper-glycemic (>0.2g/l). This is important to consider as cells *in vitro* have no means to remove the high amounts of metabolic waste products produced owing to excessive glucose levels and it is intriguing to speculate that the Warburg's effect might have merely been a cell culture artifact (Warburg, 1956). Fourthly; the cells are cultured routinely in 20% oxygen levels *in vitro*, which may induce oxidative stress and other irreversible phenotypes (Bourseau-Guilmain et al., 2012; Halliwell, 2003). Taken together,

A

Name	Fold change PDX vs. CDX	p value adjusted
MIR210HG	-1,797	3,330E-06
MIR600HG	1,246	1,580E-05
MIR133A1	2,163	0,003
MIR22HG	-1,036	0,003
MIR24-2	-0,626	0,003
MIR4435-1HG	-0,896	0,003
MIR3150B	1,586	0,005
MIR497HG	0,956	0,005
MIR31HG	-4,524	0,016
MIR503HG	1,049	0,016
MIR181A1HG	1,469	0,022
MIR3916	0,417	0,022
MIR296	-1,437	0,025
MIR181A2HG	1,090	0,027
MIR1255B1	-5,232	0,034

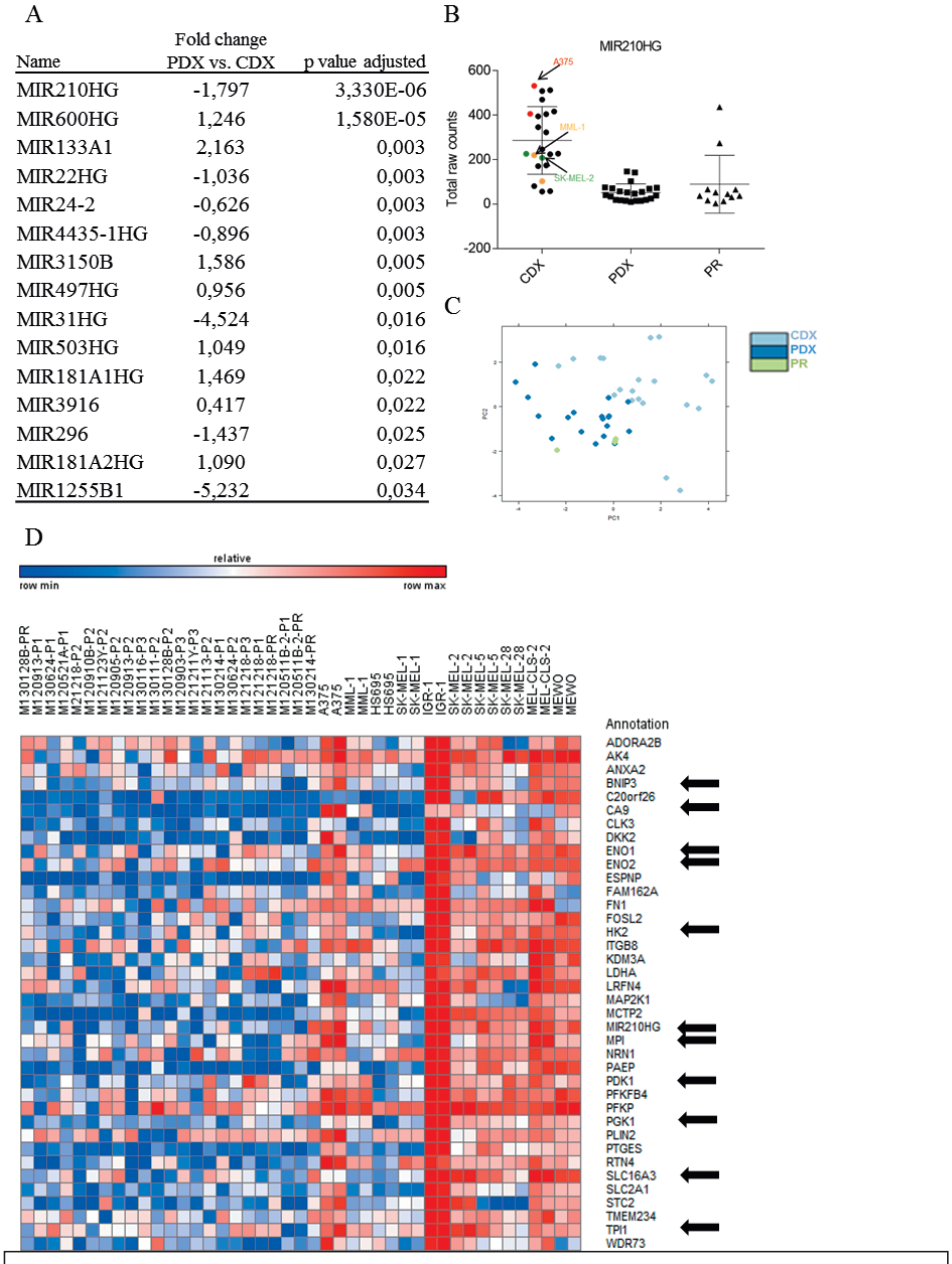


Figure 21: Even miRNAs are differentially regulated between PDXs and CDXs (A) Table showing significantly differentially expressed miRNA's and miRNA-HG's between the PDX and CDX. (B) Graph showing raw read counts of miR-210HG between Primary, PDX and CDX .(C) PCA plot of PDX and CDX. (D) Pearson correlation analysis showing top 40 genes that correlated with mir-210HG expression. Arrows mark genes regulated in hypoxia and/or metabolism.

it is not unexpected to see an altered transcriptome wide wiring between PDXs and CDXs possibly owing to pseudo-hypoxia.

To further probe the role of hypoxia in mediating the marked differences observed between PDXs and CDXs, we interrogated the role of hsa-miR-210 in this setting. Marked difference in expression of Carbonic Anhydrase IX (CA9) was seen between PDX's and CDX's. Moreover, when the *in-vitro* culture accustomed cells lines were injected sub-cutaneously into mice, these were bound to encounter physiological oxygen condition which is generally much lower than the *in-vitro* used concentration. Owing to the standard use of 20% oxygen in cell culture conditions, the differential expression pattern of CA9 between PDXs and CDXs is not unexpected. Taken together, these findings hint towards the fact that prolonged culture of cells *in-vitro* might lead to a completely rewired transcriptome.

The analysis so far indicates a general role of hypoxia in mediating the differences seen between PDX's and CDX's, probably regulated by hsa-miR-210. To interrogate this aspect further, we subjected three metastatic melanoma cell lines to 20% and 5% oxygen levels for 24 hours and performed mRNA and protein expression analysis. We not only show the potent induction of hsa-miR-210HG when subjected to low oxygen conditions, we also convincingly recapitulate a similar magnitude of expressional differences between the cell lines comparable to the RNA sequencing data (Figure 21B, 22A-B, and see Suppl. Table S5 (Bhadury et al., 2016)). Moreover, as expected qRT-PCR analysis of select genes across the cell lines show differential expression pattern in low oxygen conditions (See Suppl. figure S2 (Bhadury et al., 2016)). We next performed western blot analysis to check the protein expression across the cell lines in the above-mentioned conditions. Unexpectedly, besides A375 both MML-1 and SK-MEL-2 cell lines showed marked reduction in all proteins probed (Figure 22B). Here, phosphoRB (S780) (Kitagawa et al., 1996) and GEMININ (McGarry and Kirschner, 1998) were used as makers of G1 and S-G2M phase of cell cycle, p4E-BP1 (Pause et al., 1994) was the marker for cap dependent translation and CA9 the marker for hypoxic response respectively. CA9 is a transmembrane enzyme regulated by HIF1 orchestrated pathway in response to hypoxia (Wykoff et al., 2000). Contrary to our expectations, we did not see any upregulation of CA9 in either MML-1 or SK-MEL-2 cell lines in response to 5% oxygen level (Figure 22B), despite having significant up regulation in mRNA levels (Suppl. figure S2,(Bhadury et al., 2016)) .

Moreover, many studies have shown that deregulated expression of CA9 often leads to poor prognosis and co-relates with drug resistant and metastatic phenotypes across malignancies (Olive et al., 2001; Shin et al., 2011; Wykoff et al., 2000). It is intriguing to note that for all genes analyzed using qRT-PCR, we see a marked upregulation of expression under low oxygen conditions but that did not always implicate a higher protein level (in two out of three cell lines tested), as quantified using western blots. Moreover, for MML-1 and SK-MEL-2 cell lines the cap dependent translation machinery under hypoxic conditions seems to be severely affected (Figure 22B).

Having noted the up-regulation of hsa-miR-210HG upon low oxygen conditions and suppression of certain proteins, it was obvious to challenge the system by inhibiting the regulation deployed by the miRNA. It is well-accepted fact that miRNA's regulate gene expression by regulating them either post-transcriptionally or post-translationally (Filipowicz et al., 2008). To this end, we equipped all the three cells lines with hsa-miR-210 decoy (Mullokandov et al., 2012) and performed the same experiments as mentioned above. As expected, the cell lines engineered with the decoy showed potent induction of hsa-miR-210HG, if anything there seems to be much higher expression of most genes mainly in MML-1 and SK-MEL-2 (Figure 22B ; Suppl. figure S3 (Bhadury et al., 2016)) cell lines. Surprisingly, both MML-1 and SK-MEL-2 cell lines engineered with miR decoy did not down regulate the proteins probed under low oxygen conditions (Figure 22C), suggesting the impairment of hypoxic response in these decoy engineered cells. Moreover, A375 cell line with or without the decoy showed no visible difference in the protein expression level among the conditions tested. Taken together, this data suggests an indispensable role of hsa-miR-210 in maintaining the transcriptome homeostasis in these cells. Once the same is being challenged using miR decoy; it renders the cell with a complete loss of various controls deployed by hsa-miR-210.

Inhibition of hsa-miR-210 function using the miR decoy hints towards a contrary role of the miRNA in this context. To date, hsa-miR-210 expression has been linked to either aggressive tumors and/or poor prognosis across malignancies thereby making it and/or its downstream targets an apt candidate for therapeutic intervention (Hong et al., 2013; Noman et al., 2015; Wang et al., 2014). Contrary to the various published data to date, we see a complete reversal of all proteins probed in cell lines engineered with the miR decoy.

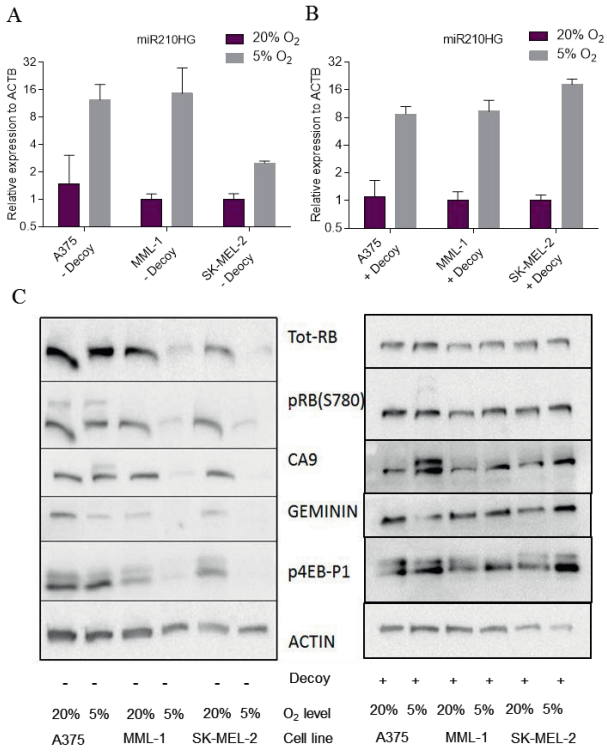


Figure 22: Abrogation of hsa-miR-210 regulation using miRNA decoy completely reverses the hypoxia-induced phenotypes. (A) qRT-PCR analysis of miR-210HG expression across cell lines in response to 5% O_2 for 24h. (B) qRT-PCR analysis showing induction of hsa-miR-210HG in response to 5% O_2 for 24h across cell lines engineered with hsa-miR-210 decoy. (C) Western blot analysis of the cell lines under normoxic and hypoxic condition after 24h with or without miR decoy.

This is not an isolated event where a miRNA's loss of function results in contrary phenotype than expected. Notably, miRNA-26 can act either as an oncogene (Huse et al., 2009) or tumor suppressor (Kota et al., 2009). To date, various studies have shown the two-faced roles of the same miRNA in either promoting or inhibiting tumor progression (Garofalo et al., 2012; Zeitels et al., 2014) depending on the cell type and/or the micro-environment.

Owing to the function of proteins reversed in decoy engineered cells, it seems pretty obvious that the same will ultimately lead to accelerated tumor growth. As speculated, MML-1 cells carrying the decoy show significant increase in the tumor volume when compared to the controls (Figure 23A). Moreover, mirRNA's has been previously linked to confer resistance across malignancies (Allen and Weiss, 2010). Despite development of various targeted therapy specifically against mutated BRAF, emergence of resistance in advance melanomas develop fairly quickly (Johannessen et al., 2010; Montagut et al., 2008). It was obvious to investigate the effect of MEK inhibition in the MML-1

cells engineered with the miR decoy, as MEK inhibition in combination and/or standalone agent has been effective in both BRAF mutated and naïve tumors (Grimaldi et al., 2014; Salama and Kim, 2013). As seen in figure 23B, all untreated mice carrying the miR decoy succumbed to the disease faster than their corresponding control. Moreover, mice carrying the decoy-engineered cells despite the treatment died significantly faster compared to mice carrying the wildtype MML-1 cells. Reassuringly, the MML-1 wildtype cells receiving the treatment survived significantly longer compared to all other groups. To further investigate the underlying cause of this aggressive tumor growth, we performed reactome pathway analysis of established hsa-miR-210 targets.

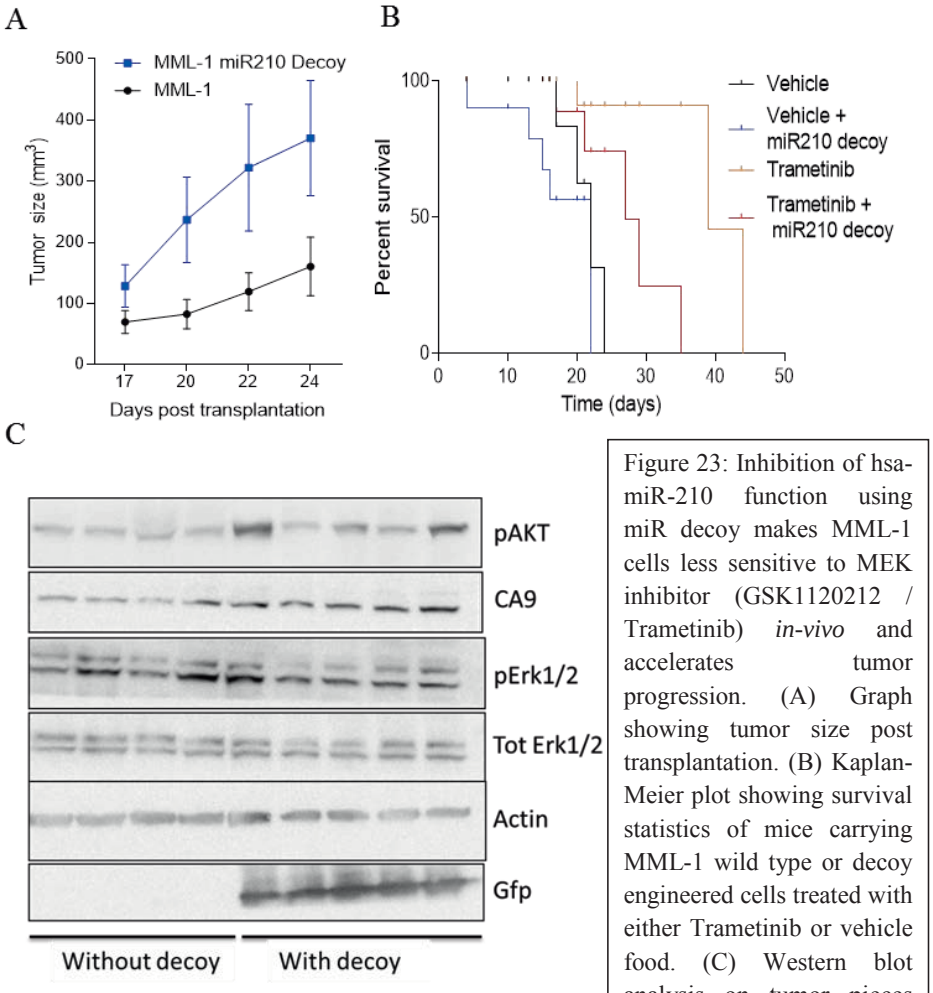


Figure 23: Inhibition of hsa-miR-210 function using miR decoy makes MML-1 cells less sensitive to MEK inhibitor (GSK1120212 / Trametinib) *in-vivo* and accelerates tumor progression. (A) Graph showing tumor size post transplantation. (B) Kaplan-Meier plot showing survival statistics of mice carrying MML-1 wild type or decoy engineered cells treated with either Trametinib or vehicle food. (C) Western blot analysis on tumor pieces from untreated mice.

We next performed western blot analysis on the tumor pieces from the vehicle controls of MML-1 wild type or decoy engineered cells to validate a few candidate targets from the MAPK pathway. As seen in figure 23C, the decoy engineered cells have marginally higher levels of CA9 compared to the controls. We speculated that using of the decoy may in fact increase the level of total and phosphorylated ERK1/2, as they are known targets of hsa-miR-210 (see Suppl. figure S1B (Bhadury et al., 2016)). We observed negligible differences between the phosphorylated (T202/Y204) and total levels of ERK1/2 between the groups. We then probed for phosphorylated AKT (T308), and indeed we saw marginally higher levels in the decoy engineered group (Figure 23C). The central role of AKT in conferring transformation capabilities among others, either due to membrane translocation or phosphorylation is very well document (Testa and Bellacosa, 2001). Moreover, the primary stress response induced by cells in conditions like hypoxia is the minimal resources and the same may be accomplished by up-regulation of HIF targets including miR-210. It is known that CAP dependent translation is one of the first processes to be affected in stress conditions like hypoxia (Liu et al., 2006; Pause et al., 1994). It is very intriguing to hypothesize, that in the decoy engineered cells the reversal of various proteins could be because of increased levels of AKT, which in turn overrides protein synthesis machinery (Dai et al., 2013) and helps this cell survive (Song et al., 2005), thereby relieving the control mechanism enforced by the miRNA.

To summarize, we demonstrated the inherent transcriptome wide difference between cell line and patient derived xenografts. Moreover, we also show that the difference between the two groups could be because of *in vitro* culture conditions. Finally, we show that targeting of hsa-miR-210 in metastatic melanoma cells might have lethal consequences, as the cells become more aggressive and in fact less sensitive to MEK inhibition *in vivo*.

3.4. iBET- It's Not MYC (Paper IV)

Our lab has previously shown that by targeting molecules in DNA damage response pathway (like Chek1 protein kinase), MYC over-expressing cells could be forced to undergo apoptosis (Höglund et al., 2011a; Höglund et al., 2011b). Couple of years ago there was no pharmaceutical agent available for MYC inhibition (McKeown and Bradner, 2014; Soucek and Evan, 2010). Recently, BETi has been shown as a preferred means of directly inhibiting MYC.

In collaboration with a Canadian company (Zenith Epigenetics Corp), we set out to test their novel and orally bio-available BET inhibitor RVX2135. RVX2135 is designed on the chemical scaffold of RVX208 (currently in late stages of clinical trial for arteriosclerosis) and is structurally unrelated to the benzodiazepine compound derivatives (McLure et al., 2013; Nicholls et al., 2011; Picaud et al., 2013). As seen in Figure 24A, RVX2135 inhibited all four BET proteins as assed by *in vitro* FRET assay in micromole potency. Moreover, RVX2135 could displace all BET proteins probed in the cell lines to similar extent as compared to the prototype (+)-JQ-1 (Filippakopoulos et al., 2010), hereafter referred to as JQ1 (see figure 1B and S1A (Bhadury et al., 2014)). Often, BET protein inhibitors (especially JQ1) are considered indirect Myc inhibitors (Alderton, 2011; Dawson et al., 2011; Delmore et al., 2011; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011). Here, we wanted to test the new BET inhibitor in transgenic mouse models of Burkitt lymphoma and the cell lines derived from them, where pathogenicity of the disease may be solely attributed to the over-expression of Myc/MYC respectively (Adams et al., 1985; Kovalchuk et al., 2000).

To investigate the anti-proliferative effect of BET protein inhibition in Burkitt lymphoma, we used cell lines derived from the aforementioned mouse models. As seen in figure 24B, cell count analysis showed that 24h post treatment cell growth was severely affected by the lower concentrations of BETi (JQ1=100nM and RVX2135=1μM). At 48 hours post treatment, severe cell death was seen at the high concentration (JQ1=1μM and RVX2135=10μM) of BETi, but not much death was observed for the lower concentrations. To better understand the lethal effect of BET inhibition in this cells, we treated the cells for 24h with both the BETi and performed western blot analysis. Indeed, the cells responded to BETi by elevating the levels of both cleaved caspase III and cleaved PARP, which are bonafide targets of mitochondria mediated apoptosis pathways

(Figure 24C) (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Oliver et al., 1998; Satoh and Lindahl, 1992). To assess if the effect of BETi was selective against malignant cells, we treated passage 2 mouse embryonic fibroblasts (MEF) with the high concentrations of either the BETi for 48h and analyzed using FCM. As seen in figure 24D, not much effect was observed in either S-phase or the Sub-G1 phase of the cell cycle. A drastic reduction, however, was observed in S-phase 24h post treatment of the λ820 cells with either of the BETi in the higher concentrations (Figure 24E). Having tested the specificity of BETi primarily for the malignant cells, we wanted to see if we could rescue the apoptotic effect in these cells. Towards this end, we pre-treated the cells with a pan caspase inhibitor

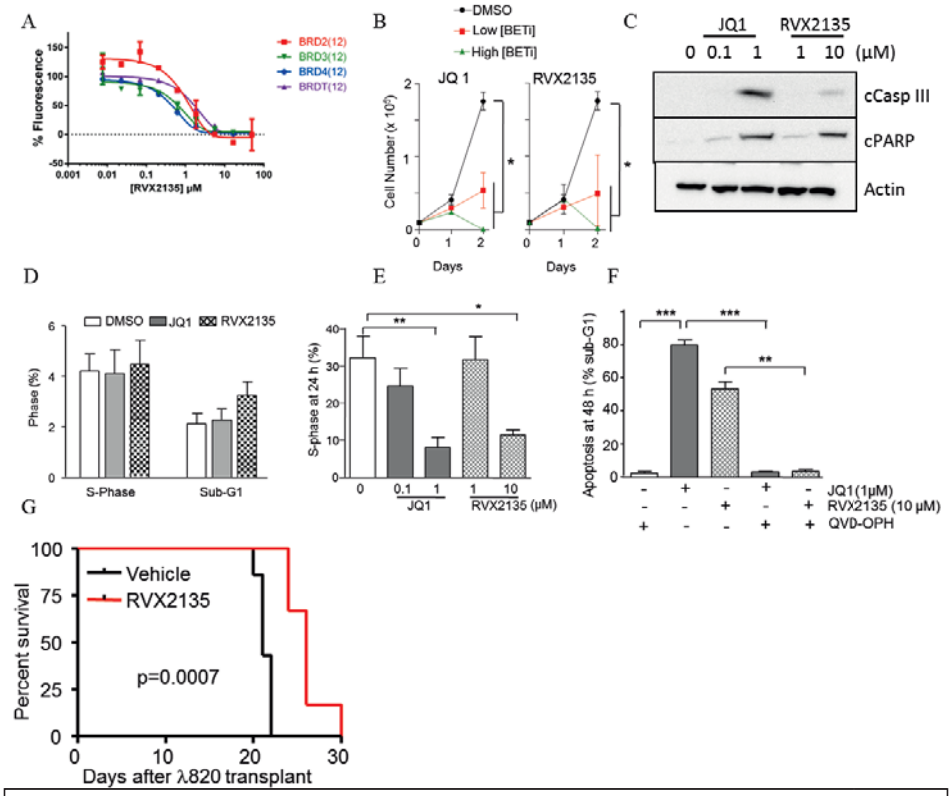


Figure 24: RVX2135 is a potent BETi: (A) FRET assay showing displacement of BET proteins. (B) Cell count analysis shows dosage dependent effect of BETi in λ 820 cells. (C) Western blot analysis shows caspase mediated apoptosis pathway activation by BETi. (D) No significant effect on MEFs by BETi. (E & F) Time and dosage dependent effect of BETi on cell cycle and apoptosis rescue by Q-VD-OPh, as assessed by FCM. (G) *In vivo* efficacy of BETi on λ820 tumors.

Q-VD-OPh (Caserta et al., 2003), prior to the BETi treatment. As expected, we could completely rescue the caspase mediated apoptosis cascade mediated by BETi treatment in this cells (Figure 24F) and the cells seemed to be completely arrested in G1 phase (See figure 2B, S2B-C and E (Bhadury et al., 2014)). To test if RVX2135 was orally bio-available, we transplanted C57BL/6 mice with λ820 cells and treated the mice 4 days post-transplant. As seen in figure 24G, mice receiving RVX2135 by oral gavage (75mg/kg, b.i.d), survived for an extra week compared to the controls. This data is in complete accordance with the previously published data of JQ1, where similar effects were observed in other malignancies BETi was administered by IP injections as compared to oral gavage used here (Delmore et al., 2011; Herrmann et al., 2012; Ott et al., 2012; Zuber et al., 2011).

In order to further strengthen our claims, we repeated the above mentioned *in vivo* experiment but with another syngeneic transplantable tumor arising from λMYC mouse model (hereafter referred to as #2749). This tumor model #2749 was established from a spontaneously arising tumor in a mouse bearing the ID#2749. The primary tumor was chopped into single cell suspension and ever since has only been propagated *in vivo* by serial transplantation in C57BL/6 recipient mice. We convincingly show that RVX2135 administration could significantly prolong the survival of mice transplanted with #2749 tumors (Figure 25A). Compared to λ820 cells, this was a very significant extension in their life expectancy (compare figure 24G and 25A). To gain more insight into the reason for this strong response in #2749 compared to 820 cells, we performed whole exome sequencing of these cells. Interestingly, we found that #2749 had an intact *Trp53* compared to the λ820 cells and this might explain the effect seen in these cells (see figure S4 (Bhadury et al., 2014) and data not shown). To rule out the possibility of any clonal expansion and/or selection as the primary reason for the anti-tumor effect in the #2749 tumor model, #2749 cells were injected into new mice and twelve days post transplantation mice were administered with ^{18}FDG probe to measure the tumor metabolic baseline values by PET scan. Thereafter, mice were treated with RVX2135 for two days. As seen in figure 25B (a representative image of the same mouse before and after treatment from the experimental set), marked reduction in PET signal were observed (see figure 3C for complete data (Bhadury et al., 2014)). Interestingly, majority of the reduction in PET signal was seen primarily from the spleen (indicated by a blue arrow) as compared to the lymph nodes (indicated with red arrow). Its intriguing to speculate that because of this limited removal of the

tumor cells from the lymph nodes (Figure 25B), this mice despite showing robust anti-tumor response from BETi may have finally succumbed to the lethal disease (Figure 24G and 25A).

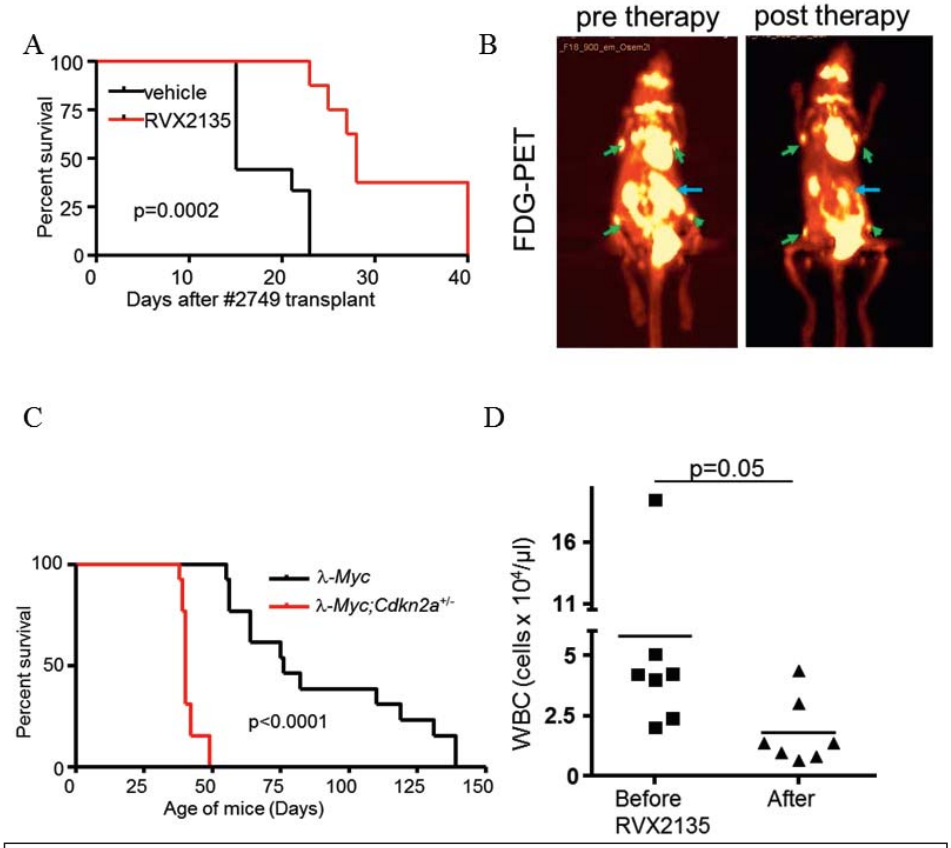


Figure 25: RVX2135 is orally bio-available (A) #2749 cells treated with RXV2135 75mg/kg b.i.d. (B) PET scan using [^{18}F]FDG probe of same mouse before and after treatment with RVX2135. (C) Plot showing tumor penetrance and development dynamics in λMYC and λ*MYC*::*Cdkn2a*$^{+/-}$ mice. (D) WBC count before and after treatment with RVX2135 in λ*MYC*::*Cdkn2a*$^{+/-}$ mice.

We wondered if the observed effects of BET inhibition could be recapitulated in spontaneous and transgenic mouse models of Burkitt lymphoma *in situ*. To this end, we made a λ*MYC*::*Cdkn2a*$^{+/-}$ mouse, which develops palpable tumors at an average age of 40 days compared to λMYC mice (Figure 25C). Moreover, the tumor development rate in these mice is similar to the Eμ-*Myc*::*Cdkn2a*$^{+/-}$, λ-*Myc*::*Trp53*$^{+/-}$ and Eμ-*Myc*::*Trp53*$^{+/-}$ mice (Eischen et al., 1999; Hsu et al., 1995; Nilsson et al., 2012; Schmitt et al., 1999). To assess the effect of BET protein

inhibition in this model, we used leukocytosis (>20 WBCs/nL) as a surrogate for tumor/disease progression. When all seven mice in the group showed signs of leukocytosis, they were treated with RVX2135 for three days. Blood collected from these mice before and after the end of the treatment showed efficient and repaid reduction of WBC count (Figure 25D), once again displaying the potent anti-tumor effect of RVX2135 in the spontaneous tumors arising in λ*MYC*::*Cdkn2a*$^{+/-}$ mice.

Having shown the potent anti-tumor effect of this novel BET inhibitor, we wanted to elucidate the molecular insights. Unexpected and contrary to the previously published literature (Alderton, 2011; Dawson et al., 2011; Delmore et al., 2011; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011), we didn't see any down regulation of *MYC*/*Myc* transcript levels in the cell lines tested post 24h of treatment with either of the BETi (Figure 26A). In line with the transcript levels, we see no significant decrease in MYC/Myc levels (Figure 26B), as assessed by western blot. At best we saw a modest decrease in MYC levels in cells lines derived from λ-*MYC* mouse and no decrease whatsoever in the lines derived from Eμ-*Myc* mouse models. If MYC inhibition was the rationale of BETi, then restoration of MYC levels should rescue the phenotype. We restored MYC levels by using GSK3β inhibitor CHIR-99021 (Bennett et al., 2002); as GSK3β is known to tag MYC for proteasome mediated degradation (Gregory et al., 2003). As seen in figure 26C, indeed treating the cells in presence of CHIR-99021 did rescue and in fact increase the levels of MYC even in the higher concentrations of BETi. But despite this reversal of protein levels, we couldn't rescue the cells (data not shown). This is not an isolated instance, as ectopic expression of MYC or FOSL1 couldn't rescue the BETi effect (Delmore et al., 2011; Lockwood et al., 2012). It is thus correct to assume that the reduction of MYC/Myc levels seen here is primarily because of destabilization of the MYC protein probably mediated via GS3β/FBW7/Proteasome (Sears et al., 2000) and not due to the transcriptional repression of *MYC/Myc* per se.

To further investigate the genes and/or networks alerted due to BET inhibition, we subjected two cell lines (one each from Eμ-*Myc* and λ*MYC* mouse model) with the higher concentration of BETi. In order to get an understanding of the molecular events triggering the apoptosis cascade among others, all cells were pretreated with Q-VD-OPh. Figure 26D shows the 50 most up and down-regulated genes in either of the cell lines tested.

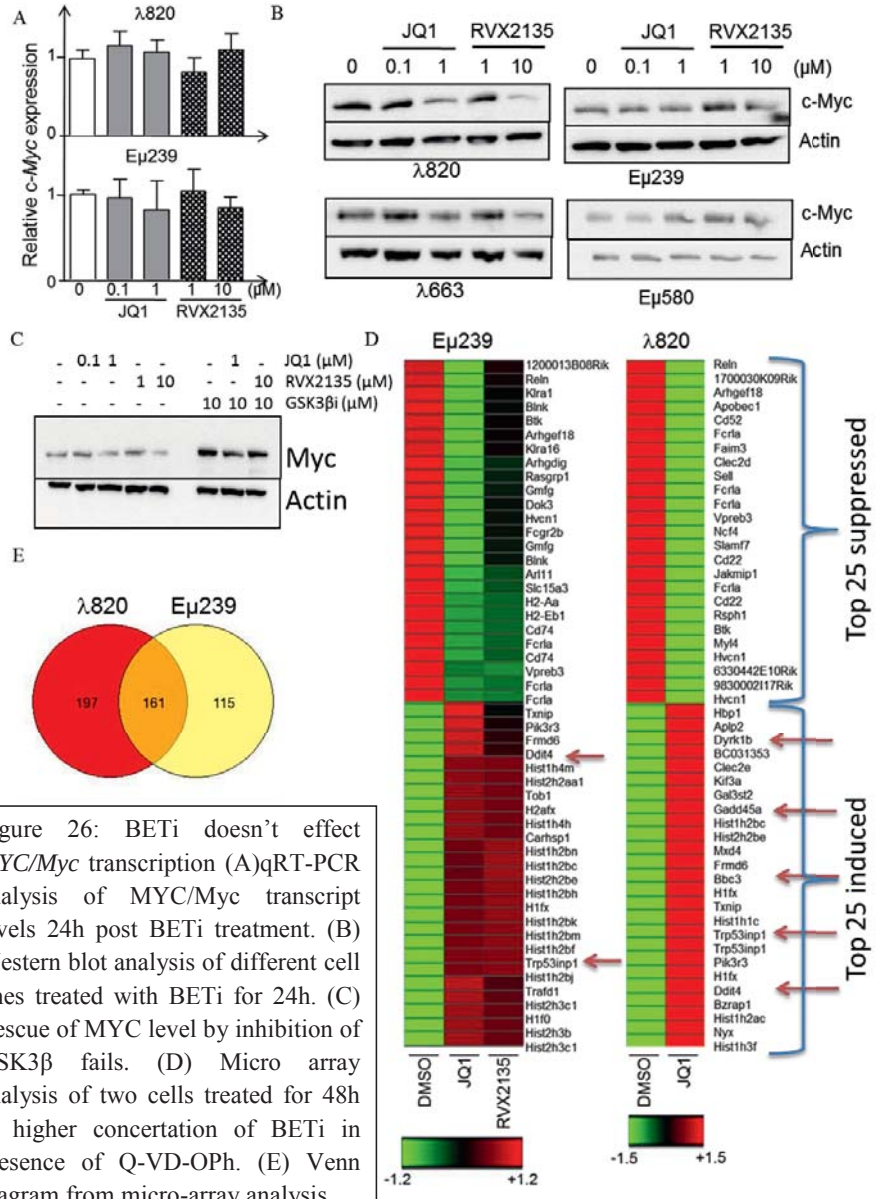


Figure 26: BETi doesn't effect *MYC/Myc* transcription (A)qRT-PCR analysis of MYC/Myc transcript levels 24h post BETi treatment. (B) Western blot analysis of different cell lines treated with BETi for 24h. (C) Rescue of MYC level by inhibition of GSK3β fails. (D) Micro array analysis of two cells treated for 48h in higher concertation of BETi in presence of Q-VD-OPh. (E) Venn diagram from micro-array analysis.

In line with our data, we see no Myc regulated signature (see figure 5C (Bhadury et al., 2014)) in our data set as the top hits from GSEA analysis. If anything, we do see few Myc targets genes being downregulated only in λ820 cells; not surprising given the fact reduced MYC levels were seen in the highest

concentrations of BETi. Moreover, there was an overlap of 161 genes that were significantly downregulated across the cell lines (Figure 26E). As mentioned before, to date most studies focused primarily on the genes downregulated by BETi, primarily the Myc regulated gene networks among others (Dawson et al., 2011; Delmore et al., 2011; Herrmann et al., 2012; Lockwood et al., 2012; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011). Instead, we were interested in the genes that were up-regulated by BETi. Primarily, several histones were significantly upregulated in both the cell lines. Moreover, as pointed out in figure 26D with red arrows, many of the p53-regulated genes were significantly induced. This is surprising, given the fact that either of the cell lines don't have a functional p53. In line with the up-regulation of p53 target genes, we do observe a significant upregulation of Egr1; which induces apoptosis cascade independent of p53 in Myc-overexpressing cells (Boone et al., 2011; Zhang et al., 2013a). We next performed GSEA analysis on the significantly upregulated genes from our data set. Surprisingly, only the genes up-regulated by BETi were similar to the ones up-regulated by histone deacetylase inhibitor (HDACi) and not the down regulated genes (Heller et al., 2008).

To further scrutinize if the genes up-regulated by BETi and HDACi share similar responses, we treated couple of the above mentioned cell lines with Vorinostat/SAHA (Richon et al., 1998) or LBH589/Panabinostat (Peter T. Lassota, 2004). As seen in figure 27A, either of the HDACi inhibitors severely affected the cell growth. We next performed cell cycle analyses on cells treated with HDACi. Once again we could recapitulate a trend similar to BETi; that is in certain dosage of all the HDACi tested the cells first responded by potent cell cycle arrest in G1 phase by 24h and later died via apoptosis by 48h post treatment (see figure 7A, S10A (Bhadury et al., 2014)). Moreover, western blot analysis showed results similar to that of BETi (Figure 27B). As expected, we see a potent increase in phosphorylation γH2ax by HDACi (Bhaskara et al., 2008), a phenomenon also seen upon BET inhibition (Floyd et al., 2013). We could confirm both the findings in our system with BETi and HDACi (Figure 25B; see figure S8C (Bhadury et al., 2014)). As seen in figure 27B, MYC levels were lower in the higher concentrations of either of the HDACi treated cells, yet again phenocopying effects similar to BETi. Furthermore, there was not much significant decrease in the MYC transcript levels (see figure S8A (Bhadury et al., 2014)). These observations pointed towards a significant correlation shown by the two different classes of inhibitors.

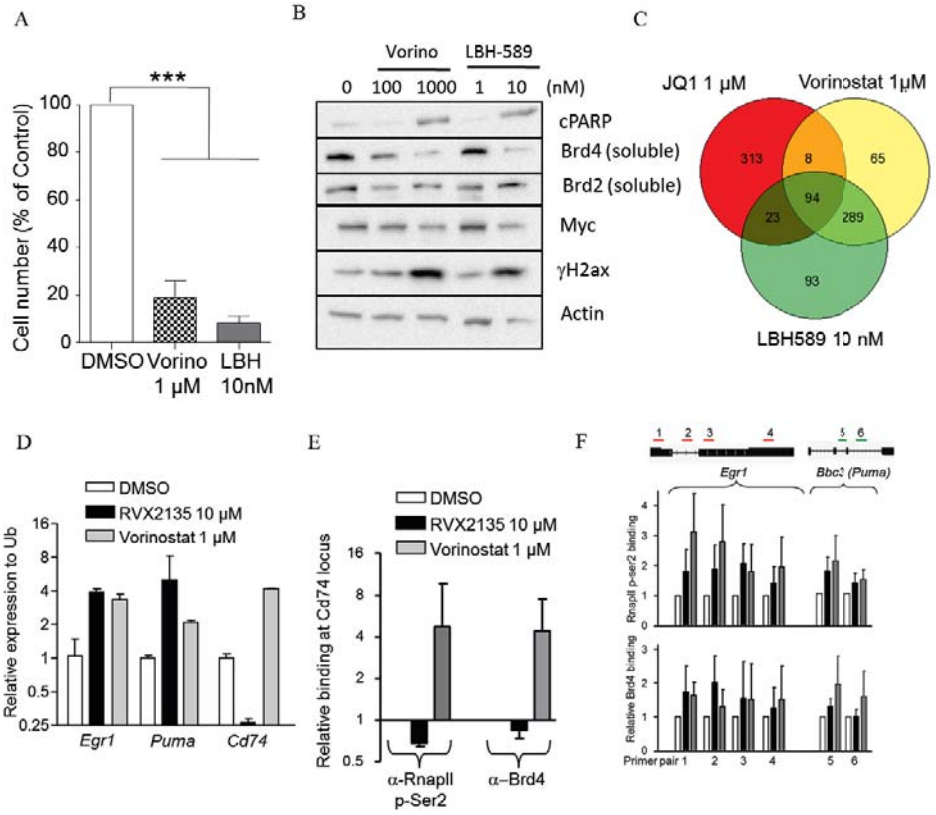


Figure 27: HDACi induces similar effects as BETi. (A) Cell count of λ820 cells treated with HDACi. (B) Western blot analysis of λ820 cells. (C) Venn diagram showing shared upregulated genes between BEti and HDACi. (D) qRT-PCR showing genes regulated similarly and differentially by BETi and HDACi (E) Cd74 occupancy by BRD4 and pS2-RNA Pol-II post treatment by BETi and HDACi, as assessed by ChIP-qRT-PCR. (F) ChIP-qRT-PCR assement of genes similarly regulated by BETi and HDACi.

We also performed gene expression analysis of λ820 cells treated with Vorinostat and LHB589. Indeed, as seen in figure 27C, there was around 25% overlap of genes up-regulated by HDACi with BETi. Interestingly, many of the genes up-regulated by HDACi (like *Egr1*, *Trp53inp1*, *Gadd45a* and *Bbc3*) are known to regulate pro-apoptotic cascade. Moreover, genes like *Cd74* were selectively downregulated in HDACi. Select genes were verified by qRT-PCR analysis (Figure 27D). In order to verify the molecular events, we performed ChIP assay on select genes using RVX2135 and Vorinostat. As seen in figure 27E, there was indeed a decreased occupancy of both Brd4 and p-Ser2-RNA POL-II in Cd74 gene locus when treated with HDACi (Vorinostat) as compared

to increased occupancy with BETi (RVX2135). Moreover, we also observed that both BETi and HDACi did in fact have increased occupancy of Brd4 and p-Ser2-RNA Pol-II, manifesting the active role of positive elongation complex (Figure 27F) (Jang et al., 2005; Marshall et al., 1996; Yang et al., 2005).

Having shown that both BETi and HDACi shared similar and unique response to gene networks, we wanted to investigate if the two may be combined to get synergistic combinations for treatment regimes. Indeed, as seen in figure 28A there was a significant reduction in cell number in the combination treatment. This combination also resulted in drastic cell death as compared to single agent dosage which merely results in potent cell cycle arrest (see figure S10A and B (Bhadury et al., 2014)). Having verified this strong synergistic effect, we wanted to check if the same may be translated *in vivo*. We then transplanted mice with #2749 cells and waited for the mice to show higher WBC counts compared to healthy mice. Mice were treated in groups as mentioned in figure 28B. As expected, RVX2135 and mainly the combination treatment drastically reduced the leukocytosis.

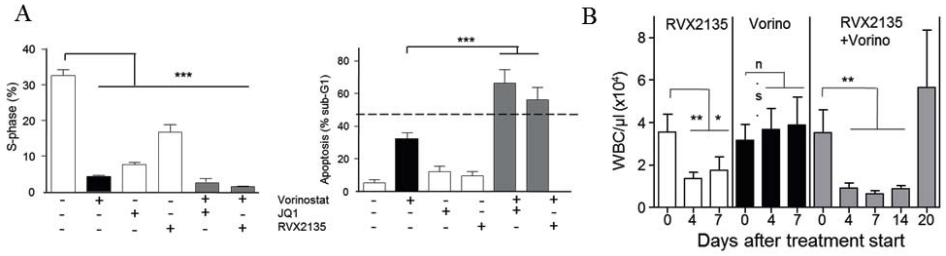


Figure 28: BETi and HDACi gives synergetic combination *in vivo* (A) i*n vitro* validation of synergistic effect by vorinostat and BETi in λ820 cells, data assessed using FCM (B) Mice bearing #2749 tumors treated with Vorinostat (40mg/kg) or RVX2135 75mg/kg or in combination. Shown are WBC count before, during and end of treatment.

One week post starting the treatment, the therapy was ceased as most of the mice in the single agent treatment group started to show signs of disease progression. Unfortunately, in two weeks post treatment termination all mice in the combination group succumbed to the disease. This shows that the entire population of malignant cells couldn't be effectively removed by the treatment.

Taken together, we have not only unraveled a novel and orally bio-available BETi; we have also clarified the widespread notion of considering BETi to be a Myc inhibitor. We have shown that BET protein inhibition results in both

downregulation and upregulation of genes and a surprising number of genes being similarly upregulated in BETi and HDACi. Interestingly, we found a synergistic combination dosage that was effective *in vivo*. We speculate that either of the drugs can be used to activate p53 target genes independent of p53 mutational status.

3.5. ATR Damage's The BET (Paper V)

In our previous study, we showed that the novel and orally bio-available BETi RVX2135 can displace the BET proteins from chromatin and ultimately trigger apoptocic cascade to kill lymphoma cells. We also show that there were many genes that were potently upregulated by both BETi and HDACi. Finally, we demonstrated that BETi and HDACi could be combined to obtain synergistic benefits *in vivo* in aggressive murine Burkitt lymphoma transplanted models.

Despite these exciting findings, we don't clearly understand why the lower concentration of either of the BETi resulted in severely hampered cell growth but not death (Figure 24B). Moreover, as we serendipitously found the synergistic combination of BETi and HDACi, could we find more combinations which could widen the range of malignancies targeted?

We treated λ820 cells with both concentrations of either of the inhibitors and performed cell cycle analysis and thymidine incorporation assay. As seen in the left panel of figure 29A, consistent with our previous data, we found modest reduction with low concentrations and significant reduction with the higher concentrations of BETi. Furthermore, we added radio labelled thymidine to the media 4 hours prior to final harvest time for the treatment duration. Surprisingly, as seen in the left panel of figure 29A low concentrations of BETi which had no apparent effect on cell cycle; showed marked reduction in thymidine incorporation. This indicated that the cells are struggling to complete the DNA synthesis phase (S-Phase) of cell cycle and might explain the reason for the slow growth of cells in this concentration. As a next step, we used P493-6 cells which are engineered with Tet-off MYC system (Pajic et al., 2000), in other words these cells generally have high levels of MYC; but addition of tetracycline for 72h reduces MYC levels below detectable limits in western blots and leads to G1 arrest. By washing off tetracycline, these cells can be brought back to active cell cycle. Using the P493-6 cells we wanted to interrogate if the used dosage of BETi were affecting entry or progression through S-Phase. As seen in figure 29B, there were dramatic effects observed on cell cycle and DNA synthesis with either concentrations of the BETi on continuously proliferating P493-6 cells. Having observed the profound effect on DNA synthesis in both the cell lines, we wanted to investigate if the cells are even in S/G2-M phase of cell cycle. As seen in figure 29C, higher concentrations of BETi, dramatically reduced the levels of Geminin; whereas not much effect was seen on the lower concentrations. Western blot analysis on

synchronized P493-6 cells show no effect on MYC levels with either concentrations of BETi (Figure 29D); similar to the observed effects in murine Burkitt lymphoma cells λ820 (Bhadury et al., 2014). Having observed the aforementioned effect, we wanted to interrogate the transcriptome in greater details. We next performed micro array analysis using two concentrations of BETi.

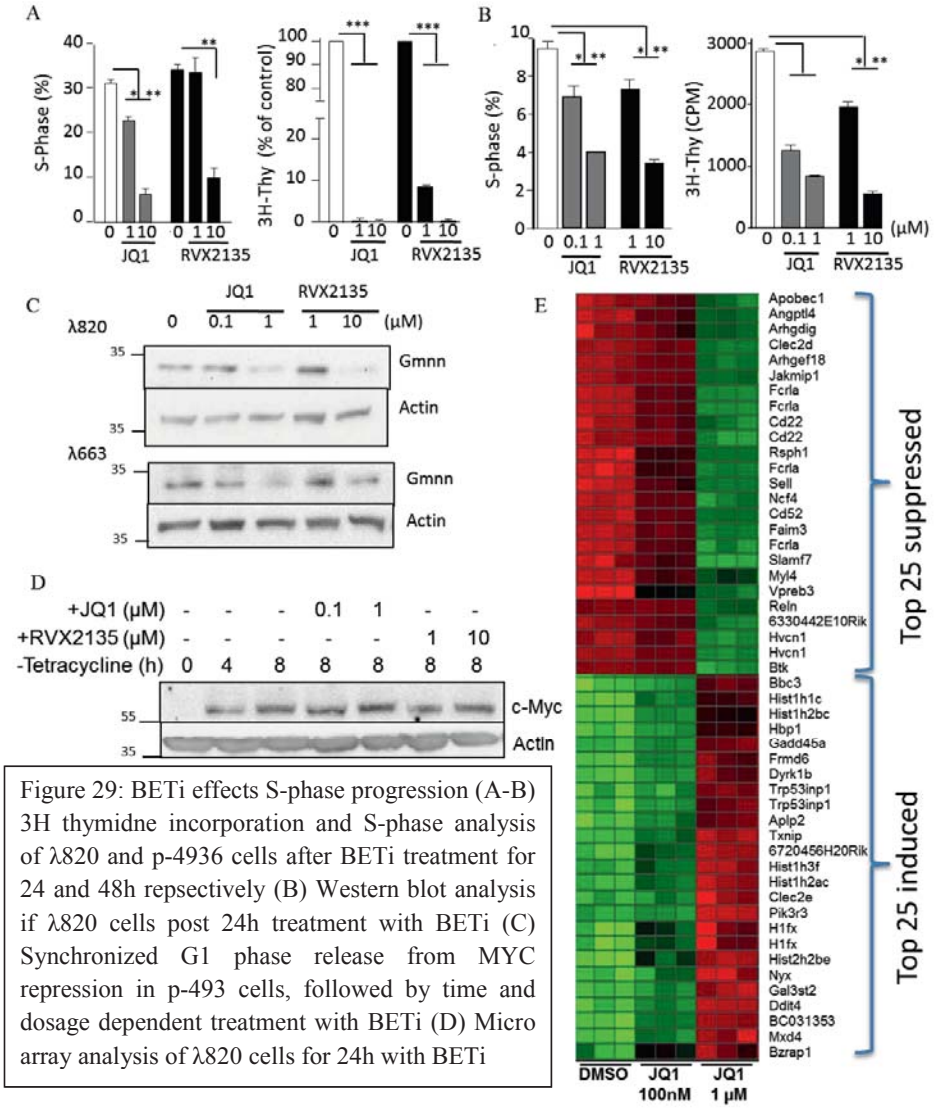


Figure 29: BETi effects S-phase progression (A-B) 3H thymidne incorporation and S-phase analysis of λ820 and p-4936 cells after BETi treatment for 24 and 48h repsectively (B) Western blot analysis if λ820 cells post 24h treatment with BETi (C) Synchronized G1 phase release from MYC repression in p-493 cells, followed by time and dosage dependent treatment with BETi (D) Micro array analysis of λ820 cells for 24h with BETi

As seen in figure 29E, there was not much affect in the transcriptome at the lower concentration of BETi despite the fact that there was significant reduction DNA synthesis at this concentration. As expected, there were marked differences at the higher concentrations (Figure 29E). Interestingly, there was no effect on DNA synthesis or replication inhibition when using BETi in cell free systems (see Suppl. figure S1D (Muralidharan et al., 2016)). Taken together, this data hints towards the fact that BETi enforces its effect primarily via regulation of DNA synthesis/replication than by directly inhibiting replication.

It was very interesting to note that transcriptome remained greatly unaltered after treating the cells with lower concentrations of BETi. Having previously shown that indeed BETi could be coupled with other inhibitors to get synergistic effects, we wanted to investigate if this can be the case even with the low dosages. We used the previously described small molecule library (Bhadury et al., 2013) and treated both cells (λ820 and Eμ239) either in presence or absence of the low concentrations of BETi (100nM JQ1). We could find many compounds that resulted in cell death (as assed by CellTiter-Glo® assay) (see figure 1D and E (Muralidharan et al., 2016). Among these molecules, many were found to be either inhibiting the Aurora Kinase and/or the PI3K/mTOR molecules. Given the role of BRD4 in replication, the synergistic effect with Aurora kinase inhibitor was not completely unexpected (Dey et al., 2000). Moreover, recently it has been shown that BETi has synergistic effect with PI3K/mTOR inhibitors (Boi et al., 2015; Stratikopoulos et al., 2015).

Intrigued by the synergy of BETi with PI3K/mTOR inhibitors, we wanted to interrogate it further. A point to note is that the small molecule library used here was at a final concertation of 1μM of each drug and at this concentration some drugs may show off-targets effects, especially for kinases. The PI3K-like (PIKK) protein kinase family is in fact very similar to the PI3K family of proteins; thus, the off-target effects maynot be overlooked. The PI3K-like protein family consists of P13K, mTOR; ATM, ATR and DNA-PK. The role of the DNA damage response kinase ATR is well known, especially in oncogene induced replication stress (Lecona and Fernandez-Capetillo, 2014). Data from our lab and others have previously shown that inhibition of ATR or even its bonafide downstream targets have lethal consequences, especially on MYC over-expressing cells (Ferrao et al., 2012; Höglund et al., 2011a; Höglund et al.,

2011b; Murga et al., 2011). Interestingly, one of the PI3K/mTOR inhibitor (NVP-BEZ235) synergizing with low concentrations of BETi was shown to be a pan PIKK family inhibitor (Shortt et al., 2013; Toledo et al., 2011). This called for further and in-depth scrutiny in this direction.

Indeed as expected, when treating λ820 cells with varying concentrations of NVP-BEZ235 in presence of low dosage of either of the BETi, we could revalidate the results from the drug screen (see figure 2A and B (Muralidharan et al., 2016)). To decipher if the seen affect was mediated via PI3K/mTOR pathway or via inhibition of ATR, we procured two recently developed selective ATR inhibitors (ATRi) (VE-821 and AZ20) (Foote et al., 2013; Reaper et al., 2011) and compared them against our PI3K/mTOR inhibitors. As can be seen in figure 30A, reduction of phospho-Chek1 and induction of phosphorylated H2Ax was specifically seen in the selective ATR inhibitors. Whereas, both the PI3K/mTOR inhibitor resulted in reduction of phosphorylation of Chek1, 4EBP1 and S6, showing inhibition of ATR and mTOR targets respectively. The PI3K/mTOR inhibitor didn't induce the phosphorylation of H2Ax most likely due to the inhibition of ATM. In events of replication fork collapse, generally double stranded DNA breaks are generated and molecularly this event is marked by the phosphorylation of H2Ax by ATM kinase (Reaper et al., 2011).

The obvious next was to test if BETi and ATRi may be combined to get synergistic effects. As seen in figure 30B, indeed BET inhibitors can be combined with inhibition of ATR-Chek1 pathway to get synergistic effects (also see figure 2D and Suppl. figure S3A,B,D,E; S3C,D,E and S4A,B,C (Muralidharan et al., 2016). We then wanted to see if this combination was active *in vivo*. Towards this end, we used AZ-20 in combination with RVX2135 to treat the serially transplantable λMYC derived tumor #2749. As seen in figure 30C, there was significant reduction of WBC count post treat. Moreover, the combination treatment of BETi and ATRi markedly increased the survival of this lethal MYC induced lymphomas (Figure 30D). This marked increase in survival is the longest survival benefit we have seen across any combinations of drug tested to date against this tumors.

Having noted the marked survival benefits, it was obvious to interrogate further into the molecular mechanism governing this effects. We subjected the λ820 cells with either AZ-20 or RVX2135 or both in presence of the pan-caspase

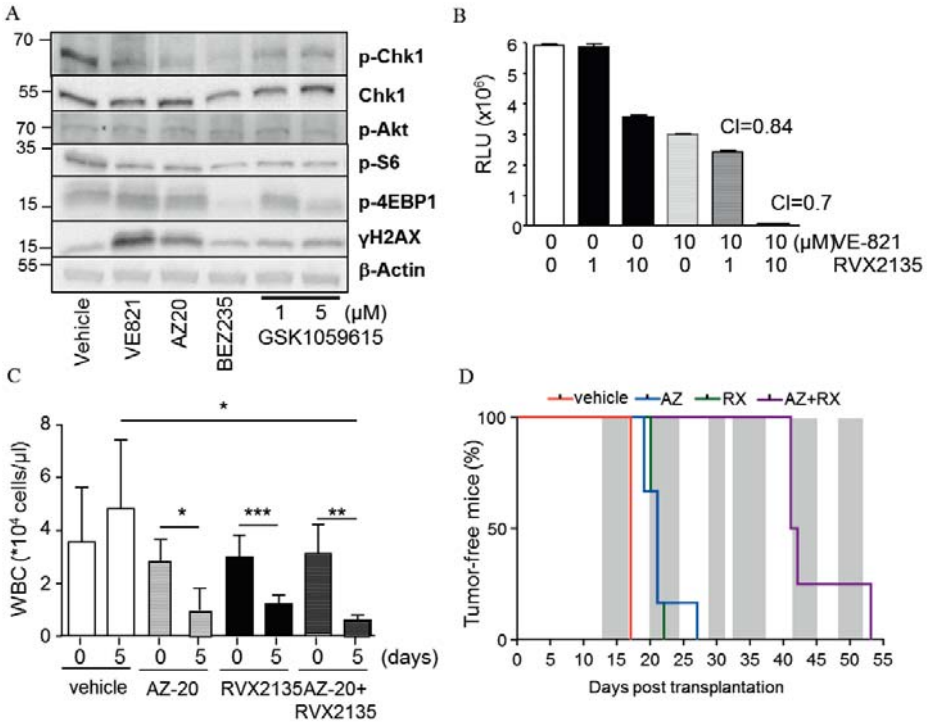


Figure 30: BETi and ATRi synergizes both *in vitro* and *in vivo* (A) Western blot analysis using mTOR/PI3K or ATR specific inhibitor for 24 h treament in λ820 cells (B) Cell titer Glo® analysis showing synergy between BETi and ATRi (C & D) #2749 tumor bearing mice treate with either ATRi or BETi in combination. WBC count and Kaplan-Miere plot are shown respectively

inhibitor Q-VD-OPh and performed micro-array analysis. Initial analysis revealed different completely different components effect either by the single agent BETi and ATRi or in combination (Figure 31A). Furthermore, potent effects of both inhibitors individually on different pathways related to replication machinery and chromosome maintenance were observed (see figure 4B (Muralidharan et al., 2016). Surprisingly, the combination treatment hinted towards increased levels of gene involved in senescence-associated secretory pathway. The genes from the NF-κB pathway were also significantly upregulated (Figure 31B) given the fact that in MYC induced lymphomas this pathway is downregulated (Keller et al., 2010; Keller et al., 2005; Klapproth et al., 2009). As seen in figure 31C (also see Suppl figure S6A (Muralidharan et al., 2016)), DDI3T/CHOP and ATF4 (involved in ER stress related pathways), Cxcl2 and Cxcl1 (in senescence secretory pathway) were markedly increased in

either ATRi or in the combination treatment only (Chien et al., 2011; Dorr et al., 2013; Jing et al., 2011). In line with the increased transcript levels of CHOP and ATF4 were increased both murine (Figure 31D and see Suppl. figure S6A (Muralidharan et al., 2016)) and human (Figure 31E) Burkitt lymphoma cell lines. Interestingly, a protein specifically degraded in autophagy (p62) by LC3

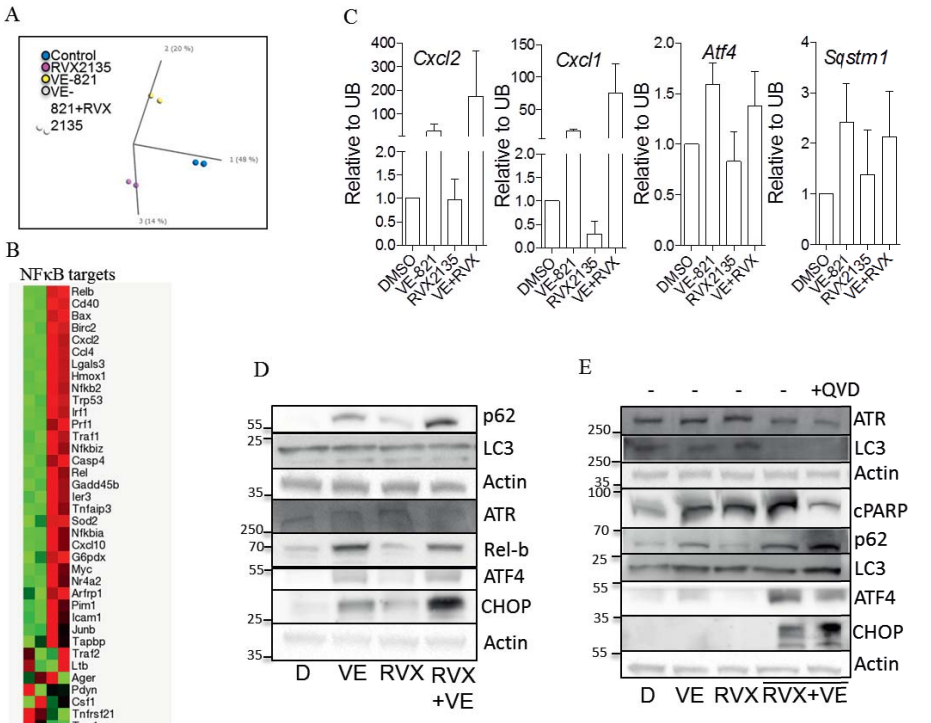


Figure 31: BETi and ATRi combination (A) PCA plot from single or combination treatment of λ820 cells with BETi and/or ATRi (B) Heatmap showing differential regulation of NFκB target genes post combination treatment of BETi and ATRi combination in λ820 cells (C) qRT-PCR validation of NFκB target genes post combination treatment of BETi and ATRi combination in λ820 cells (D) Western blot analysis λ820 cells post single or combination treatment with BETi and/or ATRi for 24h in presence of Q-VD-OPh (E) Western blot analysis of Daudi cells post single for combination treatment with BETi and/or ATRi for 24h

(unaltered here) was significantly upregulated in the combination treatment compared to the single agents (Figure 31D &E). This accumulation was most likely mediated via the up regulation of NK-κB mediated pathway and not because of a blocked autophagy response (Fang et al., 2014; Moscat and Diaz-Meco, 2009; Sanz et al., 2000).

Taken together, we show that BETi and ATRi may be combined to get synergistic benefits both *in vitro* and *in vivo*, especially in MYC induced lymphoma models. In addition, because of the lethality seen in the ATR-Chek1 pathway with BETi, many of the already known interactors of BET proteins, which are known to be important in replication machinery, may also be considered as potential therapeutic targets.

4. MATERIALS AND METHODS

4.1. Genomic DNA and plasmid DNA extraction

Genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Plasmid DNA was extracted either with NucleoSpin® Plasmid (Macherey-Nagel GmbH & Co. KG, Düren, Germany) or ZR Plasmid Miniprep™ - Classic (Zymo Research, Irvine, CA) for 3-5ml cultures and using Qiagen Plasmid Plus Midi Kit (Qiagen, Hilden, Germany) for 30-40 ml cultures. All isolations were performed as per manufacturer's protocols.

When growing the commonly used *E. coli* strains for regular plasmid preparations, use of Millers-LB is advised. Transformations were done using lab propagated cells made competent using Mix & Go reagent (Zymo Research, Irvine, CA). Using this reagent, the overall time for transformation from mixing to platting the cells is less than five minutes for plasmids containing ampicillin resistance gene as the selection marker.

4.2. RNA extraction

RNA was extracted using either Quick-RNA™ MiniPrep (Zymo Research, Irvine, CA) or NucleoSpin® RNA (Macherey-Nagel GmbH & Co. KG, Düren, Germany) kit as per the manufacturer's protocol.

4.3. cDNA synthesis

cDNA was synthesized from total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Thereafter, samples were diluted 1:1 with sterile MQ. The amount of total RNA was so used that post dilution with sterile MQ water, the amount of total RNA equivalent was within 10-20ng/µl.

4.4. qRT-PCR

Primers were either procured from KiCqStart® SYBR® Green Primers (Sigma-Aldrich, St Louis, MI) or made using PRIMER3 (Untergasser et al., 2012) and AmpliFX (Jullien, http://crn2m.univ-mrs.fr/pub/amplifx-dist). qRT-PCR was performed using KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix

(Kapa Biosystems, Inc, Woburn, MA, USA) using 10-20ng of total RNA equivalent per well. Data analyses were performed by comparing ΔΔCt values, using the reference gene and with a control sample set as relative expression 1.

4.5. Western blot Analysis

Most of the SDS PAGE systems used today are based on Laemmli's discontinuous buffer system (Laemmli, 1970) with few optimizations and modifications (Schagger and von Jagow, 1987). Typically, buffers of varying pH makes up the typical buffering system used today, where the stacking gel is around ~pH 6.8, separating gel around ~pH 8.8 and the inner tank buffer around ~pH 8.3.

SDS being an anionic detergent binds most proteins (but not all) at a constant ratio of 1.4g SDS/g of protein and imparts a global negative charge across the proteins. This results in an almost uniform mass:charge ratio which is used by the molecular sieving property of polyacrylamide in the gel for separation based solely on the mass of the protein when voltage is applied.

Glycine is one of the smallest amino acid and can exist either as positively, negatively or neutrally charged. This is typically dictated by the pH of the surroundings. In short, as the power is switched on, the negative charged glycine ions in the tank buffer (~pH 8.8) start entering the stacking gel as they move towards the cathode. The pH in the stacking gel (~pH6.8) turns this negatively charged glycine ions mostly to neutral charged (Zwitterion state). This change in state leads to their slower movement through the stacking gel. Simultaneously, the chloride ions (Cl^-) from Tris-HCL start migrating rapidly towards the cathode. These creates a small barrier with the Cl^- towards the front, the proteins in the middle (there mobility is somewhere in between Cl^- and glycine Zwitterion) and finally followed by the glycine ions as they head towards the boundary of stacking and separating gel. The stacking gels are made with low percentage of polyacrylamide and has low conductance, which promotes the proteins to move somewhat irrespective of the molecular mass and the entire samples loaded into the wells get compacted into a thin line.

Once they reach the separating gel (~pH8.3), glycine ions now becomes negatively charged or deprotonated. This causes glycine to move faster as

compared to the proteins. On the other hand, the proteins are now no more restricted within the barrier created in the stacking gel, thereby allowing them to resolve solely based on their mass. In most separating gels, a varying percentage of polyacrylamide is used which helps to get better resolution across a molecular size range. Moreover, tricine is now frequently used instead of glycine as it has a higher net negative charge and is known to better resolve proteins of 1-100kDa range (Schagger and von Jagow, 1987).

It is strongly advised not to adjust the pH of the buffers. This is because the addition of more ions directly influences their movement into the stacking gel, thereby making the same significantly less efficient because of the aforementioned reasons. The same applies to samples containing high salt concentrations.

When transferring (wet transfers) the gels onto a membrane, SDS and methanol/ethanol in the transfer buffer plays an opposing role. Methanol/ethanol helps to attach proteins into the membrane but at the same time it also shrinks the gel, thereby making the transfer of proteins from the gel more difficult. On the other hand SDS makes the proteins elute out of gel easily but opposes their transfer to the membrane. So a delicate balance of methanol/ethanol and SDS needs to be maintained. In most cases the use of Towbin's buffer (Towbin et al., 1979) with 20% methanol/ethanol (may be adjusted if required) with or without SDS (not exceeding 0.1% Wt/Vol of the total volume) is recommended.

Briefly, frozen cells pellets were lysed as previously described (Bhadury et al., 2014). Protein was quantified using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc, Hercules, CA) and 20-45µg of protein per lane was loaded onto ClearPAGE gels (C.B.S. Scientific, San Diego, CA). Gels were then transferred to 0.22µM nitrocellulose membranes (Protran; GE Healthcare Bio-Sciences) and successful transfer was confirmed by Ponceau-S (0.2% solution; Serva Electrophoresis, Heidelberg, Germany) staining. Thereafter, membranes were blocked with 5% BSA (Wt/Vol.) in TBST (Santa Cruz Biotechnology, USA) for 1h at room temperature followed by overnight incubation with specific antibodies at 4-8º C. Following washing with TBST, appropriate secondary antibody from Amersham ECL HRP-conjugated antibodies (GE Healthcare Bio-Sciences, USA) was used at 1:10000 dilution for

1hr at room temperature. Membranes were developed using Luminata Forte ECL substrate (EMD Millipore, Billerica, MA) and images were documented using the LAS-1000 imager (Fuji Films, Japan).

4.6. Flow cytometry analysis

Flow cytometry analysis was performed primarily on BD Accuri C6 (BD Biosciences, San Jose, CA). Mechanistically, the sample and sheath fluid flows through a laminar flow path at two distinct and independent velocities. In this machine, the process is accomplished using a non-pressurized method. Here, peristatic pumps are used which generally create a pulse flow but as the samples pass through the pulse dampeners, hydrodynamic focusing is maintained. This pumps also help create a vacuum in the sample sip, thereby significantly decreasing the sample volume required. Moreover, the use of peristatic pumps allows the precise measurement of liquid volume, which may be used for calculating absolute cell numbers among others parameters.

For DNA content and cell cycle analysis , Vindeløv's solution (Vindelov et al., 1983) was modified as a described (1X TBS, 1 µg/ml 7-AAD, 20 µg/ml RNase, and 0.1% IGEPAL CA-630 (AKA Nonidet-P40) adjusted to pH 8.0). No more than 1 million cells per ml of the modified Vindeløv's solution was used and incubated for 30 minutes at 37°C or 60 minutes at room temperature followed by analysis of DNA content using the FL3 channel (linear mode, cell cycle) or FL3 channel (logarithmic mode, apoptosis).

4.7. Sectioning

4.7.1. Cryostat

Tumor explants were fixed in 4% formaldehyde solution without methanol (freshly prepared and pre chilled in ice) at 4°C for 45-60 minutes. Samples were washed in ice cold PBS and put into 20% sucrose at 4°C for overnight incubation. Thereafter, samples were washed and snap frozen in OCT compound before storing in -80°C. Prior to sectioning, samples were equilibrated at -20°C for 20-30 minutes. Tissues were sectioned (parallel or serial sections of 8-10µM thickness) and placed onto positively charged slides. The slides were allowed to air dry for 10-15 minutes before further processing or were stored in -80°C.

4.7.2. Microtome

Tumor explants were fixed in 4% buffered formaldehyde in room temperature for 2-4 days and transferred to 70% ethanol solution, which allows for storage up to couple of weeks. The pieces were fixed in paraffin blocks and stored thereafter. Tissues were sectioned (parallel or serial sections of 4-6μM thickness) and placed onto positively charged slides. The slides were allowed to air dry for 10-15 minutes before further processing.

As a general practice, chilled solutions may be preferred for the purpose of fixation as the reaction is exothermic in nature.

4.8. Immunohistochemistry

4.8.1. For frozen sections

Slides were taken out from -80°C and allowed to defrost for 10 minutes in room temperature. Entire procedure hereafter is done in humidified chambers. Slides were washed with TBST before incubating with 3% H_2O_2 in methanol (Vol/Vol) for 10 minutes and washed again with TBST. Samples were then blocked with appropriate blocking solution in TBST for 45-60 minutes or as required by the specific protocols. Post blocking, 50-400μl of primary antibody in blocking solution was added to each slide and incubated over night at 4°C. To prevent evaporation, covering the slides with cover slips or paraffin paper is highly recommended. Slides were washed in TBST and thereafter incubated with appropriate secondary antibody as per the manufacturer's protocol. Slides were developed using DAB Chromogen and reaction was stopped by immersing slides in MQ. Slides were stained using appropriate counter stain and the reaction was stopped by immersing slides in tap water. Slides were then washed with PBS and mounted with xylene based mounting media and further analysis was performed as per the requirement of the experimental procedure.

4.8.2. For paraffin sections

Most paraffin blocks based procedures were outsourced to Histo-Center (Histo-Center AB, Gothenburg, Sweden). Briefly, slides were deparafinized and rehydrated. Thereafter antigen retrieval was performed and IHC was performed as per the requirement of the specific antibody used. Slides were developed

using DAB chromogen and appropriate counter staining was done. Slides were mounted using xylene based mounting media and further analysis was performed as per the requirement of the experimental procedure. Slides were either analyzed manually or scanned using Mirax automated slide scanner (Zeiss, Jenna, Germany). Scoring and/or quantification was either done manually or using BioPix IQ (Biopix AB, Gothenburg, Sweden).

4.9. Mammalian Cell Culture And Transfection

All cell lines were cultured as per the vendor's recommendations. For in house developed cell lines (Eμ, λ or M series) were cultured as described. All media were supplemented with gentamycin and/or the required additives as per the protocol. Cells were routinely tested for myco-plasma contamination.

4.9.1. Transfection using Calcium Phosphate

The method of using Calcium phosphate for introduction or transfecting foreign genetic materials into cells dates back to early 1970s (Graham and van der Eb, 1973). Today it is known that calcium phosphate precipitates are formed either by particle growth or nucleation. A multitude of factors ranging from DNA concentration, quality, pH, mixture incubation time and temperature among other factors can affect the transfection efficiency (Jordan et al., 1996; Jordan and Wurm, 2004). Briefly, endotoxin free plasmids (concentrations of ~1μg/μl), 2.5M Calcium chloride (stock solution), 2XHBS Buffer (0.28M NaCl, 0.05M HEPES, 1.5mM Na_2HPO_4 anhydrous), Buffered MQ water (2.5mM HEPES, pH ~7.2-7.3) are the only required reagents. In tube 1 take 50% of the final mixture volume of 2XHBS. In tube 2 mix the plasmid, make up the volume with buffered MQ and add calcium chloride (final concentration of 125mM). Mix the content of tube 2 into tube 1 and the mixture needs to be vortex and/or mixed immediately using pipettes. If visible precipitates are seen during the time of mixing, most likely the transfection will not work because of larger size of the precipitates. The final volume of this mixture (tube 1+2) may not exceed 10% of the total volume of media contained it the tissue culture vessel. The reaction mixture is set aside for 15-20mintues at room temperature (22 to 23°C). During the incubation time, the tissue culture media shall be replaced with a fresh media having an optimal ~pH of 7 (typically use complete DMEM media, as transfection in this case is not hampered by the presence of FBS and/or antibiotics; moreover DMEM contains higher amount of calcium and the pH ~7

enabling higher transfection efficiencies). The mixture after the incubation period, may be directly pipetted over the cells in a dropwise manner and gently mixed (avoid swirling the future vessel) and place it back into the incubator. 10 to 15 minutes post addition of the mixture, fine micro particle shall be visible under the microscope. The plates can now be placed back into the humidified incubator for overnight incubation. Despite the documentation of this precipitates bound to the cells monolayer (Graham and van der Eb, 1973), the exact mechanism of uptake of these micro particles (DNA+Calcim phosphate) is yet unknown.

4.9.2. Transfection using PEI

As an alternate and more user friendly method of transfection compared to calcium phosphate based method, PEI (either linear or branched) may be used for both transient transfection and virus production in 293T cells among others. Briefly a 25% solution of branched PEI (Cat no 408727, Sigma-Aldrich, St Louis, MI) was prepared to get a stock concentration of 250 mg/ml. Fresh working stock of 2.5 µg/µL of PEI in 150mM sodium chloride (NaCl) was prepared prior to use. In separate tubes, the plasmid DNA(s) and PEI were diluted in 150mM NaCl (Castellot et al., 1978). Typically 1:4 or 1:5 (DNA: PEI) ratios were used for transfections leading to virus production or transient transfections respectively. The PEI solution was always added to the plasmid DNA and mixed immediately. The mixture was incubated for 15-20 minutes at room temperature for allowing the complex to form, before adding the same to the cells. The total volume of this mix may not exceed 10% of the final volume of media in the culture vessel.

4.10. Virus production

pCMV-dR8.2 dvpr (a kind gift from Robert Weinberg, plasmid #8455) (Stewart et al., 2003), psPAX2 (plasmid # 12260), pMD2.G (a kind gift from Didier Trono) (plasmid #12259) , and pHCMV-EcoEnv (a kind gift from Miguel Sena-Esteves) (Plasmid #15802) (Sena-Esteves et al., 2004) were purchased from Addgene (Cambridge, MA). Plasmids were isolated as mentioned above. HEK-293T cells were seeded on 6 or 10 cm plates (Sarsted, Germany) one day prior to transfection. Calcium phosphate or PEI mediated transfection were performed using packaging:envelope:transfer plasmids at a ratio of 0.259:0.087:0.345 µg/cm^2 (adapted and modified from Recipe#1

http://tronolab.epfl.ch/webdav/site/tronolab/shared/LVPU/VectorProduction.html for 2nd generation vectors), and virus was harvested 42, 46, 50 and 66 hours post transfection. The virus containing supernatant is passed through 0.45µM low protein-binding filter (Sarstedt, Germany) filters.

To increase the virus titer, stability and prevent degradation of virus particles post thawing of aliquots from -80°C, 1% BSA (Wt./Vol.) may be supplemented to the virus harvesting media. Moreover, harvesting virus in media containing 5% FBS may also further aid in enhancing the virus titers.

4.10.1. Reverse transduction

Cells were suspended in 1ml of media and transduced with 1ml of virus containing media supplemented with 1-8 µg/ml of polybrene (Sigma-Aldrich, St Louis, MI) and seeded onto a well of a 6-well plate (Sarstedt, Germany). 12h post transduction, media was changed with fresh media and incubated for another 24h. All cell lines were either analyzed with BD Accuri C6 FCM (BD Biosciences, San Jose, CA) for reporter gene expression or selected using appropriate antibiotics before further experiments were performed.

4.11. Bio-informatics analysis

Genomic DNA (for exome sequencing) and RNA (for RNA sequencing) were extracted as described above. Library preparation and pair-end sequencing (on Illumina HiSeq 2000) was outsourced to BGI China. Briefly, for exome sequencing the SureSelect Target Enrichment System Capture Kit (Agilent Technologies) and Poly (A) enrichment method was used for RNA sequencing to prepare the respective libraries for sequencing. The raw reads data files generated post sequencing were processed by Illumina base calling software with default parameters, generating pair-end reads with appropriate read lengths as per the library enrichment methods used. “Clean files/reads” were generated by filtering out primer and adapter sequences, low quality reads and/or low quality bases from the raw reads files. The “clean files/reads” were used as the staring material for all analysis performed thereafter.

4.11.1. Exome sequencing

Clean files were assessed with FastQC (Andrews, 2010) and samples were trimmed if required (to appropriate read lengths). Makeshift genomes for Balb/c and DBA/2J were made by using the GATK package (McKenna et al., 2010). Briefly, the reference bases of the C57BL/6 mouse genome (MM9) at variation sites were replaced with the corresponding SNPs and InDels of BALB/c or DBA/2J strains available at the Mouse Genome Projects (http://www.sanger.ac.uk/resources/mouse/genomes/). The sequence reads for each sample was then mapped to their corresponding reference genome using BWA (Li and Durbin, 2009) with default parameters. PCR duplicates were marked and removed using Picard tool (http://broadinstitute.github.io/picard/). Thereafter, GATK package was used to recalibrate the quality score, InDel realignment and calling of variants (Depristo et al., 2011). The variants were annotated using Annovar (Wang et al., 2010b) and Polyphen 2 (Adzhubei et al., 2010) was used to analyze non-synonymous mutations. Control-FREEC was used to analysis CNV (Boeva et al., 2012).

4.11.2. RNA sequencing

Clean reads were first assessed with FastQC (Andrews, 2010) and for purity and/or contamination of samples (samples being possibly contaminated with murine stroma) were checked using FastQ screen (Wingett, 2011) . Reads were mapped to Hg19 (Human Genome) using TopHat 2 with the parameters (--library-type unstranded -r -40) (Kim et al., 2013). PCR duplicates were marked and removed using Picard tool (http://broadinstitute.github.io/picard/). Either RefSeq (Pruitt et al., 2014) or GENCODE V19 (Harrow et al., 2012) were used to annotate the genes. Variants were identified using GATK package by SplitNCigarReads followed by HaplotypeCaller function with default parameters (Depristo et al., 2011). Thereafter, variants were annotated using Annovar (Wang et al., 2010b). Reads were counted using HTSeq (count function) (Anders et al., 2015) using default parameters. For differential expression analysis, DESeq2 (Love et al., 2014) was used. Post normalization, variance stabilizing transformation (VSD) function was used. Heatmaps and/or PCA plots were generated using default DESeq2 functions or using Gene-E (Gould).

4.12. *In-vivo* experiments

All animal experiments were performed in accordance with E.U. directive 2010/63 (regional Gothenburg animal ethics committee approval #287/2011, 288/2011, #287/289-12 and #36-2014). All human patient samples were collected from Sahlgrenska University hospital, Gothenburg, Sweden.

5. CONCLUSION AND FUTURE PERSPECTIVE

We were among the first in the world to show the proof of concept paper using mouse exome sequencing to guide targeted therapy. In this study, we also for the first time to our knowledge made shift genomes of two very popular mouse strains namely BALB/c and DBA/2J. Furthermore, we propose the use of exome sequencing in the standard clinical setting to facilitate personalized treatment regime and development of potential biomarkers. In a subsequent paper, we could translate the same for guiding patient treatment possibly guiding patient enrollment into future clinical trials. In this study, we also convincingly show that there is indeed time to use platforms like this to aid or even guide clinical treatment regimens without any bias towards the age, disease stage or gender of the patients recruited

Thereafter, in the zest to inhibit Myc across malignancies, we collaborated with a Canadian company (Zenith Epigenetics) to test their novel and orally bio-available inhibitor against Bromodomain and extra-terminal (BET) proteins as the first BETi (JQ1) is generally dubbed by the field as an indirect Myc inhibitor. To our surprise, data obtained from the transgenic mouse models of Burkiit lymphoma suggested potent anti-tumor effects of BET inhibition independent of MYC suppression. Furthermore, we discover that BET protein inhibition not results in downregulation of genes but also seems to potentially upregulate a number of genes. Interestingly, there was surprising number only upregulated genes shared between BETi and HDACi. Moreover, we show a synergistic combination dosage of BETi and HDACi that was effective both *in vitro* and *in vivo*. From this data, it is intriguing to speculate that either of the drugs may be used to activate p53 target genes independent of p53 mutational status. In a recent follow up paper, we show lethal synergistic combinations of BET and ATR inhibitors in lymphomas. In addition, because of the lethality seen in the ATR-Chek1 pathway with BETi, many of the already known interactors of BET proteins, which are known to be important in replication machinery, may also be considered as potential therapeutic targets.

We next wanted to check the therapeutic implications of the above found synergies to other cancer types (especially metastatic melanoma). In that direction, we sequenced the transcription of metastatic melanomas derived cell line-derived xenografts (CDXs) and patient-derived xenografts (PDXs) to

decipher the shared similarities. Despite similar mutational profiles, CDXs and PDXs were inherently different irrespective of their genotype. Here, we unravel hypoxia and specifically hsa-miR-210 as a key player orchestrating the differences. We serendipitously show that abrogating miR-210 function makes this cells become less sensitive to MEK inhibition *in vivo*, suggesting a potential role of hsa-miRNA-210 in conferring resistance to MEK inhibitors.

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It is well said that, “You never know the true value of the moment, until it becomes a memory”. The memories I have made here are unparalleled and it’s not possible to jot them down within few pages. Still let me give it a try

I would like to thank Jonas and Lisa for allowing me to be a part of their lab. Jonas without your continuous support, fantabulous mentorship and unparalleled knowledge, I would not have had successful theses. In fact, I have to admit it can sometimes be overwhelming to see a couple so dedicated to science. Their greatest strength lies in the fact that both Jonas and Lisa complement each other in every aspect.

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Lisa -
until enough blossoms have been visited by the bee,
until a boat has hit enough waves in open sea,
until a runner has had enough bruises on his knee,
the bee can't have enough honey to store
the boat hasn't travelled enough to deserve the shore
the runner isn't the best, he should run, fall and run some more.

Even though we had very strong difference of opinions, end of the day we managed to pull off all the stuff. Thanks a lot for teaching me the techniques

and suggesting some of the most crucial experiments that many times both Jonas and I had missed.

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APPENDIX